Immune responses to HIV-1 Tat in natural infections and experimental immunizations: implications for vaccine design

A thesis submitted in the partial fulfillment of the requirement of the degree of

Doctor of Philosophy

By

Venkatesh Prasanna Kashi

Molecular Biology and Genetics Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
(A Deemed University)
Bangalore 560064 (India)

Table of Contents

Abbreviations

µM	Micro Molar
ADCC	Antibody Dependent Cellular Cytotoxicity
AICD	Activation Induced Cell Death
AIDS	Acquired Immunodeficiency Syndrome
BD	Basic Domain
BSA	Bovine Serum Albumin
CD	Core Domain
CFSE	Carboxyfluorescin Diacetate Succinimidyl Ester
CMV	Cytomegalovirus
CNS	Central Nervous System
CRD	Cystein Rich Domain
CRF	Circulating Recombinant Forms
CTD	Carboxy Terminal Domain
CTL	Cytotoxic T-lymphocyte
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immunosorbant Assay
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GALT	Gut Associated Lymphoid Tissue
HAART	Highly Active Anti-Retroviral Therapy
HAD	HIV Associated Dementia
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRPO	Horse Radish Peroxidase
HTL	Helper T-Lymphocyte
IFN-γ	Interferon gamma
IL-2	Interleukin-2
IL-7	Interleukin-7
KS	Kaposi’s Sarcoma

LTNP	Long-Term Non-Progressors
MAb	Monoclonal Antibodies
MHC	Major Histocompatibility Complex
mM	milli Molar
NK Cells	Natural Killer Cells
nM	nano Molar
NMR	Nuclear Magnetic Resonance
NTD	Amino Terminal Domain
OD	Optical Density
PADRE	Pan DR Epitope
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD-1	Programmed Death domain-1
PHA	Phytohaemagglutinin
PI	Propidium Iodide
RNA	Ribonucleic Acid
SHIV	Simian/Human Immunodeficiency Virus
SI	Stimulation Index
TAR	Transactivation responsive region
Th1	T-helper 1
Th2	T-helper 2
TNF-α	Tumor Necrosis Factor Alpha

Synopsis of the thesis entitled

Immune responses to HIV-1 Tat in the natural infection and experimental immunization: Implications for Tat vaccine design

Submitted by

Venkatesh P Kashi

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Jakkur, Bangalore 560064

Thesis advisor: Prof. Udaykumar Ranga

Human Immunodeficiency Virus-1 (HIV-1) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS), is the worst pandemic of mankind. As of 2007, nearly 33 million people are estimated to be living with the viral infection globally. In India, according to the National AIDS Control Organization (NACO), 2.3 million people have been estimated to be infected. Given the precarious situation of the global viral pandemic and the serious limitations of chemotherapy, there is an immediate need for vaccine development against HIV.

Transactivator of transcription (Tat) is an 86-101 amino acid long regulatory protein of HIV-1 that plays a vital role in viral replication and pathogenesis. In the natural infection, Tat is moderately immunogenic with approximately 20-40% of seropositive individuals in a population harboring humoral and/or cellular immune responses to Tat. Importantly, several studies reported a negative correlation between the presence of immune responses to Tat and disease progression and/or viral load. Furthermore, Tat being an early product in viral life cycle, immune responses targeting Tat are believed to have an advantage in controlling the viral infection. Based on these facts, Tat is believed

to be a potential candidate for multi-subunit HIV vaccine. The objective of the present study is two-fold:

- To evaluate the nature of anti-Tat humoral immune response in Indian clinical cohorts
- To examine the underlying factors influencing the immunodominant nature of the N-terminal B-cell epitope in the experimental protein immunization.

India harbors the second largest HIV incidence in the world and the epidemics are predominantly clade-C. However, no scientific studies have been reported on immune responses to Tat from the Indian populations. In ordrer to evaluate the immune response in Indian populations, we collected samples from two different clinical cohorts of southern India. One cohort from the Seva Clinic, Bangalore, the State of Karnataka while the other from the Tuberculosis Research Centre (TRC), Chennai, the State of Tamil Nadu. Tat-reactive antibodies were examined in a total of 447 plasma samples from both of the cohorts and 150 seronegative samples using recombinant Tat proteins in indirect ELISA. This analysis identified 14% (63/447) and 4.6% (7/150) of HIV seropositive and seronegative samples to be positive for Tat-reactive antibodies, respectively, at a cutoff value of mean + 1 S.D. Seropositivity of some of the control samples was expected given the presence of Tat-reactive natural IgM antibodies in healthy individuals. Noticeably, the Tat-reactive antibody titers in the two clinical cohorts did not differ significantly from each other. Importantly, a small but significant number of HIV seropositive subjects (7%, 35/447), constituting the upper outlier group, was found to be high-responders for Tat at a magnitude comparable to that of gp41. Given the low immunogenicity and immunosuppressive nature of Tat, identification of high Tat-responders is of considerable importance. A major part of the present study focuses on the detailed examination of the

nature of humoral immune responses in the high-Tat group, as this information could have direct implications for Tat vaccine design.

A direct comparative analysis of the high-Tat group with the low-Tat as well as the control groups identified qualitative as well as quantitative differences in the humoral immune responses to Tat. While in the low-Tat and the control groups we detected only IgM antibodies that reacted with Tat, only in the high-Tat group we detected IgG isotype in addition to IgM. Thus a class-switch was observed only in the high-Tat group with respect to Tat. While the natural IgM antibodies in the control group recognized only the basic domain, as reported previously by others, only in viral infection, a novel epitope in the cysteine-rich domain (CRD) was identified. The CRD was the most immunodominant and the IgG antibodies targeting the CRD epitope were of higher avidity. Importantly, , we found that the plasma antibodies of the high-Tat group efficiently blocked Tat-mediated transactivation of the provirus in HLM1 cells. Further, we were able to demonstrate high degree of cross-reactivity high-Tat group antibodies by using recombinant Tat from different clades as the antigen.

In the second section of the work, strategies to improve Tat as a vaccine candidate have been evaluated. While DNA vaccination is desirable to elicit a cell-mediated immune response, the limited amount of antigen synthesized *in vivo* following DNA administration is directly correlated to low quality immune responses elicited. In addition, low humoral immune response is elicited in genetic immunizations. A possible solution to this problem is to adapt a DNA-prime-protein-boost immunization regimen which is also likely to enhance the breadth of the immune responses. Our initial attempts at Tat protein immunization in mouse identified the problem of immunodominance of the amino-terminal domain (NTD) epitope to the extent that no other epitopes in the viral

antigen were recognized.. Efforts to understand the immunodominant nature of the NTD on the one hand and to develop strategies to extend the immunogenicity to sub-dominant epitopes in Tat on the other hand have been made in this section. We generated several constructs of Tat and immunized mice with these proteins to understand the immunodominant nature of the NTD of Tat. The immunodominance of NTD was retained in all constructs harboring the epitope irrespective of its location in Tat suggesting an inherent immunodominance of NTD. Other subdominant epitopes were recognized only when the NTD was deleted, but the response however was of lower magnitude. Further, using CFSE-dye dilution in lymphoproliferation assay we mapped the natural T-helper epitope in Tat to the basic domain.

Grafting of universal T-helper epitopes (HTL) enhance immune response and enhance immunogenicity of subdominant epitopes. We grafted two different universal T-helper epitopes (HTL) pan DR epitope (PADRE) from Tetanus toxoid and Pol_{711} from reverse transcriptase of HIV into two different domains of the viral antigen to enhance immunogenicity of Tat. Both the CRD and BD of Tat were disrupted by grafting of the HTL into these domains in both the orientations (PADRE in CRD/ Pol_{711} in BD and Pol_{711} in CRD/PADRE in BD). Furthermore, two additional Tat constructions with either CRD or BD disrupted with only PADRE-HTL were also constructed. All the four Tat constructs were compared with wild type Tat in BALB/c mice using subcutaneous protein immunization with complete Freund's adjuvant. HTL grafting not only improved the safety profile of Tat for immunization but also higher magnitude immune responses were elicited against Tat. Importantly, in all the 4 Tat constructs with HTL grafting, immune responses to a novel epitope in exon-2 of Tat were observed at a magnitude comparable to NTD which still remained the immunodominant epitope. The sera of the mice immunized with the HTL grafted Tat demonstrated higher potential for Tat

neutralization as evaluated in virus-rescue and apoptosis assays. Although the antibody isotypes were of Th2 (IgG1) predominantly, the levels of Th1 (IgG2A) isotype also were elevated as compared to wild-type C-Tat.In summary, our work demonstrates for the first time that although Tat is considered to be non-immunodominant and even immunosuppressive, it could behave as a strongly immunodominant antigen in a small but significant number of natural infections. Further studies delineating the factors underlying the immunodominant nature of Tat in this small subset of people are necessary. We also demonstrate a molecular strategy to spread immunogenicity to subdominant epitopes in Tat in experimental immunization. The present study is not only the first evaluation of host immune response to Tat in the Indian clinical cohorts but our findings have direct implications for HIV-1 Tat vaccine design.

1. Introduction

1.0 Human Immunodeficiency Virus

Human Immunodeficiency Virus-1 (HIV-1) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS), the worst pandemic of mankind. Despite the enormous amount of effort dedicated on restricting the spread of the infection, HIV expansion shows no sign of slowing. WHO/UNAIDS estimates that nearly 30 million people are living with HIV infection as of 2007 with as many as 5 million fresh infections each year. The Sub-Saharan regions are the most affected with an estimated 21.6 to 27.4 million people currently living with HIV and India has the second largest infections, with 2.5 million infections (0.36% of the adult population). Although the introduction of effective antiretroviral drugs has had its impact on controlling the infection in the developed nations, their use in the developing nations has been limited and hence less likely to control the pandemic effectively. Despite the increased efforts, a vaccine for HIV/AIDS still remains elusive.

HIV is thought to have originated in non-human primates in Sub-Saharan Africa and transferred to humans in the early 20^{th} century (Worobey et al. 2008). Two types of HIV have been shown to infect humans namely, HIV-1 and HIV-2 with HIV-1 causing most of the global infections. HIV-1 is believed to have jumped from chimpanzees (*Pan troglodytes troglodytes)* to humans while HIV-2 is considered to have originated from Sooty Mangabeys (*Cercocebus atys*) from Cameroon. HIV-1 is further classified into major (M), outlier (O), new (N) and a recently identified 'P' group that may have been transmitted from gorillas (Plantier et al. 2009). The group O is restricted to west-central Africa, and group N is rare. More than 90% of the global infections are caused by strains

belonging to the M group. The strains/clades/subtypes of M group are genetically distinct and are named A to K and the number of circulating recombinant forms (CRF) is expected to rise (Bertha Cecilia Ramirez et al. 2008). The strains of the viruses differ in their geographical distribution as well as pathological properties. Subtype C is the most dominant strain in the world accounting for more than 50% of the infections (Hemelaar et al. 2006). Furthermore, reports on the increasing incidence of subtype C from regions dominated by other strains have been on the rise (Fontella, Soares, and Schrago 2008; Bello et al. 2008). In India more than 90% of the infections are caused by subtype C strains (Siddappa et al. 2004), however, evidence exists for the emergence of several recombinant viruses in several parts of India (Lakhashe et al. 2008; Neogi et al. 2008; R. Sarkar et al. 2009; Srikanth P Tripathy et al. 2005; Siddappa et al. 2005).

HIV is a member of the genus Lentivirus and the family Retroviridae. Its genetic material is composed of two sense RNA strands consisting of nine genes and coding 19 proteins. The genetic material of nearly 10 kilo bases along with reverse transcriptase and integrase enzymes is packaged into a capsid composed of 2,000 copies of p24. The conical capsid is surrounded by matrix made of p17 matrix protein. This is subsequently enclosed in a double layer of phospholipids which is hijacked from the host cell membrane. The viral envelope is studded with about 70 copies of viral envelope proteins composed of three molecules of gp120 non-covalently attached to the transmembrane gp41 (Figure 1.1). A general outline of the viral replication cycle has been schematically shown in Figure 1.1 and has been elaborately reviewed in (W K Wang et al. 2000) and (Retroviruses. CSHL Press 1997).

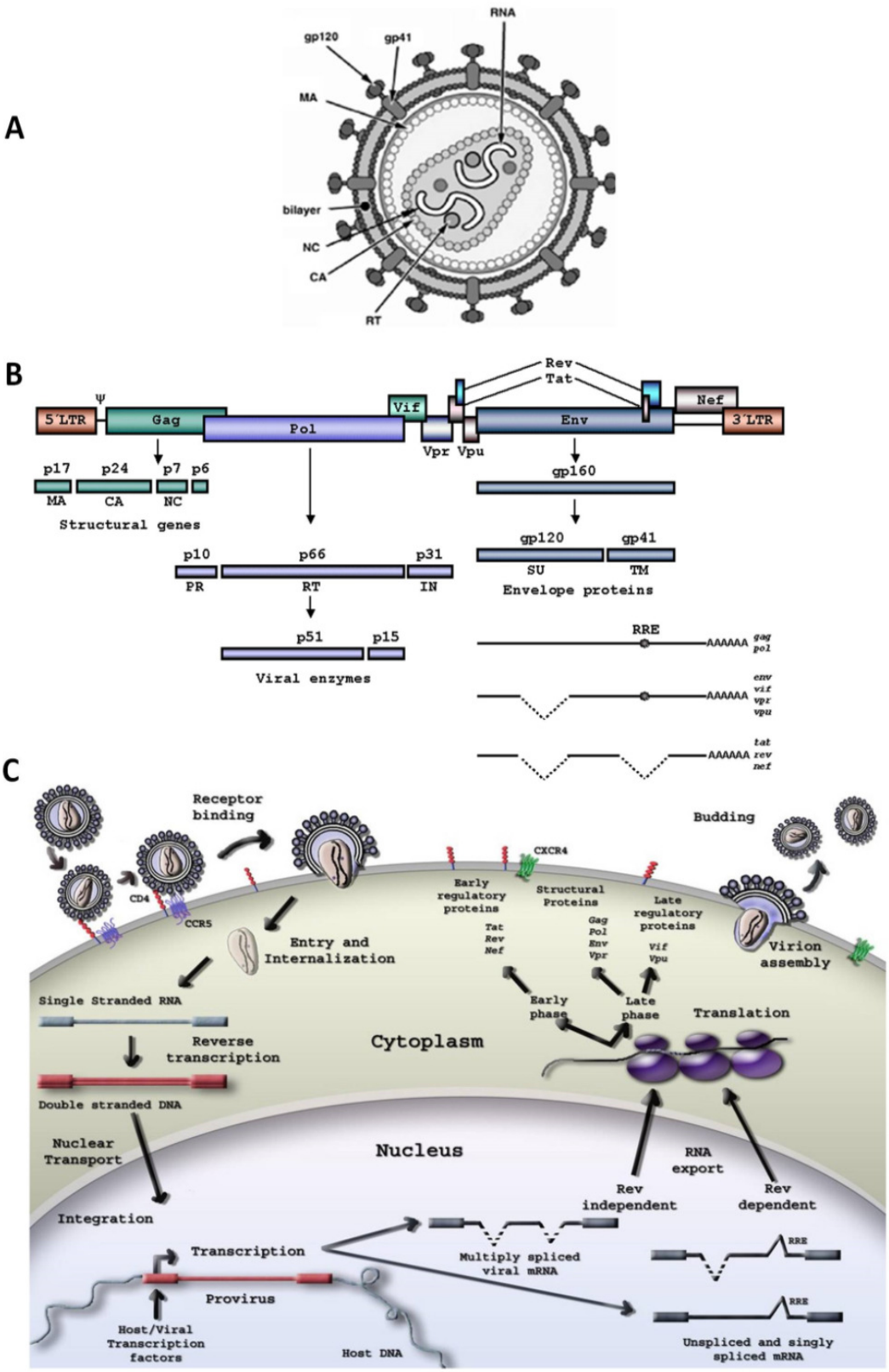


Figure 1.1 (A) Structure of HIV virion **(B)** Genome organization **(C)** Overview of viral life cycle

The viral RNA of HIV-1 codes for three classes of proteins:

1. The major structural proteins (Gag and Env)
2. Regulatory proteins (Rev and Tat)
3. Accessory proteins (Nef, Vpr, Vpu and Vif)

A brief description of the proteins is provided below:

HIV-encoded proteins and their functions

Protein	Size (kDa)	Function and properties
Gag	p24	Structural capsid protein
Gag	p17	Myristoylated matrix protein
Gag	p7	Nucleocapsid protein; helps in reverse transcription
Gag	p6	Role in budding (L domain)
Polymerase (Pol)	p66, p51	Reverse transcriptase; RNAse H; found inside the core
Protease (PR)	p10	Posttranslation processing of viral proteins
Integrase (IN)	p32	Viral cDNA integration
Envelope	gp120	Envelope surface protein
Envelope	gp41	Envelope transmembrane protein
Tat[A]	p14	Transactivation
Rev[A]	p19	Regulation of viral mRNA expression
Nef	p27	Pleiotropic effects, can increase or decrease virus replication
Vif	p23	Increases virus infectivity and cell-to-cell transmission; helps in proviral DNA synthesis and/or in virion assembly
Vpr	p15	Helps in virus replication; transactivation
Vpu[A,B]	p16	Helps in virus release; disrupts gp160-CD4 complexes
Vpx[C]	p15	Helps in viral entry and infectivity
Tev[A]	p26	Tat and Rev activities

[A]Not found associated within the virion. [B]Only present with HIV-1. [C]Only encoded by HIV-2; may be a duplication of Vpr. Reproduced from ref. 146.

Table 1.1 Proteins encoded by HIV. (Levy, J.A. 2007, HIV and the pathogenesis of AIDS. 3rd edition. ASM Press. Washington, DC)

1.1 Immune responses to HIV in the course of infection

The hallmark of HIV infection is severe and gradual depletion of the CD4+ cells as a function of disease progression. Another phenomenon of HIV infection is the chronic activation of the immune system. Innate immune responses are the first line of defense against foreign pathogens. It is believed that initial interaction between the components of innate immune system and the pathogens plays a vital role in determining the adaptive immune response. However, the protection against viral infection has been known to be conferred mainly by the adaptive immune response, both humoral and cell-mediated.

While the antibodies that are neutralizing in nature prevent infections by the free viruses, the cell mediated responses are essential for the removal of pathogen-bearing cells. Furthermore, the important differences between the humoral and the cell mediated responses are in the stages of infection in which they are believed to be effective. While neutralizing antibodies are important to block acute viral infections, their ineffectiveness in chronic infections is yet to be established. Although cell mediated immune response cannot prevent an infection, it is important to control chronic infections.

1.1.1 HIV-specific cell-mediated responses

A large number of studies supporting the protective role of cell-mediated responses to control the viral replication and eliminate infected cells have been performed. Both CD4+ and CD8+ responses have been implicated in protection against disease progression and lower viral loads. Vigorous CD4+ proliferative responses correlate with lower levels of viral load and with the control of viral replication in primary infection. However, the evidences in favor of CD8+ responses being able to control viral replication are higher than that for CD4+ responses (Harari and Pantaleo 2008). This is expected for the following reasons: 1. Strong proliferative and CTL responses to HIV proteins seen in long-term non-progressors (LTNP) 2. High CTL responses found in exposed but uninfected individuals 3. Fall in viral load to a set-point, which coincides, with the rise in HIV-specific CTL responses and 4. Increase in viral load following the emergence of CTL epitope escape mutants.

HIV-1 specific T-cell responses are associated with the primary infection (Gandhi and Walker 2002). In the acute phase of the viral infection the total viral load has been reported to be 10^6 per milliliter of blood both in natural infections and in SIV models. In

response to this uncontrolled replication of the virus, the HIV-1 specific CD8+ replicates at least 15 times and these cells make up to as much as 1-10% of the total CD8+ cells (J D Wilson et al. 2000). This rise in the CD8+ numbers coincides with the dip in viral load. Although the number of HIV-specific CD8+ cells appears to be high, it is less as compared to the immune response elicited by other viruses causing acute infections, where the numbers can reach 40% of the total CD8+ numbers. The CD8+ cells isolated from the acute phase of infection appear to be at the final-stage of life as they undergo apoptosis when removed from the blood and cultured (Tan et al. 1999). The initial rise in the CD8+ cells subsequently subsides as the viral loads also appear to dip and reach a set-point. This rise in CD8+ response to the HIV epitopes leads to the rise in escape variants. However, it has been established that the CD8+ cells recognize only a small repertoire of epitopes in the initial stages of infection and the reason for the limited breadth of the immune response is not clear (Jianhong Cao, McNevin, Holte, et al. 2003).

Substantial number of changes takes place in the chronic phase of infection, where the breadth of epitope recognition by the CD8+ cells is larger (M Altfeld et al. 2001) and the frequency of escape variants is lower. This suggests that the selection pressure mediated by the HIV-specific CD8+ T-cells is limited and is mainly present in the acute phase. Recent studies using MHC-tetramer technology also show that the CD8+ cells in the chronic infection stage also bind to the epitopes with lower avidities as compared to the CD8+ cells of the acute phase (Lichterfeld et al. 2007). Perhaps one of the most important findings with respect to CD8+ cell dysfunction in chronic infection is the specific up-regulation of programmed death domain-1 (PD-1) on HIV specific CD8+ T-cells. The interaction of PD-1 on CD8+ T-cells with its ligands PDL-1 and -2 is believed to deliver negative signaling, leading to failure of the cells in responding to antigenic stimuli (Day et al. 2006). However, antibody-mediated blocking of the PD-1 and ligand

interaction could reverse this effect. The reason for the up-regulation of the immune suppressive receptor is perhaps due to the continuous exposure of the immune cells to viral antigens in the chronic phase.

The kinetics of the HIV-1 specific CD4+ response is greatly influenced by the initial attrition in the numbers caused by the primary infection (Mattapallil et al. 2005; Brenchley et al. 2004). Nevertheless, HIV-specific CD4+ cells secreting IFN-γ (Pitcher et al. 1999) are present and these cells can control the viral proliferation more efficiently (E S Rosenberg et al. 2000). Though the number of virus-specific T-cells in seropositive subjects is substantial, the frequency of such cells is significantly smaller than those against other persisting viruses such as cytomegalovirus (CMV). Despite the obvious presence of HIV-specific CD4+ cells *in vivo*, their functional significance could be demonstrated only in a small subset of individuals known as the elite controllers. The CD4+ response is predominantly directed against structural proteins; however, the CD4+ T-cells are lost rapidly due to viral pathogenesis (Kaufmann et al. 2004). The phenotypic and functional profile of these HIV-specific T-cells has been the subject of various studies to identify the immune correlates of disease control.

1.1.2 Humoral immune responses

The immune correlates of effective vaccines developed so far are mainly represented by neutralizing antibodies. Antibodies to various proteins of HIV-1 appear within 15-30 days of infection, however, in certain cases the seroconversion is considerably delayed and detectable only 6 months later (Ranki et al. 1987). The most immunogenic antigens of HIV constitute the structural proteins gp120, gp41, and p24. Despite being immunogenic in nature, the antibody response to HIV structural proteins is

slower as compared to other viral infection. However, the presence of anti-structural protein antibodies is the best diagnostic tool for HIV that is currently available.

Though a vigorous antibody response to the viral proteins is elicited, a majority of these antibodies lack the neutralizing activity. Neutralizing antibodies have not been found in patients who progress to AIDS stage, while the titers are high in the sera of patients who do not progress rapidly, that is, in long-term non-progressors (LTNPs) (Carotenuto et al. 1998). As is true for several viruses, the neutralization determinant is in the envelope of HIV. While antibodies to gp120 block the attachment of free viruses to CD4+, antibodies to gp41 act at the post attachment step (Ugolini et al. 1997). Therefore, gp41 is widely believed to be a potential vaccine candidate (Norman L Letvin 2006; Pantophlet and Dennis R Burton 2006). In experimentally immunized chimpanzees, neutralizing antibodies were elicited and the antibody titers correlated with protection and against disease progression (M Girard et al. 1995). Furthermore, protection in non-human primates was achieved using passive immunization of immunoglobulins isolated from HIV-1 infected individuals and also with monoclonal antibodies (Prince et al. 1991; Emini et al. 1992; Igarashi et al. 1999; Baba et al. 2000; Mascola et al. 2000). In passive immunization studies protection against disease progression was attained through intravenous and mucosal immunization routes. Despite promising results at the experimental immunization levels in animal models, the results obtained with individual vaccines that have already been tested in phase III efficacy trials have been disappointing in terms of magnitude and of the neutralizing ability.

There are three major problems with respect to eliciting neutralizing antibodies at desirable levels. Firstly, the magnitude which is believed to be essential for protection has not been achieved (titers of >1:100,000 have been considered to be a prerequisite); secondly, low or absence of neutralizing ability and finally, absence of broad

neutralization ability. These technical limitations have further complicated the attempts at developing a potent vaccine against HIV. The nature of the screening assay could be one reason why many neutralizing antibodies have not been identified thus far. A typical screening assay uses recombinant proteins and indirect ELISA, rather than the virus-neutralization assay itself, for the detection of a neutralizing antibody. To overcome this limitation, recently a high-throughput neutralization assay has been applied and new antibodies with broad neutralization potential have been identified (Laura M Walker et al. 2009). Interestingly, recent studies reveal that the low titer antibodies are also able to neutralize the virus efficiently (Hessell, Rakasz, et al. 2009; Hessell, Poignard, et al. 2009). Studies have also shown that administration of high quantities of monoclonal antibodies 2F5, 4E10 and 2G12 delayed the viral rebound after cessation of antiviral therapy (Alexandra Trkola et al. 2005). Nevertheless, problems like antigenic variation and epitope masking continue to pose a serious challenge at the attempts to elicit neutralizing antibodies.

In addition to antibodies to Env that neutralize the virus at entry level, antibodies directed against the internal proteins can also confer protection against viral proliferation (Sarin et al. 1986; Virgin, Mann, and Tyler 1994). Apart from blocking the virus, antibodies have been implicated in antibody dependant cellular cytotoxicity (ADCC) and removal of infected cells (Brenner, Gryllis, and M A Wainberg 1991). However, the protective role of these antibodies in ADCC, complement fixing and phagocytosis is unclear (M Huber and A Trkola 2007).

Nef, Rev and Tat proteins constitute the regulatory proteins of HIV-1 of which Nef is the most immunodominant one. Nef-specific antibodies arise early in the infection and occasionally before those against the structural proteins of the virus. Nearly 70% of the infected individuals harbor antibodies against Nef and high levels of antibodies to Nef

are negatively correlated with disease progression (G Franchini et al. 1987). However, conflicting data failing to demonstrate a correlation between anti-Nef antibodies and proviral load or disease progression also exist (Ranki et al. 1990). Nef shares its sequence similarity with various host proteins and hence false-positive results to Nef are common in HIV-negative donors (Samuel et al. 1987). An evaluation of Nef for B-cell epitopes using monoclonal antibodies demonstrated the presence of potential antigenic sites on the entire length of the protein perhaps underlying the broad-range antibody response seen to this protein in experimental animals and natural infection (Bahraoui et al. 1990; Moureau et al. 1999; Siakkou et al. 1993).

Rev plays an important role in the transportation of viral transcripts from the nucleus to cytoplasm. Immunogenicity of Rev has been demonstrated in natural infection but with a large variation, ranging from 40 to 100% in the incidence of sero-positivity, which is typical of immune response to regulatory proteins (Chanda, Ghrayeb, and Wong-Staal 1988; P Reiss et al. 1989; Dairaku et al. 1989). The most antigenic domain of Rev has been identified to be the Arginine-rich region which also serves as the nuclear localization signal and as the RNA binding domain (Pardridge, Bickel, Buciak, J Yang, and Diagne 1994; Pardridge, Bickel, Buciak, J Yang, Diagne, and Aepinus 1994; Y Wu et al. 1996).

Tat, an important regulatory factor of HIV, has been proposed to be one of the important vaccine candidates as immune responses to Tat have been implicated in slowing disease progression and reducing the viral load. The literature on the immunogenicity of Tat has been reviewed in an independent section (please see p. 45, section 1.5) given that this theme remains the focus of the present study.

1.3 The elusive HIV vaccine

The most desirable way to combat HIV infection would be to potentiate the immune system against the virus. In natural immune responses to HIV, the major problem is the persistence of varying virus and the viral escape from the immune system. The main aim of a vaccine is to potentiate the immune system to provide efficient and prolonged control of the viral load and also to preclude the emergence of the escape mutants. An ideal vaccine should elicit neutralizing antibodies and prevent viral infection at the portal of entry. The technical limitation of the neutralizing antibodies, however, is their inability to prevent the spread of virus by cell-to-cell infection in the *in vivo* context. In such an event, vaccine-induced cell-mediated immune response becomes important since the CTL could destroy the infected cells. In an ideal situation, humoral immune response should prevent viral infection and cell-mediated immune response should control *in vivo* spread of the viral proliferation.

To date there is no evidence for spontaneous clearance of HIV-1 infection. The existence of exposed but uninfected people, nevertheless, presents a ray of hope. These people make up to 5% of the subjects with high risk behavior (Langlade-Demoyen et al. 1994). Many studies have shown that these individuals harbor HIV-specific CD8+ and/or CD4+ cell responses and mucosal IgA (T Hirbod and K Broliden 2007; Taha Hirbod et al. 2008; Rowland-Jones et al. 1995). However, the varying magnitudes of these responses add to the complexity of finding an immune correlate of protection. Several hypotheses have been proposed to explain why these individuals are not infected. Host genetic factors such as homozygous Δ32 allele of CCR5 and MHC-I haplotypes, immunological factors such as presence of polyfunctional HIV-specific CD4+ and CD8+ cells and strongly neutralizing antibodies have been proposed as possible factors (Baker et al. 2009).

Vaccine against HIV is the most desirable therapy and enormous amount of effort has been channelized in this direction. The vaccine strategies for HIV-1 currently being employed are divided into two forms, namely, traditional strategies and novel strategies (Table 1.3). The traditional vaccine approaches include the use of live attenuated viruses, whole killed viruses and protein subunits (Marc P Girard, Osmanov, and Marie Paule Kieny 2006). Although these strategies have been found to be successful for other viruses, they provide little utility for HIV vaccine (Daniel et al. 1992). Attenuated-live viruses although have been shown to be effective in monkey models, safety concerns prevent their use in humans (Learmont et al. 1999). Subunit and killed viruses are less efficient in eliciting strong neutralizing antibody response and CD8+ T-cell responses but are safer than other virus mediated strategies.

