Recent Advances in the Development of HIV-1 Tat-Based Vaccines

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Abstract: Over the last two decades most of the efforts in HIV vaccine development have been based on the use of the HIV Env with the goal to induce sterilizing immunity. However, as a result of Env variability disappointing results have been obtained in preclinical and phase III clinical trials. Although the objective of a preventive immunity still remains a priority, secondary endpoints (e.g. block of virus replication and disease onset) are being considered at the present as more achievable end-points in HIV vaccine development. This is based on accumulating evidence indicating that low viral load correlates with maintenance of immune functions and slow progression to disease, and that cell-mediated immunity plays a major protective role in the absence of sterilizing immunity. The promising results obtained in non-human primates with a vaccine based on a native Tat protein (B-clade), which is an early regulatory protein key for HIV replication and AIDS pathogenesis, highlights the importance of targeting the virus very early after infection. In particular, the immune response against Tat appears to modify the virus-host interactions at the very beginning of infection, thus containing the depletion of critical immune cells and the progression of infection. Moreover, since Tat targets and induces maturation of dendritic cells, has immunomodulatory activities and drives Th-1 and CTL responses, immunization with Tat may drive or increase these immune responses also against other HIV antigens to support an effective, long-lasting and hopefully even sterilizing antiviral immunity. Finally, Tat B-clade is similarly recognized by sera from individuals infected by different virus clades (A, B, C, D) supporting the concept of a cross-clade vaccine. Therefore, the Tat-vaccine should contain virus replication protecting from disease progression (non-sterilizing immunity) or even favoring an abortive infection. Although only a phase III clinical trial will establish the efficacy of this vaccine strategy, the Tat-vaccine has recently entered preventive and therapeutic phase I clinical testing in Italy to establish safety (primary-end-point) and immunogenicity (secondary end-point) and phase II studies are being prepared.

Keywords: HIV-1 Tat; HIV vaccine; Rodents; Non-human primates; Humans; Anti-Tat immune responses; clinical trials.

.I. NEW PERSPECTIVES FOR AN EFFECTIVE HIV VACCINE

WHO and UNAIDS estimate that more than 40 million people have been infected with the human immunodeficiency virus (HIV) since the beginning of the epidemic. During the year 2003, the global HIV/acquired immune deficiency syndrome (AIDS) pandemic killed more than 3 million people, and an estimated 5 million acquired HIV, bringing to 40 million the number of people living with the virus around the world, mostly in the developing countries. Sub-Saharan Africa remains by far the region worst-affected by the HIV infection and AIDS. An estimated 26.6 million people in this region were living with the virus, including the 3.2 million who became infected, and approximately 2.3 million people killed, during the year 2003. The epidemic is fast-spreading into areas and countries where, until recently, there was little or no HIV present, including China, Indonesia and Viet Nam, Eastern Europe, the Middle East and Latin America, providing a clear evidence of how an HIV/AIDS epidemic can erupt suddenly wherever the virus is introduced in the population (http://www.unaids.org). Although antiretroviral therapy has proven to be effective in the treatment of HIV-infected patients, it has major drawbacks, including severe side effects, short drug half-life, increasing prevalence of drug-resistant viruses, high costs and difficulty of management in poor Developing Countries. In addition, it cannot eradicate the virus and its spreading among individuals [231]. Most of all, the pharmacological therapy is unlikely to become available for routine use in developing countries because of strict treatment guidelines, which are hard to be met where infrastructures, medical and economic resources are limited or absent [177]. Since the inexorable spreading of HIV pandemic is unabated, the urgency for designing an effective, safe and inexpensive vaccine to protect people from HIV or AIDS remains a priority. Ever since 1983, when HIV was discovered to be the cause of AIDS, an enormous effort has been devoted to this goal by the scientific community. However, after two decades there is still no efficacious vaccines [139]. These attempts to develop a vaccine have extraordinary advanced our understanding in both the biology of HIV and the human immune system. This has led to new concepts and strategies that will hopefully bring in the near future to the development of an effective vaccine against HIV infection and AIDS.

Most of the efforts in HIV vaccine development has been based on the use of structural proteins, which constitute the envelope (Env) of HIV and are responsible for the binding and entry of the virus, with the rationale of generating neutralizing antibodies capable of blocking virus adsorption to the target cells and protecting from infection (sterilizing immunity) [206]. Unfortunately, the Env protein-based vaccines have shown so far to be inefficacious at eliciting

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durable, cross-clade and high titers neutralizing antibodies capable of blocking virus entry and infection of primary HIV isolates derived from patients [146]. Most of all, the inefficacy of these vaccine strategies has been recently demonstrated by the first two phase III clinical trials of gp120 envelope subunits, AIDSVAX B/B and AIDSVAX B/E by VaxGen, tested in over 5,000 at-risk volunteers, in the United States and in Thailand, respectively [75; http://www.vaxgen.com]. Indeed, these vaccines did not prevent infection by HIV (primary endpoint), neither slowed the progression of the disease among those who were vaccinated but became later infected with HIV (secondary endpoint). Although several reasons could account for these failures, they can be mostly due to the fact that HIV has developed ingenious ways to dodge attack by broadly neutralizing antibodies. For instance, it hides its antibody binding sites under the protein loops and the heavily glycosylated sites on its surface, hampering recognition of relevant, mostly conformational, epitopes by neutralizing antibodies [234]. The observation that in the course of natural infection the neutralizing antibody titers are generally low, underscores that the relevant envelope epitopes are poorly expressed or weekly immunogenic and that neutralization is difficult. In addition, HIV mutates rapidly, particularly in its structural genes, and changes its glycosylation profile [180], leading to a rapid *in vivo* evolution of HIV variants [180, 192]. This renders HIV a hard target for neutralizing antibodies, but also for cellular immunity raised by the primary infection or by vaccination [37, 139, 175]. In fact, during natural infection this allows the rapid establishment of a chronic infection with virus reservoirs in T-cells, macrophages and monocytes, where HIV replicates releasing viral particles and/or producing immunosuppressive products or proteins that eventually compromise the host defenses [172]. The escape of the host immune response helps to maintain the HIV reservoir cells and contributes to the inefficiency of actual antiretroviral therapies to eradicate HIV [79].

Despite this scenario, there is evidence that the host can be protected [24, 66, 67, 101]. This includes studies in chimpanzee with HIV Env-based vaccines or in monkeys with live attenuated simian immune deficiency virus (SIV) vaccines, that protected from homologous and heterologous challenge [66, 67]; the observation that some highly exposed persons have remained uninfected [121]; the relatively low incidence of mother to child (fetus) transmission [108, 119]; the initial effective immune response that significantly, if temporally, reduces viral load [62, 63, 81]; the observation that super infection is rare and that some infected persons are long-term non-progressors [130, 131]; the results of passive immunization studies with human monoclonal antibodies against relevant neutralizing epitopes that have proven effective in experimental animals [73, 191, 209]; and finally, successful vaccine development against other viral infections. Thus, although a sterilizing immunity against different HIV virus strains has not been achieved as yet with Env-based vaccines, evidence exists that it could be gained and, undoubtedly, the objective of inducing a preventive immunity still remains a priority, and the final challenge to the scientific community is to find strategies of eliciting such crucial and cryptic neutralizing antibodies, long-lasting and at high titers, and capable of blocking HIV infection.

Experimental evidence indicates, however, that cellular immunity can play a protective role even in the absence of neutralizing antibodies [66, 67, 90, 139, 206]. To this goal, second generation vaccines have been developed based on structural proteins, such as Gag, which seems to be the most immunogenic in HIV infection and is more conserved in its immunodominant epitopes than Env, with the concept of inducing strong and broad T helper and cytotoxic responses, which, although incapable of preventing infection, would, at least, mitigate or abort the effects of HIV infection by limiting virus replication, providing protection from disease progression and reducing virus transmission to healthy individuals [139, 221]. To this respect, the key role of CTL responses in controlling acute infection has been clearly demonstrated in monkeys, where depletion of CD8+ T cells, following SIV challenge, resulted in reduced suppression of the initial plasma viremia and acceleration of disease progression [107, 193]. Similarly, in humans the appearance of HIV-1 specific CTLs during acute infection coincides with the rapid decline of plasma viremia and with the increase of CD4+ T cells [31, 117, 118, 165]. Vigorous HIV-1-specific CTL responses contain the infection in vaccinated animals, and persist in most long-term non-progressors [31, 117, 118, 160], in opposition to their decline in subjects progressing to AIDS [44, 51, 89, 115, 183]. Accumulating evidence indicates that an inverse correlation between CD4+ T cell responses and viral load exists in chronic infected individuals [187, 188], and that strong HIV-specific T helper responses are required for maintaining functional memory CTL responses [10, 109, 199, 211]. This is a clear trick of HIV, since CD4+ T cells are susceptible to direct damage by the virus, which preferentially infects HIV-specific CD4+ T lymphocytes [61], and therefore, if CD4+ T cells are impaired, the secondary responses may become defective. Thus, based on this knowledge, different vaccine approaches, capable of eliciting CTL responses are being developed. These are based on recombinant proteins, DNA plasmids or expression vectors, and are currently being tested in animal models or human volunteers. In particular, these strategies have proven to be effective in controlling viremia and progression to AIDS in non-human primates [11, 21, 26, 139, 151, 186, 202]. However, evidence indicates that the targeting of T-cell responses on immunodominant epitopes can facilitate the insurgence of viral mutants that escape the vaccine-induced immune control [20, 102]. This may represent a concern when immunity permits low levels of virus replication.