Table 1.3 Current vaccine strategies for HIV

Strategies	Immune responses elicited	Advantages	Disadvantages	References
Traditional strategies				
Live attenuated viruses	Cellular and humoral	Strong immune response elicited. Previously tested method	Low levels of persistent infections in monkeys observed. No protection against super infection	Daniel MD et. al, 1992, Hoffman-Lehmann R., 2003, Murphey-Corb, 1997
Whole killed viruses	Cellular and humoral	Safer than attenuated virus as the viral particles are inactivated	Chemical and heat treatments destroy antigenicity of proteins	Poon B et al., 2005, Rossio JL et al., 1998
Protein sub units	Mainly humoral	Safe, amenable, potential to raise neutralizing antibodies	Sequence variation of proteins, stability and epitope masking, cross-neutralization not achieved	Karlsson Hedestam et al. 2008
Novel strategies				
Plasmid DNA	Mainly cell mediated, humoral also elicited	Easy to engineer and elicits strong cellular response	Delivery of vaccine, amount of DNA needed and multiple doses requires	Kutzler and Weiner, 2008 Rossi, June, and Kohn, 2007
Recombinant viral vectors	Mainly cell mediated	Highest percent responders recorded so far in humans to these vaccines. Most commonly used vaccine currently	Pre-existing immune response to the vector, immunodominant nature of vector antigens, safety concerns	Liniger, Zuniga, and Naim 2007; Hanke 2008
Virus like particles (VLP)	Humoral and cell-mediated	High magnitude of response due to adjuvant property, no immune suppression caused	Not well established system. Further studies necessary	(Ludwig and Wagner 2007; Young et al. 2006)

Novel strategies for vaccine development include gene delivery by naked plasmid DNA, recombinant viruses expressing HIV-1 antigens, virus-like particles and engineered viral proteins to enhance immune responses. DNA vaccines are gaining importance mainly due to their ease of use and engineering. However, the immune response elicited despite multiple doses with high amounts of DNA is low. New and promising strategies to enhance the immunogenicity have been reported that can play a significant role in vaccine design (Chong et al. 2007; D H Barouch et al. 2000). Improved delivery systems are projected as a possible answer to the low immune responses elicited by DNA vaccines (Luckay et al. 2007). Bacterial vectors such as the mycobacterium, *Salmonella, Listeria*

and Bacillus Calmette-Guerin (BCG) (Hone et al. 1996; Lieberman and Fred R Frankel 2002; Aldovini and R A Young 1990) are also being explored.

Despite considerable progress made in understanding viral pathogenesis, a vaccine for HIV remains elusive. Some of the factors for the failure of the vaccines are virological in nature while others are due to lack of good study models (Dan H Barouch 2008). Important factors for the failure of vaccines are mentioned below.

1. Clade and sequence diversity
2. Establishment of latent reservoirs
3. Viral escape from humoral and cellular immune responses
4. Inadequately understood immune correlates of protection
5. Absence of well-established small animal model that emulates natural infection

The variation in the sequences of HIV-1 is an important scientific challenge that needs to be addressed on a priority basis as the virus is known to be continually evolving and new recombinant forms are emerging. One of the pertinent questions that require consideration is whether it would be possible to design a universal vaccine which can offer protection against all subtypes of HIV, or would it be imperative to design vaccines for a viral subtype that is predominant in a geographical region. Although, the former is desirable, it has proved to be a herculean task thus far. The viral classification currently employed is based on the genomic sequence and not on the pathogenic and/or immunological properties of the virus and hence studying the different pathogenic and immunological properties of different clades is essential (Barbara S Taylor et al. 2008).

HIV integrates into the genome of various cell types and remains hidden from immune surveillance despite reduction in peripheral viral load by ART (Blankson, Persaud, and Siliciano 1999). According to one estimate, a complete eradication of the viral latent reservoir would take a minimum of 60 years (Robert C Gallo 2005). The virus

also has various mechanisms of evading the immune system (Klenerman, Ying Wu, and Rodney Phillips 2002). The Nef protein down modulates the surface expression of MHC-I while Tat is known to suppress expression of several host genes. Furthermore, Tat has also been proposed to cause immune suppression.

Neutralizing antibody based vaccines have failed to provide protection against infection because of the heavy glycosylation of the trimeric gp120 spike on the viral surface. These antibodies generally bind to the hypervariable region which is subjected to variation and not to the receptor binding regions, as these are masked by the protein structure. Although in the proof of concept experiments passive immunization with neutralizing antibodies provided protection against SHIV infection, the efficiency of the neutralizing antibodies in human clinical trials has been disappointing (Jon Cohen 2003). In addition to circumventing viral genetic diversity, vaccine strategies for HIV must also take into account the genetic variation of the human beings. Some HLA-types such as HLA-B57, B27 and B6 have been associated with slower disease progression whereas some others with rapid progression (Piacentini et al. 2009; Wertheim, Kutkowska-Kaźmierczak, and Bal 2008).

The lack of clarity on the immune correlates of protection remains unresolved mainly because there has been no reported incidence of a spontaneous viral clearance (Pantaleo and Richard A Koup 2004). Although certain important clues have emerged in this direction, only clinical studies can offer definitive evidence. The presence of neutralizing antibodies has not been a good correlate of protection. Polyfunctional T-cells secreting IL-2 and IFN-γ are considered to be good correlates of protection against disease progression. Importantly, the presence of polyfunctional cells in the gut associated lymphoid tissues (GALT) is considered to be critical (Ferre et al. 2009).

The development of an efficient vaccine is hindered by the lack of small animal models. Small experimental animals are relatively easy to handle and economical to maintain. Unfortunately, they are not infected by HIV due to blocks at various levels. Recent advancements in the generation of humanized mice (hu-SCID), and engineering of HIV by pseudotyped envelopes has been of significant importance (Van Duyne et al. 2009). Currently SIV infection of the Indian Rhesus macaques is considered to be the most appropriate model for testing vaccine efficacy as the pathogenic effects of SIV in these animals are believed to be similar to the human infection including the loss of CD4+ CCR5+ cells in the GALT and the heterogeneous immune responses seen in the chronic phase (B M Nath, Schumann, and J D Boyer 2000). The use of the SHIV models where the SIV backbone is cloned to harbor a HIV envelope suffers from some limitations. Especially the chimera viral strain SHIV89.6, the most commonly used challenge virus, is being questioned as it is an X4 virus and hence easily neutralizable. A new chimera viral strain containing subtype C Env which is R5 tropic has been reported and is being projected as an important model for neutralization studies of transmitted viruses (Siddappa et al. 2009). However, the laboratory adapted viruses differ significantly from natural viral strains at multiple levels hence precautions must be taken while interpreting data from such experiments.

Recombinant viral vectors including attenuated pox viruses and adenoviruses and these in combination with the DNA prime/boost were the most promising vaccine regimens so far. However, the recent failure of the STEP trial which used adenovirus 5 (Ad5) as the vector has been a major setback for viral vectors suggesting that more precaution and care must be taken in terms of pre-existing immune responses to vectors (Corey et al 2009). The trial involved 3000 healthy and uninfected volunteers who were randomized to receive either a placebo or the vaccine. These volunteers were further

categorized into high, moderate, low and seronegative to immune response the vector based on the antibody titers to the Ad5. The disconcerting finding of this study was that the individuals who were immunoreactive for the vector had two-fold higher infection rate for HIV as compared to those who were not exposed to the virus before. Further, a multivariate study revealed that uncircumcised men who were Ad5 seropositive had a four-fold higher incidence of HIV acquisition. Many possible reasons have been proposed for the failure of the STEP trial, two important ones being:

1. Boosting of prior existing immune response to the vector, which in turn renders the CD4+ cells susceptible to HIV infection
2. Prior Ad5 responses may skew the immune system to respond to the vector leading to higher acquisition rates in sexually exposed men.

Though studies attempting to disprove the first hypothesis have been published, (Hutnik et al 2009), contrasting reports also exist (Benlahrech A et al 2009). Despite the setback, attempts are presently on to redesign the vectors to better suit HIV vaccines studies (Lasaro and Ertl 2009). The failure of the STEP trial has been a major setback for vaccine research that has posed various questions. Answers for some of these questions are being attained with rigorous analysis of the data at the preclinical stage.

While the failure of STEP trial was a major setback, the results from the recently concluded vaccine trial in Thailand (RV144) have been very encouraging. In this trial a combination of two vaccines (ALVAC-HIV and AIDSVAX B/E) which were ineffective by themselves were used. The outcome is hailed to be very significant as the risk for contracting HIV was reduced by a third (Rerks-Ngarm et al. 2009). The Thai trial was a community-based, randomized, multicenter, double-blind, placebo-controlled efficacy trial of the prime–boost with Thai men and women between the age 18 and 30 years and

no prior infection were recruited. The ALVAC-HIV is a live attenuated recombinant canarypox that has been genetically engineered to express subtype E HIV-1 gp120 (92TH023) linked to transmembrane anchoring portion of gp41 (strain LAI), and HIV-1 gag and protease (LAI strain) while the AIDSVAX B/E is a bivalent vaccine containing recombinant gp120 from two strains of HIV, namely, subtype B and E. The results of the study was revealed by the U.S Army Surgeon General and demonstrated that the vaccine regimen lowered the rate of infection by 31.2% when compared with the placebo. The immunogenicity of the vaccine was evaluated by ELISPOT assay detection IFN-y and intracellular staining IL-2 and IFN-y to Gag and Env. Further, lymphroproliferation assay to gp120 and antibody binding to gp120 and p24 was performed.

The findings of this study though have instilled hope of developing a vaccine against HIV, some of the experts believe the results of the study are weak (Letvin 2009).

1.4 Trans –activator of transcription (Tat)

HIV-1 Tat protein is a 14-15 kDa protein synthesized at both early and late stages in the viral life cycle and necessary for efficient viral replication (A I Dayton et al. 1986; Fisher et al. 1986). Two forms of the protein are generated from a multiply spliced transcript, where one form is 72 amino acid long encoded by exon-1 and the second form of 101 amino acid long is encoded by both of the exons. Differences in the position of the translational stop codons in the second coding exon account for the range of size of two-exon Tat i.e. 86-101 amino acids found in different viral clades. It has also been reported that the expression of the two forms of Tat is temporally different in that, two-exon Tat is expressed early in infection whereas the 72 amino acid Tat is expressed after the Rev expression is established in the infected cell (Malim et al. 1988). Although trans-activation can be observed *in vitro* with the first exon (1-72 amino acids), the second exon that codes for 14-34 amino acids at the C-terminal has been proposed to be necessary for trans-activation *in vivo* (K T Jeang, Xiao, and Rich 1999).

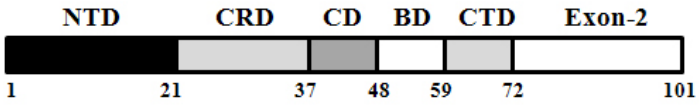


Figure 1.2 Domain organization of Tat protein

Tat is demarcated into five functional domains (Kuppuswamy et al. 1989) as illustrated in Figure 1.2. These domains include (1) The N-terminal domain (NTD, 1-21 amino acids) which contains acidic amino acids. This domain forms a stable structure sandwiched between the glutamine-rich and the core domains (Bayer et al. 1995). The N-terminus has been implicated in playing a role in preventing mitogen- and antigen-mediated proliferation of peripheral mononuclear cells (PBMC) and T-cells (Chirmule et al. 1995; D Zagury et al. 1998) and thus leading to immune suppression. Extracellular Tat has been implicated in various pathogenic functions and for most of them it needs to

reach the cytosol through endocytosis. Trp11 plays a key role in the endosomal membrane fusion at low pH and the subsequent entry of Tat into the cytosol (Yezid et al. 2009). (2) The Cysteine-rich domain (CRD, 22-37 amino acids) contains conserved cysteine residues at 22, 25, 27, 30, 31, 34 and 37 which form intramolecular disulphide bonds. However, subtype C Tat is an exception with a conserved C31S mutation (Udaykumar Ranga et al. 2004). The CRD has also been attributed with chemotactic property on monocytes (A Albini et al. 1998) and is essential for Tat to function as a transactivator. (3) The core domain (CD, 38-48 amino acids) is known to play crucial role in Tat-mediated transactivation (Carroll, Martarano, and Derse 1991). (4) The basic domain (BD, 49-59 amino acids) conserved among the viral clades is made up of six Arg and two Lys residues (RKKRRQRRR). It is known to play a pivotal role in Tat-TAR interaction (Dingwall et al. 1989) and for nuclear localization (Siomi et al. 1990; Berkhout, Silverman, and K T Jeang 1989). (5) The C-terminal domain (60-72 amino acids) has the highest degree of sequence variation; however, it contains conserved glutamine residues. The N-terminus, CRD and the core domain together form the activation domain of Tat. The second exon of Tat is known to have the RGD motif in clades B and D (K T Jeang, Xiao, and Rich 1999) and is known to be involved in interaction with integrin receptors on the cell surface and furthermore, in regulating host gene expression (Brake, C Debouck, and Biesecker 1990).

Structurally, Tat has 30% beta-turn structure and almost no alpha-helix in aqueous solutions (J M Péloponèse et al. 1999). However, in the native form Tat is known to be a flexible protein and the secondary structure alters considerably based on the environment. It has been reported that alpha-helix becomes the major secondary structure when Tat is in hydrophobic solvents and this property of Tat has been implicated in its ability to cross cell membranes (Campbell and Erwann Loret 2009). X-ray crystallographic studies so far

have not been carried out on Tat protein. However, NMR studies on four variants have been performed. These studies reveal that the Tat variants have similar folding in the aqueous solution characterized by a core region composed of a part of the N-terminus. The core is surrounded by other regions that are well exposed to the solvent with the BD adopting an extended structure while the CRD, CD and C-terminal domain have beta-turns in most variants (Bayer et al. 1995; Shojania and O'Neil 2006; Jennifer D Watkins et al. 2008).

1.4.1 Tat as a transcription factor of HIV

The gene expression of HIV happens in two phases, an early Tat-independent phase and a late Tat-dependant phase. In the presence of Tat, the gene expression is known to increase several hundred-fold (Garber and K A Jones 1999; Mauro Giacca 2004). This regulatory function of Tat was realized when accumulation of RNA polymerases downstream of HIV promoter in the presence of Tat was discovered (Kao et al. 1987; Laspia, Rice, and Mathews 1989). It was also noted that in the absence of Tat short transcripts predominate whereas in the presence of Tat a dramatic increase in the long transcripts occurs leading to the belief that Tat acts exclusively at the transcriptional elongation (Laspia, Rice, and Mathews 1990; S Ghosh, Selby, and Peterlin 1993; Marciniak and Sharp 1991). Unlike most transcription factors, Tat functions by binding to a cis-element in the mRNA called the transactivation responsive region (TAR) present at the +1 to +59 positions (Brigati et al. 2003) by binding to a tri-nucleotide bulge in the stem-loop structure of the TAR RNA and recruiting P-TEFb, a positive elongation factor composed of cyclin-T1 and CDK9 (Marshall and D H Price 1992). Interaction of Tat with TAR and P-TEFb leads to the hyperphosphorylation of the C-terminal domain (CTD) of

RNA polymerase II which facilitates the elongation step of transcription (Bieniasz et al. 1998).

Although Tat, was considered to act at the elongation step of transcription, many findings suggested a possible role for Tat in transcriptional initiation (F Kashanchi et al. 1994; Karn 1999). The role of Tat in assembling transcription complex at the LTR was further based on the observations that Tat recruited RNA pol II, TATA Binding Protein (TBP) and other general transcription factors (GTFs) (Raha, Cheng, and Michael R Green 2005; John Brady and Fatah Kashanchi 2005). Furthermore, it has also been suggested that Tat contributes for chromatin remodeling by recruiting specific histone acetyl transferases (HAT) to the viral promoter facilitating acetylation of histone H3 and H4 (Lusic et al. 2003). It has also been suggested that Tat-mediated nucleosome remodeling is the rate limiting step and not the acetylation of histones (Kiefer et al. 2004). More recent studies implicate the interaction of non-acetylated Tat with CBP/p300 and CDK9/CyclinT1 in initiation of gene transcription, whereas acetylated Tat dissociates from the TAR and recruits p/CAF and SWI/SNF and possibly facilitates elongation of transcription (Emmanuel Agbottah et al. 2006). Taken together, it is possible that Tat facilitates chromatin modifications, assembly of initiation complex and transcription elongation in a series of sequential and ordered events that lead to high levels of HIV gene expression.

In addition to regulating the gene expression of HIV, Tat is also involved in various other processes which enhance viral infectivity and development of AIDS. Using Tat-gene deleted constructs it was established that efficient reverse transcription was absent, which was subsequently restored when Tat-expressing plasmids were co-transfected to cells (Harrich et al. 1997; Ulich et al. 1999). Tat has also been proposed to facilitate the binding of tRNA primer to the viral genome and promote the first strand

transfer in reverse transcription (Kameoka et al. 2002). Further studies have led to the hypothesis that Tat can augment virion infectivity by suppressing reverse transcriptase activity before viral maturation and hence prevent incorporation of immature viral DNA into virions (M Kameoka et al. 2001; Masanori Kameoka et al. 2002).

1.4.2 Multifaceted functions of Tat: Pathogenic effects of intracellular Tat

Tat is known to participate in development of AIDS by modulating the expression of several cellular and viral genes (Huigen, Kamp, and Nottet 2004). Tat released from infected cells is able to enter other cells through interactions with heparin sulfate proteoglycans expressed on the cell surface and is translocated to the nucleus in an active form (Tyagi et al. 2001). In uninfected cells, Tat is able to transactivate genes in a paracrine fashion and this action on uninfected cells may play a crucial role in the pathogenesis of HIV infection. Tat is able to up-regulate the expression of immunomodulatory cytokines including IL-2 and TNF-α (Ehret et al. 2001; Sastry et al. 1990). The repressor in the promoter of IL-2 is reportedly replaced by an activator, leading to up-regulation of the gene expression. This up-regulation was also suggested to increase viral infection since IL-2 is an important growth factor for T-cells. However, some reports proposed the contrary, that Tat mediates inhibition of IL-2 and IL-2 receptor expression thus resulting in immune suppression (Vacca et al. 1994; Purvis et al. 1992; Puri, Leland, and Aggarwal 1995). Tat is also able to up-regulate the expression of IL-6 by binding to a specific UCU sequence present in the stem-loop structure of IL-6 transcript. Up-regulation of IL-6 has been associated with AIDS-related disorders such as Kaposi's sarcoma, psoriasis and B-cell lymphoma (Ambrosino et al. 1997). On one hand while Tat up-regulates inflammatory cytokines, it is also known to up-regulate one of the immune suppressive cytokines TGF-β (Nabell et al. 1994; Cupp et al. 1993); this has

been implicated in dampening of immune responses against HIV. Other important cytokines that are regulated by Tat include IL-8, IL-10 and TGF- β (Mahieux et al. 2001; Badou et al. 2000; Cupp et al. 1993). Cell-adhesion molecules that may facilitate the extravasation of HIV-infected cells have been noted to be up-regulated by Tat (Dhawan et al. 1997). The expression levels of fibronectin and collagen types I and II, which play a vital role in many cellular process including cell migration and adhesion were found to be up-regulated in glial-derived cells (J P Taylor et al. 1992). Another important molecule which is up-regulated by Tat is CD95-ligand. This elevated expression has been shown to have a role in Tat-mediated death of CD4+ cells and hence in HIV pathogenesis (Westendorp et al. 1995). Interestingly, Tat is also known to induce the expression of anti-apoptotic gene *BCL2* (G Zauli et al. 1995) hence exemplifying pleiotropic effects.

Apart from inducing the expression of several host genes, Tat can down modulate genes that are important for immune responses and cell survival. p53, the master regulator of cell cycle and a tumor suppressor, is down modulated at the transcriptional level (C J Li et al. 1995). While some of the cytokines are up-regulated, the expression of IL-12 which is known to stimulate NK-cells and induce Th-1 response has been reported to be impaired (Poggi, Rubartelli, and Zocchi 1998). Another important gene that is down modulated by Tat is the MHC class I gene. The repression of MHC-I molecules provides HIV-1 with another mode of escape from the immune system. The repression of MHC-I gene expression is inflicted by the interaction of Tat with $TAF_{II}250$, which is a component of the TAFIID general transcription factor and exon-2 of Tat has been shown to be essential for this interaction (Verhoef et al. 1998) although uncertainty remains on the role of the second exon as other workers have failed to establish the same effect (Matsui et al. 1996). Tat can act on the RNA processing complex such as cleavage and polyadenylation specificity factor 3 (CPSF-3) and lead to up-regulation of host and viral

genes (Calzado, Sancho, and Muñoz 2004). CPSF-3 has been shown to bind to the LTR and reduce the basal level expression of HIV genes and this effect is countered by Tat protein thus elevating gene expression levels (de la Vega et al. 2007). Recently, it has been demonstrated that Tat can interact with Dicer and impair the RNAi mechanism against the HIV genome hence rendering innate cellular defense mechanisms ineffective against HIV (Yamina Bennasser et al. 2005; Yamina Bennasser and Kuan-Teh Jeang 2006).

1.4.3 Pathogenic effects of extra-cellular Tat

In addition to the major role of cellular Tat as a trans-activator, it is also known to be secreted in to the extracellular milieu by the infected cells where it brings about a plethora of effects and hence contributing to HIV pathogenesis (K T Jeang, Xiao, and Rich 1999). The release of the Tat protein in to the extra-cellular milieu occurs when the expression of the protein is very high in the infected cell and when it is predominantly localized to the cytoplasm (B Ensoli et al. 1993). Tat is found in the sera of HIV-1 infected patients at a low concentration between 0.1 and 1.0 ng/ml (Westendorp et al. 1995). The secretion of Tat has been proposed to be independent of the cell-death, in that, Tat is exported into the extracellular milieu from live cells without any sign of change in membrane permeability and is not inhibited by protein transport inhibitors such as brefeldin A. Moreover, the release of the protein is believed to be via a leaderless secretory pathway which is temperature and serum concentration dependant *in vitro*. A fraction of the released protein binds to the heparin sulfate proteogylcans via its basic domain and the rest is found to be present in a soluble fraction (Chang et al. 1997). This soluble Tat protein is known to enter the by-stander cells and bring about a multitude of

effects leading to angiogenesis, apoptosis, suppression of immune response and neurotoxicity.

Kaposi's sarcoma (KS) is one of the major AIDS-associated diseases and the first disorder to which the role of extra-cellular Tat was attributed to (B Ensoli et al. 1990). KS is a neoplasm caused by human herpes virus-8 (HHV-8) and is not life threatening. However, AIDS-related KS has been found to be dramatically different, in that, it is aggressive and is more frequent (B Ensoli et al. 1994). Using transgenic mice it was shown that recombinant Tat protein is able to induce the aberrations in the skin that resembled those of KS (S K Huang et al. 1993). Further it was shown that the growth of mesenchymal cells from AIDS patients with KS was enhanced in the presence of Tat and furthermore was abrogated in the presence of anti-Tat antibodies (B Ensoli et al. 1990). Tat induces the expression of inflammatory cytokines and these cytokines in turn lead to the expression of basic fibroblast growth factor (bFGF). Tat binds to heparin sulfate proteoglycans with high affinity with a K_d of 10-30 nM (M Rusnati et al. 1999) and hence it competes with bFGF for the heparin-binding sites. This subsequently leads to elevated levels of soluble bFGF which enhances the growth of spindle cells and endothelial cells (Barillari, Sgadari, V Fiorelli, et al. 1999; Barillari, Sgadari, Palladino, et al. 1999). The RGD motif in the exon-2 of Tat has been suggested to be important for this function of Tat.

Infection-associated depletion of the CD4+ cells, the hallmark of HIV infections, is believed to be to the consequence of rapid destruction of CD4+ cells by diverse mechanisms including direct viral infection, apoptosis, autophagy, impaired production of new T-cells, and activation induced cell death. *In vitro* co-culture assays have shown that while HIV-infected cells are resistant to apoptosis, the uninfected bystander cells undergo apoptosis (Nardelli et al. 1995). Tat is one of the factors inducing bystander cell death

with some reports suggesting death due to activation induced cell death (AICD) (Bartz and Emerman 1999; Gülow et al. 2005), while a few other reports claim that activation of the cells is not essential for Tat to induce apoptosis in resting CD4+ T-cells (Campbell et al. 2004; Campbell et al. 2005; CJ Li et al. 1995; Patki and M M Lederman 1996). Several groups have identified that cell lines expressing Tat show enhanced proliferation rate and also that these cells show resistance to apoptosis (G Zauli et al. 1993). This has been suggested to be a strategy by the virus to protect the infected cell in the early stages of viral life cycle and since Tat is one of the early gene products, it is considered to play a pivotal role. Even when present extracellularly in picomolar concentrations, Tat is known to stimulate important cell survival signals such as AKT, MAPK, transcription factors such as CREB and AP-1, and also up-regulate survival genes like BCL-2 (Borgatti et al. 1997; D Gibellini et al. 1997; D Gibellini et al. 1998; D Milani et al. 1998; Selliah and Finkel 2001; Z Wang et al. 1999; G Zauli et al. 1995; G. Zauli et al. 1995; Mingjie Zhang et al. 2002; Zidovetzki et al. 1998). Tat has been implicated in rendering the cells of immune system susceptible to HIV-1 infection. Experimentally when primary cells were treated with low concentrations of Tat, they were shown to be stimulated for proliferation and subsequently progressed to G1 phase of cell cycle. Actively dividing cells have been shown to be susceptible for productive HIV infection. Consistent with this claim, it has been shown that in the presence of Tat, PBMCs support growth of viruses better when incubated with Tat, so much so that, it has been suggested to be better than when stimulated with PHA/IL-2 (Sahaf et al. 2008). Co-receptor molecules, CXCR4 (Mondal et al. 2005) and CCR5 (Lin Zheng et al. 2005), which are needed for the entry of HIV into the host, are known to be up-regulated on CD4+ cells by Tat (L Huang et al. 1998). By increasing the density of co-receptors on the surface of the cell, Tat promotes infection of both M-tropic and T-tropic viruses. This effect was noted to be dose-dependent and that a

concentration in the picomolar range was sufficient to enhance the expression of co-receptors (L Huang et al. 1998). Further studies showed that the increase in the levels of the co-receptors needed de novo synthesis and that it was not by altered transport mechanism of the cell (Secchiero et al. 1999).

Interestingly, these activation signals were inhibited when Tat protein was used at higher concentrations in peripheral blood mononuclear cells (PBMCs) or purified lymphocytes. This inhibition of the activation signals in turn abrogates CD3-mediated and antigen-specific proliferation of T-cells (Viscidi et al. 1989; Subramanyam et al. 1993; Chirmule et al. 1995). Furthermore, apoptosis of T-cell lines and primary cells in the presence of TNF-α and CD3-stimulation was enhanced when exogenous Tat was added (CJ Li et al. 1995; Westendorp et al. 1995). Micromolar concentrations of Tat have been shown to up-regulate CD95-ligand and this effect was proposed as one of the important modes by which Tat suppresses immune responses in HIV-1 infection. Enhanced tubulin polymerization into cellular microtubules induced by Tat has also been demonstrated to lead to apoptosis via the mitochondrial apoptotic pathway (Dan Chen et al. 2002; de Mareuil et al. 2005). It has also been shown that exposure to Tat leads to the activation of B-cells (L Huang, C J Li, and A B Pardee 1997). Subsequent studies revealed that Tat has profound influence on the differentiation of B-lymphocytes. While Tat inhibited the proliferation of B-lymphocytes when activated by the B-cell receptor, it enhanced the proliferation of B-cells activated by CD4+ antibody and IL-4 (Lefevre et al. 1999).

Neurological manifestations have been known to be associated with HIV infection (McArthur et al. 1994) and the symptoms including progressive loss of cognitive functions, decreased memory, difficulty in concentrating and psychiatric dysfunction with others are collectively called HIV-1 associated dementia (HAD) or AIDS dementia complex (ADC). Tat is known to be released extracellularly not only by

the infected lymphocytes but also by the glial cells (Tardieu et al. 1992) making it a mediator of neurotoxicity along with gp120 (Lipton 1993) and Vpr (Patel, Mukhtar, and R J Pomerantz 2000) proteins of HIV-1. Further, injection of full length Tat protein or a peptide encompassing the basic domain to the hippocampus can cause neurotoxicity (M Jones et al. 1998). Tat can bring about the neurotoxic manifestations in two ways: direct and indirect. The mechanism of direct effect of neurotoxicity is believed to be involving the increase of intracellular Ca^{2+}. This increase in calcium ions is followed by the mitochondrial Ca^{2+} uptake, generation of reactive oxygen species (ROS) which subsequently leads to the activation of caspases and eventually apoptosis (Pocernich et al. 2005). Indirect neurotoxicity of Tat is rendered upon CNS as Tat stimulates the production of inflammatory cytokines (TNF-α, IL-6, IL-1, IL-8) and chemokines (RANTES and MCP-1) in glial cells and macrophages (P Chen et al. 1997; A Nath et al. 1999). TNF-α releases excitatory amino acid like glutamate from astrocytes and this leads to increased accumulation of glutamate leading to hyperactivation of NMDA receptors, calcium influx and apoptosis. MCP-1 may facilitate the transmigration of leukocytes and monocytes across the blood-brain barrier, which in turn leads to CCR5 up-regulation rendering the astrocytes susceptible to infection by HIV-1 (Weiss et al. 1999). Astrocytes are programmed by Tat to release inducible nitric oxide (iNOS) which in turn causes elevation in the nitric oxide (NO) levels and hence an increase in the oxidative-stress. Excess of NO can again lead to the release of glutamate and in turn leading to NMDA activation (Bal-Price, Moneer, and Guy C Brown 2002). The neurotoxic capacity of Tat is also attributed to its ability to cross the blood-brain barrier (Banks, Robinson, and Avindra Nath 2005).

While extracellular Tat has neurotoxic effects, surprisingly, intracellular Tat protects astrocytes from internal injury induced by 3-nitroprionic acid (3-NP) (Chauhan et

al. 2003). Another intriguing aspect of Tat in terms of dementia is the subtype-specific differences in the ability to induce neurological manifestations. Tat has been attributed with chemotactic properties (A Albini et al. 1998; Benelli et al. 2000) and this is attributed to the presence of a conserved Cys-Cys motif at positions 30 and 31respectively in subtype B. However, subtype C Tat lacks this motif and instead has Cys-Ser motif and hence has been proposed to be a defective chemokine (Udaykumar Ranga et al. 2004). It has also been shown that the subtype C Tat protein fails to induce intracellular calcium influx in monocytes and also fails to induce up-regulation of TNF-α (Campbell et al. 2007). Recent studies have demonstrated a subtype specific difference in neuropathogenesis. While subtype B protein activates the NMDA receptor, subtype C Tat protein is less efficient in this function (Wenxue Li et al. 2008). Further, experiments carried out in human fetal CNS progenitor cell-derived astrocytes and neurons demonstrated subtype C Tat protein to cause lower levels of cell death as compared to subtype B Tat, further establishing the importance of the role of Cys-Cys motif in neuropathogenesis and subtype differences (Mamata Mishra et al. 2008).

Taken together, Tat plays a pivotal role in viral replication and pathogenesis not only by providing higher rate of gene expression for the virus, but also by suppressing immune responses, inducing anergy and apoptosis of immunologically important cells. It also brings about other HIV related pathogenic symptoms such as KS and dementia. Since Tat is one of the early viral proteins to be expressed and an immune response to a multi faceted protein like Tat could abrogate many HIV related pathogenic effects, thus making it important for the scientific community to channelize efforts towards a better understanding of the protein and its functions. In conjunction to basic understanding, devising novel ways to tackle the effects of Tat on the host and importantly exploring Tat as a vaccine candidate is essential.

1.5 Tat as a potential vaccine candidate

The choice of Tat as a vaccine candidate is mainly because it is a key player in HIV infection, as in the absence of Tat no infectious virus is made and also because it plays a vital role in AIDS pathogenesis (see 1.4.2 and 1.4.3). Additionally, Tat elicits a humoral and cell mediated immune response in experimental models as well as natural infection and importantly this response is negatively correlated with viral loads and disease progression. Tat is also known to have immunomodulatory properties, where it modifies the proteasome complex which in turn leads to enhanced CTL responses to multiple epitopes (Gavioli et al. 2004). Tat protein, though is predominantly an intracellular factor, is also secreted into the extracellular milieu, and as an extracellular protein could access antigen presenting cells including dendritic cells and is presented in the MHC-I context. Tat protein is relatively well conserved across viral clades making it an attractive vaccine candidate. Owing to these properties Tat has been formulated in various forms and used as a vaccine (Antonella Caputo, Gavioli, and Barbara Ensoli 2004). Apart from being a vaccine candidate, Tat is also projected as a potential therapeutic target (Mauro Giacca 2004; Fulcher and Jans 2003). Owing to these facts clinical trials with Tat as the antigen has been initiated in Italy. Further, Tat in combination with regulatory and structural proteins of HIV is also being evaluated in multicomponent vaccine (Caputo A et al. 2009). The following sections will discuss the immune responses elicited by Tat and also provide evidence to establish Tat as an important vaccine candidate.

1.5.1 Cell-mediated immune responses to Tat in natural infections

CTL responses against the proteins of HIV-1 have been highlighted to be important to control viral replication (Masopust D 2009). Detailed screening for CTL epitopes using over lapping peptides identified CTL responses to Tat in humans during

primary (Cao, J et al. 2003) and chronic stages and in subtype C and B infections (M M Addo et al. 2001; M M Addo et al. 2002; M M Addo et al. 2003; Chouquet et al. 2002; Lamhamedi-Cherradi et al. 1992; Novitsky et al. 2002). CTL-epitopes have been mapped to three different domains in Tat, namely, N-terminus, Cys-rich domain and core-domain and these epitopes are recognized by multiple MHC-I alleles (M M Addo et al. 2002; M M Addo et al. 2003; Oxenius et al. 2002; Novitsky et al. 2002). Importantly, reports on inverse correlation with disease progression and presence of CTL against Tat are abundant in both monkey models and natural human infections suggesting that these responses play a vital role in retarding the disease progression by eliminating infected cells (T M Allen et al. 2000; Todd M Allen et al. 2002; A. Cafaro et al. 1999; A Cafaro et al. 2001; Maria Teresa Maggiorella et al. 2004; Bianca R Mothé et al. 2002). Notably, Tat and Rev are targeted by CD8+ cells in the initial stages of infection (M M Addo et al. 2001). Since Tat is an early viral product a strong CD8+ response to Tat can prevent establishment of an HIV infection. Indeed, Allen TM et al. elegantly demonstrated that a CTL response to Tat in the primary infection imparts a selection pressure on the virus which gives rise to viral escape mutants (Allen TM et al. 2000). In this study, the authors identified a new CTL epitope (Tat_{28-35}) in macaques and found that the response to this epitope declined rapidly after the acute phase of infection. The study further demonstrates that the loss of the CTL response to Tat in later stages of infection is due to the mutation in this epitope as a result of selection pressure. Sequence analysis of the virus post acute phase (6-8 weeks post infection) revealed amino acid substitution in as many as 86% of the clones. Interestingly, very few mutations were identified outside this epitope. This implies that an effective CTL response to Tat can control the virus efficiently in the acute phase and hence is an important vaccine candidate. More so because Tat is expressed before Nef down modulates the MHC class I molecules and hence dampening the

immune responses (Gruters, van Baalen, and Osterhaus 2002). A study comparing the CTL responses to Tat and structural proteins in individuals at different disease spectrum identified that presence of Tat-specific CD8 cells strongly correlated with lack of disease progression (van Baalen et al. 1997). Another striking observation made in this study was that while a robust CTL response to RT, Gag and Nef was present in both progressors and non-progressors, only the latter group harbored Tat and Rev-specific response. Interestingly the frequency of CTLs response against Tat was lower than that against Gag, Nef and RT suggesting that immune response to Tat even when present at a lower frequency can play a major role in controlling disease progression.

The low frequency of Tat-specific response can be due to various reasons and an important being found to be lower than that of structural proteins the variability in the sequence of the proteins play an important role in the detection of antigen specific CTLs (Betts MR et al. 2002). Since the sequence of proteins vary 5-15% amongst the strains, it is possible that the lower frequency of detection is due to the methodology of detection (reviewed in Yu MR et al. 2005). M M Addo et al. have also shown that CTL responses to Tat are more frequent in patients controlling viremia. It is general belief that the presence of CTL response controls the viral load and indeed, presence of Tat-specific CTLs in the acute phase negatively correlated with viral loads (Cao, J et al. 2003).

The T-helper responses to Tat have not been adequately characterized, although lymphoproliferation assays conducted on cells from HIV-1 infected individuals demonstrate responses to full-length protein as well as individual peptides. The most dominant of the T-helper epitopes have been mapped to 17-32, 33-48, and 65-80 of Tat (Blazevic et al. 1993). These epitopes were not only dominant, but also were found to be presented by many HLA-DR molecules (Castelli et al. 2008). This suggests that Tat induces antigen-specific CD4+ T-helper responses in HIV infections.

1.5.2 Humoral immune response to Tat in natural infections

The initial reports on immune responses to regulatory proteins of HIV in various stages of infection (G Franchini et al. 1987) or seroconversion (Ranki et al. 1987) demonstrated that the antibody response was directed not only to the structural proteins of the virus but also to Tat. Antibodies to Tat have been identified in 10-50% of the HIV infected individuals (J F Zagury et al. 1998; Stefano Buttò et al. 2003; Campbell et al. 2007; Kashi VP et al. 2009). The importance of antibodies to Tat was underscored when it was discovered that antibodies to Tat were elicited even before to Gag and Env (Krone et al. 1988) and importantly before p24 antigenemia, suggesting a possible role of anti-Tat antibodies in controlling viral replication. The fact that Tat-antibodies have a physiological role was further established when it was observed that in the presence of high-titers of antibodies to Tat in the early stages of infection was associated with lack of rapid progression to AIDS (Peter Reiss et al. 1990). The presence of antibodies to non-structural proteins and particularly to Tat, as an important correlate for lack of disease progression was further established when rate of disease progression after 39 months of observation was lower in individuals who harbored antibodies to Tat as opposed to those who lacked it (P Reiss et al. 1991).

The significance of Tat-antibodies was established initially by *in vitro* studies using monoclonal antibodies (Steinaa et al. 1994). The monoclonal antibody that reacted with the amino-terminal epitope of Tat was found to inhibit the viral replication in a dose-dependent fashion. Further analysis showed that various functions of Tat including immunosuppression of hematopoiesis (G Zauli et al. 1991), transactivation of transcription factors (Westendorp et al. 1994; D Gibellini et al. 1997; D Gibellini et al. 1998), promotion of growth of Kaposi's sarcoma spindle cells (B Ensoli et al. 1994) and

protection against apoptosis (G Zauli et al. 1995) were completely abolished. More importantly, the rate of viral replication was significantly lower when PBMCs infected with HIV were co-cultured with PHA activated donor cells in the presence of low concentrations of a monoclonal antibody. This suggests that the autocrine effect of Tat secreted into the supernatant is important for viral replication hence blocking Tat could be beneficial in slowing disease progression. Furthermore, an inverse correlation between natural anti-Tat antibodies and p24 antigenemia was demonstrated in hemophiliacs in a retrospective analysis (G. Zauli et al. 1995; M C Re et al. 1995; Mhashilkar et al. 1997). In a study involving two groups, rapid-progressors and non-progressors showed that anti-Tat antibody was inversely correlated with viral load in the peripheral blood. Importantly, the inverse correlation was restricted to only anti-Tat antibodies and not to antibodies against Env, Nef and Gag (J F Zagury et al. 1998). Epitope mapping analysis of Tat-antibodies in asymptomatic individuals and also those with Kaposi's sarcoma revealed different patterns. While the asymptomatic samples had antibodies to functional domains of Tat, there was complete absence of antibodies to the functional domains in samples from individuals with Kaposi's sarcoma (Demirhan et al. 2000; Demirhan et al. 1999). An account of the epitopes identified in Tat in clinical studies has been enlisted at http://www.hiv.lanl.gov/content/immunology/ab_search. In addition to these initial studies, various groups have carried out studies implicating anti-Tat antibodies with negative correlation to disease progression. Interestingly, the inverse correlation his seen not only in HIV-1 but also in HIV-2 infected individuals (Shaun K Rodriguez et al. 2006; Rezza et al. 2005). Importantly, studies carried out in well-defined clinical cohorts in Africa consisting of a group of 25 women followed for two years presents an interesting case. Twenty three of the 25 seropositive individuals seroreverted, became exposed but non-infected individuals and did not progress to AIDS (Huet et al. 1989). The subtype of

virus infecting these women was called HIV Oyi which is similar to regular HIV clades but for Tat (Gregoire and E P Loret 1996). Immunological assays showed that these individuals harbored CTL responses to HIV and contained antibodies to p24. Ten years following the first report on this cohort, the 23 women remained healthy and traces of HIV were not detectable. These data provide strong evidence that an immune response when present against Tat, has the potential to control the disease progression and as in the case of Tat Oyi, serorevert individuals who were exposed to HIV.

Apart from the HIV infected individuals, anti-Tat antibodies have been identified in HIV negative individuals and the isotype of these antibodies have been identified to be IgM. Importantly, these antibodies have been found in a high frequency in HIV negative people and are lost as the infection ensues (Rodman et al. 1992). Monoclonal antibodies generated against Tat were found to interact with several tissues, hence suggesting that Tat has some sequence similarities with host proteins which might elicit the natural IgM antibodies (Parmentier et al. 1992).

1.5.3 Immune response to native Tat in vaccine studies

Given that Tat plays an important role in HIV life cycle and that immune response to Tat are negatively correlated with disease progression, many preclinical studies have been carried out. Systemic and mucosal immunization strategies which were developed to evaluate the safety, efficacy and immunogenicity of Tat are being evaluated. Tat in various formats including biologically active or inactive proteins, DNA and synthetic peptides are currently under evaluation. So far toxicity at the systemic and local level has not been observed (reviewed in Caputo et al. 2009).

In its native form Tat protein has been tested in vaccine studies in preclinical and clinical studies and has been shown to elicit strong immune response and attenuate disease (Borsutzky et al. 2003; A. Cafaro et al. 1999; A Cafaro et al. 2001; Calarota et al. 1998; Caputo et al. 2003; Caselli et al. 1999, Borsetti A et al. 2009). Borsetti A et al. have recently demonstrated Tat vaccinated (native Tat of 86 aa or plasmid) cynomolgus monkeys had lower viral load upon intravenous rechallenge with pathogenic SHIV89.6 (cy243) and this correlated with a stable CTL and humoral response to Tat. Interestingly, responses to Gag and Env were nearly undetectable. In their previous report, the authors had demonstrated that Tat-vaccinated monkeys controlled the virus for up to 2 years of follow up (Maggiorella M T et al. 2004). Immunization of rabbits with Tat Oyi elicited a strong antibody response and the antibodies recognized different Tat variants with variation as much as 38%, a phenomenon not seen with other variants (Opi et al. 2002) and in macaques heterologous challenge with $SHIV_{BX08}$ offered protection (Watkins et al. 2006). In this study, adjuvants that are approved for human usage were also tested and Montanide ISA720 and Calcium phosphate were found to be most suitable. Tat peptides have also been used to elicit antibody response to Tat and have shown to elicit response to multiple epitopes when a pool of peptide conjugates was used to immunize experimental animals and were challenged with $SHIV_{33}$. Interestingly, the antibodies blocked not only the apoptotic activity of Tat but also controlled viremia significantly and prevented CD4+ attrition rate although failed in controlling the acute infection (Belliard et al. 2005; G Goldstein et al. 2000).

In contrast to the results that demonstrate efficacy of Tat as a vaccine, studies exist where partial (Pauza et al. 2000) or no protection (Allen et al. 2002; Silera et al. 2002) was observed upon challenging intrarectally or intravenously with $SHIV_{89.6P}$. Also, a replication-competent adenovirus (Ad5) expressing Tat (86 aa) failed to protect rhesus

monkeys against homologous challenge with $SHIV_{89.6P}$ (Demberg et al. 2007). Although the reasons for these contrasting results are not known, it is likely that difference in the viral strain, Tat sequence, challenge route and dose and host factors could be important. In a recent comparative study, biologically active Tat was tested in cynomolgus and rhesus monkeys and the association of MHC-I and MHC-II haplotypes with the control of plasma viremia after intravenous challenge of $SHIV_{89.6P}$ was indentified. This study suggests the importance of challenge dose and also of genetic makeup in vaccine outcome (Florese et al. 2008).

Immunogenicity and safety of Tat as a mucosal vaccine has been evaluated in mice after intranasal immunization either as native protein alone or with adjuvants. These studies proved to be safe and also elicited cellular and humoral immune responses (Borsutzky et al. 2003; Marinaro et al. 2003; Morris et al. 2001). Recently three independent studies on Tat-transgenic plants to deliver Tat orally have been reported. While no immunogenicity studies were reported in potato transgenic plant (Kim & Langridge 2004), mice fed with spinach expressing Tat elicited a response only when boosted with DNA expressing Tat (Karasev et al. 2005). In another study, Tat was expressed in tomato and mice fed orally with the tomato extracts developed IgA in the mucous tissue and IgG in the serum (Partidos et al. 2004).

1.5.3.1 Approaches to improve stability and immunogenicity of Tat

Tat oxidizes when exposed to light and air and is also labile at tome temperature due to the presence of Cys residues and this oxidation leads to multimerization and loss of biological activity (B Ensoli et al. 2006). As it has been shown that it is important for Tat to be in the monomeric form to retain its immunomodulatory property it is considered important to maintain the native forms of Tat. To enhance the shelf-life, increase immunogenicity and to abrogate possible toxic properties of the protein, Tat vaccines

with novel delivery systems is being tested. Exploiting the highly basic region of Tat consisting of arginine and lysine, novel anionic biocompatible core-shell nano- and micro-spheres were developed consisting of poly (methylmethacrylate) (PMMA) core and an hydrophilic shell composed of poly (methacrylic acid-*st*-ethyl acrylate) copolymer. These particles bind Tat, prevent it from oxidation and release it both *in vitro* and *in vivo*. Importantly, these particles are well tolerated in mice and macaque models and vaccine formulations with these particles elicit both humoral and cell mediated immune responses (Caputo et al. 2008; Sparnacci et al. 2005; Voltan et al. 2007). Molecular adjuvants such as C3d-tagged, ubiquitin-tagged and codon-optimized Tat as DNA developed in our laboratory elicited strong cell mediated immune responses in mice (Ramakrishna et al. 2004). Recently, Goldstein G & Chicca J J conjugated the immunodominant amino-terminal epitope of Tat protein to a universal T-helper epitope and TLR2 agonist and demonstrated that the humoral immune response is augmented in immunized mice (Goldstein G & Chicca J J. 2010). However, none of the methods mentioned have addressed the possible means to both augment immune responses and also abrogate the possible toxic properties of Tat. To this end, our lab has developed a new strategy of addressing these shortcomings by inserting universal T-helper epitopes into the CRD and BD of Tat. This strategy not only abrogates the apoptotic and transactivation property of Tat but also enhances the cell mediated and humoral immune response (Anand KK et al. manuscript in preparation; present study).

1.5.4 Clinical trials with native Tat protein

Based on these positive results in the preclinical studies, biologically active Tat as a vaccine has been forwarded to clinical trials. In a phase I trial, Tat-vaccine was parallel tested as a preventive as well as a therapeutic vaccine. The primary end-point of the study

being safety and the secondary end-point being immunogenicity. Both the studies were randomized, double-blinded and placebo controlled. Vaccination was carried out in two different immunization routes with one being sub-cutaneous (s.c) and the other being intradermal (i.d). The vaccination was found to be safe and also elicited IgM, IgG and IgA responses in therapeutic protocol (B Ensoli et al. 2008; Longo et al. 2009). Further, 100% of the vaccinated individuals demonstrated cellular response to Tat quantified by measuring IFN-gamma and or IL-4 and or proliferation. Remarkably, the circulating CD4+ cells were preserved and there was no significant viral rebound in therapeutic vaccination. Also, a correlation was observed with IgA titers and the viral loads in both the forms of immunization (s.c and i.d). Boosted by the results of the phase I trial, the Tat-vaccine has been pushed to phase II therapeutic trial in Italy (http://www.hiv1tat-vaccines.info/). Although, the Tat-vaccine study has been forwarded, several scientists believe that using biologically active Tat can prove to be derogatory as Tat is immunosuppressive and also can lead to death of immune cells. Few others believe that a Tat-based vaccine may not work as the existence of extracellular Tat has not been proved beyond doubt. Interestingly, a recent report by Rayne et al. demonstrates a high-affinity interaction between Tat and phosphotidylinositol-4,5-bisphosphate (PI-[4,5]-P2). The authors go on to show that by binding to PI-(4,5)-P2, Tat accumulates in the plasma membrane and is exported to the extracellular milieu in large amounts (~50% of total Tat synthesized in an infected cell) with the extracellular concentrations reaching up to 0.25 nM (Rayne et al. 2010).

In spite of the demonstration that Tat as a native protein is safe, concerns regarding its safety profile persist. To make Tat non-toxic, various strategies have been employed, one of them being the use of transdominant Tat protein, which lacked the transactivation property but retained the immunogenicity which has been tested and found

to be efficient (A Caputo et al. 1996; Caselli et al. 1999). Tat protein has also been rendered non-toxic by various chemical treatments such as carboxymethylation (Max W Richardson et al. 2002), and the Tat-toxoid elicited immune responses similar to that of native Tat. However, this efficiency has not been reproduced by other groups and conflicting results have been reported (Silvera et al. 2002).

1.5.5 Multi-component Tat vaccines

Various strategies to broaden the immune responses in sub-unit vaccine have been devised and both structural and regulatory proteins have been tried in combination with Tat. Strategies to elicit humoral and cell-mediated immune response to a greater number of strains and also possibly multiple target antigens have are now being evaluated.

Tat has been combined with Gag and Env and the preclinical and clinical evaluation of this combination is being evaluated. The logic for using the structural proteins with the Tat is to target an early protein (Tat) as well as late proteins (Gag and Env) and therefore broadening the immune response. To this end, vaccines with Tat, Gag and Env or V2-deleted Env protein were tested in mice and humans. A strong humoral and cellular immune response was elicited against the co-administered antigens as well as Tat (Wahren et al. 2002; Cellini et al. 2008). Further, this combination has also been tried as a mucosal vaccine with LTK63 adjuvant by intranasal administration in mice and a long lasting local and systemic humoral and cellular response was elicited (Caputo et al. 2008). This combination was tested in macaques in a DNA prime/protein boost regimen and a strong antibody and cellular response controlled the loss of CD4+ cells and disease progression upon SHIV89.6P challenge (Mooij et al. 2004). However, in a study where in an ALVAC-formulation (canary pox) based vaccine containing gag-pol or gag-pol-env

was boosted with Tat protein, little or no effect on the protection of CD4+ depletion and viral load was observed (Pal et al. 2006). The possible explanation for this could be the timing of Tat administration, as in \this study Tat was not co-administered; the immunomodulatory effect might not have been elicited and further due to the immunodominance of the Gag and Env, administering Tat post Gag and Env immunization may not elicit a good response. In another study Gupta et al. showed that immunizing mice with DNA co-expressing Tat and gp120 led to immune suppression in an IL-10 dependant manner (Gupta et al. 2008). Although, the reasons for this is not clearly understood, it is hypothesized that in this study while screening for response to gp120 only few peptides were used which do not correspond to the entire length of the protein.

Tat along with Rev or Nef has been found to be efficient in eliciting B- and T-cell responses in a DNA immunization regimen. In this therapeutic vaccination study HIV infected individuals who had very low or no response to these antigens prior to immunization were subjected to genetic immunization and the response was monitored for antibodies and CD8+ responses (Hinkula et al. 1997; Calarota 1998). Furthermore, DNA vaccination of mice with subtype C Tat, Rev and Nef elicited a strong CD8+ response (Scriba et al. 2005). A recombinant Semiliki Forest virus or modified virus Ankara coding for Tat and Rev from SIV_{mac32H} was used to vaccinate cynomolgus monkeys and was followed with a homologous viral challenge. At the time of evaluation, no cell associated virus was identified. Furthermore, it was shown that the vaccination with regulatory proteins performed better than the same vectors coding for Gag and Pol (Stittelaar et al. 2002). More recently, a chimeric gene of the three regulatory proteins, called the ‘Retanef’ encoding Rev, Tat and Nef of SIV was evaluated as a eukaryotic expression plasmid, poxvirus based vectors or a lentiviral vector (Hel et al. 2002; Scott G

Hansen et al. 2009). Vaccination of monkeys with Retanef not only established effector memory cells but also controlled viral loads.

More recently, alternative and attractive approaches have been developed where in the vaccine formulations contain multiple HIV antigens, including structural, enzymes, regulatory and accessory genes. The strategy to include of several antigens from multiple clades seems attractive as the immune response can be expected to be broad and hence prevent the out-growth of escape variants. However, caution should be exercised when including sub-dominant antigen such as Tat in a multi-component, multi-clade HIV vaccine along with the immunodominant structural proteins as this could lead to immune interference against Tat as was demonstrated in the case of a HCV vaccine recently (Riedl et al. 2009). A multi-domain DNA vaccine which co-expressed immunodominant and sub-dominant epitopes was used to vaccinate mice and it was observed that, unless the immunodominant epitope were removed, response to the sub-dominant epitope was poor. In a similar vein, co-administration of an immunodominant Gag peptide suppressed the CTL responses to an Env peptide (Manuel et al. 2009) thus abrogating a broad immune response which is essential for not only the control of the viral load, but also to circumvent the emergence of escape mutants. In the design of multi-component vaccines for HIV, the problem of immune suppression against sub-dominant antigens like Tat must be carefully considered.

1.6 Motivation for the present work

Literature currently available supports Tat as an important vaccine candidate owing mainly to its role in viral life cycle and pathogenesis and for the fact that immune response to Tat is negatively correlated with disease progression. Although use of Tat

alone as a vaccine has met with opposition, Tat in multi-subunit and multi-component vaccines has provided much promise. However, certain aspects of the Tat-based vaccines are yet to be addressed and in the present study efforts are made to contribute to the present understanding of immune response to Tat in natural infections and also experimental animals. The key motivating factors for the present study include the following:

- Immune response to Tat in Indian scenario has not been addressed so far. India harbors the second largest number of HIV positive individuals and Tat being an important vaccine candidate it is imperative to understand the response elicited to Tat in natural infections. Further, subtype C, which accounts for nearly 56% of global infection is the predominant strain in this population. Studies attempting to evaluate the contribution of host factors in susceptibility and protection to infection have shown that the HLA alleles in India represent a different pattern as compared to other races (Umapathy et al. 2007; Umapathy et al. 2004) and hence underscoring the need of studying immune response to Tat in different geographical locations which may represent genetically distinct population. This information has direct implications in designing an effective and rational vaccine against HIV to circumvent the spread of the virus.

- Despite several studies evaluating Tat as a vaccine candidate, issues pertaining to the toxic property and moderate immunogenicity are yet to be satisfactorily addressed. Previous studies from our lab have demonstrated that engineering universal T-helper epitopes into the CRD and BD of Tat not only abrogates the toxic properties, but also enhances the cell-mediated responses to Tat in DNA immunization format. However, while DNA immunizations elicit cell-mediated immune responses, protein immunizations predominantly elicit a strong humoral response and humoral immune responses are

essential to block the pathogenic effects of extracellular Tat. Further, it is increasingly becoming clear that both the wings of immune system are essential for an effective vaccine against HIV. Hence, in chapter 3, the efficacy of Tat harboring universal T-helper epitopes in eliciting a strong CD4+ and antibody response has been evaluated. Additionally, monoclonal antibodies which are directed to different domains of Tat have been generated. These antibodies can be important tools in understanding the functions of Tat.

Bibliography

Abrams, D. et al., 2009. Interleukin-2 therapy in patients with HIV infection. The New England Journal of Medicine, 361(16), 1548-1559.

Addo, M.M. et al., 2001. The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. Proceedings of the National Academy of Sciences of the United States of America, 98(4), 1781-1786.

Addo, M.M. et al., 2002. Cytotoxic T-lymphocyte (CTL) responses directed against regulatory and accessory proteins in HIV-1 infection. DNA and Cell Biology, 21(9), 671-678.

Addo, M.M. et al., 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. Journal of Virology, 77(3), 2081-2092.

Agbottah, E. et al., 2006. Effect of SWI/SNF chromatin remodeling complex on HIV-1 Tat activated transcription. Retrovirology, 3, 48.

Albini, A. et al., 1998. HIV-1 Tat protein mimicry of chemokines. Proceedings of the National Academy of Sciences of the United States of America, 95(22), 13153-13158.

Aldovini, A. & Young, R.A., 1990. Development of a BCG recombinant vehicle for candidate AIDS vaccines. International Reviews of Immunology, 7(1), 79-83.

Allen, T.M. et al., 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature, 407(6802), 386-390.

Allen, T.M. et al., 2002. STI and beyond: the prospects of boosting anti-HIV immune responses. Trends in Immunology, 23(9), 456-460.

Allen, T.M. et al., 2002a. Tat-Vaccinated Macaques Do Not Control Simian Immunodeficiency Virus SIVmac239 Replication. J. Virol., 76(8), 4108-4112.

Altfeld, M. et al., 2001. Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. The Journal of Experimental Medicine, 193(2), 169-180.

Ambrosino, C. et al., 1997. HIV-1 Tat induces the expression of the interleukin-6 (IL6) gene by binding to the IL6 leader RNA and by interacting with CAAT enhancer-binding protein beta (NF-IL6) transcription factors. The Journal of Biological Chemistry, 272(23), 14883-14892.

Arduino, R.C. et al., 2004. CD4 cell response to 3 doses of subcutaneous interleukin 2: meta-analysis of 3 Vanguard studies. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 39(1), 115-122.

Baba, T.W. et al., 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nature Medicine, 6(2), 200-206.

Badou, A. et al., 2000. Tat protein of human immunodeficiency virus type 1 induces interleukin-10 in human peripheral blood monocytes: implication of protein kinase C-dependent pathway. Journal of Virology, 74(22), 10551-10562.

Bahraoui, E. et al., 1990. Immunogenicity of the human immunodeficiency virus (HIV) recombinant nef gene product. Mapping of T-cell and B-cell epitopes in immunized chimpanzees. AIDS Research and Human Retroviruses, 6(9), 1087-1098.

Baker, B.M. et al., 2009. Elite control of HIV infection: implications for vaccine design. Expert Opinion on Biological Therapy, 9(1), 55-69.

Bal-Price, A., Moneer, Z. & Brown, G.C., 2002. Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. Glia, 40(3), 312-323.

Banks, W.A., Robinson, S.M. & Nath, A., 2005. Permeability of the blood-brain barrier to HIV-1 Tat. Experimental Neurology, 193(1), 218-227.

Barillari, G., Sgadari, C., Fiorelli, V. et al., 1999. The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the alpha5beta1 and alphavbeta3 integrins and by mobilizing sequestered basic fibroblast growth factor. Blood, 94(2), 663-672.

Barillari, G., Sgadari, C., Palladino, C. et al., 1999. Inflammatory cytokines synergize with the HIV-1 Tat protein to promote angiogenesis and Kaposi's sarcoma via induction of basic fibroblast growth factor and the alpha v beta 3 integrin. Journal of Immunology (Baltimore, Md.: 1950), 163(4), 1929-1935.

Barouch, D.H. et al., 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science (New York, N.Y.), 290(5491), 486-492.

Barouch, D.H., 2008. Challenges in the development of an HIV-1 vaccine. Nature, 455(7213), 613-619.

Bartz, S.R. & Emerman, M., 1999. Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. Journal of Virology, 73(3), 1956-1963.

Bayer, P. et al., 1995. Structural studies of HIV-1 Tat protein. Journal of Molecular Biology, 247(4), 529-535.

Belliard, G. et al., 2005. Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. Vaccine, 23(11), 1399-407.

Bello, G. et al., 2008. Origin and evolutionary history of HIV-1 subtype C in Brazil. AIDS (London, England), 22(15), 1993-2000.

Benelli, R. et al., 2000. Human immunodeficiency virus transactivator protein (Tat) stimulates chemotaxis, calcium mobilization, and activation of human polymorphonuclear leukocytes: implications for Tat-mediated pathogenesis. The Journal of Infectious Diseases, 182(6), 1643-1651.

Benlahrech, A. et al., 2009. Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. Proceedings of the National Academy of Sciences of the United States of America, 106(47), 19940-19945

Bennasser, Y. & Jeang, K., 2006. HIV-1 Tat interaction with Dicer: requirement for RNA. Retrovirology, 3, 95.

Bennasser, Y. et al., 2005. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity, 22(5), 607-619.

Berkhout, B., Silverman, R.H. & Jeang, K.T., 1989. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. Cell, 59(2), 273-282.

Betts, M.R., Yusim, K. & Koup, R.A., 2002. Optimal Antigens for HIV Vaccines Based on CD8+ T Response, Protein Length, and Sequence Variability. *DNA and Cell Biology*, 21(9), 665-670.

Bieniasz, P.D. et al., 1998. Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. The EMBO Journal, 17(23), 7056-7065.

Blankson, J., Persaud, D. & Siliciano, R.F., 1999. Latent reservoirs for HIV-1. Current Opinion in Infectious Diseases, 12(1), 5-11.

Blazevic, V. et al., 1993. Helper T-cell recognition of HIV-1 Tat synthetic peptides. Journal of Acquired Immune Deficiency Syndromes, 6(8), 881-890.

Borgatti, P. et al., 1997. Extracellular HIV-1 Tat protein activates phosphatidylinositol 3- and Akt/PKB kinases in CD4+ T lymphoblastoid Jurkat cells. European Journal of Immunology, 27(11), 2805-2811.

Borsetti, A. et al., 2009. Containment of infection in tat vaccinated monkeys after rechallenge with a higher dose of SHIV89.6P(cy243). Viral Immunology, 22(2), 117-124

Borsutzky, S. et al., 2003. Efficient mucosal delivery of the HIV-1 Tat protein using the synthetic lipopeptide MALP-2 as adjuvant. European Journal of Immunology, 33(6), 1548-1556.

Brady, J. & Kashanchi, F., 2005. Tat gets the "green" light on transcription initiation. Retrovirology, 2, 69.

Brake, D.A., Debouck, C. & Biesecker, G., 1990. Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type 1 transactivation protein, tat. The Journal of Cell Biology, 111(3), 1275-1281.