More recently, based on new concepts of "reverse vaccinology", novel strategies have been developed, aimed at targeting virus regulatory and accessory genes, which are essential for HIV replication, maturation and infectivity, are capable of inducing a broad immunity and are highly conserved among the HIV clades [38, 184]. Therefore, these approaches have the potential to be effective for both preventive and therapeutic vaccine strategies. A number of pre-clinical and clinical studies based on HIV-1 and SIV regulatory genes have been performed and the results have shown that they are safe and immunogenic in mice, monkeys and in HIV-1 infected individuals [39-43, 52, 99, 100, 136, 163, 166, 203]. Of note, they have been shown to induce a long-lasting protection from infection in monkeys after challenge with pathogenic simian/human

immunodeficiency (SHIV) or SIV [39-41, 136, 163, 166, 210].

II. WHY HIV-1 TAT IS A PROMISING VACCINE CANDIDATE?

Among the early proteins of HIV-1, the Tat protein possesses very attractive features which make it a relevant antigen for the development of an anti-HIV/AIDS vaccine. These include its early expression and key role in the virus life cycle and pathogenesis of AIDS, the evidence that the anti-Tat immune response correlates with non-progression to AIDS in infected patients and in monkeys, the properties of selectively targeting dendritic cells and of being efficiently internalized by and to be presented in the context of the major histocompatibility complex (MHC) class I, the conservation among different geographically distinct virus clades, the safety and immunogenicity in different animal models and in humans, and the safety, immunogenicity and efficacy of Tat vaccine (DNA and protein) in non-human primates and in humans. For these reasons, Tat is considered a potential and promising vaccine candidate and phase I clinical trials on both seronegative and seropositive individuals with the Tat protein-vaccine are ongoing in Italy. In addition, the Tat antigen will be tested in combination with other HIV structural and regulatory genes within the European AIDS Vaccine Integrated Project (AVIP), and it has been included in combined vaccine formulations that are currently under clinical evaluation in the United States (http://chi.ucsf.edu.vaccines). These features of Tat and the advances in the development of Tat-based vaccines will be reviewed below.

Tat Plays a Key Role in the Virus Life Cycle and Pathogenesis of AIDS

Tat is a small polypeptide of 14 to 16 kDa molecular weight, containing 86-102 amino acids (depending on the virus strain), which is encoded by two exons and translated from multiply spliced 2 kb mRNAs [15, 74]. Tat is produced very early after infection, even prior to HIV integration, and it is essential for virus gene expression and replication and cell-to-cell transmission [15, 48, 54, 64, 65, 68, 74, 233]. In particular, Tat trans activates the HIV-1 long-terminal repeat promoter increasing the expression of all HIV transcripts [15, 74]. This effect is mediated by translocation of Tat from the cytoplasm into the cell nucleus, where it interacts with host factors and the Tat responsive element (TAR), present at the 5' end of all viral mRNA, leading to increased initiation and elongation of HIV transcription [124]. In the absence of Tat no infectious virus is made. However, the replication of *tat*-defective proviruses can be rescued by the addition of exogenous Tat [65, 133]. Tat-mediated trans activation requires the first exon (amino acids 1-72), and in particular the first 58 amino acids, which include the N-terminal proline-rich (aa 1-20), the cysteine-rich (aa 21-37 containing 7 cysteines) and the core (aa 38-48) regions, representing Tat activation domain, and the basic region (aa 49-58) for nuclear localization and binding to TAR RNAs. The C-terminal region of Tat, comprising the second exon, is not necessary for Tat-trans activation of HIV-1 gene expression [78]. However, this region contains the arginine-glycine-aspartic acid (RGD) sequence that enables extracellular Tat to bind and to signal through the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which are the physiological receptors for fibronectin and vitronectin, respectively [19, 48, 53, 68]. In fact, during acute infection Tat is released by the infected CD4+ T lymphocytes in the extra-cellular compartment, in the absence of cell death or cell permeability changes via a leaderless exocytosic pathway [53, 64, 65]. Release of Tat occurs at the moment of maximal *tat* gene expression, very early after infection, and is undetectable in HIV-1 chronically infected cells or *tat*-transfected cells in which Tat expression is low [18, 53, 64, 65, 98]. Tat is released also *in vivo* both in the tissues and in the blood of HIV-1 infected patients [68, 133, 198, 232]. The release of extracellular Tat is key for the biology of the virus. In fact, extracellular Tat can act both from outside and inside the cells, exerting effects that depend on its concentration and on the target cells [18]. Tat binds to the heparan sulfate proteoglycans at the cell surface through its basic region, enters and translocates into the nucleus of both infected cells and uninfected cells, in which it enhances replication of HIV-1 or *trans* activates the replication of *tat*-defective or latent proviruses, and/or modulates the activity of cellular genes, including transcription factors, cytokines and genes involved in cell-cycle regulation whose expression is critical for HIV replication [18, 48, 54, 65, 76, 168]. These effects are prevalent at high concentrations of Tat (nanomolar to micromolar). On the other hand, experimental evidence indicates that the biological effects of low (picomolar) concentrations of extracellular Tat on infected and uninfected cells mainly occur through the interaction of Tat with cell surface receptors and the engagement of specific signal transduction pathways [18]. It has been shown that Tat binds the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins receptors and VEGF receptor 2 [18] and that phosphorylates tyrosine kinases [80, 143, 144] resulting in downstream intracellular signaling, such as the induction of phosphoinositide 3-kinase activity [142-144, 241]. Furthermore, extracellular Tat induces the expression of the chemokine receptors (and HIV-1 co-receptors) CCR5 and CXCR4 [103, 195] responsible for the transmission of macrophage- and T-cell-tropic HIV-1 strains, respectively, thus favoring the spreading of infection. Consistent with these autocrine and paracrine activities of Tat on virus replication, experimental evidence shows that the addition of anti-Tat antibodies or immune sera from Tat-vaccinated monkeys to the culture medium of *tat*-transfected or HIV-1 infected cells blocks entry of Tat into cells and/or transactivation of HIV-1 LTR and virus production [34, 35, 39, 65, 98, 177, 208, 240]. In addition, cross-sectional and longitudinal studies indicate that the presence of anti-Tat antibodies in HIV infected individuals correlates with low or undetectable viral load and with non-progression to AIDS [120, 177-179, 185, 238, 239, Ensoli et al., unpublished].

Finally, the key role played by Tat in HIV infection and AIDS progression is underscored by the evidence that few mutations in this protein can generate more or less efficient Tat variants, which correlate with a more or less HIV virulent phenotype, such as the Tat protein of the highly virulent African sybtype D Mal, isolated from a rapid progressor during a dramatic increase of AIDS in central Africa [4], or the Tat protein of the low virulent subtype B Oyi variant, isolated from long-term non-progressor patients in Gabon, in which a single mutation in the cysteine-rich

domain generates a trans-activation inactive Tat variant and a less virulent virus [92, 105, 161, 167]. In agreement with these results, other studies indicate that nucleotide changes in the *tat* gene are responsible for the generation of more or less virulent SIV or SHIV variants, which are associated with a more or less rapid CD4+ T cell decline and progression to AIDS-like disease in monkeys infected with these viruses [7, 110].

Tat is Immunogenic

Recent evidence highlights the importance of the CTL responses against the proteins of HIV that are produced early during infection, in particular Tat and Rev, which are the only two viral proteins produced before Nef down-modulates MHC I expression [56]. Indeed, the selective pressure that CD8+ T cells exert on the virus, during the acute phase of the infection, pushes HIV to mutate in its immunodominant CTL epitopes faster and more frequently in the early proteins than in the late proteins [9, 87], thus favoring immune escape and rapid establishment of chronic infection [20, 32, 45, 69, 88, 89, 95, 96, 116, 169, 218, 235, 236]. Mathematical models and *in vitro* studies suggest that CTL responses directed against HIV early proteins suppress virus production more effectively [96, 218, 221, 235, 236], and this is confirmed by studies in monkeys showing that CTL responses directed to Tat and Rev are more efficacious to control SIV replication than CTL responses against Gag and Pol [210].

CTL responses to Tat have been identified in humans after primary infection [44] and in individuals chronically infected with subtypes B and C [1-3, 8, 45, 55, 122, 156, 157, 164, 220, 237]. Tat-CTL epitopes, presented by A6801, B15, B53 and Cw1203 HLA alleles, have been mapped at the N-terminal, cysteine-rich and core regions of the protein, in particular at residues 2-11 [1, 2], 30-37 [44, 45], 38-48 [164], and 36-47 [157]. There is evidence that the presence of anti-Tat CTLs correlates with non-progression, both in HIV-positive individuals and in monkeys infected with SIV [158, 159, 220, 222]. Furthermore, the detection of Tat-CTL escape mutants early after infection in humans and in monkeys [5-7, 45, 158] highlights the selective pressure that the immune system exerts on this early protein and the importance of anti-Tat CTLs early after infection. Finally, Tat-specific CTL responses represent the major correlate of protection in monkeys vaccinated with Tat [5-7, 39, 41, 136, 149, 158]. Altogether these studies indicate that the control of HIV replication by CTL targeted to early proteins, and in particular to Tat, is relevant for containment of infection and for non-progression to AIDS.

Although T helper responses against Tat have been poorly characterized, a proliferative T cell response to Tat and Tat peptides has been described in HIV infected individuals [30]. In particular, three immunodominant T helper epitopes have been mapped at amino acids 17-32, 33-48 and 65-80 of Tat, and shown to be presented in association with several HLA-DR molecules (amino acids 17-32 and 33-48) or restricted to the HLA DR-2 allele (amino acids 65-80). This evidence indicates that Tat induces antigen-specific CD4+ T helper responses during infection, although at present it is unclear whether they are directly associated with the control of viral load, by releasing cytokines or chemokines with antiviral activity, and/or whether they are required to support efficient Tat-specific CTL responses.

The humoral response to Tat has also been correlated with the containment of HIV infection. Tat is released by HIV-1 infected cells and is able to induce antibodies, which are detected in 10-20% of the infected individuals [38, 58, 120, 213, Rezza et al. submitted]. Several studies have shown that antibodies to Tat, or to functional domains of Tat, inhibit its effects on virus replication and cell functions *in vitro* [36, 64, 65, 68, 177, 178, 208, 219]. Most importantly, cross-sectional and longitudinal studies indicate that the presence, and the titers, of anti-Tat antibodies is associated with the asymptomatic stage of infection and with non progression to AIDS [38, 179, 182, 185, 239, Rezza et al., submitted]. Consistent with these findings, monkeys vaccinated with Tat peptides, which are known to contain B-cell epitopes, significantly reduce the plasma viremia after challenge with $SHIV_{33}$ [84]. The predominant antibody responses to Tat have been mapped at amino acids 1-20 and 36-50, which are highly conserved among different HIV subtypes [38], are key for Tat function and contain B [85] and CTL [1, 156] epitopes. However, antibody responses against other functional domains of Tat are also detected during infection, including the cysteine, the basic, and the carboxyterminal regions [38, 58, 120, 170, 179, 182, 213]. Of note, a large portion of the humoral response against Tat is against conformational epitopes whose detection requires a native, monomeric Tat protein [38, 179, our unpublished results]. This is demonstrated by the evidence that the majority of HIV-1 infected patients seropositive for Tat, or of Tat-immunized animals, have higher titers of conformational anti-Tat IgG as compared with antibody titers directed against linear epitopes [38, our unpublished results]. Along with these observations, Tat immunization elicits antibody responses in rodents, monkeys and humans with similar epitope mapping profile and which block the activity of extracellular Tat on cellular entry, gene expression and replication [25, 36, 39, 41, 52, 53, 65, 155, 162, 203, 216, 219, our unpublished results]. Interestingly, recent evidence in monkeys and in humans indicate that immunization with a chemically modified Tat protein (Tat toxoid) restricts the pattern of anti-Tat antibody recognition, both in terms of reactivity to linear epitopes and of intra- and inter-clade reactivity, as compared to immunization with an unmodified biologically active Tat protein [155, 216]. This indicates that the native conformation of Tat is essential for the induction of broadly reacting antibodies. In fact, conformational antibodies are most prevalent in anti-Tat sera [38, 161, 162]. Finally, recent evidence show that HIV-1 infected patients, which are fast-progressors [182] or have developed Kaposis's sarcoma [58, Ensoli et al., unpublished results] have lower or absent anti-Tat antibodies whose epitope profile is more restricted as compared to HIV-infected and asymptomatic individuals. Altogether, these results suggest that the presence of high titers antibodies, directed against a broad spectrum of Tat functional domains neutralize the effects of extracellular Tat, playing an important role in the control of the HIV infection and in disease progression.

Taken together, the experimental evidence suggests that an early and broad immune response against Tat, both

cellular and humoral, is relevant for the containment of virus replication, and supports the idea that vaccination with Tat might impact or abort early infection by HIV-1. Indeed, recent studies in monkeys showed that immunization with Tat protein or DNA controlled primary infection of a highly pathogenic $SHIV_{89.6P}$ virus at its early stages and allowed a long-term containment of virus replication and spread in blood and tissues up to 2 years after the challenge [136] or after two tetanus toxoid boosts capable of increasing replication of residual virus.

Tat has Immunomodulatory Activity

Extracellular Tat is taken up by the cells and, unlike most soluble proteins, enters MHC class I pathway and elicits CTL activities [111, 150]. This is a feature which Tat shares with very few other proteins called "penetratins" [59]. Purified, endotoxin-free and biologically active Tat targets and is taken up by monocyte-derived dendritic cells (MDDC) at doses as low as 0.01 ng/ml. This process occurs in a time-, dose- and maturation-dependent fashion and it is hampered by the oxidation and inactivation of the protein or by low temperature. Uptake is more efficient upon MDDC maturation suggesting a receptor-mediated pathway which become up-regulated upon cell maturation [70, 71]. Moreover, active (but not oxidized) Tat induces maturation of MDDC at relatively higher doses (1-20 μg/ml), as indicated by the dose-dependent increase of surface expression of MHC, CD40, CD80 and CD86 costimulatory molecules, and of production of IL-12, TNFα, and of the β-chemokines MIP-1α , MIP-1β and RANTES, which drive Th-1 type immune responses. Consistently, Tat increases the capability of MDDC to present both allogenic and recall antigens potentating T cell responses against heterologous antigens [70, 71]. Apparently, these results are in contrast with those described in a more recent report showing that Tat expressed by an adenovirus vector induces interferon-responsive gene expression in immature MDCC without inducing their maturation [106]. Although it is reported that the adenovirus delivery system used to express Tat does not induce MDDC maturation and leaves the cells susceptible to other maturation stimuli [205], the different results may be due to the different experimental systems. In fact, cells with low CD1 expression were used in that system as compared to the previous study [Ensoli et al., in preparation], and CD1 must be highly expressed to have MDDC maturation upon induction with stimuli.

Consistent with an immunomodulatory activity of Tat, other experimental evidence shows that the basic region (amino acids 47-59) of Tat acts as a protein transduction domain (PTD), enabling conjugated proteins to efficiently enter the cells in an energy- and receptor-independent fashion [72, 194]. Thus, the PTD peptide or the Tat protein have been largely used to deliver conjugated antigens to the MHC class I restricted-antigen presentation pathway, and to increase CTL responses to the conjugated antigen [111-113, 200, 201, 214]. Accumulating evidence, however, indicates that the higher immunogenicity of these Tat-delivered antigens is not only due to increased cellular uptake, but to their greater presentation onto MHC class I molecules, suggesting that Tat possesses not yet defined immunomodulatory properties, including increase of T helper immune responses and modulation of the antigen processing machinery [14, 82, 104, 127-129, 196, Gavioli et al., submitted].

Taken together these data indicate that Tat is not only an antigen but also a potent T cell adjuvant capable of modifying CTL responses against heterologous antigens.

Tat is Conserved Among Different HIV-1 Clades

The Tat protein is highly conserved in its immunogenic epitopes among all M subtypes [23, 38, 85, 141, 216]. Of note, recent data show an effective serological cross-recognition of Tat protein from the BH10 clone of $HTLV_{IIIB}$ lab-adapted clade B strain, which was isolated almost 20 years ago [176], by individuals infected with HIV-1 B-clade viruses circulating in Italy and with HIV-1 subtypes A, B, C, D, F and G circulating in Africa [38]. Similar anti-Tat antibody prevalence, titers and epitope mapping were reported, irrespective of the geographic distribution. This observation is confirmed by the results of sequence analysis, demonstrating that the predicted amino acidic sequence of Tat is well conserved among the circulating viruses belonging to distinct HIV-1 clades and presents a high degree of homology with the BH10 Tat sequence [38]. Homology is specifically high in the first exon-encoded portion of Tat, and, particularly, in the 1-58 amino acids region, which contains the activation domain of Tat and most of the B, T-helper and CTL epitopes so far identified [38, 147, 161, 216]. In fact, epitope mapping studies using linear peptides from the BH10 Tat sequence indicate the same pattern of recognition by sera of individuals from Italy and Africa, with a major B cell epitope of Tat mapping within the amino terminal region, and a large portion of anti-Tat antibodies represented by conformational antibodies. These findings are in agreement with other data indicating that Tat variants from HIV-1 clades A, B, C, D and AE, identified in different parts of the world, have a high degree of sequence homology and similar protein activity [161]. Furthermore, circular dichroism measurements, molecular modeling and NMR spectroscopy studies show that similar folding exists among different Tat variants [17, 23, 77, 91, 92, 161, 167], and that non-homologous Tat proteins are cross-recognized by antibodies mainly directed against conformational epitopes [161, 162]. Consistent with these data, other studies show that sera from macaques immunized with a recombinant Tat protein of clade B cross-react with HIV-1 Tat peptides from B and C clades, block the cellular uptake of extracellular Tat and neutralize its activity in transactivation assays [39, 216]. Finally, other recent reports showed that anti-Tat sera from a large cohort of HIV-1 seropositive Caucasian individuals cross-react with Tat from different viral isolates, including HIV-1 subtype E, $SHIV_{89.6P}$ and SIV_{mac251} [182], and that sera collected in France from HIV-infected individuals recognize equally well Tat proteins from distinct HIV strains of clades A, B and D [25]. These results indicate that the overall identity of Tat is preserved at the sequence, functional and conformational level and provide strong formal evidence that a Tat-based vaccine may indeed represent a universal anti-HIV tool capable of inducing immune responses effective against multiple virus clades.