Brenchley, J.M. et al., 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. The Journal of Experimental Medicine, 200(6), 749-759.

Brenner, B.G., Gryllis, C. & Wainberg, M.A., 1991. Role of antibody-dependent cellular cytotoxicity and lymphokine-activated killer cells in AIDS and related diseases. Journal of Leukocyte Biology, 50(6), 628-640.

Brigati, C. et al., 2003. HIV Tat, its TARgets and the control of viral gene expression. FEMS Microbiology Letters, 220(1), 57-65.

Buttò, S. et al., 2003. Sequence conservation and antibody cross-recognition of clade B human immunodeficiency virus (HIV) type 1 Tat protein in HIV-1-infected Italians, Ugandans, and South Africans. The Journal of infectious diseases, 188(8), 1171-80.

Cafaro, A. et al., 1999. Control of SHIV-89.6 P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. Nature medicine, 5(6), 643-650.

Cafaro, A. et al., 2001. Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). Vaccine, 19(20-22), 2862-2877.

Calarota, S. et al., 1998. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. Lancet, 351(9112), 1320-1325.

Calzado, M.A., Sancho, R. & Muñoz, E., 2004. Human immunodeficiency virus type 1 Tat increases the expression of cleavage and polyadenylation specificity factor 73-kilodalton subunit modulating cellular and viral expression. Journal of Virology, 78(13), 6846-6854.

Campbell, G. & Loret, E., 2009. What does the structure-function relationship of the HIV-1 Tat protein teach us about developing an AIDS vaccine? Retrovirology, 6(1), 50.

Campbell, G.R. et al., 2004. The Glutamine-rich Region of the HIV-1 Tat Protein Is Involved in T-cell Apoptosis. J. Biol. Chem., 279(46), 48197-48204.

Campbell, G.R. et al., 2005. The C terminus of HIV-1 Tat modulates the extent of CD178-mediated apoptosis of T cells. The Journal of Biological Chemistry, 280(46), 38376-38382.

Campbell, G.R., Senkaali, D. et al., 2007. Tat mutations in an African cohort that do not prevent transactivation but change its immunogenic properties. Vaccine, 25(50), 8441-8447.

Campbell, G.R., Watkins, J.D. et al., 2007. Human immunodeficiency virus type 1 subtype C Tat fails to induce intracellular calcium flux and induces reduced tumor necrosis factor production from monocytes. Journal of Virology, 81(11), 5919-5928.

Cao, J., McNevin, J., Holte, S. et al., 2003. Comprehensive analysis of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-secreting CD8+ T cells in primary HIV-1 infection. Journal of Virology, 77(12), 6867-6878.

Cao, J., McNevin, J., Malhotra, U. et al., 2003. Evolution of CD8+ T cell immunity and viral escape following acute HIV-1 infection. Journal of Immunology (Baltimore, Md.: 1950), 171(7), 3837-3846.

Capitini, C.M., Chisti, A.A. & Mackall, C.L., 2009. Modulating T-cell homeostasis with IL-7: preclinical and clinical studies. Journal of Internal Medicine, 266(2), 141-153.

Caputo, A. et al., 1996. Inhibition of HIV-1 replication and reactivation from latency by tat transdominant negative mutants in the cysteine rich region. Gene Therapy, 3(3), 235-245.

Caputo, A. et al., 1997. Studies on the effect of the combined expression of anti-tat and anti-rev genes on HIV-1 replication. Gene Therapy, 4(4), 288-295.

Caputo, A. et al., 2003. Immunization with low doses of HIV-1 tat DNA delivered by novel cationic block copolymers induces CTL responses against Tat. Vaccine, 21(11-12), 1103-11.

Caputo, A., Gavioli, R. & Ensoli, B., 2004. Recent advances in the development of HIV-1 Tat-based vaccines. Current HIV Research, 2(4), 357-376.

Caputo, A. et al., 2008. Functional polymeric nano/microparticles for surface adsorption and delivery of protein and DNA vaccines. Current Drug Delivery, 5(4), 230-242.

Caputo, A., Brocca-Cofano, E. et al., 2008. Characterization of immune responses elicited in mice by intranasal co-immunization with HIV-1 Tat, gp140 DeltaV2Env and/or SIV Gag proteins and the nontoxicogenic heat-labile Escherichia coli enterotoxin. Vaccine, 26(9), 1214-1227.

Caputo, A. et al., 2009. HIV-1 Tat-based vaccines: an overview and perspectives in the field of HIV/AIDS vaccine development. International Reviews of Immunology, 28(5), 285-334.

Carotenuto, P. et al., 1998. Neutralizing antibodies are positively associated with CD4+ T-cell counts and T-cell function in long-term AIDS-free infection. AIDS (London, England), 12(13), 1591-1600.

Carroll, R., Martarano, L. & Derse, D., 1991. Identification of lentivirus tat functional domains through generation of equine infectious anemia virus/human immunodeficiency virus type 1 tat gene chimeras. Journal of Virology, 65(7), 3460-3467.

Caselli, E. et al., 1999. DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. Journal of Immunology (Baltimore, Md.: 1950), 162(9), 5631-5638.

Castelli, F.A. et al., 2008. Immunoprevalence of the CD4+ T-cell response to HIV Tat and Vpr proteins is provided by clustered and disperse epitopes, respectively. European Journal of Immunology, 38(10), 2821-2831.

Cellini, S. et al., 2008. Identification of new HIV-1 Gag-specific cytotoxic T lymphocyte responses in BALB/c mice. Virology Journal, 5, 81

Chanda, P.K., Ghrayeb, J. & Wong-Staal, F., 1988. Nonrandom distribution of antibodies to the TRS protein of human immunodeficiency virus in infected people with different clinical status. AIDS Research and Human Retroviruses, 4(1), 11-16.

Chang, H.C. et al., 1997. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS (London, England), 11(12), 1421-1431.

Chauhan, A. et al., 2003. Intracellular human immunodeficiency virus Tat expression in astrocytes promotes astrocyte survival but induces potent neurotoxicity at distant sites via axonal transport. The Journal of Biological Chemistry, 278(15), 13512-13519.

Chen, D. et al., 2002. HIV-1 Tat targets microtubules to induce apoptosis, a process promoted by the pro-apoptotic Bcl-2 relative Bim. The EMBO Journal, 21(24), 6801-6810.

Chen, P. et al., 1997. The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. The Journal of Biological Chemistry, 272(36), 22385-22388.

Chirmule, N. et al., 1995. Human immunodeficiency virus Tat induces functional unresponsiveness in T cells. Journal of Virology, 69(1), 492-498.

Chong, S. et al., 2007. Comparative ability of plasmid IL-12 and IL-15 to enhance cellular and humoral immune responses elicited by a SIVgag plasmid DNA vaccine and alter disease progression following SHIV(89.6P) challenge in rhesus macaques. Vaccine, 25(26), 4967-4982.

Chouquet, C. et al., 2002. Correlation between breadth of memory HIV-specific cytotoxic T cells, viral load and disease progression in HIV infection. AIDS (London, England), 16(18), 2399-2407.

Cohen, J., 2003. Public health. AIDS vaccine trial produces disappointment and confusion. Science (New York, N.Y.), 299(5611), 1290-1291.

Corey, L., McElrath, M.J. & Kublin, J.G., 2009. Post-step modifications for research on HIV vaccines. AIDS (London, England), 23(1), 3-8.

Cupp, C. et al., 1993. Evidence for stimulation of the transforming growth factor beta 1 promoter by HIV-1 Tat in cells derived from CNS. Oncogene, 8(8), 2231-2236.

Dairaku, M. et al., 1989. Incidence and titer of anti-rev antibody in HIV infection. AIDS (London, England), 3(11), 765-766.

Day, C.L. et al., 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature, 443(7109), 350-354.

Dayton, A.I. et al., 1986. The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. Cell, 44(6), 941-947.

de la Vega, L. et al., 2007. The 73 kDa subunit of the CPSF complex binds to the HIV-1 LTR promoter and functions as a negative regulatory factor that is inhibited by the HIV-1 Tat protein. Journal of Molecular Biology, 372(2), 317-330.

de Mareuil, J. et al., 2005. HIV-1 Tat protein enhances microtubule polymerization. Retrovirology, 2,5.

Demirhan, I. et al., 1999. Detection of distinct patterns of anti-tat antibodies in HIV-infected individuals with or without Kaposi's sarcoma. Journal of Acquired Immune Deficiency Syndromes (1999), 22(4), 364-368.

Demirhan, I. et al., 2000. Antibody spectrum against the viral transactivator protein in patients with human immunodeficiency virus type 1 infection and Kaposi's sarcoma. Journal of Human Virology, 3(3), 137-143.

Dhawan, S. et al., 1997. Human immunodeficiency virus-1-tat protein induces the cell surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human endothelial cells. Blood, 90(4), 1535-1544.

Dingwall, C. et al., 1989. Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. Proceedings of the National Academy of Sciences of the United States of America, 86(18), 6925-6929.

Ehret, A. et al., 2001. The effect of HIV-1 regulatory proteins on cellular genes: derepression of the IL-2 promoter by Tat. European Journal of Immunology, 31(6), 1790-1799.

Emini, E.A. et al., 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature, 355(6362), 728-730.

Ensoli, B. et al., 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature, 345(6270), 84-86.

Ensoli, B. et al., 1993. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. Journal of Virology, 67(1), 277-287.

Ensoli, B. et al., 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature, 371(6499), 674-680.

Feldblum, P.J. et al., 2008. SAVVY vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria. PloS One, 3(1), e1474.

Ferre, A.L. et al., 2009. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. Blood, 113(17), 3978-3989.

Fisher, A.G. et al., 1986. The trans-activator gene of HTLV-III is essential for virus replication. Nature, 320(6060), 367-371.

Florese, R.H. et al., 2008. Comparative study of Tat vaccine regimens in Mauritian cynomolgus and Indian rhesus macaques: influence of Mauritian MHC haplotypes on susceptibility/resistance to SHIV(89.6P) infection. Vaccine, 26(26), 3312-3321

Fontella, R., Soares, M.A. & Schrago, C.G., 2008. On the origin of HIV-1 subtype C in South America. AIDS (London, England), 22(15), 2001-2011.

Franchini, G. et al., 1987. Spectrum of natural antibodies against five HTLV-III antigens in infected individuals: correlation of antibody prevalence with clinical status. Blood, 69(2), 437-441.

Fulcher, A.J. & Jans, D.A., 2003. The HIV-1 Tat transactivator protein: a therapeutic target? IUBMB Life, 55(12), 669-680.

Gallo, R.C., 2005. The end or the beginning of the drive to an HIV-preventive vaccine: a view from over 20 years. Lancet, 366(9500), 1894-1898.

Garber, M.E. & Jones, K.A., 1999. HIV-1 Tat: coping with negative elongation factors. Current Opinion in Immunology, 11(4), 460-465.

Gavioli, R. et al., 2004. HIV-1 tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity. Journal of Immunology (Baltimore, Md.: 1950), 173(6), 3838-3843.

Ghosh, S., Selby, M.J. & Peterlin, B.M., 1993. Synergism between Tat and VP16 in trans-activation of HIV-1 LTR. Journal of Molecular Biology, 234(3), 610-619.

Ghosn, J. et al., 2005. No benefit of a structured treatment interruption based on genotypic resistance in heavily pretreated HIV-infected patients. AIDS (London, England), 19(15), 1643-1647.

Giacca, M., 2004. The HIV-1 Tat protein: a multifaceted target for novel therapeutic opportunities. Current Drug Targets. Immune, Endocrine and Metabolic Disorders, 4(4), 277-285.

Gibellini, D. et al., 1997. Upregulation of c-Fos in activated T lymphoid and monocytic cells by human immunodeficiency virus-1 Tat protein. Blood, 89(5), 1654-1664.

Gibellini, D. et al., 1998. Extracellular HIV-1 Tat protein induces the rapid Ser133 phosphorylation and activation of CREB transcription factor in both Jurkat lymphoblastoid T cells and primary peripheral blood mononuclear cells. Journal of Immunology (Baltimore, Md.: 1950), 160(8), 3891-3898.

Girard, M. et al., 1995. Vaccine-induced protection of chimpanzees against infection by a heterologous human immunodeficiency virus type 1. Journal of Virology, 69(10), 6239-6248.

Girard, M.P., Osmanov, S.K. & Kieny, M.P., 2006. A review of vaccine research and development: the human immunodeficiency virus (HIV). Vaccine, 24(19), 4062-4081.

Goldstein, G. et al., 2000. Minimization of chronic plasma viremia in rhesus macaques immunized with synthetic HIV-1 Tat peptides and infected with a chimeric simian/human immunodeficiency virus (SHIV33). Vaccine, 18(25), 2789-2795.

Goldstein, G. & Chicca, J.J., 2010. A universal anti-HIV-1 Tat epitope vaccine that is fully synthetic and self-adjuvanting. Vaccine, 28(4), 1008-1014

Goulder, P.J. et al., 2001. Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. The Journal of Experimental Medicine, 193(2), 181-194.

Gregoire, C.J. & Loret, E.P., 1996. Conformational heterogeneity in two regions of TAT results in structural variations of this protein as a function of HIV-1 isolates. The Journal of Biological Chemistry, 271(37), 22641-22646.

Gruters, R.A., van Baalen, C.A. & Osterhaus, A.D.M.E., 2002. The advantage of early recognition of HIV-infected cells by cytotoxic T-lymphocytes. Vaccine, 20(15), 2011-2015.

Gülow, K. et al., 2005. HIV-1 trans-activator of transcription substitutes for oxidative signaling in activation-induced T cell death. Journal of Immunology (Baltimore, Md.: 1950), 174(9), 5249-5260.

Gupta, S. et al., 2008. HIV-1 Tat suppresses gp120-specific T cell response in IL-10-dependent manner. Journal of Immunology (Baltimore, Md.: 1950), 180(1), 79-88.

Hansen, S.G. et al., 2009. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nature Medicine, 15(3), 293-299.

Harari, A. & Pantaleo, G., 2008. HIV-1-specific immune response. Advances in Pharmacology (San Diego, Calif.), 56, 75-92.

Harrich, D. et al., 1997. Tat is required for efficient HIV-1 reverse transcription. The EMBO Journal, 16(6), 1224-1235.

Hel, Z. et al., 2002. A novel chimeric Rev, Tat, and Nef (Retanef) antigen as a component of an SIV/HIV vaccine. Vaccine, 20(25-26), 3171-86.

Hemelaar, J. et al., 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS (London, England), 20(16), W13-23.

Hessell, A.J., Poignard, P. et al., 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nature Medicine. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19525965 [Accessed July 7, 2009].

Hessell, A.J., Rakasz, E.G. et al., 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathogens, 5(5), e1000433.

Hirbod, T. & Broliden, K., 2007. Mucosal immune responses in the genital tract of HIV-1-exposed uninfected women. Journal of Internal Medicine, 262(1), 44-58.

Hirbod, T. et al., 2008. HIV-neutralizing immunoglobulin A and HIV-specific proliferation are independently associated with reduced HIV acquisition in Kenyan sex workers. AIDS (London, England), 22(6), 727-735.

Hone, D.M. et al., 1996. Optimization of live oral Salmonella-HIV-1 vaccine vectors for the induction of HIV-specific mucosal and systemic immune responses. Journal of Biotechnology, 44(1-3), 203-207.

Huang, L. et al., 1998. Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. Journal of Virology, 72(11), 8952-8960.

Huang, L., Li, C.J. & Pardee, A.B., 1997. Human immunodeficiency virus type 1 TAT protein activates B lymphocytes. Biochemical and Biophysical Research Communications, 237(2), 461-464.

Huang, S.K. et al., 1993. Extravasation and transcytosis of liposomes in Kaposi's sarcoma-like dermal lesions of transgenic mice bearing the HIV tat gene. The American Journal of Pathology, 143(1), 10-14.

Huber, M. & Trkola, A., 2007. Humoral immunity to HIV-1: neutralization and beyond. Journal of Internal Medicine, 262(1), 5-25.

Huet, T. et al., 1989. A highly defective HIV-1 strain isolated from a healthy Gabonese individual presenting an atypical western blot. AIDS (London, England), 3(11), 707-715.

Huigen, M.C.D.G., Kamp, W. & Nottet, H.S.L.M., 2004. Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection. European Journal of Clinical Investigation, 34(1), 57-66.

Hutnick, N.A. et al., 2009. Baseline Ad5 serostatus does not predict Ad5 HIV vaccine-induced expansion of adenovirus-specific CD4+ T cells. *Nature Medicine*, 15(8), 876-878.

Igarashi, T. et al., 1999. Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. Nature Medicine, 5(2), 211-216.

Imamichi, H., Sereti, I. & Lane, H.C., 2008. IL-15 acts as a potent inducer of CD4(+)CD25(hi) cells expressing FOXP3. European Journal of Immunology, 38(6), 1621-1630.

Jeang, K.T., Xiao, H. & Rich, E.A., 1999. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. The Journal of Biological Chemistry, 274(41), 28837-28840.

Jones, M. et al., 1998. Intraventricular injection of human immunodeficiency virus type 1 (HIV-1) tat protein causes inflammation, gliosis, apoptosis, and ventricular enlargement. Journal of Neuropathology and Experimental Neurology, 57(6), 563-570.

Kameoka, M. et al., 2001. Role for human immunodeficiency virus type 1 Tat protein in suppression of viral reverse transcriptase activity during late stages of viral replication. Journal of Virology, 75(6), 2675-2683.

Kameoka, M. et al., 2002. The Tat protein of human immunodeficiency virus type 1 (HIV-1) can promote placement of tRNA primer onto viral RNA and suppress later DNA polymerization in HIV-1 reverse transcription. Journal of Virology, 76(8), 3637-3645.

Kao, S.Y. et al., 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature, 330(6147), 489-493.

Karn, J., 1999. Tackling Tat. Journal of Molecular Biology, 293(2), 235-254.

Karasev, A.V. et al., 2005. Plant based HIV-1 vaccine candidate: Tat protein produced in spinach. Vaccine, 23(15), 1875-1880

Kashanchi, F. et al., 1994. Direct interaction of human TFIID with the HIV-1 transactivator tat. Nature, 367(6460), 295-299.

Kaufmann, D.E. et al., 2004. Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. Journal of Virology, 78(9), 4463-4477.

Kiefer, H.L.B. et al., 2004. Retinoic acid inhibition of chromatin remodeling at the human immunodeficiency virus type 1 promoter. Uncoupling of histone acetylation and chromatin remodeling. The Journal of Biological Chemistry, 279(42), 43604-43613.

Kim, T. & Langridge, W.H.R., 2004. Synthesis of an HIV-1 Tat transduction domain-rotavirus enterotoxin fusion protein in transgenic potato. Plant Cell Reports, 22(6), 382-387.

Klenerman, P., Wu, Y. & Phillips, R., 2002. HIV: current opinion in escapology. Current Opinion in Microbiology, 5(4), 408-413.

Krone, W.J.A. et al., 1988. Natural antibodies to HIV-tat epitopes and expression of HIV-1 genes in vivo. Journal of Medical Virology, 26(3), 261-270.

Kuppuswamy, M. et al., 1989. Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. Nucleic Acids Research, 17(9), 3551-3561.

Lamhamedi-Cherradi, S. et al., 1992. Qualitative and quantitative analysis of human cytotoxic T-lymphocyte responses to HIV-1 proteins. AIDS (London, England), 6(11), 1249-1258.

Langlade-Demoyen, P. et al., 1994. Human immunodeficiency virus (HIV) nef-specific cytotoxic T lymphocytes in noninfected heterosexual contact of HIV-infected patients. The Journal of Clinical Investigation, 93(3), 1293-1297.

Lasaro, M.O. & Ertl, H.C.J., 2009. New insights on adenovirus as vaccine vectors. Molecular Therapy: The Journal of the American Society of Gene Therapy, 17(8), 1333-1339.

Laspia, M.F., Rice, A.P. & Mathews, M.B., 1989. HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. Cell, 59(2), 283-292.

Laspia, M.F., Rice, A.P. & Mathews, M.B., 1990. Synergy between HIV-1 Tat and adenovirus E1A is principally due to stabilization of transcriptional elongation. Genes & Development, 4(12B), 2397-2408.

Laus, M. et al., 2001. Complex associates of plasmid DNA and a novel class of block copolymers with PEG and cationic segments as new vectors for gene delivery. Journal of Biomaterials Science. Polymer Edition, 12(2), 209-228.

Lawrence, J. et al., 2006. Disadvantages of structured treatment interruption persist in patients with multidrug-resistant HIV-1: final results of the CPCRA 064 study. Journal of Acquired Immune Deficiency Syndromes (1999), 43(2), 169-178.

Learmont, J.C. et al., 1999. Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort. The New England Journal of Medicine, 340(22), 1715-1722.

Lefevre, E.A. et al., 1999. Cutting Edge: HIV-1 Tat Protein Differentially Modulates the B Cell Response of Naive, Memory, and Germinal Center B Cells. J Immunol, 163(3), 1119-1122.

Letvin, N.L., 2006. Progress and obstacles in the development of an AIDS vaccine. Nature Reviews. Immunology, 6(12), 930-939.

Letvin, N.L., 2009. Moving Forward in HIV Vaccine Development. Science, 326(5957), 1196-1198.

Levin, R. et al., 1997. Inhibition of early and late events of the HIV-1 replication cycle by cytoplasmic Fab intrabodies against the matrix protein, p17. Molecular Medicine (Cambridge, Mass.), 3(2), 96-110.

Li, C. et al., 1995. Induction of aphoptosis in uninfected lymphocytes by HIV-1 Tat protein. Science, 268(5209), 429-431.

Li, C.J. et al., 1995. Reciprocal modulations between p53 and Tat of human immunodeficiency virus type 1. Proceedings of the National Academy of Sciences of the United States of America, 92(12), 5461-5464.

Li, W. et al., 2008. NMDA receptor activation by HIV-Tat protein is clade dependent. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 28(47), 12190-12198.

Lichterfeld, M. et al., 2007. Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. Journal of Virology, 81(8), 4199-4214.

Lieberman, J. & Frankel, F.R., 2002. Engineered Listeria monocytogenes as an AIDS vaccine. Vaccine, 20(15), 2007-2010.

Lipton, S.A., 1993. Human immunodeficiency virus-infected macrophages, gp120, and N-methyl-D-aspartate receptor-mediated neurotoxicity. Annals of Neurology, 33(2), 227-228.

Longo, O. et al., 2009. Phase I therapeutic trial of the HIV-1 Tat protein and long term follow-up. Vaccine, 27(25-26), 3306-3312.

Luckay, A. et al., 2007. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. Journal of Virology, 81(10), 5257-5269.

Lundholm, P. et al., 2002. DNA mucosal HIV vaccine in humans. Virus Research, 82(1-2), 141-145.

Lusic, M. et al., 2003. Regulation of HIV-1 gene expression by histone acetylation and factor recruitment at the LTR promoter. The EMBO Journal, 22(24), 6550-6561.

Maggiorella, M.T. et al., 2004. Long-term protection against SHIV89.6P replication in HIV-1 Tat vaccinated cynomolgus monkeys. Vaccine, 22(25-26), 3258-3269.

Mahieux, R. et al., 2001. Cell cycle regulation of human interleukin-8 gene expression by the human immunodeficiency virus type 1 Tat protein. Journal of Virology, 75(4), 1736-1743.

Malim, M.H. et al., 1988. Immunodeficiency virus rev trans-activator modulates the expression of the viral regulatory genes. Nature, 335(6186), 181-183.

Manuel, E.R. et al., 2009. Dominant CD8+ T lymphocyte responses suppress expansion of vaccine-elicited subdominant T lymphocytes in rhesus monkeys challenged with pathogenic simian-human immunodeficiency virus. Journal of Virology.

Marciniak, R.A. & Sharp, P.A., 1991. HIV-1 Tat protein promotes formation of more-processive elongation complexes. The EMBO Journal, 10(13), 4189-4196.

Marinaro, M. et al., 2003. Mucosal delivery of the human immunodeficiency virus-1 Tat protein in mice elicits systemic neutralizing antibodies, cytotoxic T lymphocytes and mucosal IgA. Vaccine, 21(25-26), 3972-81.

Marshall, N.F. & Price, D.H., 1992. Control of formation of two distinct classes of RNA polymerase II elongation complexes. Molecular and Cellular Biology, 12(5), 2078-2090.

Mascola, J.R. et al., 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nature Medicine, 6(2), 207-210.

Masopust, D., 2009. Developing an HIV cytotoxic T-lymphocyte vaccine: issues of CD8 T-cell quantity, quality and location. *Journal of Internal Medicine*, 265(1), 125-137.

Matsui, M. et al., 1996. Effects of HIV-1 Tat on expression of HLA class I molecules. Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology: Official Publication of the International Retrovirology Association, 11(3), 233-240.

Mattapallil, J.J. et al., 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature, 434(7037), 1093-1097.

McArthur, J.C. et al., 1994. HIV dementia. Incidence and risk factors. Research Publications - Association for Research in Nervous and Mental Disease, 72, 251-272.

Mhashilkar, A.M. et al., 1997. Inhibition of human immunodeficiency virus type 1 replication in vitro by a novel combination of anti-Tat single-chain intrabodies and NF-kappa B antagonists. Journal of Virology, 71(9), 6486-6494.

Milani, D. et al., 1998. HIV-1 Tat induces tyrosine phosphorylation of p125FAK and its association with phosphoinositide 3-kinase in PC12 cells. AIDS (London, England), 12(11), 1275-1284.

Mishra, M. et al., 2008. Clade-specific differences in neurotoxicity of human immunodeficiency virus-1 B and C Tat of human neurons: significance of dicysteine C30C31 motif. Annals of Neurology, 63(3), 366-376.

Mondal, D. et al., 2005. The HIV-1 Tat protein selectively enhances CXCR4 and inhibits CCR5 expression in megakaryocytic K562 cells. Experimental Biology and Medicine (Maywood, N.J.), 230(9), 631-644.

Mooij, P. et al., 2004. Qualitative T-helper responses to multiple viral antigens correlate with vaccine-induced immunity to simian/human immunodeficiency virus infection. Journal of Virology, 78(7), 3333-3342.

Morris, C.B. et al., 2001. Identification of a peptide capable of inducing an HIV-1 Tat-specific CTL response. Vaccine, 20(1-2), 12-15.

Mothé, B.R. et al., 2002. Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. Journal of Virology, 76(2), 875-884.

Moureau, C. et al., 1999. Specificity of anti-Nef antibodies produced in mice immunized with DNA encoding the HIV-1 nef gene product. Vaccine, 18(3-4), 333-341.

Mueller, Y.M. et al., 2003. IL-15 enhances survival and function of HIV-specific CD8+ T cells. Blood, 101(3), 1024-1029.

Nabell, L.M. et al., 1994. Human immunodeficiency virus 1 Tat stimulates transcription of the transforming growth factor alpha gene in an epidermal growth factor-dependent manner. Cell

Growth & Differentiation: The Molecular Biology Journal of the American Association for Cancer Research, 5(1), 87-93.

Nardelli, B. et al., 1995. CD4+ blood lymphocytes are rapidly killed in vitro by contact with autologous human immunodeficiency virus-infected cells. Proceedings of the National Academy of Sciences of the United States of America, 92(16), 7312–7316.

Natarajan, V. et al., 2002. Increased peripheral expansion of naive CD4+ T cells in vivo after IL-2 treatment of patients with HIV infection. Proceedings of the National Academy of Sciences of the United States of America, 99(16), 10712-10717.

Nath, A. et al., 1999. Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. The Journal of Biological Chemistry, 274(24), 17098-17102.

Nath, B.M., Schumann, K.E. & Boyer, J.D., 2000. The chimpanzee and other non-human-primate models in HIV-1 vaccine research. Trends in Microbiology, 8(9), 426-431.

Novitsky, V. et al., 2002. Magnitude and frequency of cytotoxic T-lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C. Journal of Virology, 76(20), 10155-10168.

Opi, S. et al., 2002. Tat HIV-1 primary and tertiary structures critical to immune response against non-homologous variants. The Journal of biological chemistry, 277(39), 35915-9.

Oxenius, A. et al., 2002. Residual HIV-specific CD4 and CD8 T cell frequencies after prolonged antiretroviral therapy reflect pretreatment plasma virus load. AIDS (London, England), 16(17), 2317-2322.

Pal, R. et al., 2006. Systemic Immunization with an ALVAC-HIV-1/Protein Boost Vaccine Strategy Protects Rhesus Macaques from CD4+ T-Cell Loss and Reduces both Systemic and Mucosal Simian-Human Immunodeficiency Virus SHIVKU2 RNA Levels. Journal of Virology, 80(8), 3732-3742.

Pantaleo, G. & Koup, R.A., 2004. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. Nature Medicine, 10(8), 806-810.

Pantophlet, R. & Burton, D.R., 2006. GP120: target for neutralizing HIV-1 antibodies. Annual Review of Immunology, 24, 739-769.

Paranjape, R.S. et al., 1998. Cross-reactive HIV-1-specific CTL in recent seroconverters from Pune, India. The Indian Journal of Medical Research, 108, 35-41.

Pardridge, W.M., Bickel, U., Buciak, J., Yang, J. & Diagne, A., 1994. Enhanced endocytosis and anti-human immunodeficiency virus type 1 activity of anti-rev antibodies after cationization. The Journal of Infectious Diseases, 169(1), 55-61.

Pardridge, W.M., Bickel, U., Buciak, J., Yang, J., Diagne, A. & Aepinus, C., 1994. Cationization of a monoclonal antibody to the human immunodeficiency virus REV protein enhances cellular uptake but does not impair antigen binding of the antibody. Immunology Letters, 42(3), 191-195.

Parmentier, H.K. et al., 1992. Epitopes of human immunodeficiency virus regulatory proteins tat, nef, and rev are expressed in normal human tissue. The American Journal of Pathology, 141(5), 1209-1216.

Partidos, C.D. et al., 2004. A synthetic HIV-1 Tat protein breaches the skin barrier and elicits Tat-neutralizing antibodies and cellular immunity. European Journal of Immunology, 34(12), 3723-373

Patel, C.A., Mukhtar, M. & Pomerantz, R.J., 2000. Human immunodeficiency virus type 1 Vpr induces apoptosis in human neuronal cells. Journal of Virology, 74(20), 9717-9726.

Patki, A.H. & Lederman, M.M., 1996. HIV-1 Tat protein and its inhibitor Ro 24-7429 inhibit lymphocyte proliferation and induce apoptosis in peripheral blood mononuclear cells from healthy donors. Cellular Immunology, 169(1), 40-46.

Pauza, C.D. et al., 2000. Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. Proceedings of the National Academy of Sciences of the United States of America, 97(7), 3515-3519.

Péloponèse, J.M. et al., 1999. Full peptide synthesis, purification, and characterization of six Tat variants. Differences observed between HIV-1 isolates from Africa and other continents. The Journal of Biological Chemistry, 274(17), 11473-11478.

Pett, S.L., 2009. Immunotherapies in HIV-1 infection. Current Opinion in HIV and AIDS, 4(3), 188-193.

Piacentini, L. et al., 2009. Genetic correlates of protection against HIV infection: the ally within. Journal of Internal Medicine, 265(1), 110-124.

Pitcher, C.J. et al., 1999. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nature Medicine, 5(5), 518-525.

Plantier, J. et al., 2009. A new human immunodeficiency virus derived from gorillas. Nature Medicine, 15(8), 871-872.

Pocernich, C.B. et al., 2005. HIV-dementia, Tat-induced oxidative stress, and antioxidant therapeutic considerations. Brain Research. Brain Research Reviews, 50(1), 14-26.

Poggi, A., Rubartelli, A. & Zocchi, M.R., 1998. Involvement of dihydropyridine-sensitive calcium channels in human dendritic cell function. Competition by HIV-1 Tat. The Journal of Biological Chemistry, 273(13), 7205-7209.

Prince, A.M. et al., 1991. Prevention of HIV infection by passive immunization with HIV immunoglobulin. AIDS Research and Human Retroviruses, 7(12), 971-973.

Puri, R.K., Leland, P. & Aggarwal, B.B., 1995. Constitutive expression of human immunodeficiency virus type 1 tat gene inhibits interleukin 2 and interleukin 2 receptor expression in a human CD4+ T lymphoid (H9) cell line. AIDS Research and Human Retroviruses, 11(1), 31-40.

Purvis, S.F. et al., 1992. Suppression of interleukin-2 and interleukin-2 receptor expression in Jurkat cells stably expressing the human immunodeficiency virus Tat protein. Cellular Immunology, 144(1), 32-42.

Raha, T., Cheng, S.W.G. & Green, M.R., 2005. HIV-1 Tat stimulates transcription complex assembly through recruitment of TBP in the absence of TAFs. PLoS Biology, 3(2), e44.

Ramakrishna, L. et al., 2004. Codon optimization and ubiquitin conjugation of human immunodeficiency virus-1 Tat lead to enhanced cell-mediated immune responses. Vaccine, 22(20), 2586-2598.

Ramirez, B.C. et al., 2008. Implications of recombination for HIV diversity. Virus Research, 134(1-2), 64-73.

Ranga, U. et al., 1998. Enhanced T cell engraftment after retroviral delivery of an antiviral gene in HIV-infected individuals. Proceedings of the National Academy of Sciences of the United States of America, 95(3), 1201-1206.

Ranga, U. et al., 2004. Tat protein of human immunodeficiency virus type 1 subtype C strains is a defective chemokine. Journal of Virology, 78(5), 2586-2590.

Ranki, A. et al., 1987. Long latency precedes overt seroconversion in sexually transmitted human-immunodeficiency-virus infection. Lancet, 2(8559), 589-593.

Ranki, A. et al., 1990. Antibodies to recombinant HIV-1 nef protein detected in HIV-1 infection as well as in nonrisk individuals. Journal of Acquired Immune Deficiency Syndromes, 3(4), 348-355.

Rayne, F. et al., 2010. Phosphatidylinositol-(4,5)-bisphosphate enables efficient secretion of HIV-1 Tat by infected T-cells. EMBO J, advance online publication. Available at: http://dx.doi.org/10.1038/emboj.2010.32

Re, M.C. et al., 1995. Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology: Official Publication of the International Retrovirology Association, 10(4), 408-416.

Reiss, P. et al., 1989. Seroconversion to HIV-1 rev- and tat-gene-encoded proteins. AIDS (London, England), 3(2), 105-106.

Reiss, P. et al., 1990. Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. Journal of Medical Virology, 30(3), 163-168.

Reiss, P. et al., 1991. Contribution of antibody response to recombinant HIV-1 gene-encoded products nef, rev, tat, and protease in predicting development of AIDS in HIV-1-infected individuals. Journal of Acquired Immune Deficiency Syndromes, 4(2), 165-172.

Rerks-Ngarm, S. et al., 2009. Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand. The New England Journal of Medicine. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19843557 [Accessed November 16, 2009].

Rezza, G. et al., 2005. The presence of anti-Tat antibodies is predictive of long-term nonprogression to AIDS or severe immunodeficiency: findings in a cohort of HIV-1 seroconverters. The Journal of infectious diseases, 191(8), 1321-4.

Richardson, M.W. et al., 2002. Immunogenicity of HIV-1 IIIB and SHIV 89.6P Tat and Tat toxoids in rhesus macaques: induction of humoral and cellular immune responses. DNA and Cell Biology, 21(9), 637-651.

Riedl, P. et al., 2009. Elimination of immunodominant epitopes from multispecific DNA-based vaccines allows induction of CD8 T cells that have a striking antiviral potential. Journal of Immunology (Baltimore, Md.: 1950), 183(1), 370-380.

Rodman, T.C. et al., 1992. Human immunodeficiency virus (HIV) Tat-reactive antibodies present in normal HIV-negative sera and depleted in HIV-positive sera. Identification of the epitope. The Journal of experimental medicine, 175(5), 1247-53.

Rodriguez, S.K. et al., 2006. The absence of anti-Tat antibodies is associated with risk of disease progression in HIV-2 infection. The Journal of Infectious Diseases, 194(6), 760-763.

Rosenberg, E.S. et al., 2000. Immune control of HIV-1 after early treatment of acute infection. Nature, 407(6803), 523-526.

Rowland-Jones, S. et al., 1995. HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. Nature Medicine, 1(1), 59-64.

Rusnati, M. et al., 1999. Multiple interactions of HIV-I Tat protein with size-defined heparin oligosaccharides. The Journal of Biological Chemistry, 274(40), 28198-28205.

Sahaf, B. et al., 2008. Culturing of human peripheral blood cells reveals unsuspected lymphocyte responses relevant to HIV disease. Proceedings of the National Academy of Sciences of the United States of America, 105(13), 5111-5116.

Samuel, K.P. et al., 1987. The 3'-orf protein of human immunodeficiency virus shows structural homology with the phosphorylation domain of human interleukin-2 receptor and the ATP-binding site of the protein kinase family. FEBS Letters, 218(1), 81-86.

Sarin, P.S. et al., 1986. Neutralization of HTLV-III/LAV replication by antiserum to thymosin alpha 1. Science (New York, N.Y.), 232(4754), 1135-1137.

Sastry, K.J. et al., 1990. HIV-1 tat gene induces tumor necrosis factor-beta (lymphotoxin) in a human B-lymphoblastoid cell line. The Journal of Biological Chemistry, 265(33), 20091-20093.

Scriba, T.J. et al., 2005. Functionally-inactive and immunogenic Tat, Rev and Nef DNA vaccines derived from sub-Saharan subtype C human immunodeficiency virus type 1 consensus sequences. Vaccine, 23(9), 1158-1169.

Secchiero, P. et al., 1999. Extracellular HIV-1 tat protein up-regulates the expression of surface CXC-chemokine receptor 4 in resting CD4+ T cells. Journal of Immunology (Baltimore, Md.: 1950), 162(4), 2427-2431.

Selliah, N. & Finkel, T.H., 2001. Biochemical mechanisms of HIV induced T cell apoptosis. Cell Death and Differentiation, 8(2), 127-136.

Shankarkumar, U., 2004. HIV and human leukocyte antigen association--the Indian scenario. Journal of HIV Therapy, 9(3), 60-64.

Shojania, S. & O'Neil, J.D., 2006. HIV-1 Tat is a natively unfolded protein: the solution conformation and dynamics of reduced HIV-1 Tat-(1-72) by NMR spectroscopy. The Journal of Biological Chemistry, 281(13), 8347-8356.

Siakkou, H. et al., 1993. Fine mapping of HIV-1 Nef-epitopes by monoclonal antibodies. Archives of Virology, 128(1-2), 81-95.

Siddappa, N.B. et al., 2004. Identification of subtype C human immunodeficiency virus type 1 by subtype-specific PCR and its use in the characterization of viruses circulating in the southern parts of India. Journal of clinical microbiology, 42(6), 2742-51.

Siddappa, N.B. et al., 2009. Neutralization-sensitive R5-tropic simian-human immunodeficiency virus SHIV-2873Nip, which carries env isolated from an infant with a recent HIV clade C infection. Journal of Virology, 83(3), 1422-1432.

Silvera, P. et al., 2002. Outcome of simian-human immunodeficiency virus strain 89.6p challenge following vaccination of rhesus macaques with human immunodeficiency virus Tat protein. Journal of Virology, 76(8), 3800-3809.

Siomi, H. et al., 1990. Effects of a highly basic region of human immunodeficiency virus Tat protein on nucleolar localization. Journal of Virology, 64(4), 1803-1807.

Sparnacci, K. et al., 2005. Core-shell microspheres by dispersion polymerization as promising delivery systems for proteins. *Journal of Biomaterials Science. Polymer Edition*, 16(12), 1557-1574.

Sportès, C. et al., 2008. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. The Journal of Experimental Medicine, 205(7), 1701-1714.

Steinaa, L. et al., 1994. Antibody to HIV-1 Tat protein inhibits the replication of virus in culture. Archives of Virology, 139(3-4), 263-271.

Stittelaar, K.J. et al., 2002. Comparison of the efficacy of early versus late viral proteins in vaccination against SIV. Vaccine, 20(23-24), 2921-2927.

Strayer, D.S. et al., 2005. Current status of gene therapy strategies to treat HIV/AIDS. Molecular Therapy: The Journal of the American Society of Gene Therapy, 11(6), 823-842.

Subramanyam, M. et al., 1993. Mechanism of HIV-1 Tat induced inhibition of antigen-specific T cell responsiveness. Journal of Immunology (Baltimore, Md.: 1950), 150(6), 2544-2553.

Tan, R. et al., 1999. Rapid death of adoptively transferred T cells in acquired immunodeficiency syndrome. Blood, 93(5), 1506-1510.

Tardieu, M. et al., 1992. Human immunodeficiency virus type 1-infected monocytic cells can destroy human neural cells after cell-to-cell adhesion. Annals of Neurology, 32(1), 11-17.

Taylor, B.S. et al., 2008. The challenge of HIV-1 subtype diversity. The New England Journal of Medicine, 358(15), 1590-1602.

Taylor, J.P. et al., 1992. Activation of expression of genes coding for extracellular matrix proteins in Tat-producing glioblastoma cells. Proceedings of the National Academy of Sciences of the United States of America, 89(20), 9617-9621.

Thakar, M.R. et al., 2005. Cytolytic T lymphocytes (CTLs) from HIV-1 subtype C-infected Indian patients recognize CTL epitopes from a conserved immunodominant region of HIV-1 Gag and Nef. The Journal of Infectious Diseases, 192(5), 749-759.

Trkola, A. et al., 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nature Medicine, 11(6), 615-622.

Trono, D., Feinberg, M.B. & Baltimore, D., 1989. HIV-1 Gag mutants can dominantly interfere with the replication of the wild-type virus. Cell, 59(1), 113-120.

Tsomides, T.J. et al., 1994. Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. The Journal of Experimental Medicine, 180(4), 1283-1293.

Tyagi, M. et al., 2001. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. Journal of Biological Chemistry, 276(5), 3254-3261.

Ugolini, S. et al., 1997. Inhibition of virus attachment to CD4+ target cells is a major mechanism of T cell line-adapted HIV-1 neutralization. The Journal of Experimental Medicine, 186(8), 1287-1298.

Ulich, C. et al., 1999. Functional domains of Tat required for efficient human immunodeficiency virus type 1 reverse transcription. Journal of Virology, 73(3), 2499-2508.

Umapathy, S., Pawar, A. & Ghosh, K., 2007. Specific human leukocyte antigen alleles associated with HIV-1 infection in an Indian population. Journal of Acquired Immune Deficiency Syndromes (1999), 44(4), 489-490.

Vacca, A. et al., 1994. Human immunodeficiency virus type-1 tat enhances interleukin-2 promoter activity through synergism with phorbol ester and calcium-mediated activation of the NF-AT cis-regulatory motif. Biochemical and Biophysical Research Communications, 205(1), 467-474.

van Ballegooijen, M. et al., 2003. AIDS vaccines that allow HIV-1 to infect and escape immunologic control: a mathematic analysis of mass vaccination. Journal of Acquired Immune Deficiency Syndromes (1999), 34(2), 214-220.

Van Damme, L. et al., 2008. Lack of effectiveness of cellulose sulfate gel for the prevention of vaginal HIV transmission. The New England Journal of Medicine, 359(5), 463-472.

Van Duyne, R. et al., 2009. The utilization of humanized mouse models for the study of human retroviral infections. Retrovirology, 6(1), 76.

Verhoef, K. et al., 1998. On the role of the second coding exon of the HIV-1 Tat protein in virus replication and MHC class I downregulation. AIDS Research and Human Retroviruses, 14(17), 1553-1559.

Virgin, H.W., Mann, M.A. & Tyler, K.L., 1994. Protective antibodies inhibit reovirus internalization and uncoating by intracellular proteases. Journal of Virology, 68(10), 6719-6729.

Viscidi, R.P. et al., 1989. Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. Science (New York, N.Y.), 246(4937), 1606-8.

Voltan, R. et al., 2007. Preparation and characterization of innovative protein-coated poly(methylmethacrylate) core-shell nanoparticles for vaccine purposes. *Pharmaceutical Research*, 24(10), 1870-1882.

Wahren, B. et al., 2002. HIV subtypes and recombination strains-strategies for induction of immune responses in man. Vaccine, 20(15), 1988-1993

Walker, L.M. et al., 2009. Broad and Potent Neutralizing Antibodies from an African Donor Reveal a New HIV-1 Vaccine Target. Science (New York, N.Y.). Available at: http://www.ncbi.nlm.nih.gov/pubmed/19729618 [Accessed September 29, 2009].

Wang, W.K. et al., 2000. Molecular biology of human immunodeficiency virus type 1. Journal of Microbiology, Immunology, and Infection = Wei Mian Yu Gan Ran Za Zhi, 33(3), 131-140.

Wang, Z. et al., 1999. Activation of Bcl-2 promoter-directed gene expression by the human immunodeficiency virus type-1 Tat protein. Virology, 257(2), 502-510.

Watkins, J.D. et al., 2008. Homonuclear 1H NMR and circular dichroism study of the HIV-1 Tat Eli variant. Retrovirology, 5, 83.

Weiss, J.M. et al., 1999. HIV-1 Tat induces monocyte chemoattractant protein-1-mediated monocyte transmigration across a model of the human blood-brain barrier and up-regulates CCR5 expression on human monocytes. Journal of Immunology (Baltimore, Md.: 1950), 163(5), 2953-2959.

Wendt, K. et al., 2007. Interleukin-21 differentially affects human natural killer cell subsets. Immunology, 122(4), 486-495.

Wertheim, K., Kutkowska-Kaźmierczak, A. & Bal, J., 2008. [Genetically determined human susceptibility to selected infectious diseases]. Medycyna Wieku Rozwojowego, 12(3), 738-747.

Westendorp, M.O. et al., 1994. Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. Journal of Virology, 68(7), 4177-4185.

Westendorp, M.O. et al., 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature, 375(6531), 497-500.

Wilson, J.D. et al., 2000. Direct visualization of HIV-1-specific cytotoxic T lymphocytes during primary infection. AIDS (London, England), 14(3), 225-233.

Woffendin, C. et al., 1996. Expression of a protective gene-prolongs survival of T cells in human immunodeficiency virus-infected patients. Proceedings of the National Academy of Sciences of the United States of America, 93(7), 2889-2894.

Worobey, M. et al., 2008. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. Nature, 455(7213), 661-664.

Wu, Y. et al., 1996. Binding of intracellular anti-Rev single chain variable fragments to different epitopes of human immunodeficiency virus type 1 rev: variations in viral inhibition. Journal of Virology, 70(5), 3290-3297.

Yezid, H. et al., 2009. Mechanism for HIV-1 TAT insertion into the endosome membrane. The Journal of Biological Chemistry. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19549783 [Accessed June 26, 2009].

Yu, X.G. et al., 2005. Regulatory and accessory HIV-1 proteins: potential targets for HIV-1 vaccines? Current Medicinal Chemistry, 12(6), 741-747.

Zagury, D. et al., 1998. Interferon alpha and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. Proceedings of the National Academy of Sciences of the United States of America, 95(7), 3851-3856.

Zagury, J.F. et al., 1998. Antibodies to the HIV-1 Tat protein correlated with nonprogression to AIDS: a rationale for the use of Tat toxoid as an HIV-1 vaccine. Journal of Human Virology, 1(4), 282-292.

Zauli, G. et al., 1991. Evidence for an HIV-1 mediated suppression of in vitro growth of enriched (CD34+) hematopoietic progenitors. Journal of Acquired Immune Deficiency Syndromes, 4(12), 1251-1253.

Zauli, G. et al., 1993. Human immunodeficiency virus type 1 Tat protein protects lymphoid, epithelial, and neuronal cell lines from death by apoptosis. Cancer Research, 53(19), 4481-4485.

Zauli, G. et al., 1995. An autocrine loop of HIV type-1 Tat protein responsible for the improved survival/proliferation capacity of permanently Tat-transfected cells and required for optimal HIV-1 LTR transactivating activity. Journal of acquired immune deficiency syndromes and human retrovirology: official publication of the International Retrovirology Association, 10(3), 306.

Zauli, G. et al., 1995. The human immunodeficiency virus type-1 Tat protein upregulates Bcl-2 gene expression in Jurkat T-cell lines and primary peripheral blood mononuclear cells. Blood, 86(10), 3823-3834.

Zhang, M. et al., 2002. Bcl-2 upregulation by HIV-1 Tat during infection of primary human macrophages in culture. Journal of Biomedical Science, 9(2), 133-139.

Zheng, L. et al., 2005. Extracellular HIV Tat and Tat cysteine rich peptide increase CCR5 expression in monocytes. Journal of Zhejiang University. Science. B, 6(7), 668-672.

Zidovetzki, R. et al., 1998. Human immunodeficiency virus Tat protein induces interleukin 6 mRNA expression in human brain endothelial cells via protein kinase C- and cAMP-dependent protein kinase pathways. AIDS Research and Human Retroviruses, 14(10), 825-833.

2. Evaluation of the humoral immune response to Tat in two different clinical cohorts from southern states of India

There have been no studies evaluating immune responses to Tat in Indian populations. Such studies are essential for two reasons: 1. India harbors the second largest incidence of HIV infection and 2. the vast majority of the infections in India are caused by HIV-1 subtype C, which is also the most dominant strain globally. Furthermore, such knowledge could be useful not only for vaccine design but also for the understanding of the viral pathogenesis. To this end, we studied humoral immune response to Tat in two different clinical cohorts from southern states of India. One of the cohorts was derived from the Seva Free Clinic, Bangalore and the Tuberculosis Research Centre (TRC), Chennai located in two different states of Karnataka and Tamil Nadu, respectively.

2.1 Materials and methods:

2.1.1 Plasmids: Tat, from subtypes C and B was cloned in our laboratory previously (Siddappa et al. 2006). Reference molecular clones of subtypes A (P92UG037.1), D (P94UG114.1.6) and AE (P90CF402.1) were procured through the NIH AIDS-research and reference program. Exon-1 of Tat was PCR amplified from the reference plasmids using gene-specific primers and sub-cloned into pET21b+ bacterial expression vector (Invitrogen). List of the primers used for the cloning are illustrated in Table 2.1.

Subtype C rev of INDIE was sub-cloned into pET21b+ using target-specific primers (Table 2.1). HEK 293T cells were transfected with pINDIE using Ca_2PO_4 method, total RNA isolated using Trizol (Sigma) and first-strand synthesis was carried out using Superscript II (Invitrogen) followed by second-strand synthesis using Taq DNA polymerase (Microtest Innovations, Bangalore).

Clone	Primer no.	Sequence	Cloning enzyme
Tat			
A	**N674F**	GAAGAG**CATATG**GATCCGGTAGATCCT	NdeI
	N675R	TACGAT**CGGCCG**CTGCTTTGGTATAGGATT	EagI
D	**N670F**	GAAGAA**CATATG**GAGCCAGTAGATCCC	NdeI
	N671R	TACTCA**AAGCTT**CTGCTTTGGTACAGGATC	HindIII
E	**N672F**	GAAGAG**CATATG**GAGCTGGTAGATCCT	NdeI
	N673R	TACACT**CTCGAG**CTGCTCTGGTATAGGATA	XhoI
Rev			
	N1117F	CAGCG**CATATG**AGGGCTTCCCAACTGCTG	NdeI
	N1116R	CAGCGA**AAGCTT**ATGGCAGGAAGAAGCGGAG	XbaI

Table 2.1 Primers used for molecular cloning of bacterial expression plasmids.

2.1.2 Clinical cohorts: Samples for this study have been collected from two different clinical cohorts, the Seva Free Clinic, Bangalore and the Tuberculosis Research Centre

(TRC), Chennai located in two different states of Karnataka and Tamil Nadu, respectively, in southern India. Although a large number of the volunteers hailed from these two states, a few donors from the other two southern states, Andhra Pradesh and Kerala also constituted the cohorts. The present study has been approved by the institutional bioethics committees of JNCASR and TRC. An informed consent was obtained from each participant prior to blood collection. Clinical profile of the Seva Clinic samples has been described previously (Siddappa et al. 2006). Briefly, from the Seva Clinic, a total of 200 peripheral blood samples were collected from HIV-seropositive volunteers (119 men, mean age 32.4 years, range 17–54; 40 women, mean age 29.76 years, range 20–56; 15 subjects below 15 years of age; for the rest of the participants details were not recorded). Most of the blood samples from the Seva Clinic were collected over a period of 4 years (2001–2004)**.** Of the 247 samples obtained from TRC, clinical information is available for 212 samples (Appendix 1). The cohort constituted of 84 males (mean age of 36.6, SD = 6.9) and 128 females (mean age of 29.3, SD = 6.5). Mean CD4 counts per micro liter for the males and females were 283 (SD = 188.7) and 425 (SD = 278), respectively. The seropositive status of the participants was confirmed by multiple ELISA kits. A single peripheral blood sample of 8 ml was collected from each volunteer, into vaccutainers (BD Biosciences) containing EDTA. Plasma from the whole blood was separated and stored at -80^{0}C in aliquots of 500 µl.

2.1.3 Tat sequence determination from primary clinical samples: Genomic DNA was isolated from cryo-preserved peripheral blood mononuclear cells (PBMC) using blood genomic DNA isolation kit (Sigma). A nested PCR approach was applied to amplify Tat exon-1 and exon-2 using three sets of primers. The first round of PCR was carried out with N817F and N418R which amplifies a fragment of 3.9 kb. The inner PCR was carried

out using first round PCR product as the template with primers N111F and N1156R for exon-1 and N1169F and N847R for exon-2 of Tat, respectively. All the PCRs were carried out using Phusion high fidelity enzyme (Finnzymes). Amplicons obtained from each of the second round were cleaned using PCR-cleanup columns and sequenced (Applied Biosystems). To prevent carry over contamination in the PCR reactions, the reagents and template were spatially separated. The PCR reaction was set up in a clean room (no plasmid or genomic DNA was brought into the room) while the template was added to the reaction in a PCR hood which was decontaminated with UV and 0.4% bleach wipes. The PCR products were run on agarose gels in a separate room to prevent contaminations from the amplified products. Further, filter tips were used at every step to prevent contaminations from micropipettes. BLAST analysis was carried out on all of the sequences as a measure of quality assurance and to rule out the possibility of laboratory contamination.

Sequences of Tat from the clinical samples were compared with representative HIV-1 Tat sequences of subtypes A, B, C, D, F, G, H, J and K downloaded from the HIV sequence database. A neighbor-joining tree was constructed on the basis of the hidden Markov model nucleotide alignment of reference Tat sequences. Branch lengths were drawn to scale with the scale bar representing 0.02 nucleotide substitution per site.

2.1.4 Indirect ELISA for screening of Tat-antibodies: We used indirect ELISA to quantitate antigen-specific antibody levels in the plasma samples using the recombinant Tat and Rev proteins essentially as described previously (Siddappa et al. 2006). Briefly, the first step of purification was based on Ni-NTA (Amersham) affinity purification following which the protein was passed through SP-Sepharose (Amersham) columns. DTT at a final concentration of 1 mM was included in all the steps to prevent oxidation of

Tat. The plastic ware was siliconized to prevent adherence of the protein to the surfaces. For the immunoassay, microtiter wells (Nunc Maxisorp) were coated with 400 ng of subtype C Tat protein (C-Tat) in 50 mM carbonate buffer (pH 9.6) and incubated at 37^0C for 2 h. The wells were blocked with 3% BSA in PBS at 37^0C for 1 h. Plasma samples diluted 1:100 in PBS containing 5% sheep serum and 0.2% Tween 20 were added to appropriate wells. The strips were incubated at 37^0C for 1 h and washed thoroughly with the wash buffer (50 mM Tris, pH 8.0; 100 mM NaCl; 0.2% Tween 20). Goat anti-human polysera conjugated to Horse-radish peroxidase, (Catalog # 401455, Calbiochem, CA) was diluted 1:10,000, added to each well and the strips were incubated at 37^0C for 1 h. After thorough washing, 100 µl of substrate solution (0.1 M citrate monohydrate, 0.2 M disodium hydrogen phosphate, 1 mg/ml OPD and 0.3% hydrogen peroxide) was added and incubated in dark at room temperature for 15 min. Enzyme reaction was stopped by adding 100 µl of 1 N HCl. The absorbance was recorded at 490 nm using an ELISA reader (BioRad, USA). The specificity of the assay was evaluated in a competition assay where 1:100 diluted plasma samples were incubated for 30 min with 2 µg of Tat or p24 proteins followed by the indirect ELISA method as described above. For cross-clade reactivity determination, Tat from different clades was coated on to the microtiter plate and the subsequent steps were performed as described above. Samples showing an absorbance above the mean value of the control group plus 1 SD (0.26 + 0.14) were considered to be positive for Tat- reactive antibodies. Antibodies against gp41 were determined using a commercial kit (Xcyton, Bangalore, India). With the exception of the isotype determination experiments, in all other assays, the ELISA detected all of the antibody isotypes as the secondary antibody was expected to detect both of the light chains kappa (κ) and lambda (λ) as well as the gamma (γ) heavy chain. Recombinant Tat from different viral clades and Rev from clade C used in various assays have been

purified essentially as described previously (Siddappa et al. 2006). With the exception for cross-reactivity analysis, full-length, subtype C Tat protein was used in all other experiments.

2.1.5 Isotype determination of the Tat-reactive antibodies: The isotype profile of the Tat-reactive antibodies in the plasma samples was determined using commercially available isotype-specific secondary antibodies conjugated to peroxidase (Catalog # 202005, 905205, 906005, 921005 and 920005 for IgM, IgG1, IgG2, IgG3 and IgG4, respectively; Southern Biotech, Birmingham, UK). Briefly, 400 ng of C-Tat protein was coated into microtiter wells and blocked with 3% BSA following which plasma samples diluted 1:100 were added and incubated at 37^0C for 1 h. Following extensive washes, isotype-specific secondary antibodies were added at dilutions as recommended by the manufacturer and incubated for 1 h at 37^0C. Subsequent steps of the assay were performed as described above for the indirect ELISA. The cutoff value for each secondary antibody was individually determined as mean of control $\pm$ 1 SD.

2.1.6 Pepscan analysis for B-cell epitope mapping: Synthetic peptides (Genemed, San Francisco, CA), 20 amino acids in length, with an overlap of 10 amino acids and spanning the full-length of Tat were used for the pepscan analysis (see Fig. 2.7). The peptides represented the consensus sequence of C-Tat (Ranga et al. 2004). One micro gram of each peptide was coated into microtiter plates and the subsequent steps of the assay were performed essentially as described above for the indirect ELISA. A 21-mer non-specific peptide (QASALAPAPPQVLPQAPAPAC residues 362–381 plus an additional cysteine residue added at the C-terminus) derived from p65, a Rel-family member, was used as a control in the assay to determine background levels in the assay.

2.1.7 Antibody avidity determination: The avidity of anti-Tat antibodies was determined by using increasing concentrations of NaSCN as described previously (Pullen et al. 1986). Briefly, plasma samples diluted 1:100 were incubated in wells coated with 400 ng of C-Tat protein. Following which, 100 µl of defined concentrations of NaSCN solution (0.5, 1.0, 2.0 and 4.0 M) prepared in PBS were added to appropriate wells, the plates were incubated at room temperature for 15 min and thoroughly washed. Subsequent steps of the assay were as described above for ELISA.

2.1.8 Virus-rescue assay: HLM1 cells were seeded into 96-well plates, 20,000/well, and incubated overnight in 200 µl of DMEM (Sigma-Aldrich, USA) supplemented with 5% equine serum (Gibco). Plasma samples were diluted 100-fold in serum-free medium, 500 ng of Tat protein was added to 100 µl of each plasma dilution and the samples were incubated at 37^0C for 30 min. The samples were added to appropriate wells containing cells and incubated for 3 h. The supernatant was removed and 200 µl of complete medium was added to each well. The plates were incubated for 72 h and the levels of viral antigen p24 were determined using a commercial kit (Perkin Elmer, Waltham, MA). As a positive control for Tat neutralization, we used 50 µg/ml of an IgG1 monoclonal antibody E6.4 raised in-house against C-Tat that recognizes the N-terminal 20 amino acid residues and blocks extracellular-Tat with high efficiency.

2.1.9 Peptide competition assay: Plasma samples were diluted 1:100 times with serum-free medium and incubated for 1 h at 37^0C with 1 µg of peptides 3 or 5 representing the B-cell epitopes in the CRD or BD of Tat, respectively. To this solution, 500 ng of Tat protein was added and incubated for 30 min at 37^0C and this mixture was subsequently transferred to appropriate wells containing 20,000 HLM1 cells and incubated for 3 h. The supernatant was replaced with DMEM supplemented with 5% equine serum and the cells

were cultured for 72 h. The p24 levels in the culture supernatant were monitored as mentioned above.

2.1.10 Tat antigenicity prediction: The antigenicity of the Tat proteins derived from different viral subtypes was predicted using an online antigenicity prediction tool available at http://immunax.dfci.harvard.edu/Tools/antigenic.pl. This tool is based on a semi-empirical method of detection of antigenic determinants. The method of prediction is reported to be up to 75% accurate (Kolaskar & Tongaonkar 1990).

2.1.11 Statistical methods: All the statistical analyses were performed with the GraphPad Prism 4 software. Experiments were performed two or three times, and values obtained from three replicate samples were averaged in each experiment. Data are presented as mean value with the standard deviation (± 1 S. D.). Statistical significance was tested using Student's t-test. Differences were considered significant at $p<0.05$. The correlation coefficient (R) and *p*-value (two-tailed) were calculated at 95% confidence interval.

2.2 Results

2.2.1 A small but significant number of subjects are high-responders to Tat

We collected clinical samples from two independent clinical cohorts, from the metropolitan cities of Bangalore (n = 200) and Chennai (n = 247) in southern India. Additionally, samples from 150 seronegative volunteers were also included in the analyses for comparison. We used full-length, recombinant, subtype C Tat protein in the assay. The assay detected all the antibody isotypes, including IgM and IgG, as the secondary antibody was targeted to bind the light as well as the γ heavy chains (# 401455, Calbiochem, USA) and the assay specificity was confirmed by protein competition analysis using free Tat protein (Fig 2.1).