Table 1. Results of "SAFETY" in Vaccination Studies with the HIV-1 Tat Protein or Tat DNA

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Toxic effects	References
Mice	HIV-1 Tat DNA	1-100 μg (3 times)	None	i.m., i.d.	None	198
	HIV-1 Tat DNA	1-100 μg (1-3 times)	None	i.m., g.g (i.d.), i.p., i.d.	None	100
	HIV-1 Tat protein	5-50 μg (2 times)	Incomplete Freund's, RIBI	s.c.	None	100
	HIV-1 Tat DNA	5-10 μg (3 times)	None	i.m., i.d., i.n., intra toungue	None	99
	HIV-1 Tat DNA	20 μg	None	i.n.	None	16
	HIV-1 Tat DNAmutants	5-100 μg (6 times)	bupivacaine	i.m.	None	52
	HIV-1 Tat protein	10 μg (4 times)	LTR192G	i.n.	None	148
	HIV-1 Tat DNA in a self-replicating vector	1-150 μg (6 times)	None	g.g., i.m., i.d.	None	213
	HIV-1 Tat DNA	10-50 μg (twice) or 20-100 μg (twice)	None	i.m.	None	114
	HIV-1 Tat protein	10 μg (4 times)	LTR72, Alum, Incomplete Freund's	i.n., s.c.	None	138
	HIV-1 Tat protein	10 μg (4 times)	None, MALP-2	i.n., i.d.	None	33
	HIV-1 Tat protein	40 ng-10 μg (4 times)	Complete Freund's, Red blood cells	i.p.	None	60
	HIV-1 Tat protein	0.5-2 μg (3 times)	None, Anionic microparticles, Alum	i.m.	None	Footnote[1]
	HIV-1 Tat DNA	1-30 μg (6 times)	Cationic block copolymers	i.m.	None	49
	HIV-1 Tat protein	10 μg (3 times)	Alum	s.c.	None	57
	HIV-1 Tat protein	5 μg (twice)	Wax anionic nanoparticles, Alum, Lipid A	s.c.	None	57
	HIV-1 Tat DNA (codon-optimized, and ubiquitin-tagged codon-optimized)	100 μg (4 times)	none	i.m.	None	174
Rabbits	HIV-1 Tat protein	100 μg (3 times)	Complete Freund's	i.d.	None	162
Monkeys	SIV Tat DNA in SFV and MVA viral vectors	SFV-tat (10^{8-9} $TCID_{50}$ 2 times); MVA-tat (10^{8-9} $TCID_{50}$ 2 times)	SFV, MVA	i.m.	None	163
	SIV Tat DNA in SFV and MVA viral vectors	SFV-tat (10^{8-9} $TCID_{50}$ 2 times); MVA-tat (10^{8-9} $TCID_{50}$ 2 times)	SFV, MVA	i.m.	None	210
	HIV-1 Tat DNA and protein	10 μg (4 times) +2 boosts with 50 μg of Tat protein	Golden Particles, RIBI	g.g. (i.d.)	None	173
	HIV-1 Tat protein	6-10 μg (8 times + 1 boost with 16 μg of Tat protein with ISCOMs)	RIBI, Alum	s.c., i.d.	None	39

(Table 1). contd.....

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Toxic effects	References
	HIV-1 Tat DNA	0.2-1 μg (8 times + 1 boost with 16 μg of Tat protein with ISCOMs)	Methylparaben + bupivacaine	i.d., i.m.	None	41
	HIV-1 Tat protein	10-40 μg (5 times)	Incomplete Freund's, polyphosphazene	i.d., i.m.	None	166
	HIV-1 Tat DNA	1 mg (7 times + 2 boosts with 25 μg of Tat protein with ISCOMs)	None	i.m.	None	Footnote[2]
	HIV-1 Tat protein	6 μg (11 times)	None	i.d.	None	Footnote[2]
	HIV-1 Tat protein	20 μg (3 times)	Alum, MPL	s.c.	None	Footnote[3]
	HIV-1 Tat DNA in SFV and MVA viral vectors	SFV-tat (10^8 $TCID_{50}$) (twice), MVA-tat (10^8 $TCID_{50}$) (twice)	SFV, MVA	s.c., i.m.	None	223
	HIV-1 NEF-tat fusion protein	20 μg (3 times)	AS02A	i.m.	None	225
	SIV Tat DNA	32 μg (3 times) + 1 MVA boost (5 x 10^8 pfu)	Golden particles	g.g., i.d., i.r.	None	6
	HIV-1 Tat DNA	500 μg (3 times) + 2 boosts with 25 μg of Tat protein in ISCOMs	Methylparaben + bupivacaine	i.m.	None	145
Humans	HIV-1 Tat DNA	100 μg (3 times)	None	i.m.	None	42

[1]Caputo et al, unpublished
[2]Ensoli et al, unpublished
[3]Shiver J. HIV vaccines: some lessons learned from nonhuman primate models. 18th Annual Symposium on Nonhuman Primate Model for AIDS, 2000. Madison, Wisconsis

III. TAT-BASED VACCINES

Based on the experimental evidence described above, immunization to Tat has been proposed as a novel strategy of immune intervention to prevent AIDS, aimed at blocking very early steps of infection, modifying the virus-host dynamics and controlling infection and disease progression (non sterilizing immunity), or even favoring the occurrence of an abortive infection [39, 79, 86]. Thus, systemic and mucosal immunization approaches with both biologically active or inactivated Tat are being evaluated in animal models and in humans for safety, immunogenicity and efficacy. The results of these studies are discussed below and summarized in Tables 1-3.

Vaccines Based on Biologically Active Tat

Several experiments or trials performed in rodents, non-human primates and humans indicate that immunization with a native, biologically active Tat protein or wild-type *tat* DNA is safe. In fact, no local nor systemic toxic effects were ever detected at the biochemical, hematological or immunological levels in mice, in monkeys (both infected or uninfected) and humans [6, 33, 39, 41, 42, 49, 52, 60, 100, 114, 138, 154, 163, 166]. Moreover, no enhancement of virus replication nor further CD4+ T cell decline was observed in $SHIV_{89.6P}$-infected monkeys with AIDS after vaccination with Tat protein or *tat* DNA (our unpublished results), nor in HIV-1 seropositive individuals immunized with *tat* DNA, even though vaccination boosted pre-existing B- and T-cell immune responses to Tat [42, 43].

Vaccination with a biologically active Tat protein or with *tat* DNA elicits Tat-specific humoral and cellular (including CTLs) immune responses in mice, monkeys and humans both after systemic and mucosal administration [6, 33, 39, 41, 42, 49, 52, 60, 93, 94, 99, 114, 138, 166, 203, 212, 213]. Tat is immunogenic also when administered together with other viral regulatory or structural genes [42, 43, 135, 145, 154, 163, 210, 223-225].

To evaluate the efficacy of the biologically active Tat vaccine in controlling virus replication, preclinical trials

Table 2. Results of "IMMUNOGENICITY" in Vaccination Studies with the HIV-1 Tat Protein or Tat DNA

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Ab response	Cellular responses		References
						T-helper response	CTL response	
Mice	HIV-1 Tat DNA	1-100 μg (3 times)	None	i.m., i.d.	+	+	ND	198
	HIV-1 Tat DNA	1-100 μg (1-3 times)	None	i.m., g.g. (i.d.), i.p., i.d.	+/-	+	ND	100
	HIV-1 Tat protein	5-50 μg (2 times)	Incomplete Freund's, RIBI	s.c.	+	ND	ND	100
	HIV-1 Tat DNA	5-10 μg (3 times)	None	i.m., i.d., i.n., intra toungue	+	ND	ND	99
	HIV-1 Tat DNA	20 μg	None	i.n.	+	+	ND	16
	HIV-1 Tat DNA (wild-type amd mutants)	100 μg (6 times)	Bupivacaine	i.m.	+/-	+	ND	52
	HIV-1 Tat protein	10 μg (4 times)	LTR192G	i.n.	ND	ND	+	148
	HIV-1 Tat DNA in a self-replicating vector	1-150 μg (6 times)	None	g.g., i.m., i.d.	-	+	+	213
	HIV-1 Tat DNA	10-50 μg (twice) or 20-100 μg (twice)	None	i.m.	+	+	ND	114
	HIV-1 Tat protein	10 μg (4 times)	LTR72, Alum, Incomplete Freund's	i.n., s.c.	+	+	+	138
	HIV-1 Tat protein	10 μg (4 times)	None, MALP-2	i.n., i.d.	+	+	+	33
	HIV-1 Tat protein	40 ng-10 μg (4 times)	Complete Freund's, Red blood cells	i.p.	+	+	+	60
	HIV-1 Tat protein	0.5-2 μg (3 times)	None, anionic microparticles, Alum	i.m.	+	+	+	Footnote[1]
	HIV-1 tat DNA	1-30 μg (6 times)	Cationic block copolymers	i.m.	-	+/-	+	49
	HIV-1 Tat protein	10 μg (3 times)	Alum	s.c.	+	+	ND	57
	HIV-1 Tat protein	5 μg (twice)	Wax anionic nanoparticles, Alum, Lipid A	s.c.	+	+	IFNγ ELISA	57
	HIV-1 Tat DNA (codon-optimized, and ubiquitin-tagged codon-optimized)	100 μg (4 times)	none	i.m.	ND	+	+	174
Rabbits	HIV-1 Tat protein	100 μg (3 times)	Complete Freund's	i.d.	+	ND	ND	162
Monkeys	SIV Tat DNA in SFV and MVA viral vectors	SFV-tat (10^{8-9} $TCID_{50}$, 2 times) MVA-tat (10^{8-9} $TCID_{50}$, 2 times)	SFV, MVA	i.m.	ND	ND	+	163
	SIV Tat DNA in SFV and MVA viral vectors	SFV-tat (10^{8-9} $TCID_{50}$, 2 times) MVA-tat (10^{8-9} $TCID_{50}$, 2 times)	SFV, MVA	i.m.	+	+	+	210
	HIV-1 Tat DNA and protein	10 μg (4 times + 2 boosts with 50 μg of Tat protein)	Golden particles, RIBI	g.g.-i.d. (DNA), i.m. (protein)	+	+	ND	173

(Table 2). contd.....

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Ab response	Cellular responses		References
	HIV-1 Tat DNA protein	6-10 μg (8 times + 1 boosts with 16 μg of Tat protein with ISCOMs)	RIBI, Alum	s.c., i.d.	+	+	+	39
	HIV-1 Tat DNA	0.2-1 mg (8 times + 1 boost with 16 μg of Tat protein with ISCOMs)	Methylparaben + bupivacaine	i.d., i.m.	+	+	+	41
	HIV-1 Tat DNA protein	10-40 μg (5 times)	Incomplete Freund's, polyphospazene	i.d., i.m.	+	+	ND	166
	HIV-1 Tat DNA	1 mg (7 times + 2 boosts with 25 μg of Tat protein with ISCOMs)	None	i.m.	+	+	+	Footnote[2]
	HIV-1 Tat DNA protein	6 μg (11 times)	None	i.d.	+	+	+	Footnote[2]
	HIV-1 Tat protein	20 μg (3 times)	Alum, MPL	s.c.	+	+	+	Footnote[3]
	HIV-1 Tat DNA in SFV and MVA viral vectors	SFV-tat (108 $TCID_{50}$) (twice), MVA-tat (10^8 $TCID_{50}$) (twice)	SFV, MVA	s.c., i.m.	-	ND	ND	223
	HIV-1 NEF-tat fusion protein	20 μg (3 times)	AS02A	i.m.	+	ND	ND	225
	SIV Tat	32 μg (3 times) + 1 MVA boost (5 x 10^8 pfu)	Golden particles	g.g., i.d., i.r.	ND	+	+	6
	HIV-1 Tat DNA	500 μg (3 times) + 2 boost with 25 μg of Tat protein with ISCOMs)	Methylparaben+ bupivacaine	i.m.	+	+	+	145
Humans	HIV-1 Tat DNA	100 μg (3 times)	None	i.m.	+/-	+	+	42

[1]Caputo et al., unpublished

[2]Ensoli et al., unpublished

[3]Shiver J. HIV vaccines: some lessons learned from nonhuman primate models. 18th Annual Symposium on Nonhuman Primate Model for AIDS, 2000. Madison, Wisconsis

with the Tat protein or *tat* DNA (BH10 clone, clade B) were carried out in 12 cynomolgus monkeys (*Macaca fascicularis*) as two arms of the same protocol [39, 41]. In the Tat protein protocol, monkeys were immunized subcutaneously (s.c.) with 10 μg of Tat in RIBI (three monkeys) or Alum (three monkeys), and intradermally (i.d.) with 6 μg of Tat in the absence of adjuvant (one animal). In the *tat* DNA protocol, monkeys were vaccinated i.m. with 1 mg (three animals) or 0.5 mg (one animal) of plasmid pCV-*tat*, or i.d. with 0.2 mg of pCV-*tat* DNA (one animal). Monkeys were boosted eight-nine times over 36-42 weeks. Controls were represented by three monkeys inoculated s.c. with Ribi (one monkey) or with Alum (one monkey) and i.m. with the pCV-0 backbone vector (one monkey), and by two naive macaques. At week 14-18 after the last boost, all macaques were challenged intravenously (i.v.) with 10 MID_{50} of $SHIV_{89.6P}$, a chimeric virus that contains the *tat* gene of HIV-1 and is highly pathogenic in macaques, with the exception of the two naive control monkeys, which were inoculated with a dose 3-fold lower (2.8 MID_{50}) or higher (28 MID_{50}). After the challenge, the five controls and three vaccinated monkeys (two vaccinated with Tat protein, and one vaccinated with *tat* DNA, given *tat* i.d.) were infected, as indicated by the presence of high levels of p27 antigen, plasma viremia, proviral DNA copies, cytoviremia or positive virus isolation and a rapid CD4+ T cells decline. In contrast, all these parameters were negative for the other nine vaccines, with the exception of SIV proviral DNA that was only sporadically and barely detected (<10 copies/μg of DNA) in a few animals [39, 41]. Protection correlated (100%) with the presence, prior to challenge, of anti-Tat specific CTLs and with TNFα production by $CD8^+$ T cells upon Tat stimulation. However, the 2 infected monkeys vaccinated with Tat and RIBI or Tat and Alum (that had high anti-Tat antibody titers but not CTLs) developed lower (2 logs) and delayed anti-SIV antibody titers as compared to the controls, suggesting that they were exposed to a lower antigen load. No correlation of protection was observed with the production of β-chemokines at post-challenge time (RANTES, MIP1α, MIP1β) [Ensoli et al., unpublished]. Further experimental evidence indicate that both the Tat protein and *tat* DNA vaccines elicit long-term memory T-cell responses in the absence of any vaccine boosting, and that protection is long-lasting, even after activation of the immune system with two tetanus toxoid boosts given 3 weeks apart approximately one year after the challenge [136]. Indeed, in all protected vaccinated monkeys neither CD4+T cell decline nor active virus replication was observed, neither in blood nor in lymph nodes, up to week 104 after the challenge. All vaccinated animals controlling infection (9/12) were negative for plasma viremia, proviral DNA,

Table 3. Results of "EFFICACY" in Vaccination Studies with the HIV-1 Tat Protein or Tat DNA

Model	Immunogen	Dose (schedule)	Adjuvant	Route	Antibody Response	Cellular responses		Challenge			Control of infection	References
						T-helper	CTL	Virus	Dose (MID_{50})	Route		
Monkeys	SIV Tat DNA in SFV and MVA vectors	SFV-tat ($10^{8-9}TCID_{50}$ 2 times); MVA-tat ($10^{8-9}TCID_{50}$ 2 times)	SFV, MVA	i.m.	ND	ND	+	SIV_{mac32H}	50	i.v.	YES	163
	SIV Tat DNA in SFV and MVA vectors	SFV-tat ($10^{8-9}TCID_{50}$ 2 times); MVA-tat ($10^{8-9}TCID_{50}$ 2 times)	SFV, MVA	i.m.	+	+	+	SIV_{mac32H}	50	i.v.	YES	210
	HIV-1 Tat DNA and protein	10 µg (4 times) + 2 boosts with 50 µg of Tat protein	Golden particles, RIBI	g.g. (i.d.)	+	+	ND	SHIV-4	10 (MID_{100})	i.v.	NO	173
	HIV-1 Tat protein	6-10 µg (8 times + 1 boost with 16 µg of Tat protein with ISCOMs)	RIBI, Alum	s.c., i.d.	+	+	+	SHIV 89.6P	10	i.v.	YES	39
	HIV-1 Tat DNA	0.2-1 µg (8 times + 1 boost with 16 µg of Tat protein with ISCOMs)	Methylparaben + bupivacaine	i.d., i.m.	+	+	+	SHIV 89.6P	10	i.v.	YES	41
	HIV-1 Tat protein	10-40 µg (5 times)	Incomplete Freund's, polyphosphazene	i.d., i.m.	+	+	ND	SHIV 89.6P	2500 $TCID_{50}$	i.r.	YES	166
	HIV-1 Tat protein	20 µg (3 times)	Alum, MPL	s.c.	+	+	+	SHIV 89.6P	≤100	i.v.	NO	Footnote[1]
	SIV Tat	32 µg (3 times) + 1 MVA boost (5×10^8 pfu)	Golden particles	g.g., i.d., i.r.	ND	+	+	SIV_{mac239}	3160 $TCID_{50}$	i.r.	NO	6
	HIV-1 Tat DNA	500 µg (3 times) + 2 boosts with 25 µg of Tat protein with ISCOMs	Methylparaben + bupivacaine	i.m.	+	+	+	SHIV 89.6P	50	i.v.	NO	145
	HIV-1 Tat DNA in SFV and MVA viral vectors	SFV-tat (10^8 $TCID_{50}$) (twice), MVA-tat (10^8 $TCID_{50}$) (twice)	SFV, MVA	s.c. i.m.	-	ND	ND	SHIV BX08	10	i.r.	YES	223
	HIV-1 NEF-tat fusion protein	20 µg (3 times)	AS02A	i.m.	+	ND	ND	SHIV 89.6P	5-50	i.v.	NO	225
Humans	HIV-1 Tat DNA	100 µg (3 times)	None	i.m.	+/-	+	+	NA	NA	NA	NA	42

[1]Shiver J. HIV vaccines: some lessons learned from nonhuman primate models. 18th Annual Symposium on Nonhuman Primate Model for AIDS, 2000. Madison, Wisconsis

virus isolation and circular viral DNA (E-DNA), a parameter more sensitive than evaluation of plasma viremia. In contrast, the 3 vaccinated monkeys unable to control virus replication and the control monkeys were positive for all assays in blood and tissues [136]. Humoral and cellular immune responses against Tat persisted high and stable only in the 9 protected monkeys. In contrast, in the vaccinated and in the control monkeys that did not control primary replication, humoral and cellular responses to SIV Env and Gag were persistently high. The control monkey infected with 28 MID_{50} of SHIV and the monkey immunized i.d.

with *tat* DNA showed disease progression and died at weeks 35 and 75, respectively [136].

Altogether this experimental evidence indicates that immunization with a biologically active Tat or *tat* DNA is safe, induces a long-lasting immune response capable of blocking virus replication to undetectable levels, preventing the CD4+ T cell decline and disease onset. The capability of Tat of being presented in association with MHC class I molecules and its immunomodulatory activity on dendritic cell functions may explain the type of immune response and the protective role exerted by vaccination with Tat. Although the Tat strategy does not block virus entry, it can efficiently control virus replication and render «abortive» the natural infection. This is likely to occur against different virus strains as suggested by studies in HIV-infected individuals, described in the previous section, that although infected by different HIV clades recognize the Tat from clade B used in the vaccine formulation [38]. Thus, based on the results of the preclinical studies, phase I clinical trials with the biologically active Tat protein vaccine are ongoing in Italy. In particular, the Tat vaccine is currently being tested in 32 low risk HIV-negative individuals and in 56 untreated HIV-positive volunteers to determine the safety and immunogenicity of both a preventive and therapeutic Tat-vaccination. The protocol consists of 6 months treatment and 6 months observation, and at the end of this period the results will be announced by the Istituto Superiore di Sanità (http://www.iss.it). In addition virologic, immunologic and feasibility studies are ongoing also in African countries where subsequent phase II/III clinical trials are being organized.