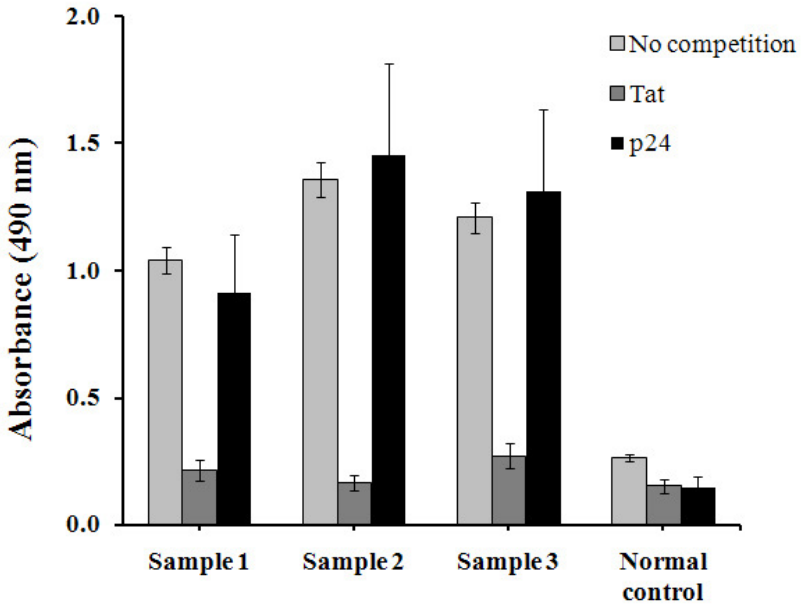


Figure 2.1: Antibodies in clinical samples interact with Tat-protein specifically. The specificity of antibody interaction with Tat protein was evaluated using competition ELISA. The grey bars correspond to absorbance in the absence of a competing protein; filled dark grey bars represent the absorbance in the presence of competing Tat protein while the striped bars correspond to absorbance in the presence of p24. The low absorbance value in the presence of Tat and not p24 confirms the specificity of the assay. As a negative control a normal sample was included in the assay.

As the Tat-reactive antibody levels in the two cohorts did not differ significantly from each other (Fig 2.2), data for both of the cohorts were pooled and presented together (Fig 2.3).

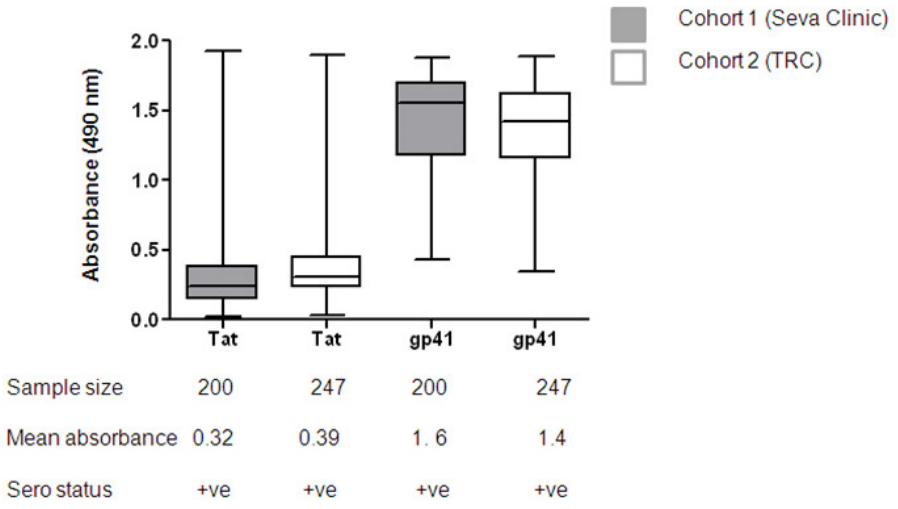
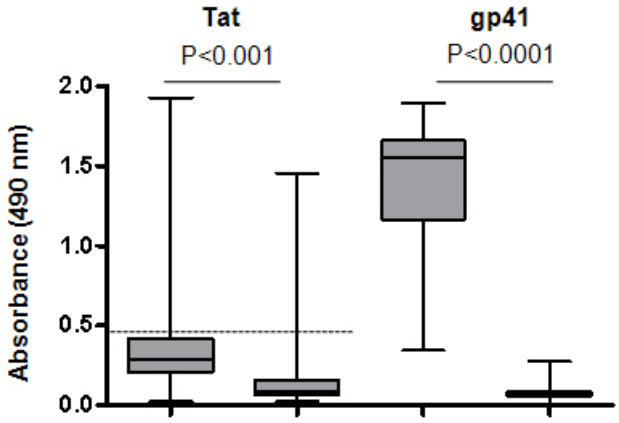


Figure 2.2: Humoral immune response to viral antigens in south Indian cohorts. The boxes represent the interquartile range, the line inside each box the median of the samples and the whiskers the range of the data. The grey boxes correspond to antibody levels in the cohort from Seva Clinic, Bangalore, and open boxes correspond to the cohort from TRC, Chennai.

Of the samples tested, 14% (63/447) and 4.6% (7/150) of seropositive and control samples contained Tat-reactive antibodies at a 100-fold plasma dilution above the cut off value. Consistent with the non-dominant nature of Tat, the antibody levels varied over a broad range in both of the groups with a mean absorbance value of 0.37 and 0.26, respectively (Fig.2.3). Although there was a considerable overlap between the groups, they differed significantly from each other in mean absorbance values (Fig. 2.3, left panel, $p<0.001$). In contrast, anti-gp41 antibodies between these two groups, with mean absorbance values of 1.56 and 0.14, differed from each other significantly without an overlap (Fig. 2.3, right panel, $p<0.0001$). These data ascertained the non-immunodominant nature of Tat in the Indian cohorts consistent with the previous reports from other cohorts. Interestingly, a small but significant number of seropositive samples

(7%, 31/447) contained anti-Tat antibody levels higher than the 75th percentile of the box plot thus representing the upper outlier group. The mean absorbance value, 1.33, of this upper outlier group is not only higher than that of the entire group, 0.37, but also comparable to that of the gp41 group which is 1.56. A few samples in the Tat upper outlier group even contained higher absorbance values than the mean absorbance value of the gp41 group.



Sample size	447	150	447	150
Mean absorbance	0.37	0.26	1.56	0.14
Sero status	+ve	-ve	+ve	-ve
n above cut off	85	15		
% above cut off	14	4.6		
Upper outlier	31	3		

Figure 2.3: Humoral immune response to viral antigens in south Indian cohorts. Comparison of antibody reactivity against Tat and gp41. Full-length, recombinant subtype C Tat protein was used in the assays. The gp41 commercial kit used a pool of peptides representing the immunodominant epitopes in the ectodomain of gp41. The boxes represent the interquartile range, the line inside each box the median of the samples and the whiskers the range of the data. The cutoff value is represented by the dashed horizontal line. In the assay, all the antibody isotypes are expected to be detected by the secondary antibody conjugated to peroxidase.

We further compared antibody levels to Tat and Rev, both proteins of subtype C origin, in 31 high-, 22 moderate- and 31 low-Tat samples. A significant correlation existed in antibodies to these two non-immunodominant antigens only in the low-Tat group (Fig. 2.4, right panel, R = 0.48, p = 0.006) but not in the moderate (Fig. 2.4, middle panel, R = 0.20, p = 0.377) or high-Tat (Fig. 2.4, left panel, R = 0.10, p = 0.48) groups. An absence of significant correlation between Tat and Rev antibodies in the high-Tat group is suggestive of these subjects being high-responders to Tat specifically.

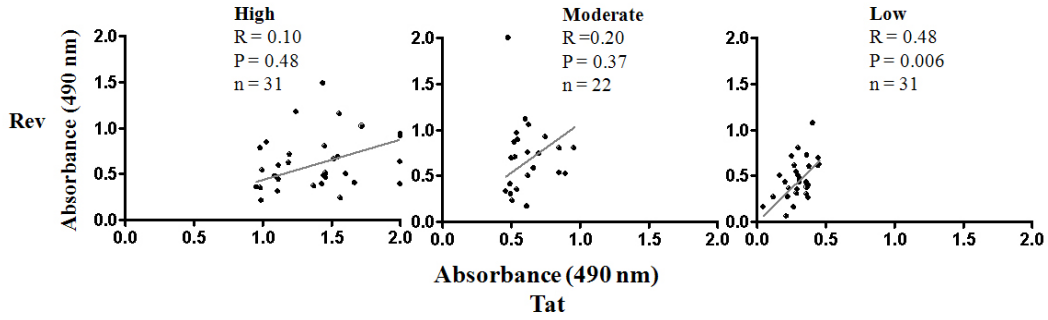


Figure 2.4: Comparison of antibody reactivity against Tat and Rev. Both the viral antigens used in the assay are full-length and are of subtype C origin. Samples were divided into three categories based on anti-Tat antibody levels. The correlation coefficient values (R), the p values and the samples size (n) have been shown.

2.2.2 Only in the high-Tat group a class-switch to IgG was manifested

Presence of Tat-reactive antibodies in control plasma samples was not unexpected since natural IgM antibodies that constitute an important component of the innate immune response, have been identified previously from other clinical cohorts. The focus of the present study, however, is on the upper outlier group with significantly high magnitude of anti-Tat antibodies. To examine if the exposure of the immune system to the viral antigen promoted isotype switching of the Tat-reactive antibodies, we determined the isotype profile of the anti-Tat antibodies in three different groups, high-Tat, low-Tat and control groups. Full-length, recombinant, subtype C Tat protein was used in the assay. All the 14

outlier plasma samples tested in the assay contained significant levels of Tat-reactive IgM as well as IgG antibodies (Fig. 2.5.A, left panel). A few samples within this group also contained IgG3 and/or IgG2. In contrast, all the samples in the low-Tat group and the control group contained only Tat-reactive IgM and no IgG (Fig. 2.5.A, middle and right panels). We also determined the isotype profile of the anti-gp41 antibodies in the high-Tat and low-Tat groups simultaneously (Fig. 2.5.B). The isotype profile of the anti-gp41 antibodies in these two groups was identical to each other and to that of the high-Tat group. This similarity in the antigen-specific antibody isotype profile between anti-Tat and anti-gp41 antibodies is quite striking and suggestive of Tat being immunodominant in this subset of subjects.

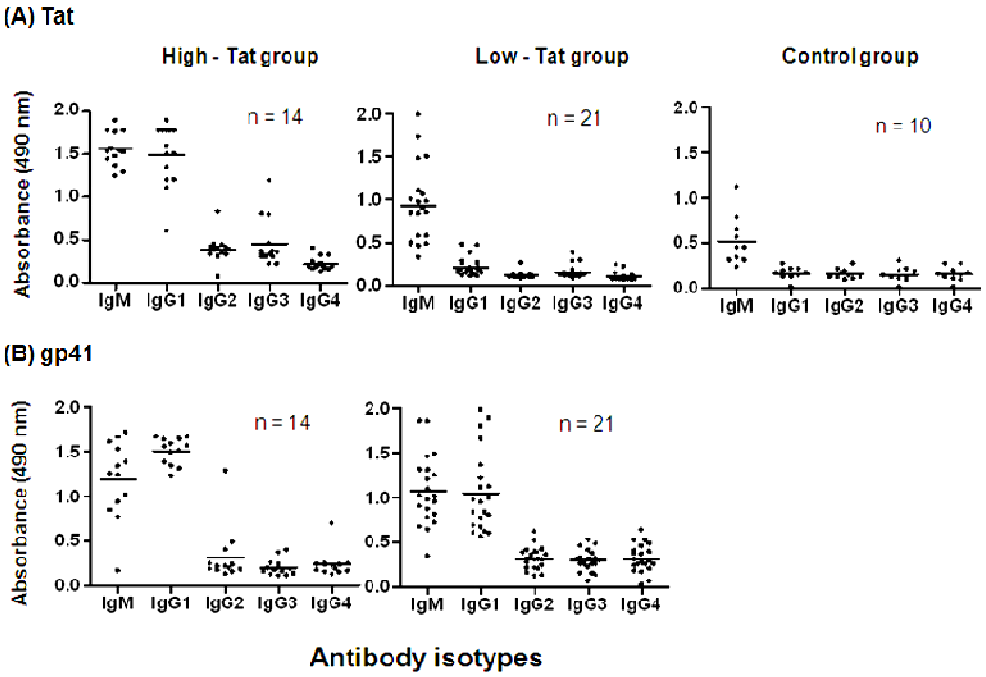


Figure 2.5: Isotype profiles of antigen-reactive antibodies in HIV-1 seropositive plasma samples. (A) Isotype profile of the Tat-reactive antibodies. Full-length, recombinant subtype C Tat protein was used in these assays. Each filled circle represents an individual sample. The mean absorbance value is represented by a horizontal line. The dotted horizontal lines represent cutoff values for each of the individual secondary antibodies. The dotted lines correspond to the cut-off values for each isotype specific antibody. **(B)** Isotype profile of anti-gp41 antibodies. The analysis was performed on high-Tat and low-Tat groups.

Importantly, an isotype switch is not only indicative of T-help but also of possible affinity maturation and more efficient neutralization of extra-cellular Tat. With increasing concentrations of NaSCN as previously reported (Pullen et al. 1986), we measured the avidity of the anti-Tat antibodies in the three different groups using full-length subtype C Tat protein in the assay. All of the samples in the high-Tat group, but none from the low-Tat and the control groups, withstood NaSCN treatment at a concentration as high as 2 M (Fig. 2.6) suggesting the presence of high avidity antibodies in the high-Tat group alluding to T-helper activity. At 4 M concentration of NaSCN, nearly all the samples showed significant reduction in antigen binding.

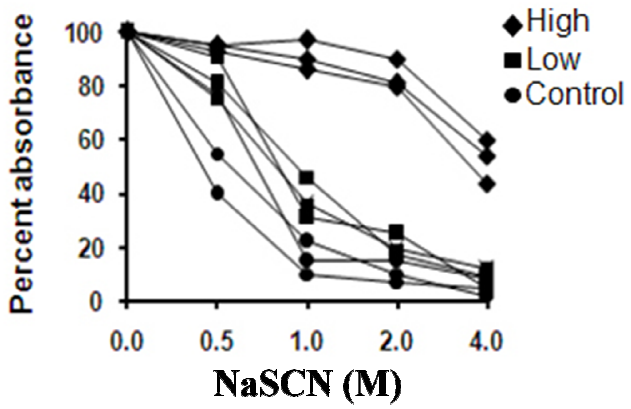
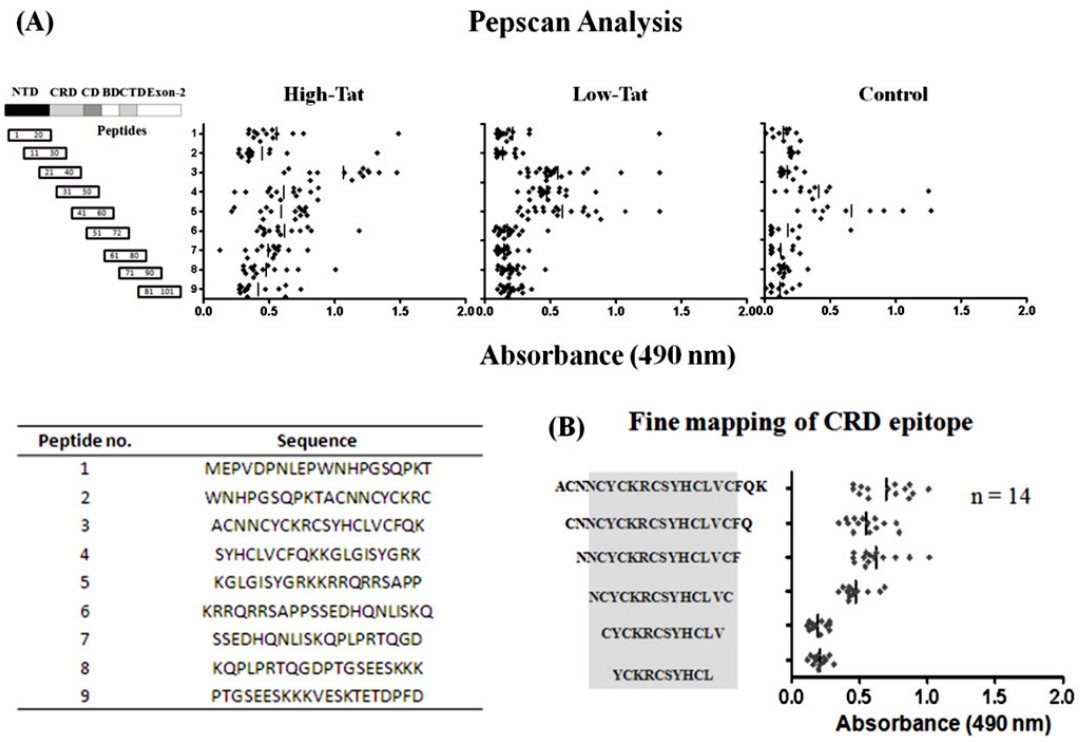


Figure 2.6: Avidity determination of anti-Tat antibodies in HIV plasma samples. Three samples each were randomly drawn from high-Tat (diamonds), low-Tat (squares) and the control (circles) groups. Each curve represents an individual clinical sample. The x-axis represents the concentration of NaSCN while and y-axis the percent absorbance.

2.2.3 A novel epitope in the cysteine-rich domain is recognized only in the viral infection

We performed a pepscan analysis using randomly selected subsets of samples from the three groups to examine which domains of Tat were targeted by the Tat-reactive antibodies in the respective groups (Fig. 2.7.A). Synthetic oligopeptides of 20 amino acid residues, with 10 residue overlap between adjoining peptides, representing subtype C Tat consensus sequence and spanning the full-length of Tat were used in the analysis. The secondary antibody is targeted to detect all the antibody isotypes including IgM and IgG. The control plasma samples identified only the basic domain (peptide 5) but not any other domains in Tat (Fig. 2.7.A, right panel). The samples of the low-Tat group too recognized the BD. Additionally, these samples also targeted the cysteine-rich domain (CRD) of Tat (peptide 3) with equal intensity suggesting the presence of two different Tat-reactive IgM species (Fig. 2.7.A, middle panel). In the high-Tat group, the antibodies primarily targeted the CRD epitope although other domains of Tat including BD were also recognized at a considerably lower magnitude (Fig. 2.7.A, left panel). Thus, in high-Tat samples, we identified three different species of Tat-reactive antibodies, the natural IgM targeting the BD, the infection-induced IgM and IgG both recognizing the CRD. Further, using a new set of synthetic peptides, we fine-mapped the precise limits of the B-cell epitope in the CRD to 14 amino acids NCYCKRCSYHCLVC between residues 24 and 37 (Fig. 2.7.B). Taken together, the viral infection appears to recruit T-help primarily to Tat CRD resulting in the de novo generation of IgM to this specific domain in a large majority of the samples with a further class-switch to IgG1 in a small but significant number of samples.



Peptide no.	Sequence
1	MEPVDPNLEPWNHPGSQPKT
2	WNHPGSQPKTACNNCYCKRC
3	ACNNCYCKRCSYHCLVCFQK
4	SYHCLVCFQKKGLGISYGRK
5	KGLGISYGRKKRRQRRSAPP
6	KRRQRRSAPPSSEDHQNLISKQ
7	SSEDHQNLISKQPLPRTQGD
8	KQPLPRTQGDPTGSEESKKK
9	PTGSEESKKKVESKTETDPFD

Figure 2.7: Immunodominant epitopes in Tat as recognized by Tat-reactive antibodies. **(A)** Pepscan analysis. The synthetic peptides used in the assay represent the consensus sequence of subtype C Tat. The assay is expected to detect all the antibody isotypes including IgM and IgG. The schematic diagram of Tat and the peptide location have been presented on the left side. The peptide sequences have been presented in the table below. The pepscan analysis was performed on samples drawn randomly from three different groups. Each dot represents an individual sample. The mean absorbance value of each group is represented by a horizontal line. **(B)** Fine-mapping of the B-cell epitope in high-Tat plasma samples. The core CRD epitope is highlighted by shading.

2.2.4 Cross-clade reactivity of anti-Tat antibodies

Given the high magnitude of genetic variation of HIV-1, one of the objectives of a potential HIV vaccine is to generate broad range immune protection against diverse viral subtypes. We previously demonstrated the predominance of subtype C in India (Siddappa et al. 2005) and subtype-specific signature residues in Tat. Cross-clade immune reactivity of the Tat antibodies has been reported previously from other clinical cohorts (Grant R. Campbell et al. 2007). The ability of these antibodies in the Indian clinical samples to

cross-react with Tat of five major viral subtypes was analyzed in indirect ELISA. Tat proteins consisting of exon-1 (all five clades) or full-length (only B and C) were purified as reported previously (Siddappa et al. 2006). Only the plasma samples from the high-Tat group were evaluated in this analysis. Interestingly, these samples demonstrated a broad range of cross-reactivity with three of the five subtype Tat proteins C, D and AE, moderate reactivity with A and low reactivity with B (Fig. 2.8.A). Unexpectedly, the reactivity of the antisera was higher for heterologous Tat proteins of subtypes AE and D as compared to homologous clade C even though all the plasma samples were confirmed subtype C infections. Of note, with the exception of subtype B Tat, antibody reactivity differences among other subtypes were not statistically significant. Furthermore, the antisera also showed a very broad range of reactivity with the Tat proteins of C, D and AE clades. Full-length Tat proteins demonstrated marginally higher reactivity compared to exon-1 of the corresponding protein suggesting that exon-2 did not contribute significantly towards the immune reactivity in these cohorts unlike in a previous report. SDS-PAGE analysis of the recombinant Tat proteins confirmed the quality and quantity of the recombinant proteins (Fig. 2.8.B). The previous study also demonstrated identical data where samples derived from clades A and D infections of Uganda showed higher reactivity with Tat of clades C, D and AE (Grant R. Campbell et al. 2007). Put together, these data seem to suggest that regardless of the heterogeneous nature, clade AE Tat is highly cross-reactive closely followed by clade D and C proteins in that order.

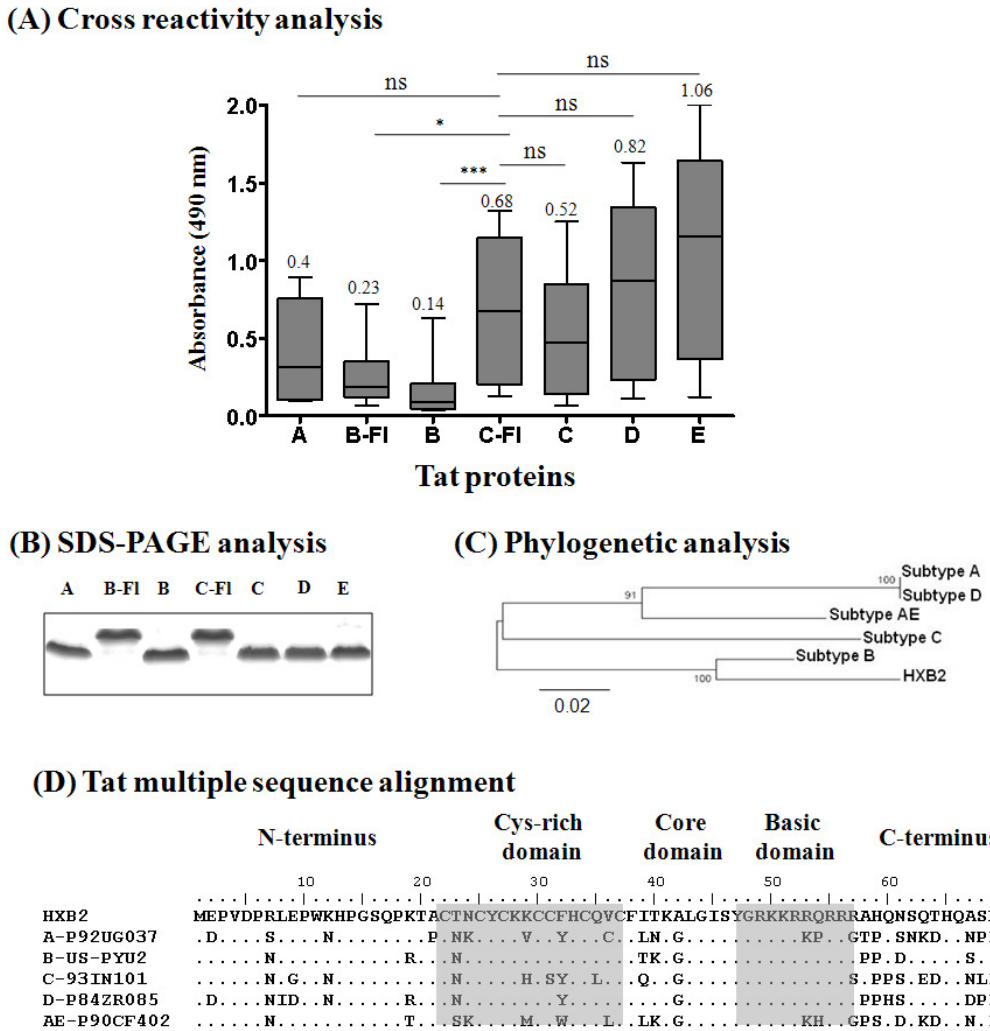


Figure 2.8: Cross-clade reactivity of anti-Tat antibodies (A) Immune-reactivity of high-Tat antibodies with recombinant Tat proteins of five different viral subtypes. Note that only clades B and C Tat proteins were used also as full-length (Fl). The numbers above the boxes represent mean absorbance value of the group. The secondary antibody is targeted to detect all the antibody isotypes including IgM and IgG. **(B)** SDS-PAGE analysis of the recombinant Tat proteins. **(C)** Phylogenetic relationship of Tat exon-1 of different subtypes. The tree was rooted by using HXB2 Tat. **(D)** Amino acid sequence variation in Tat representing different viral clades. Dots represent sequence homology. The core CRD B-cell epitope is boxed and important variations highlighted by shading.

The phylogenetic analysis of the Tat proteins did not provide a clue as per the superior reactivity of anti-Tat antibodies to heterologous Tat proteins (Fig. 2.8.C). Amino acid sequence alignment of Tat, especially in the CRD identified important differences within the core B-cell epitope (Fig. 2.8.D). We are presently evaluating if these variations

in the core epitope might serve as the anchoring residues for the MHC class-II presentation and influence antibody avidity. A prediction analysis of the antigenicity of all the five Tat protein amino acid sequences, using three different algorithms, identified a longer window of hydrophilic amino acids in the middle part of subtype E Tat protein, spanning the immunodominant CRD, suggesting that probably subtype E Tat protein is antigenically more accessible to the antibodies than other Tat proteins (Fig 2.9).

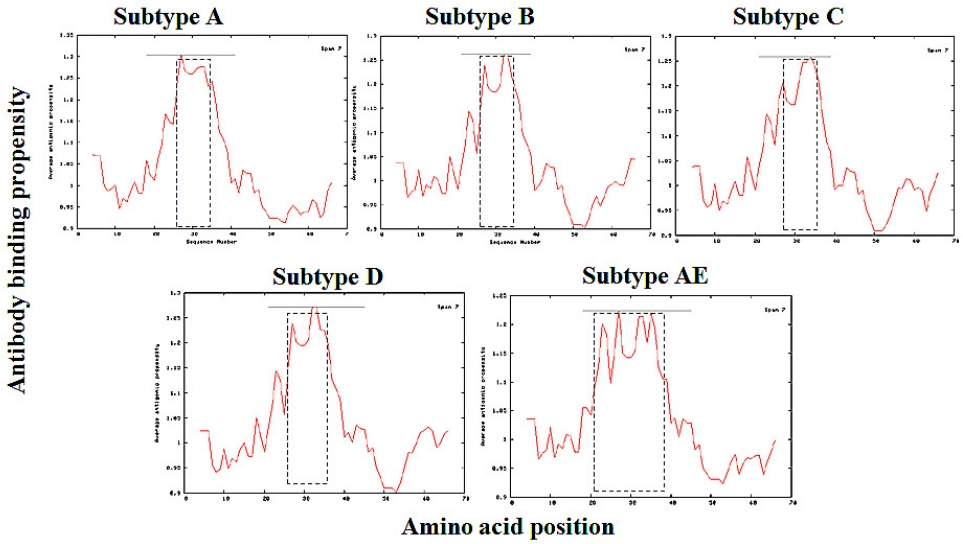


Figure 2.9: Tat antigenicity prediction: The antigenicity of the Tat proteins derived from different viral subtypes was predicted using an online antigenicity prediction tool. On the x-axis is the amino acid position and on the y-axis is the antibody binding propensity of Tat from different subtypes. The method of prediction is reported to be up to 75% accurate. The dotted line corresponds to the region of Tat that has the highest binding propensity.

2.2.5 Tat-reactive antibodies neutralize exogenous Tat protein

Tat neutralization potential of the anti-Tat antibodies in the high-Tat group was evaluated using HLM1 cells in a virus rescue assay. Recombinant subtype C Tat protein was incubated with the plasma samples prior to addition of the plasma-Tat mixture to the wells seeded with the cells. Significant quantities of p24 were secreted when the cells were supplemented with Tat and in the absence of the plasma antibody (Fig. 2.10). A Tat-specific monoclonal antibody, E6.4, reduced Tat transactivation significantly to 30% level. Only marginal reduction in Tat transactivation was observed with a representative control plasma sample due to the presence of natural antibodies. A large majority of the high-Tat plasma samples neutralized exogenous Tat with an efficiency comparable to E6.4, where Tat transactivation was inhibited by up to 50-70% as compared to the Tat control.

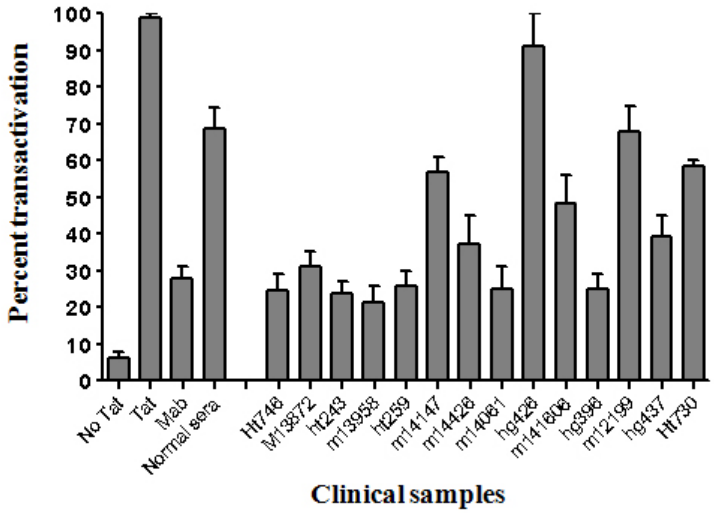


Figure 2.10: Tat transactivation is effectively blocked by anti-Tat antibodies in the plasma samples. Full-length, recombinant subtype C Tat protein was used in these assays. Only samples from the high-Tat group have been used in the analysis. Tat transactivation of the provirus in HLM1 cells could be blocked using a monoclonal antibody, E6.4, raised in-house, but not normal human plasma. The percent transactivation at 72 h was plotted on the y-axis with samples on the x-axis. The mean absorbance of each sample was plotted as percent transactivation value, considering the absorbance of 'Tat-alone' well as 100%.

2.2.6 Antibodies targeting the CRD demonstrate superior Tat-neutralization potential

To evaluate the ability of CRD binding antibodies to neutralize exogenous Tat as compared to those binding to BD, we performed viral rescue assay using HLM1 cells. Given the paucity of the individual clinical samples, we were unable to adopt the strategy of selective depletion of individual antibody species to examine the effect on Tat-neutralization. Alternatively, we used synthetic peptides representing the CRD and BD of Tat, respectively, to mask Tat-reactive antibody species selectively. Following the incubation with the peptides, the antibody species left unmasked would be the ones that should neutralize Tat from activating the provirus in HLM1 cells. In the absence of competing peptides, since all the Tat-reactive antibodies were available to block Tat, transactivation was abrogated up to 75% in high-Tat responders (Fig. 2.11, left panel). A moderate increase in transactivation, but not statistically significant, was observed in the presence of BD peptide as compared to 'no peptide' due to masking of the IgM antibodies to BD. A similar treatment of the samples with the CRD peptide enhanced Tat transactivation further suggesting that the infection-induced Tat antibodies are the most potent in neutralizing extracellular Tat as compared to the natural IgM (Fig. 2.11, left panel). Tat neutralization by infection-induced antibodies in this group was statistically significant as compared to the 'no peptide' control ($p<0.05$). Similar results were obtained with the low-Tat responder group (Fig. 2.11, middle panel). Masking the infection-induced IgM with the CRD peptide resulted in significantly higher levels of Tat transactivation as opposed to masking the natural IgM with the BD peptide. Finally, in the control group that contained only the natural IgM antibodies reacting with the BD, masking the antibodies with the BD peptide resulted in significant levels of Tat

transactivation. Furthermore, the CRD peptide did not affect the levels of Tat transactivation (Fig. 2.11, right panel) confirming the specificity of the assay.

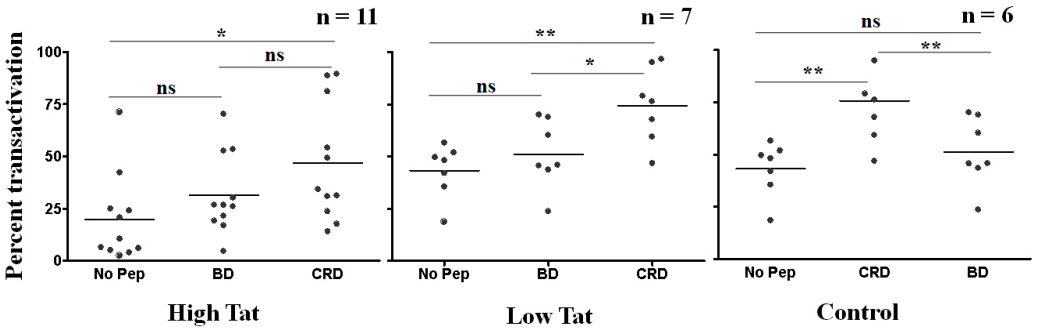


Figure 2.11: Anti-CRD antibodies block Tat effectively. Selective masking of Tat-reactive antibodies using synthetic peptides. Plasma samples belonging to three different groups were used in the assay. The number of samples constituting each group has been shown. *: $p<0.05$, **: $p<0.005$, ns: not significant at confidence interval of 95%.

2.2.7 Sequence analysis of high-Tat group shows no distinct pattern

We determined the primary sequences of 7 high-Tat and 3 low-Tat samples to examine if significant differences at the amino acid level could underlie the observed antigenicity differences of Tat with respect to humoral immune response. Tat cloned from an African individual, Tat Oyi, was shown to be broadly and highly immunogenic previously and the stronger antigenicity of this Tat has been ascribed to the primary amino acid sequence of this Tat protein (David L. Yirrell et al. 2002). Tat was amplified using target-specific primers and genomic DNA extracted from cryo-preserved cells and the DNA sequence was determined directly from the amplified PCR product. In multi-sequence alignment, all the clinical samples were found within the subtype C cluster and were closely associated with the Indian reference strains, confirming their Indian origin. Furthermore, the 6 signature amino acids of subtype C Tat (Ranga et al. 2004) were preserved in all the 10 Tat proteins (Fig 2.12, upper panel). However, the multiple sequence alignment of the sequences from the cohort failed to identify a distinct pattern to

differentiate between from the high- and low-Tat groups (Fig 2.12, lower panel). The number of samples used in this analysis is small and the possibility of Tat primary sequence influencing its antigenicity differences between the two groups appears to be small and hence suggesting a possible role of the host-genetic factors. It remains to be determined if an evaluation of T-helper epitope profile and additional examination of host factors could explain the Tat antigenicity differences between the two groups.

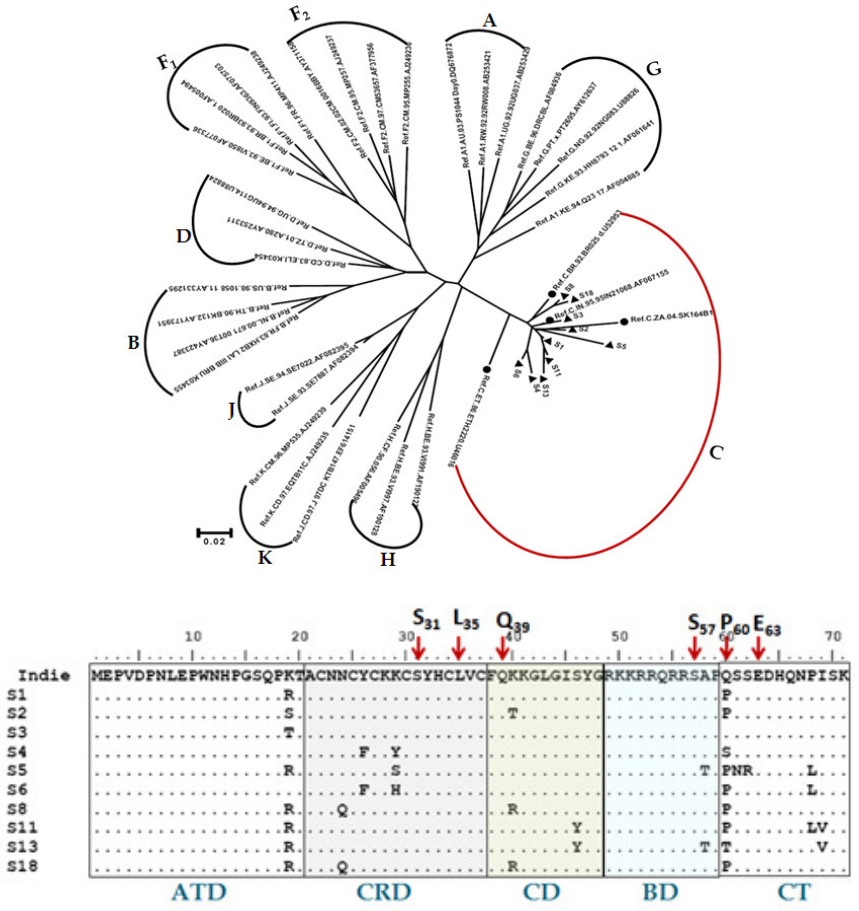


Figure 2.12: Sequence analysis of Tat from the Indian cohorts The upper panel presents the phylogenetic relationship between representative clinical samples randomly drawn from the high-Tat (n =7) and low-Tat (n = 3) groups. The reference Tat sequences of HIV-1 subtypes A-K have been taken from the HIV sequence databank. A neighbor-joining tree was constructed on the basis of the hidden Markov model nucleotide alignment of Tat sequences. Branch lengths are drawn to scale with the scale bar representing 0.02 nucleotide substitution per site. The lower panel represents Tat protein multiple sequences alignment with Indie-C1 Tat as the reference. Subtype C Tat signature amino acids have been highlighted with red arrows and illustrated.

2.3 Discussion

Several issues surrounding the role of Tat in viral disease progression and immune protection remain controversial. A large number of studies reported an indirect correlation between disease progression and/or viral load and humoral and/or cell-mediated immune responses (Wieland et al. 1990; S. S. Cohen et al. 1999; Krone et al. 1988; Peter Reiss et al. 1990; Rappaport et al. 2003; M. C. Re et al. 2001; M C Re et al. 1996; TC Rodman et al. 1992; van Baalen et al. 1997; Caputo et al. 2005; Rappaport et al. 1998; La Placa et al. 1995; P Reiss et al. 1991). One study, in contrast, documented a positive correlation while a few others failed to see any such correlation (D Senkaali et al. 2008). It is therefore controversial whether immune responses to Tat have a pathological significance.

In our clinical cohorts, the presence of a significant number of people being high-responders to Tat is paradoxical given the immunosuppressive and non-immunodominant nature of Tat although experimental evidence exists that Tat could also function as a positive immunomodulator enhancing immune responses (Cafaro et al. 2008; Fanales-Belasio et al. 2002; Cafaro et al. 2004). Understanding the mechanisms underlying the high-responsive nature of these subjects and the neutralization potential of the anti-Tat antibodies has direct relevance for Tat vaccine design. The data presented here implicate that Tat is as an immunodominant antigen in this minority of subjects. The possibility of these subjects being high responders specifically to Tat due to genetic predisposition needs to be evaluated in future studies. Importantly, Tat can tolerate significant levels of genetic variation without compromising over the transactivation properties (David L. Yirrell et al. 2002). It is critical to evaluate if some of these natural genetic variant forms of Tat could have diametrically opposite functions to the extent that some of them could function as immune activators.

The immunodominant nature of different Tat domains in natural infection appears to be highly variable. Although the N-terminal domain and the exon-2 of Tat have been primarily targeted by Tat antibodies (Rappaport et al. 2003; Pauza et al. 2004; McPhee et al. 1988; T C Rodman et al. 1993), with the exception of a few studies (Tähtinen et al. 1997; TC Rodman et al. 1992), CRD has not been identified as a common B-cell epitope. In the study by Rodman et al, although CRD was identified by IgM antibodies in both seropositive and seronegative subjects, the levels of the Tat-reactive natural antibodies were too low and importantly, these antibodies failed to recognize any other domains of Tat. In an analysis of a human Fab phage display library generated from peripheral blood lymphocytes of an individual who remained asymptomatic over 10 years of infection, Pilkington et al identified that 3 of the 4 anti-Tat Fab clones isolated were directed against the CRD (Pilkington et al. 1996). Tahtinen et al evaluated anti-Tat IgG antibody responses in a Finnish clinical cohort against recombinant Tat proteins. Out of 83 seropositive sera used in the study, only 15 samples (18%) demonstrated a strong response against Tat in ELISA suggesting isotype switch (Tähtinen et al. 1997). A pepscan analysis using a set of 8-mer Tat peptides spanning the length of Tat identified four different B-cell epitopes in Tat including CRD and BD in addition to one at the amino-terminus and the other in exon-2. None of the control sera demonstrated significant reactivity against any of the Tat epitopes perhaps because their assay measured only IgG, but not IgM, antibodies. In contrast to the report by Tahtinen et al, our study included a much larger sample size and evaluated all of the antibody isotypes including IgM and IgG in the primary analysis, as the secondary antibody detected all the isotypes. Furthermore, CRD in our cohorts was not only a novel epitope identified only in infection but also was the most immunodominant one as compared to other domains. The reasons why CRD was not identified in other cohorts are not clear. It is possible that CRD is

immunodominant in subtype C and/or in the Indian host specifically. Additionally, the primary amino acid sequence of the CRD contains multiple variations among the viral clades. We previously demonstrated signature amino acid residues in subtype C Tat at 7 different locations (Ranga et al. 2004). Of these variations, 2 of the residues at positions 29 and 31 are located right in the middle of CRD. Out of the 14 amino acids constituting the CRD, subtype C varies from the other four subtypes at as many as 6 positions as illustrated Fig. 2.8.D. It remains to be determined if the clade-specific variations within the CRD could influence immunogenicity of this domain across viral clades.

Data presented in Fig 2.8 ascertain the existence of cross-clade immune reactivity of the anti-Tat antibodies in the high-Tat group. The antibodies from the high-Tat group, broadly cross-recognized three of the five subtype Tat proteins (C, D and AE), displaying moderate reactivity with A and low reactivity with B. Of note, the observed differences in anti-Tat antibody reactivity with different Tat proteins were, however, not statistically significant with the exception that a statistical difference could be observed only between clade B Tat and clades C, D and E proteins. The observed higher level antibody cross-reactivity to heterologous Tat protein from a different clade as compared to homologous proteins is intriguing (Fig. 2.8.A). This phenomenon was also reported by other groups previously (Cafaro et al. 2003; Grant R. Campbell et al. 2007). The Campbell et al study from Uganda where clades A and D infections are prevalent demonstrated data identical to those of ours where their samples showed higher reactivity with Tat of clades C, D and AE (Grant R. Campbell et al. 2007). Interestingly, an analysis of the antigenic nature of Tat proteins of all the five major clades using three different algorithms predicted that clade-E Tat to be more accessible for antigenic interaction as compared to others (Fig. 2.9). This observation could possibly serve as a partial explanation for the noted Tat cross-clade antigenic responses of the antibodies with subtype E Tat.

It was important to examine if the anti-Tat IgG1 antibodies in the plasma samples were more efficient in neutralizing extracellular Tat as opposed to anti-Tat IgM or natural IgM. The best way to address this question was to deplete each of the antibody species specifically and evaluate Tat neutralization. Since we did not have adequate quantities of clinical samples for antibody depletion, we developed an alternative strategy of masking individual subsets of antibodies targeting a specific domain of Tat using synthetic peptides encompassing CRD or BD domains (Fig. 2.11). This strategy, however, allowed us only to compare the neutralization significance of basic domain versus the cysteine-rich domain of Tat. Our assay could not distinguish between the infection-induced IgM and IgG antibodies since these two species target the same CRD of Tat. The neutralization significance of CRD as compared to the BD is distinguishable in the low Tat-titer group than in the high Tat-titer group for two different reasons. First, in the low Tat group, there were only two species of IgM each targeting different Tat domains, the natural IgM antibody reacting with the BD and the anti-Tat IgM reacting with the CRD. Secondly, in the low Tat group, Tat domains other than BD and CRD were not recognized to a significant extent thus keeping the noise to the minimum in the assay. In contrast, in the high-Tat group, although the CRD is the most immunodominant epitope, antibodies reacted with almost all the other domains (Fig. 2.7.A, middle panel). Collectively the data presented in figure 2.7 suggested that the CRD of Tat is a potential target for antibody-mediated Tat neutralization.

In summary, for the first time from India, we carried out an evaluation of humoral immune response to HIV-1 Tat protein from two different Indian cohorts with extensive characterization of the antibodies for cross-clade reactivity, Tat-neutralizing ability, epitopes recognition and their isotypic nature. The results presented here have important implications for Tat vaccine design. Additional studies are required to examine whether

these antibodies confer protection against disease progression in these cohorts. The present study is only a cross-sectional analysis and the lack of quality clinical cohorts in India prevented us from addressing the important issue of correlation with disease progression.

Bibliography

van Baalen, C.A. et al., 1997. Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. The Journal of General Virology, 78 (Pt 8), 1913-1918.

Cafaro, A. et al., 2003. Sequence conservation and antibody cross-recognition of clade B human immunodeficiency virus (HIV) type 1 Tat protein in HIV-1-infected Italians, Ugandans, and South Africans. The Journal of Infectious Diseases, 188(8), 1171-1180.

Cafaro, A. et al., 2004. HIV-1 tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity. Journal of Immunology (Baltimore, Md.: 1950), 173(6), 3838-3843.

Cafaro, A. et al., 2008. The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: Implications for the design of new vaccination strategies against AIDS. Vaccine, 26(5), 727-737.

Campbell, G.R. et al., 2007. Tat mutations in an African cohort that do not prevent transactivation but change its immunogenic properties. Vaccine, 25(50), 8441-8447.

Caputo, A. et al., 2005. The presence of anti-Tat antibodies is predictive of long-term nonprogression to AIDS or severe immunodeficiency: findings in a cohort of HIV-1 seroconverters. The Journal of Infectious Diseases, 191(8), 1321-1324.

Cohen, S.S. et al., 1999. Pronounced acute immunosuppression in vivo mediated by HIV Tat challenge. Proceedings of the National Academy of Sciences of the United States of America, 96(19), 10842-10847.

Fanales-Belasio, E. et al., 2002. Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. Journal of Immunology (Baltimore, Md.: 1950), 168(1), 197-206.

Kolaskar, A.S. & Tongaonkar, P.C., 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Letters, 276(1-2), 172-174.

Krone, W.J.A. et al., 1988. Natural antibodies to HIV-tat epitopes and expression of HIV-1 genes in vivo. Journal of Medical Virology, 26(3), 261-270.

La Placa, M. et al., 1995. Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology: Official Publication of the International Retrovirology Association, 10(4), 408-416.

McPhee, D.A. et al., 1988. Recognition of envelope and tat protein synthetic peptide analogs by HIV positive sera or plasma. FEBS Letters, 233(2), 393-396.

Pauza, C.D. et al., 2004. Sequence Variation within the Dominant Amino Terminus Epitope Affects Antibody Binding and Neutralization of Human Immunodeficiency Virus Type 1 Tat Protein. J. Virol., 78(23), 13190-13196.

Pilkington, G.R. et al., 1996. Recombinant human Fab antibody fragments to HIV-1 Rev and Tat regulatory proteins: Direct selection from a combinatorial phage display library. Molecular Immunology, 33(4-5), 439-450.

Pullen, G.R., Fitzgerald, M.G. & Hosking, C.S., 1986. Antibody avidity determination by ELISA using thiocyanate elution. Journal of Immunological Methods, 86(1), 83-87.

Ranga, U. et al., 2004. Tat protein of human immunodeficiency virus type 1 subtype C strains is a defective chemokine. Journal of Virology, 78(5), 2586-2590.

Rappaport, J. et al., 1998. Interferon α and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. Proceedings of the National Academy of Sciences of the United States of America, 95(7), 3851-3856.

Rappaport, J. et al., 2003. Antibodies to Tat and Vpr in the GRIV cohort: differential association with maintenance of long-term non-progression status in HIV-1 infection. Biomedecine & Pharmacotherapy, 57(1), 4-14.

Re, M.C. et al., 1996. Antibody against human immunodeficiency virus type 1 (HIV-1) Tat protein may have influenced the progression of AIDS in HIV-1-infected hemophiliac patients. Clinical and Diagnostic Laboratory Immunology, 3(2), 230-232.

Re, M.C. et al., 2001. Antibodies against full-length Tat protein and some low-molecular-weight Tat-peptides correlate with low or undetectable viral load in HIV-1 seropositive patients. Journal of Clinical Virology, 21(1), 81-89.

Reiss, P. et al., 1991. Contribution of antibody response to recombinant HIV-1 gene-encoded products nef, rev, tat, and protease in predicting development of AIDS in HIV-1-infected individuals. Journal of Acquired Immune Deficiency Syndromes, 4(2), 165-172.

Reiss, P. et al., 1990. Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. Journal of Medical Virology, 30(3), 163-168.

Rodman, T.C. et al., 1993. Epitopes for natural antibodies of human immunodeficiency virus (HIV)-negative (normal) and HIV-positive sera are coincident with two key functional sequences of HIV Tat protein. Proceedings of the National Academy of Sciences of the United States of America, 90(16), 7719-7723.

Rodman, T. et al., 1992. Human immunodeficiency virus (HIV) Tat-reactive antibodies present in normal HIV-negative sera and depleted in HIV-positive sera. Identification of the epitope. J. Exp. Med., 175(5), 1247-1253.

Senkaali, D. et al., 2008. Tat-specific binding IgG and disease progression in HIV type 1-infected Ugandans. AIDS Research and Human Retroviruses, 24(4), 587-594.

Siddappa, N.B. et al., 2005. Identification of unique B/C recombinant strains of HIV-1 in the southern state of Karnataka, India. AIDS (London, England), 19(13), 1426-1429.

Siddappa, N.B. et al., 2006. Transactivation and signaling functions of Tat are not correlated: biological and immunological characterization of HIV-1 subtype-C Tat protein. Retrovirology, 3, 53.

Tähtinen, M. et al., 1997. B-cell epitopes in HIV-1 Tat and Rev proteins colocalize with T-cell epitopes and with functional domains. Biomedecine & Pharmacotherapy, 51(10), 480-487.

Wieland, U. et al., 1990. Antibodies to recombinant HIV-1 vif, tat, and nef proteins in human sera. Medical Microbiology and Immunology, 179(1), 1-11.

Yirrell, D.L. et al., 2002. Tat HIV-1 Primary and Tertiary Structures Critical to Immune Response Against Non-homologous Variants. J. Biol. Chem., 277(39), 35915-35919.

3. Immunodominance of the N-terminal domain B-cell epitope in Tat and strategies to spread the immune response to cryptic epitopes

Immune responses to Tat are considered necessary for protection and/or responsible for delayed disease progression. Tat therefore is an attractive vaccine candidate. Tat, however, is inherently a moderate immunogen (Wieland et al. 1990; Re et al. 1996). Work from our own laboratory demonstrated that in Indian clinical cohorts, antibodies to Tat are present in up to only 14-20% of HIV seropositive individuals and a class switch to IgG is manifested in even fewer individuals (Kashi et al. 2009). Various biological properties of Tat, including its nuclear localization, relatively smaller protein size and importantly the immunosuppressive quality may contribute towards the non-immunodominant nature of Tat. Tat predominantly is an intra-cellular protein, although experimental evidence suggests its secretion into the body fluids. Further, Tat is not exposed on the surface of the virus. Cell-mediated immune responses to Tat, therefore is believed to be the predominant component to restrict viral expansion *in vivo,* however antibodies do play a significant role. Majority of the previous strategies used Tat as a recombinant protein or toxoid in animal immunization studies (A. Cafaro et al. 1999; B Ensoli & A Cafaro 2000; C D Pauza et al. 2000; Max W Richardson et al. 2002; Tikhonov et al. 2003) or human clinical trial (Barbara Ensoli et al. 2006; Longo et al. 2009). As proteins, these antigens are less likely to access the MHC-I compartment to stimulate efficient anti-viral cell-mediated immune response. Although Tat protein is known to be cross-presented to MHC-I compartment (Kim et al. 1997) it is not likely to be a predominant pathway of antigen presentation. The absence of strong cellular immune responses in the previously reported studies underlies the importance of targeting Tat to

MHC-I compartment for vaccine development. Recombinant viruses could efficiently introduce encoded antigens into MHC-I pathway. Further, preexisting immune response to the viral vector is a significant problem that limits recombinant vector-mediated antigen delivery (Bangari & Mittal 2006). DNA vaccine, therefore, is considered to be an ideal medium for antigen delivery given that this form of vaccination can stimulate strong immune responses akin to viral vectors. DNA vaccines, however, have several technical challenges that must be addressed before they could be used as a reliable medium of immunization (Dean 2005). The most important limitation of the DNA vaccines is the degradation of a large quantity of the injected DNA extra- and intra-cellularly before it could reach the nucleus where it could be transcribed (Donnelly et al. 2005). DNA vaccines by themselves are known to have low adjuvanticity and studies exploring the possibilities of using several molecules as adjuvants represent an important part of DNA vaccine research (Garmory et al. 2003; Barouch et al. 2004). Promoter silencing is a significant limitation of DNA vaccines as most of the current vectors employ eukaryotic promoters of viral origin that are recognized and down modulated by the cell often leading to inconsistent gene expression (Donnelly et al. 2005). Furthermore, genetic immunizations although elicit a strong cell-mediated response, are poor in evoking humoral response and presence of antibodies to Tat is negatively correlated with disease progression (Antonella Caputo et al. 2005).

One possible solution for the low-immunization potential of Tat DNA vaccine is to adapt a prime-boost strategy using DNA for priming and subsequent booster immunizations with Tat protein. Tat protein immunization, however, might lead to a problem of a different kind. Tat protein in its native form is known to be toxic to cells (reviewed in chapter 1). The nature of Tat format in which it is delivered appears to influence the nature of epitope recognition. In Tat DNA immunization, the immune

response is typically and uniformly distributed among diverse B-cell epitopes consisting of the N-terminal domain (NTD), cysteine-rich domain (CRD), core domain (CD), basic domain (BD) and exon-2 (Caselli et al. 1999; Ramakrishna et al. 2004). In human beings, in the absence of infection, the basic domain appears to be immunodominant targeted by the natural antibodies (Max W Richardson et al. 2003; D. M. Noonan et al. 2003; Tracy J Ruckwardt et al. 2004). In a large number of seropositive subjects, the NTD, CRD and exon-2 are immunodominant (Belliard et al. 2005; Goldstein et al. 2001). In contrast, in experimental protein immunization, the NTD of Tat seems to be the most immunodominant B-cell epitope to the extent that no other epitope in Tat exon-1 is recognized. Anti-Tat IgG is predominantly focused on the 15 amino acid residue N-terminal epitope when Tat is administered as protein in various animals including human beings, mice, rats, rabbits and sheep (A. Cafaro et al. 1999; B Ensoli & A Cafaro 2000; C D Pauza et al. 2000; Max W Richardson et al. 2002; Tikhonov et al. 2003) although exceptions have been reported (Demirhan et al. 1999; Mascarell et al. 2006). Furthermore, although Tat is considered to be relatively well conserved, clade-specific sequence variations have been reported (Ranga et al. 2004). Importantly, sequence variation in the immunodominant NTD of Tat affects the ability of antibodies to bind and facilitate cross-clade neutralization (Campbell et al. 2007; Tracy J Ruckwardt et al. 2004). In order to attain cross-clade neutralization and to prevent the out-growth of escape mutants it is imperative that multiple epitopes are targeted by immune responses. Immunodominant nature of the Tat NTD was quite explicit in our own attempts at generating monoclonal antibodies to Tat (see the next chapter). In several independent fusions with Tat protein immunization of mice, several hybridomas exclusively identified only the N-terminal epitope of Tat. Only after deleting the N-terminal 15 amino acid residues, we could obtain monoclonal antibodies to the core domain of Tat. In the

presence of the NTD, the immune system appears to ignore other epitopes identified in the natural infection and in other immunization protocols. A previous publication too reported the domination of NTD of Tat in hybridoma generation (Brake et al. 1990). The skewed nature of immune response to one epitope in the protein is undesirable as immune response to a broad spectrum of epitopes is essential for efficient neutralization of Tat (Tracy J Ruckwardt et al. 2004). Further, the underlying factors which can skew the humoral response such as the T-helper epitope(s) that might influence the immune response to NTD and epitope polarity with respect to the position of T-helper epitope are not well understood.

However, despite the above concerns, Tat still remains an important vaccine candidate. The primary objective of the present work, therefore, is two-fold, to understand the factors underlining the immunodominant nature of the NTD of Tat on the one hand and to devise means to break the immunological interference and broaden the immune responses to other domains in Tat through protein immunization. Engineering the pan antigen DR epitope (PADRE), a universal HLA-DR binding peptide (Alexander et al. 1994), or other T-helper epitopes into antigens is one of the molecular strategies extensively used by many groups to enhance antigen-specific immune responses (Alexander et al. 1998). Given that peptide antigens are less immunogenic, PADRE epitope has been widely used to enhance immunogenicity of this form of vaccines (Beebe et al. 2007; Fitzmaurice et al. 1996; Decroix et al. 2002). Use of universal T-helper epitope in protein vaccines is less common although some examples are available (Greenstein et al. 1992). Carbohydrate vaccines, derived from pathogenic organisms, which are least immunogenic intrinsically, too have been shown to become immunogenic after conjugating such substrates to PADRE epitope (Bélot et al. 2005; Alexander et al. 1994). Use of PADRE for mucosal vaccines has been documented (Decroix et al. 2002).

A large quantum of effort has been directed against diverse type of cancers by generating cancer-specific peptides or antigens that are molecularly linked to T-helper epitopes (Rice et al. 2004; Mansour et al. 2007). T-helper epitopes have been engineered into DNA vaccines to augment their performance against viral infections (Hsu et al. 1999; W. Gao et al. 2004; Hung et al. 2007) including HIV-1 (Gorse et al. 2008; Newman et al. 2002). Polyclonal antisera with high antibody titers were raised in experimental animals against more than a hundred different antigens when these antigens were expressed as chimeras of PADRE epitope suggesting generic and wide application of T-help recruitment to a broad range of antigens. Recruitment of T-help through PADRE T-helper epitope has also been documented against parasite infections (Rosa et al. 2004) and even auto-immune disorders like Alzheimer's (Agadjanyan et al. 2005) or experimental autoimmune encephalitis (Uyttenhove et al. 2004).

A wide range of molecular strategies have been employed to enhance performance of different types of vaccines (Lecoq et al. 2008; Mayol et al. 2007; Mascarell et al. 2005). The strategies encompassed a wide array of formats to improve protein expression, transcript stabilization, antigen processing and presentation, antigen delivery, coadministration of immune modulatory factors, recruiting innate immune components and many more. Further, adjuvants such as nanoparticles that enhance immune response to Tat considerably have also been designed (Antonella Caputo et al. 2009). An important hurdle in eliciting strong antibody response is the susceptibility of Tat protein to enzymatic cleavage and hence strategies have been developed to increase the stability by conjugating Tat to sulfated sugar moieties (Lecoq et al. 2008).

However, most of the mentioned strategies do not address the issue of Tat toxicity and low immunogenicity of Tat. Attempts have been made to formulate Tat protein as a toxoid by chemical treatment (A Gringeri et al. 1998; H Le Buanec et al. 2001). Tat

toxoid was shown to be safe which also generated moderate immune responses in humans (A Gringeri et al. 1998; D. M. Noonan et al. 2003; Moreau et al. 2004) and primates (C. David Pauza et al. 2004; Max W Richardson et al. 2003). Although several studies demonstrated immunogenicity of Tat taxoid, often comparable to that of Tat protein, evidence also exists that Tat toxoid may generate qualitatively different kind of immune responses as compared to the native antigen (Tikhonov et al. 2003). However, native, but not oxidized, Tat promoted maturation of monocyte-derived dendritic cells and efficient antigen presentation suggesting that native Tat could be a superior vaccine candidate than the attenuated forms (Fanales-Belasio et al. 2002). Additionally, native Tat protein also modulated the subunit composition of the immunoproteasomes leading to augmented antigen processing (Gavioli et al. 2004; Remoli et al. 2006). Tat mutants inactive for transactivation have been tested in mice but no progress is reported beyond this animal model (Caselli et al. 1999; Mayol et al. 2007). Oxidized Tat was proposed to be a safe format for vaccination (S. S. Cohen et al. 1999).