Second Generation Vaccines Based on Biologically Active Tat

Alongside these studies, new delivery systems, both synthetic and biological, have been produced and are under preclinical evaluation to develop safe, efficacious and easily administrable second generation Tat (protein/DNA)-based vaccines with increased shelf-life and stability, capable of triggering broad, efficacious and long-lasting immune responses with a reduced amount of antigen and number of boosts. To this respect, novel biocompatible core-shell polymeric nanoparticles and microparticles, were recently developed by our group, made of an inner hard core of poly(methylmethacrylate) and a highly hydrophilic outer shell, constituted by hydrosoluble co-polymers bearing ionic or ionisable functional groups, which are able to reversibly associate with DNA and proteins. These novel delivery systems are capable of reversibly adsorbing the native and biologically active Tat protein at their surface, preserving its conformation and activity, and of triggering broad antigen-specific humoral and cellular immune responses after vaccination with a low dose of Tat and few booster shots in mice, in the absence of toxicity [46, and unpublished results]. These nanoparticles and microparticles are currently being investigated in monkeys trials. Within this frame, recent studies by Cui and co-workers [57] also showed that anionic nanoparticles, made of emulsifying wax and sodium-dodecyl-sulfate, potentate the cellular immune response against HIV-1 Tat after two immunization in mice. Furthermore, novel cationic block copolymers were developed by our group for efficient delivery of *tat* DNA, which have proven to be safe, to protect the DNA from enzymatic degradation, allowing immunization with a low dose of DNA and few boosts, and to potentate Tat CTL responses [47, 49, 125, and our unpublished results]. These vectors may thus represent suitable storage and delivery systems for the development of second generation Tat-based vaccines. Finally, since anti-Tat immune responses, and, in particular, cellular immune responses, are important correlates of protection against disease progression, novel strategies based on a codon-optimized and ubiquitin-tagged *tat* DNA vaccine were described and shown to significantly enhance the cellular responses against Tat in mice [174].

Mucosal Vaccines Based on Biologically Active Tat

The relative recently appreciated notion that HIV-1 infection is primarily a sexually transmitted disease has generated interest to develop immunization strategies aimed at conferring protection at the mucosal level [126]. Mucosal vaccination has proven advantageous with respect to other routes, because it ensures high compliance and easy administration. Thus, the use of the Tat as a mucosal vaccine has been investigated [33, 138, 148]. Intranasal immunization of mice with the Tat protein alone or associated with mucosal adjuvants, such as MALP-2, *Escherichia coli* heat-labile enterotoxin (LT) and LT-R72, a non-toxic mutant of LT, has proven to be safe and to elicit Tat-specific serum and secretory antibodies, and cellular proliferative and CTL responses [33, 138, 148]. In addition, oral immunization with *tat*, *rev* and *nef* genes was tested in humans and shown to be safe and induce antigen-specific mucosal and systemic IgA and IgG and Th1 type cellular responses [135]. The efficacy of a mucosal Tat-based vaccine is currently being tested in non-human primates.

Vaccines Based on Inactivated Tat

Although Tat protein or *tat* DNA inoculation was safe both in naive [39, 41, 145], in SHIV-infected monkeys (our unpublished data) and in HIV-infected humans [42], the possibility to use transdominant negative mutants of HIV-1 Tat lacking the transactivating activity, but preserving the immunogenicity of the wild-type Tat, was also investigated [52]. Intramuscular immunization of mice with plasmids encoding two transdominant negative mutants (Tat_{22} and $Tat_{22/37}$) [50] elicited an immune response to wild-type Tat protein that was comparable to that induced by inoculation of wild-type *tat* DNA, in terms of IgG subclasses, antibody titers, epitope specificity and neutralization of the biological activities of Tat, and of proliferative responses of splenocytes to wild-type Tat protein [52]. These mutants are currently being investigated in monkeys trials.

A modified Tat, inactivated by carboxymethylation, was also recently described as a vaccine candidate. Studies in rodents, monkeys, seronegative and seropositive humans indicated that the inactivated Tat, termed Tat toxoid, is safe and immunogenic, as assessed by measurements of the antibody titers and proliferative responses [93, 94]. To evaluate its efficacy, initial experiments have been performed in rhesus macaques (*Macaca mulatta*) (n=8) that were vaccinated i.d. and i.m. four times, at one week interval,

with 20-60 μg of the Tat toxoid with polyphosphazene adjuvant, and then challenged i.r. with 2.500 $TCID_{50}$ of the pathogenic $SHIV_{89.6PD}$ three weeks after the last immunization [166]. An attenuated infection was observed in vaccinees, as indicated by the reduction of p27 antigenemia and plasma viremia (<2.500 copies/ ml). In addition, the monkeys that controlled the infection exhibited at pre-challenge time both humoral and proliferative responses to Tat, suggesting that both arms of the immunity contributed at controlling disease. Moreover, these animals showed significantly reduced expression of INFα and chemokine receptors (CXCR4 and CCR5) on CD4+T cells. In this study, no other information is available on the cellular immune responses, such as presence of CTLs and CAF, to more precisely compare the Tat toxoid approach with the native Tat protein vaccine that, utilizing a biologically active Tat protein, also exploits the unique capability of Tat to be taken up by APC, to enter the MHC class I pathway of presentation, and to generate CD8+ CTLs [39, 41, 111]. This is of importance, since CTLs, but not anti-Tat antibodies, correlated with protection after vaccination with a biologically active Tat or *tat* DNA in monkeys trials [39, 41, 136]. However, these results indicate that systemic vaccination with the Tat toxoid attenuates disease upon mucosal challenge, in the apparent absence of mucosal immunity.

The efficacy of immunization with Tat toxoid versus immunization with biologically active Tat was compared in a different study by Silvera et al. [203], which, however, reported conflicting results. Four groups of rhesus monkeys (*Macaca mulatta*) (n=4) were vaccinated i.m. with 100 μg of biologically active Tat or Tat toxoid derived from $HXBc2_{IIIB}$ and from $SHIV_{89.6P}$, in the presence of incomplete Freund's adjuvant at weeks 0, 5, 9 and 29, and challenged i.v. four weeks after the last boost with 30 MID_{50} of $SHIV_{89.6P}$. After immunization all immunized animals developed high titers of anti-Tat antibodies, and in addition, Tat-specific T-helper and INFγ responses were detected in approximately 50% of the vaccinated monkeys. However, none of the vaccines conferred protection against SHIV infection as compared to control monkeys injected only with the adjuvant. Although disappointing, these different results may depend on differences in the vaccine design, such as adjuvant, dose of Tat, number of boosts, route of immunization and dose of the challenge virus and are difficult to compare with the previous studies.

Vaccines Based on Tat Peptides

The use of synthetic peptides of Tat has been proposed as an alternative approach to the use of inactivated forms of the Tat protein. Several studies have shown the safety, immunogenicity and efficacy of an immune response triggered by Tat peptides in the control of infection. Indeed, rhesus macaques (*Macaca mulatta*) (n=9) were immunized i.m. with 100 μg of synthetic peptides, containing Tat B-cell epitopes, in the presence of complete Freund's adjuvant, and boosted at weeks 3, 6 and 9 after the first immunization, with the same dose of peptide in the presence of incomplete Freund's adjuvant. Two weeks after the last immunization, monkeys were challenged i.v. with 50 MID_{50} of $SHIV_{33}$ [84]. Immunization induced anti-Tat antibodies that, although not capable of controlling acute infection after the challenge, however significantly reduced the plasma viremia and prevented CD4+ T cell decline during the chronic asymptomatic phase of infection [84], suggesting that the presence of high titers anti-Tat antibodies may impact the evolution of infection through the block of the intercellular trafficking of extracellular native Tat. In agreement with these results, immunization of mice with a multiple-peptide conjugate, comprising three functional domains of Tat from HIV-1 group M and group O, induced a broad antibody response to all three domains, which inhibited Tat-induced viral activation and cytopathic effect in monocytes infected with the HIV BaL strain and with different HIV-1 isolates [34, 35]. Moreover, recent studies demonstrated that rabbits and macaques immunized with several Tat peptides developed antibody responses reacting with the whole Tat protein and capable to inhibit Tat- induced apoptosis of T lymphocytes [25].

IV. COMBINED VACCINE APROACHES BASED ON TAT AND OTHER HIV EARLY AND LATE GENE PRODUCTS

Tat, Gag and Env

Since the induction of Th-1 responses and CTL correlates with reduced viral loads, and since native Tat has immunomodulatory activity on other antigens, it is reasonable to exploit the use of Tat (protein or DNA) to drive or to increase Th-1 immune responses and CTL activity against other HIV antigens. A recent study, using a DNA/protein prime-boost regimen, showed that i.m. immunization of rhesus macaques (*Macaca mulatta*) (n=4) with Gag and Env in combination with Tat (three immunizations with 0.5 mg of DNA at weeks 0, 2 and 6, and three boosts with 25 μg of proteins at weeks 10, 14 and 24) resulted in strong antibody and cellular responses, characterized by INFγ, IL-2 and IL-4 cytokines production by antigen-specific cells. Following the i.v. challenge with 50 MID_{50} of $SHIV_{89.6P}$, given eight weeks after the last immunization, animals were protected from CD4+T cells decline and disease progression up to 62 weeks post-challenge [145]. Containment of virus load, maintenance of CD4+ T-cell counts to levels similar to pre-challenge, and protection from progression to AIDS correlated with broad and marked Th1 and Th2 responses before challenge and with CTL responses after challenge against Env and Gag. Interestingly, one monkey resulted completely protected from infection as suggested by the absence of plasma viremia and proviral DNA in PBMCs of this animal. It is possible that this animal had a limited infection following challenge as indicated by a rapid boost of Env and Gag cell-mediated responses immediately after exposure to the virus; nevertheless, the antigen-specific immune responses were effective at eliminating the virus and/or the virus-infected cells before virus replication and dissemination could take place [145]. Novel approaches with Tat combined with Gag and Env are under preclinical and clinical evaluation within AVIP in Europe [http://www.iss.it: Press release July 12th, 2004] to develop vaccine strategies in which also Tat-adjuvant capabilities and immunomodulatory activities are exploited. These combined vaccines should be more efficacious because they activate immunity against both

regulatory and structural genes, attacking the virus on early and late products. Finally, these vaccines may be capable of inducing a sterilizing immunity that, until now, has not been possible to achieve.

Tat and Rev

The safety and protection of vaccination with Tat together with another viral regulatory gene have been described by Osterhaus and co-workers [163] using a combined Tat and Rev vaccine approach. In this study, two cynomolgus monkeys (*Macaca fascicularis*) (n=2) were immunized twice, at four weeks interval, i.m. with 10^8 and 10^9 $TCID_{50}$ recombinant Semiliki Forest virus (SFV) and twice with 10^8 and 10^9 $TCID_{50}$ recombinant modified virus Ankara (MVA), producing SIV_{mac32H} Rev and Tat, and challenged i.v. with 50 MID_{50} of SIV_{mac32H} (pJ5) two weeks after the last immunization. During the 36 weeks follow up period, plasma viremia could be detected transiently only during the first four weeks after infection, and no cell associated viremia was found during this period, whereas in control monkeys vaccinated with SFV and MVA expressing LacZ high levels of plasma viremia and productively infected cells were easily detectable. In this study no information is available on the type of immune responses elicited by vaccination, and on the correlates of protection. However, further studies from the same research group have suggested that the presence of antigen specific CTL responses are responsible of the control of virus infection [95, 210]. In particular, cynomolgus macaques (*Macaca fascicularis*) were vaccinated i.m. four times in a prime-boost regimen with SFV (weeks 0 and 6) and MVA (weeks 12 and 16) expressing SIV Tat and Rev (n=4) or SIV Pol and Gag (n=4), and challenged i.v. with 50 MID_{50} of SIV_{mac32H} (pJ5), 5 weeks after the last immunization, to compare the efficacy of vaccination with early versus late proteins. All vaccinated animals developed antigen-specific antibodies and CTLs. Interestingly, it was shown that CTL responses raised by vaccination with SFV/MVA expressing Tat and Rev appeared to be more effective at controlling SIV replication as compared to CTL responses induced by vaccination with SFV/MVA expressing Gag and Pol. Indeed, two out of four Tat/Rev vaccinated animals remained negative for plasma viremia and the other two had a short viremia with little virus production. Conversely, all Gag/Pol vaccinated monkeys developed plasma viral loads similar to control monkeys, vaccinated with SFV/MVA expressing LacZ (n=4) [95, 210].

The efficacy of a HIV-1 Tat or Rev/Tat DNA or SFV prime/MVA boost regimen was also tested in the SHIV model [223]. Twelve rhesus macaques were vaccinated s.c. with 1 mg of DNA (n=6) or 10^8 $TCID_{50}$ SFV (n=6) vectors expressing Tat and Rev at weeks 0 and 4, and boosted i.m. with 10^8 $TCID_{50}$ MVA expressing Tat and Rev three and four months later. In addition, six rhesus macaques were immunized with SVF-tat, and boosted with recombinant MVA-tat, to compare the efficacy of vaccination with one or with two regulatory genes. Nine weeks after the last boost, vaccinees were challenged i.r. with 10 MID_{50} of $SHIV_{BX08}$. At pre-challenge humoral responses were low in all vaccinated monkeys, and cellular immune responses, estimated by INF-γ Elispot, were significant only in four out of six monkeys vaccinated with Tat and Rev (SVF prime/MVA boost). In particular, 4 monkeys responded to Tat peptides and only two of them also to Rev peptides. After the challenge, the plasma viremia was reduced more than 200-fold (<4.000 copies/ml) in 30% (6/18) of vaccinated macaques, whatever the vaccine regimen they received, as compared to controls. Of note, four out of the six protected animals did not seroconvert (one vaccinated with SVF/MVA Tat, one vaccinated with SFV/MVA Tat and Rev, and two monkeys immunized with DNA/MVA Tat and Rev). Although the only difference between macaques vaccinated with Tat (SFV/MVA) and with Tat and Rev (SFV/MVA) was based on the INF-γ Elispot, and no clear conclusion could be drawn regarding the addition of Rev, these results indicate that the systemic vaccination with Tat, and apparently to a lesser degree with Rev, elicits an immune response which is sufficient to control mucosal infection in a large number of animals. From the data available, it is difficult to compare the results of these studies with those obtained with the native Tat protein and *tat* DNA vaccines, since the only data available are the analysis of antibody responses (which were barely detectable) and of INF-γ Elispot.

Taken together these two studies confirm the safety, immunogenicity and efficacy of the Tat vaccine approach alone and combined to Rev and underscore the importance of the immune targeting of early proteins in controlling the primary infection.

Tat, Rev, and Multiple Structural and Early Gene Products

HIV-1 Tat and Rev have been also included in vaccine formulations with SIV Gag, Pol, Vif, Vpx, Vpr and HIV-1 Env [11-13]. Groups of rhesus macaques (n=6) were immunized i.m. or i.d. with DNA (2.5 mg or 2.5 μg), at weeks 0 and 8, and boosted i.m. and i.d. with 2 x 10^8 pfu of MVA at week 24. Seven months after the last boost, monkeys were subsequently challenged i.r. with 20 intrarectal infectious doses of $SHIV_{89.6P}$. Although this vaccine formulation and regimen induced T cell responses to Gag, Pol and Env which correlated with a 10-fold reduction of plasma viremia up to week 20 after challenge, however no data are available on the immune responses to the other vaccine antigens, including Tat and Rev, to define the role of the immune responses to these antigens underlying the observed protective effects. Phase I clinical trials with DNA vaccine based on clade B env, gag, pro, rt, vpu, tat, and rev are currently ongoing at Emory University (pGA2/JS2 DNA) or about to start by GeoVax (product name HIVB DNA pGA2/JS7#2) (http://chi.ucsf.edu/vaccines).

Tat, Rev, Nef

Vaccination against all the three HIV regulatory genes *tat*, *rev* and *nef* has also been investigated in mice, monkeys and humans. Wahren and coworkers [227] have been the first to demonstrate that both humoral and cellular immune responses are induced in mice (4 different strains) upon immunization with plasmids encoding the HIV-1 regulatory genes *tat*, *rev*, and *nef* with no interference being detected among these plasmids, even when plasmids encoding for the structural proteins Env and Gag were associated in the vaccination protocol [173]. The epitope specificities of the

humoral response was comparable to that obtained by vaccinating with the corresponding protein. In addition, epitope-specific T cell responses and a Th-1 pattern of cytokine secretion were detected. Finally, studies in HIV-infected patients have confirmed that DNA vaccination with either *tat*, *rev* or *nef* is safe (no evidence of increased viral load) and induces B- and T-cell responses (including CD8+ CTLs) that were absent or low prior to immunization [42], demonstrating the feasibility of genetic immunization to induce new immune responses in HIV-infected patients. A more recent study [93], in which the same immunization approach was associated to the highly active antiretroviral treatment (HAART), confirmed the safety and immunogenicity of the vaccine, whereas the reduction of the viral load was dependent on HAART, indicating that the combination of Tat, Rev and Nef may improve the capacity of the immune system to deal with the virus infection. The efficacy of a vaccine based on all the three regulatory gene products together and in the absence of other viral genes (i.e. Env, Gag) has not yet been tested in non-human primates.

Tat, Rev, Nef, Gag

The efficacy of a multispecific CTL-based vaccine combining the three HIV regulatory genes (Tat, Rev, Nef) and one structural gene (Gag) was recently tested in the SIV model using a DNA/MVA prime-boost regimen [224]. Three Mamu-A*01 positive rhesus macaques were immunized epidermally three times, at 4 to 9 weeks interval, with the SIV Nef and the Mamu-A*01-restricted CTL immunodominant $Gag_{181-189}$CM9 epitope and, after a 7- to 14-week rest period, three times at intervals of 4 weeks, both epidermally and orally, with DNA vectors expressing SIV Tat and Rev and the Mamu-A*01-restricted immunodominant CTL Tat_{28-35}SL8 epitope. Fourteen to 28 weeks after the last DNA inoculation, vaccines were boosted i.d. and i.n. with 10^8 pfu of MVA vaccines expressing full-length SIV Nef, Rev, Tat proteins and the CTL $Gag_{181-189}$CM9 epitope, and after a 33- to 35-week rest period animals received a second MVA boost, but this time it was given i.v. with the aim of targeting long-lasting memory T cells in the mucosa. This vaccination strategy induced strong CTL responses to $Gag_{181-189}$CM9 and Tat_{28-35}SL8 epitopes, as well as CD8+- and CD4+-T cell responses to Tat, Rev and Nef in both systemic and mucosal tissues. Following i.r. challenge with 10 MID_{50} of SIV_{mac239}, given sixteen weeks after the last immunization, all vaccines mounted a robust anamnestic response to $Gag_{181-189}$CM9 and Tat_{28-35}SL8 epitopes, and to other regions of Tat, Rev and Nef that were recognized up to 2 weeks after challenge, and controlled acute infection with peak viral loads significantly lower as those of the controls. However, from week 3 post-infection the difference in viral loads lost statistical significance between vaccines and controls, and none of the vaccinated animals was able to control viral replication in the chronic phase of infection. Neutralizing antibodies against SIV_{mac239} Env were barely detected in the sera of both vaccinated and control monkeys at six months after challenge. Although it cannot be excluded that with a higher number of vaccines a statistically significant difference in viral loads could have been maintained during the chronic infection, these results indicate that multispecific CTLs, targeted both systematically and mucosally, provide protection against acute infection but in the absence of neutralizing antibodies, they are unable to control chronic virus replication [134]. These results appear in contrast with other studies, where $SHIV_{89.6P}$ was used as the challenge virus [11, 12, 21, 186, 202]. These conflicting results may be attributed to the fundamental differences between $SHIV_{89.6P}$ and SIV_{mac239}, such as a different cell tropism and a major sensitivity of SHIV to neutralization, which could help to prevent CD4+ T cells from rapid decline maintaining the adequate support for B cells to mount an effective neutralizing antibody response against SHIV. However, key differences in HIV-1 Tat versus SIV Tat protein may play a role in results comparability.

A phase I clinical trial is anticipated to start by Therion during the year 2004, based on the combination of clade B Tat, Rev and Nef together with Env, Gag and Pol using a MVA prime/Fowl pox boost regimen (products name TBC-M358; TBS-M335; TBC-F357; TBC-F349) (http://chi.ucsf.edu/vaccines).

Tat, Rev, Nef and Gag, Pol, Env

Recently, a chimeric gene comprising genetically modified *rev*, *tat* and *nef* genes of SIV (*Retanef*), expressed in the context of an eukaryotic expression vector or of a highly attenuated poxvirus-based NYVAC vector, was shown to be safe both in naïve rhesus macaques (*Macaca mulatta*) and in monkeys chronically infected with SIV_{mac251} undergoing antiretroviral therapy (ART) [97, 217]. Immunization i.m. and i.d. with 4 mg of DNA-SIV-*Retanef* at weeks 0, 4 and 12 followed by an i.m. boost with 10^8 pfu of NYVAC- *Retanef* at week 25, elicited Mamu-A*01 restricted Tat_{28-35}SL8 CTL responses and INFγ Elispot responses to Rev, Tat and Nef proteins, and expanded the frequency of Tat-specific Tat_{28-35}SL8 CTLs by two- to seven-fold in infected macaques [97]. In addition, vaccination of macaques, with chronic SIV_{mac251} infection (≥5 months) and undergoing ART, with 10^8 pfu of NYVAC expressing SIV-*Retanef* and SIV *gag-pol-env* genes, at weeks 42, 48 and 54 after infection, showed an expansion of virus-specific CD4+ and CD8+ T cell responses to both structural and regulatory gene products, in particular to Gag, Env, Tat and Rev. The efficacy of therapeutic vaccination before ART discontinuation was associated with a significant containment of viral replication for at least 4 consecutive months following ART interruption, and it was correlated with the vaccine-induced virus specific CD4+ and CD8+ T cell responses [217]. No data is available on the effect of vaccination with *Retanef* or with SIV *gag-pol-env* alone to define the mechanisms of action of Tat, Rev, Nef and of Gag, Pol, Env underlying the observed protective effects. Nevertheless, these data confirm the safety and immunogenicity results of Tat, Rev, and Nef vaccination and further support the rationale that targeting HIV regulatory genes by vaccination is likely important for containment of HIV infection.

Tat, Nef, Env

Since most Env-based vaccines elicit neutralizing antibodies that confer protection against homologous but not heterologous viruses, the effect of the addition of HIV-1 Tat and Nef proteins on the efficacy of gp120 (clade B) protein

vaccination was recently tested in genetically unselected rhesus macaques and shown to be required for gp120 vaccine efficacy against challenge with a heterologous virus [225]. In this study rhesus macaques (*Macaca mulatta*) (n=4) were immunized i.m. three times at 0, 1 and 3 months, with 100 μg of recombinant $gp120_{W61D}$ and 20 μg of Nef-Tat fusion protein, formulated in adjuvant AS02A. SIV-Nef protein was included in the vaccine formulation to adapt the vaccine to the SHIV challenge. At pre-challenge anti-gp120 antibodies capable of neutralizing a standard HIV-1_{MN} isolate and the homologous HIV-$1_{W6.1D}$ T-cell line adapted strain were detectable in all vaccinees, whereas only one immunized macaques was able to neutralize the challenge virus. Antibodies against Nef and Tat were also developed although their titers decreased until the day of challenge. Four weeks after the last immunization, macaques were challenged i.v. with 5 to 50 50% infectious doses for macaques of $SHIV_{89.6P}$ (clone 89.6KB9). Although a sterilizing immunity was not achieved, the vaccine formulation was able to reduce the virus load, to protect the animals from CD4+ T cell decline, and to prevent the development of AIDS for more that 2.5 years, as compared to macaques vaccinated with gp120 or Nef-Tat protein alone, in which the disease progressed rapidly in most animals. An anamnestic response of gp120, but not of Nef and Tat, antibodies capable of neutralizing SHIV was developed after virus challenge in most of the animals that remained healthy for a prolonged period of time, suggesting a role in the long-term control of viremia. However, no information is available on the cellular immune responses to the vaccine antigens to define the immune correlate of protection and the mechanisms of action of Tat and/or Nef underlying the observed protective effects. Since this vaccine appeared to be safe and efficacious in macaques, the combination of gp120 and Nef-Tat did not show any adverse effects in toxicology studies and the adjuvant AS02A has been already tested in human volunteers, a phase I clinical trial with this formulation has been carried out by Glaxo-Smith Kline and it is currently under evaluation (product name NefTat + $gp120_{W61D}$) (http://chi.ucsf.edu/vaccines).

V. CONCLUSION AND PERSPECTIVES

The results obtained in nonhuman primates with vaccines based on Tat underscore the importance of targeting an early and vital gene product, in the absence of a sterilizing immunity. A Tat-based vaccine should reduce HIV-1 replication and disease progression in seropositive patients, whereas in seronegative vaccinated individuals, exposed to the virus after vaccination, it could modify the virus-host interactions at the very beginning of the infection having an impact on the depletion of critical immune cells and on the evolution of the infection. Indeed, the level of viral load at the beginning of the infection has been shown to be a strong indicator of progression to disease [132, 140, 172, 207, 215, 229]. Also, development of an immune response to HIV-1 with no apparent infection has been reported, suggesting that exposure without infection or clearance of the virus after infection may occur [27-29, 83, 123, 171, 189, 190]. Thus, a Tat-based vaccine may likely allow a minimal virus replication, which provides enough antigen to boost the anti-Tat immunity and to generate a primary immune response against all the other viral proteins, as suggested by the results of the Tat-vaccinated and protected monkeys [39, 41, 136], leading to a long-lasting control of infection and disease progression. In contrast, the use of vaccines based on immunodominant structural proteins, such as Env or Gag, may raise immune responses towards irrelevant epitopes, and interfere with proper antigen processing and presentation pathways [22, 137, 197, 204, 230]. In this scenario, the development of an immunity against other viral components, such as the early viral antigens (Tat, Rev, and Nef) that may play a key role to control virus infection becomes inefficient because of the Env and/or Gag dominancy. This is suggested by those preclinical studies in which these viral antigens have been used together [152, 153, 228] but a low immune response was found for the regulatory genes Tat, Rev and Nef and no protection was achieved. Finally, the capability of Tat of being presented in association with MHC class I molecules and to induce Th-1 type immune responses and CTLs, that correlated with protection in vaccinated monkeys, its immunomodulatory activity on dendritic cells and on other antigens, and the evidence that Th-1 responses and CTLs correlates with reduced viral loads and containment of infection also with Env-based vaccines, suggest that Tat may be important to drive or to increase Th-1 immune responses and CTL activity against other HIV antigens. These vaccines could support an effective and long-lasting antiviral immunity capable of controlling virus replication and blocking disease onset, and may be even capable of inducing a sterilizing immunity. The goal of prophylactic and therapeutic immunization may be closer based on these recent advancements in vaccine development and in the comprehension of Tat-immune system interactions.

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LIST OF ABBREVIATIONS

AIDS	=	acquired immunodeficiency syndrome
APC	=	antigen presenting cells
ART	=	antiretroviral therapy
AVIP	=	AIDS Vaccine Integrated Project
CAF	=	chloramphenicol acetyl transferase
CTL	=	cytotoxic T lymphocyte
Env	=	envelope
g.g.	=	gene gun
HAART	=	highly active antiretroviral treatment
HIV	=	human immunodeficiency virus
i.d	=	intradermal,
i.m.	=	intramuscular
		i.n. intranasal
i.p.	=	intraperitoneal
i.r.	=	intrarectal

i.v.	=	intravenous
IL	=	interleukin
INF	=	interferon
MDDC	=	monocyte-derived dendritic cells
MHC	=	major histocompatibility complex
MID_{50}	=	50% monkey infectious dose
MVA	=	Modified Vaccinia Virus Ankara
NA	=	not applicable
ND	=	not done
PBMCs	=	peripheral blood mononuclear cells
pfu	=	plaque forming units
PTD	=	protein transduction domain
s.c.	=	subcutaneous
SFV	=	Semiliki Forest Virus
SHIV	=	simian-human immunodeficiency virus
SIV	=	simian immunodeficiency virus
TAR	=	Tat-responsive element
$TCID_{50}$	=	50% tissue culture infectious dose

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