A novel approach to simultaneously abrogate the toxic properties of Tat and to augment its immunogenicity has been devised in our laboratory (Anand KK et al. manuscript in preparation and present work). The insertion of universal T-helper epitopes (HTL) is one of the most common strategies employed to augment immune responses to proteins and is based on the principle of 'linked recognition'. We engineered universal T-helper epitopes PADRE (pan DR epitope, from tetanus toxin, AKFVAAWTLKAAA) and/or pol_{711} (from RT of HIV, EKVYLAWVPAHKGIG) into CRD (between C30 and S31) and/or BD (between K52 and R53) of Tat. The Pol epitope and the PADRE HTL have been shown to bind to various HLA-DR and also several murine MHC-II molecules and augment immune responses (Alexander et al. 2000; Alexander et al. 1998; Franke et al. 1999; Boaz et al. 2003; Haas et al. 1991). The CRD and BD domains have been

implicated in various toxic functions of Tat and introducing mutations in these domains abrogated the functions of Tat (Mayol et al. 2007). DNA immunization of mice with domain-disrupted Tat DNA augmented strong cell-mediated immune responses and controlled viral load in mice (Anand KK et al, manuscript in preparation). The presence of Tat as an extracellular protein is of pathological importance (Bartz & Emerman 1999; Campbell et al. 2005; Campbell et al. 2004) and the induction of strong humoral immune response to Tat is critical to neutralize exogenous Tat. While DNA immunizations predominantly elicit cell-mediated immune response, protein immunizations are known to elicit strong humoral responses. The insertion of the universal HTL-epitopes is expected to augment not only cell-mediated immune responses, but also antibody responses. In this section, we will present a strategy devised to study the immunodominant nature of Tat NTD and how grafting of HTL epitopes resulted in the recognition of a cryptic B-cell epitope in Tat immune responses to subdominant epitopes.

3.1 Materials and Methods:

3.1.1 Cloning of Tat constructs: N-del, C-myc and N-C variants of Tat were constructed using appropriately designed PCR primers as summarized below.

Tat variant	Primer	Sequence	Cloning enzyme
N-del	**N632F**	AT**GGATCC**CCATATGAGTCAGCCGAAACTGCTTGC	NdeI
	N495R	GCCCTCT**CTCGAG**GTCGAAGGGGTCTGTCTC	XhoI
C-myc	**N636F**	AT**CATATG**GAACAGAAACTTATTTCTGAAGAAGATCTTCCGCGG	NdeI
	N637R	AT**GGATCC**GATCCCGCGGAAGATCTTCTTCAGAAATAAGTTTCTGTTCCA	BamHI
N-C	**N634F**	**CTCGAG**ATGGAGCCAGTAGATCCTAATCTAGAGCCCTGGAATCATCCAG GAC	XhoI
	N635R	**CTCGAG**TCCTGGATGATTCCAGGGCTCTAGATTAGGATCTACTGGCTCC AT	XhoI

The forward primer (N632F) for the N-del Tat was positioned at amino acid residue 16 and the reverse primer (N495R) at the end of exon-2 thus deleting the first 15 amino acids at the N-terminus. The amplified PCR product was cloned directionally into pET21b+ between sites NdeI and XhoI. In C-myc Tat, the N-terminal 15 amino acids of Tat were replaced by the 13 amino acid residues of the C-myc-tag (MEQKLISKKDLPR). An internal SacII site present in the forward primer was used for the screening of the recombinant clones. In the N-C Tat, the 15 amino acid NTD peptide from the N-terminus was shifted to the C-terminus using N-del Tat as the source vector. The coding sequences for the 1-15 amino acids were incorporated into primers N634F and N635R and the tag was generated by extending the two complementary primers on each other. The extended PCR tag was inserted at the C-term of N-del Tat into the XhoI site located upstream of the His-Tag of the pET21b+ vector. To enable easy screening of the recombinant clones an XbaI site was engineered without altering the original amino acid sequence. The HTL-

Tat-constructs were generated essentially as described in Anand KK et al, (manuscript in preparation).

3.1.2 Protein expression and purification: The recombinant Tat proteins expressed in *E. coli* (BL21DE3) were purified essentially as described previously (Siddappa et al. 2006). Briefly, two different chromatography techniques, Nickel-NTA affinity chromatography and SP-Sepharose, were used in succession to prepare endotoxin-free Tat protein. The protein fraction eluted from the Ni-NTA columns was directly loaded to the SP-Sepharose column without removing imidazole. All the solutions used for protein purification were supplemented with 1 mM of DTT to minimize oxidation of Tat. All the materials that came in contact with Tat were siliconized to minimize protein loss by adherence to surfaces. Two ml of Ni-NTA beads (70666, Novagen, Madison, MI) were equilibrated with the lysis buffer and the bacterial cell lysate was passed through the column at least four times to ensure protein binding. The column was washed thoroughly with 40 bed volumes of the wash buffer, (2% Triton X-100; 20 mM Tris-HCl, pH 7.9; 10% glycerol; 0.4 mM EDTA; 30 mM KCl; 0.1% IGEPAL; 50 mM imidazole; 0.2 mM PMSF and 1 mM DTT). Triton X-100, at 2% concentration, was employed to remove endotoxin. The washing procedure was repeated four additional times with the same buffer without Triton X-100. An additional wash with 0.5 M NaCl in phosphate buffer (pH 7.4) was used to remove proteins non-specifically bound to the resin. Protein was eluted by passing 5 ml of the elution buffer (300 mM imidazole and 1 mM DTT in 50 mM phosphate buffer, pH 6.4) and collected in 1 ml aliquots. One ml of the SP Sepharose ion exchanger resin (#17-0729-10, Amersham Biosciences, Uppsala) was packed into a column equilibrated with 50 mM phosphate buffer. The protein eluted from the Ni-NTA column was directly applied to the SP Sepharose column. The column was washed four times with phosphate buffer containing 2% Triton X-100 and 1 mM DTT to remove

residual endotoxins. Washing was repeated for four additional times with phosphate buffer without Triton X-100. Finally a single wash with 0.2 M NaCl in 20 mM Tris buffer (pH 8.0) was used to remove proteins bound nonspecifically. Bound Tat was eluted with 2.5 ml of elution buffer (0.5 M NaCl and 1 mM DTT in 20 mM Tris buffer, pH 8.0) and the eluted protein was collected in aliquots of 0.5 ml each, lyophilized and stored at -70^{0}C.

3.1.3 Electro-elution of protein for immunizations: To ensure complete removal of bacterial antigens, we resolved recombinant Tat proteins in 15% acrylamide gel and eluted the Tat proteins from the gel pieces using an electroeluter (Biorad, model 422). Following electrophoresis, the gel was stained by negative staining method using 5% $CuCl_2$ solution. The unstained portion of the gel, corresponding to the protein, was sliced out using a sterile scalpel and the gel piece was soaked in 10 ml of a solution containing 250 mM Tris and 250 mM glycine for 5 min. This wash step was repeated 5 times to remove traces of $CuCl_2$. Subsequently the slice was cut into small pieces and placed in dialysis membrane with appropriate cut-off limit. The dialysis bag covered with western transfer buffer (without SDS) and placed in a horizontal position in the electrophoresis tank. Electroelution of the protein was carried out for 1 h at 100 V. The protein was collected into a siliconized vial and the process of eleoctro-elution was repeated twice. The protein fractions were pooled, protein quality and quantity determined and proteins were subsequently used in mouse immunization.

3.1.4 Immunization protocol: All the experimental work was approved by the Institutional Bioethics Committee of JNCASR. Four to six weeks old female BALB/c mice were procured from the animal facility at JNCASR. The mice were immunized subcutaneously on days 1, 30, 45 and 60 with 25, 20, 10 and 5 µg of protein, respectively.

The first immunization was administered with Freund's complete adjuvant and subsequent booster immunizations with incomplete adjuvant. Blood samples were collected via the tail vein artery on days 29, 44 and 59 and stored at -20^{0}C in aliquots for further analysis.

3.1.5 Analysis of the anti-Tat antibody responses: Microtiter plates were coated with 400 ng of recombinant proteins in 100 µl of carbonate buffer (pH 9.0) and incubated at 37^{0}C for 2 h. The wells were blocked with 200 µl of 2% BSA and incubating the plates at 37^{0}C for 1 h. After thorough washing with the wash buffer (PBS supplemented with 0.1% Tween 20), appropriately diluted sera (with PBS supplemented with 0.01% Tween 20) were added to wells and incubated at 37^{0}C for 1 h. Following a thorough wash, anti-mouse antibody HRPO conjugate (Calbiochem, cat. 401253, reacting with IgG, heavy and light chains) diluted 10,000 fold was added to each well and the plates were incubated at 37^{0}C for 1 h. This antibody was used in all the assays with the exception of isotype analysis. Substrate solution (0.1 M citrate monohydrate, 0.2 M disodium hydrogen phosphate, 1 mg/ml OPD and 0.3% hydrogen peroxide) was added 100 µl/well and the plates were incubated in dark for 15 min. The enzyme reaction was stopped by adding 100 µl of 1 N HCl to each well. Absorbance was measured at 490 nm using a microplate reader (Biorad, Model 680). The titer of anti-Tat antibody was defined as the reciprocal value of the maximum dilution where the absorbance value was 0.5. For the antibody isotype analysis, rabbit secondary antibodies specific for mouse IgG1 or IgG2a (Sigma) were used in the assay. Following which anti-Rabbit HRPO was added to each well and the subsequent steps were as mentioned above.

3.1.6 Pepscan analysis for B-cell epitope mapping: Peptides (Genemed, CA, USA) encompassing the full-length of C-Tat protein, each 20 amino acid long and with an

overlap of 10 amino acids, were used to map the epitopes recognized by the antisera. The lyophilized peptides were dissolved in DMSO and stored at -80^{0}C at concentration of 40 mg/ml. Peptides were coated at a concentration of 1 µg/well in micro titer wells. Antisera diluted 1:500 times were added to appropriate wells and incubated at 37^{0}C for 1 h, washed and incubated with anti-mouse HRPO conjugated antibody. Subsequent steps of ELISA were as described above.

3.1.7 Lymphoproliferation assay for T-helper epitope mapping: Lymphoproliferation assay was performed essentially as described previously (Quah et al. 2007). Briefly, splenocytes isolated from immunized mice were suspended in PBS supplemented with 5% FCS and 110 µl of the cell suspension was distributed per tube to which CFSE (carboxyfluorescein succinimidyl ester) from a 5 mM stock was added to a final concentration of 2.5 µM. The tube was capped and inverted several times to ensure thorough mixing of the cells and incubated at room temperature for 5 min in dark. Immediately following incubation the cells were washed thoroughly with PBS supplemented with 5% FCS to remove unbound CFSE. The cells were resuspended in complete RPMI and 1x10^6 cells/well were dispensed into appropriately labeled 96-well plate. Appropriate peptides were added to the wells to a final concentration of 5 µg/ml. PHA (phytohemagglutinin) was used as the positive control for T-cell activation at a final concentration of 5 µg/ml. The plates were wrapped with aluminum foil and incubated at 37^{0}C in a CO_2 in incubator. After four days, cells were harvested, washed with PBS and stained with anti-CD4-PE antibody (Southern Biotech, Birmingham). CFSE dilution data was acquired using FACSCalibur (BD Biosciences) and analyzed by gating on live cell population based on forward and side scatter profile, followed by scoring for CFSE dilution in CD4+ cells. All the analysis was performed using CellQuest Pro software (BD Biosciences).

3.1.8 Virus rescue assay: Neutralization of Tat-mediated transactivation was assayed essentially as described previously (see section 2.1.8)

3.1.9 Apoptosis assay: Jurkat cells were seeded into a 96 well-plate, at a density of 1 x 10^6/well. B-Tat protein (2μg/ml) was incubated at 37^0C for 30 min with or without the sera diluted 1000-fold in serum-free medium. Following which, the samples were added to cells in appropriate wells and incubated at 37^0C in a CO_2 incubator for 1 h. Subsequently, FCS was added to a final concentration of 10% v/v. Cells were harvested after 8 h of incubation, washed with PBS and suspended in 100 μl of Annexin V binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$) supplemented with 20 μg/ml propidium iodide (Sigma) and 1 μl of Annexin V-FITC (Southern Biotech, Birmingham). The samples were incubated in dark for 15 min, washed and immediately analyzed on FACSCalibur (BD Biosciences). Percent apoptosis was evaluated using CellQuest Pro software.

3.2 Results

3.2.1 Deletion of the Tat NTD lowers the antibody levels in immunized mice

Interference in immune response due to antigenic competition, a well documented phenomenon, where the presence of an immunodominant epitope modulates immune response to molecularly-linked cryptic or subdominant epitopes, is undesirable for the design of a multi-subunit vaccine. As detailed earlier, immune response to Tat is almost exclusively directed to the NTD in experimental immunizations. To understand the immunodominant nature of the NTD of Tat we generated three different variant constructs of Tat for immunization. In one construct (N-del Tat), the NTD epitope of Tat was deleted to examine if elimination of the most dominant epitope would reassort immune responses to sub-dominant epitopes. In a different construct (C-myc Tat); the C-myc sequence of 13 amino acids was substituted for the N-terminal 15 residues of Tat to ask if the C-myc B-cell epitope would now become the immunodominant epitope when placed into the same context as the original NTD. In the third Tat construct (N-C Tat), the NTD was transferred to the C-terminus of Tat to test the importance of the temporal location for the immune-dominance of this epitope.

The recombinant Tat proteins, including the wild-type full-length subtype C Tat, were purified to homogeneity and used for subcutaneous immunization of BALB/c mice. Antibody titers against homologous antigens were evaluated in an indirect-ELISA 14 days following the second booster immunization. Antisera diluted serially were used in the assay. Antibody titers were 2200 $\pm$ 330 for the wild-type Tat and 1200 $\pm$ 180 for the N-del Tat confirming the immunodominant nature of NTD (Fig. 3.1). NTD deletion resulted in a significant reduction in anti-Tat antibody titers ($p<0.001$, Fig. 3.1). C-myc Tat immunized mice showed similar antibody titers against homologous C-myc Tat

protein. The antibody titers were comparable to N-del Tat immunization but significantly lower than wild-type Tat immunization (p<0.001). Importantly, C-myc sequence placed at the N-terminal end of Tat did not seem to have acquired immunodominance unlike the original NTD (also see Fig 3.2, lower panel). Interestingly, moving of the NTD to the C-terminus of Tat reconstituted the immune response such that the antibody titers in wild-type Tat and N-C Tat immunized mice were not statistically different (p = 0.45). This presented an interesting situation where transferring the NTD to a different location on Tat retained immunodominance suggesting that the immunodominance is an intrinsic quality of this epitope.

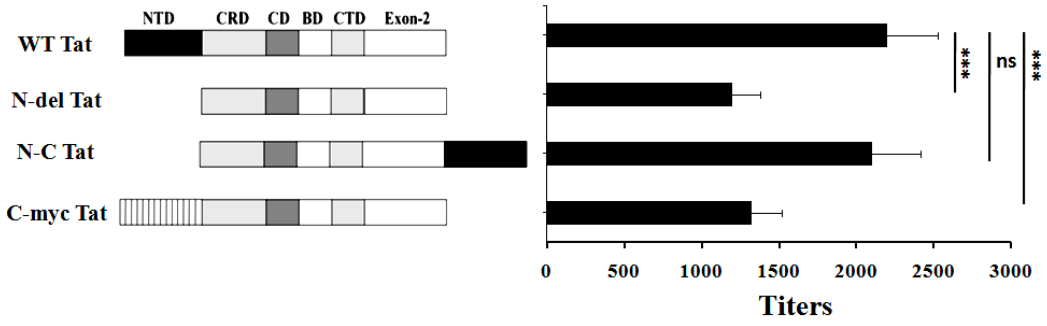


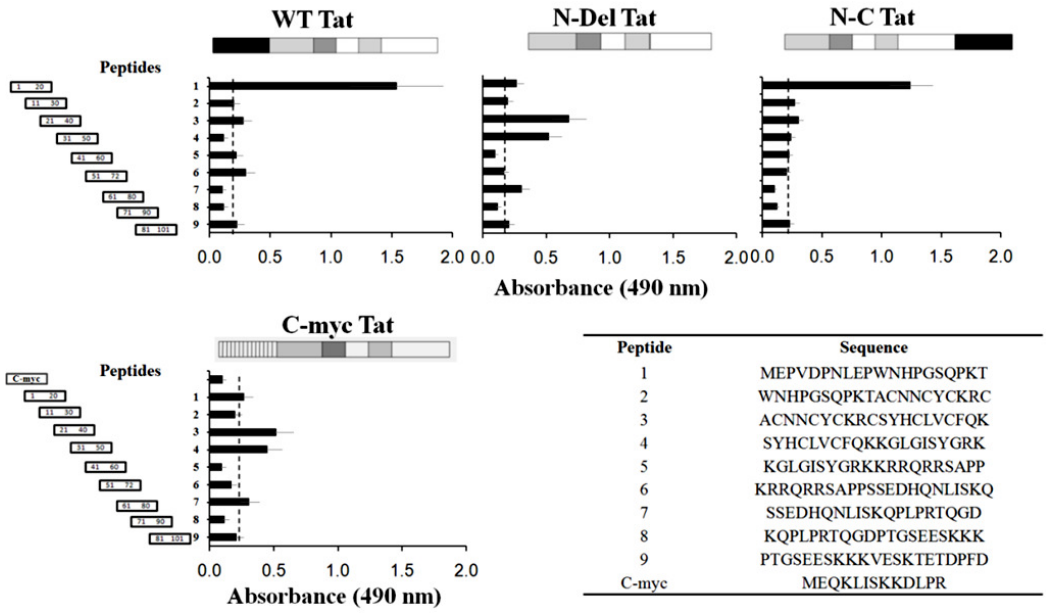
Figure 3.1: Immunogenicity of Tat variant recombinant proteins. Antigen-specific antibody titers in immunized mice were measured using indirect ELISA with homologous recombinant proteins immobilized in microtiter plates. BALB/c mice, 4 or 5 female animals per group were bled 14 days after second booster immunization. The domain structures of the recombinant proteins used in the immunization have been illustrated on the left hand side. The antisera were diluted serially (five-fold) for the assay and the inverse of the serum dilution at which the absorbance was 0.5 were defined as the antibody titers. Preimmune serum was included as negative control (not shown). The mean titer $\pm$1 SD are plotted on the x-axis. Two independent experiments were performed. Data of one experiment has been presented. ***: p<0.001, ns: not significant.

3.2.2 In the absence of NTD, two cryptic epitopes in Tat acquire immunodominance

Both of the Tat constructs lacking the NTD (N-Del and C-myc Tat) were immunogenic in mice, however, the antibody titers against these proteins were significantly lower compared to the wild-type Tat and N-C Tat proteins. This observation

suggested that immune responses to N-del Tat and C-myc Tat were possibly directed to different subdominant epitopes in Tat. To identify linear B-cell epitopes in the antigens, we performed pepscan analysis using peptide sets of 20 amino acid length, with an overlap of 10 amino acids between adjacent peptides and spanning the full-length of Tat or Tat variant proteins (Fig. 3.2). Microtiter wells were coated with individual peptides and antisera diluted 1:500 were incubated in appropriate wells. Peptide pool and Tat protein coated wells were used as controls (data not shown).

In the immunization with the wild-type Tat protein, as expected, the NTD epitope was detected by the antibodies suggesting that the immune response was exclusively directed against this single linear-epitope (Fig 3.2, upper-left panel). Antibodies in N-C Tat immunized mice too reacted exclusively with the NTD epitope, at comparable magnitude, although this epitope is at a different location in the antigen (Fig 3.2, upper-right panel) confirming that the nature of immunodominance of this epitope is intrinsic. Importantly, in both of the Tat proteins that lacked the NTD, N-Del Tat (Fig. 3.2, upper-middle panel) and C-myc Tat (Fig. 3.2, lower-left panel), the antibodies reacted with two novel epitopes in the CRD and the CD that were not recognized previously in the presence of the NTD. This implies that the antibody response to these epitopes was masked in the presence of the dominant NTD. However, the magnitude of immune response to the novel epitopes in these antisera is significantly lower. These data further confirm the immune dominance of NTD and also that engineering of Tat is necessary to elicit immune responses to a broad spectrum of epitopes.



Peptide	Sequence
1	MEPVDPNLEPWNHPGSQPKT
2	WNHPGSQPKTACNNCYCKRC
3	ACNNCYCKRCSYHCLVCFQK
4	SYHCLVCFQKKGLGISYGRK
5	KGLGISYGRKKRRQRRSAPP
6	KRRQRRSAPPSSEDHQNLISKQ
7	SSEDHQNLISKQPLPRTQGD
8	KQPLPRTQGDPTGSEESKKK
9	PTGSEESKKKVESKTETDPFD
C-myc	MEQKLISKKDLPR

Figure 3.2: Pepscan analyses to detect B-cell epitopes in Tat proteins. Microtiter wells were coated with peptides of 20 amino acid length spanning the full-length of the protein and with an overlap of 10 residues between adjacent peptides. The schematic diagram of Tat and the peptide location have been presented for each immunogen. Antisera were tested at a dilution of 500 fold. The assay is expected to detect all the antibody isotypes including IgM and IgG. The dashed line corresponds to the cut-off value, calculated as mean of control peptide $\pm$ 1 SD. The sequence of the peptides has been shown.

3.2.3 The basic domain in Tat probably contains a T-helper epitope

To identify the potential T-helper epitope in Tat that might provide help to antigen-specific B-cells, we carried out a lympho-proliferation assay using the CFSE dye and splenocytes from mice immunized with Tat proteins (one prime and two booster regimen). When splenocytes labeled with CFSE are activated *in vitro* with antigen peptides, the fluorescent dye is distributed equally between daughter cells thus providing a means to quantitate the magnitude of antigen-specific cell proliferation. Splenocytes isolated from three or more mice immunized with one of the four Tat proteins (wild-type, N-del, N-C or C-myc) were activated with Tat peptide pool or individual peptides for four days as described in section 3.1.7. Phytohemagglutinin (PHA) stimulation of the

splenocytes was used as positive control. Cells were simultaneously scored for CSFE dilution and CD4 surface expression using flow cytometry. The stimulation index (SI) was calculated as the ratio of percent stimulated cells over percent unstimulated cells. Typically, a SI value above 1.5 is considered significant for the presence of a T-helper epitope in a peptide.

A significant level of lymphoproliferation was seen in the assay when Tat peptide pool was used for stimulation in two of the three immunizations using wild-type Tat and N-C Tat with SI values of 1.92 and 2.01, respectively (Fig.3.3). Splenocytes isolated from N-del and C-myc immunized mice, however, did not demonstrate a similar stimulation suggesting the intrinsic immunodominant nature of the NTD epitope. Furthermore, absence of the first 15-amino acids may have partly modulated endosomal trafficking of Tat (Yezid et al. 2009) and hence possibly affecting the antigen processing. When individual peptides were used for stimulation of the splenocytes in the assay, only peptide 5 comprising of the BD of Tat demonstrated statistically significant SI index of 1.95 especially in wild-type Tat immunization. This result is consistent with the low quality anti-Tat immune responses observed in natural infections and suggests that Tat lacks inherent strong T-helper epitopes.

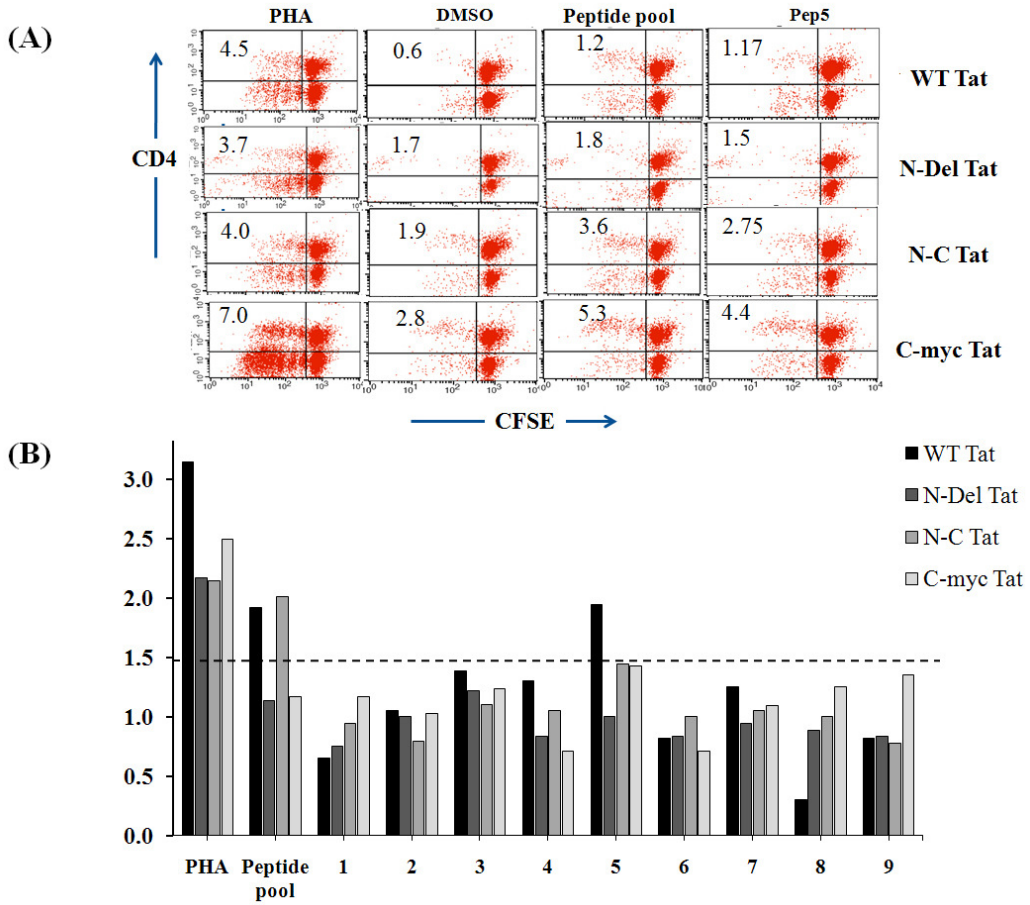


Figure 3.3: Mapping of the T-helper epitope(s) in Tat using lympho-proliferation assay. BALB/c mice, 6 animals per group, were immunized with one of the four Tat proteins shown. Splenocytes isolated from mice were loaded with CFSE, stimulated with a pool of Tat peptides or individual peptide for four days and the dilution of CFSE was analyzed by flow cytometry in cells gated on CD4+. **(A)** Representative data for CFSE-dilution. The upper-left quadrant corresponds to CD4+ cells proliferated and the numbers in the quadrant represent the percent of cells. Data for only four experimental conditions, but not others, have been presented here (PHA stimulation, unstimulated, peptide pool and peptide 5). **(B)** Splenocyte proliferation as a function of antigen-specific cell stimulation. The stimulation index (SI) was calculated as the ratio of percent stimulated over percent unstimulated. SI of ≥1.5 was considered to be positive as represented by the dotted line. Tat protein immunizations are represented by different filling and each bar represents an individual animal. The assay was carried out in duplicates.

3.2.4 Universal HTL-epitope-grafting enhances immune response to Tat

Although deletion of the NTD in Tat resulted in the immune-recognition of two other sub-dominant epitopes, this strategy could not make the new epitopes co-dominant with the NTD. Several other Tat B-cell epitopes reported previously in different experimental animals and natural infection appear (described in chapter 1) to remain sub-dominant in the presence of the NTD epitope. We explored a different experimental strategy, to graft universal T-helper epitopes into Tat domains, to extend immune responses to the subdominant epitopes in the presence of the NTD epitope.

Two different universal T-helper epitopes (HTL), PADRE and Pol_{711} (Brian Livingston et al. 2002), were grafted into CRD and/or BD of Tat disrupting either one or both of the domains in either orientation thus generating a total of 4 recombinant Tat proteins (Fig. 3.4). The immunogenicity of all the four Tat constructs was compared with the wild-type Tat protein in BALB/c mice (Fig. 3.4). After the second booster immunization, all the four HTL-Tat proteins induced significantly higher antigen-specific antibody titers as compared to wild-type Tat. Identical data were observed after other booster immunizations (data not shown). While the mean antibody titer of wild-type Tat immunization was 2200 ± 330, the HTL-Tat proteins induced 5.2 to 7 fold higher magnitude of antigen-specific antibodies suggesting that the HTL were functional in the Tat context.

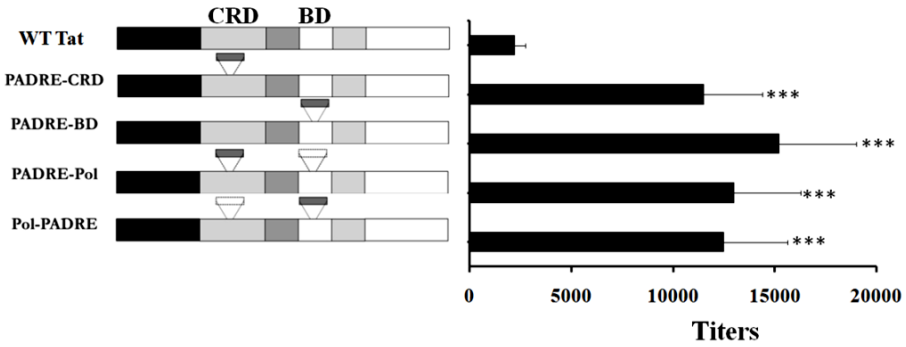


Figure 3.4: Antibody titers in the sera of mice immunized with Tat-HTL- recombinant proteins. HTL-epitopes enhance humoral immune response to Tat. The schematic diagrams of the Tat proteins used in the immunization have been depicted on the left where the grey and open boxes represent the PADRE and pol HTL epitopes. Antisera collected 14 days following the second booster immunization were diluted serially for the assay. The reciprocal dilution of the sample where the absorbance value was 0.5 was considered as the titer of the sera. Each bar represents the mean titer of 3 or more mice $\pm$ 1 SD. Statistical significance of antibody titer differences between wild-type Tat and each of the HTL-Tat proteins has been calculated using student's t-test. ***: $p<0.001$. Two independent experiments showed similar results.

Interestingly, a single insertion event of the PADRE HTL into either the CRD or the BD enhanced immune response at a magnitude comparable to two epitope insertion. An extended analysis using four each Tat proteins with or without the HTL-grafting clearly differentiated between these two sets with all the four HTL-Tat proteins inducing significantly higher levels of Tat antigen-specific antibodies as compared to the ones devoid of the HTL epitopes at all the time points of analyses (Fig. 3.5). A previous analysis in our laboratory using DNA vectors found that the PADRE-CRD construct to be significantly superior to other Tat constructs in BALB/c mice (Anand KK et al, manuscript in preparation). In contrast, protein immunization in the present work found no such differences among the recombinant Tat proteins and all HTL-Tat proteins elicited comparable levels of immune response that is superior to the wild-type Tat. Taken together, these data confirm that the HTL insertion improved immune response to Tat regardless of the Tat domain into which the HTL were grafted or the number of insertions.

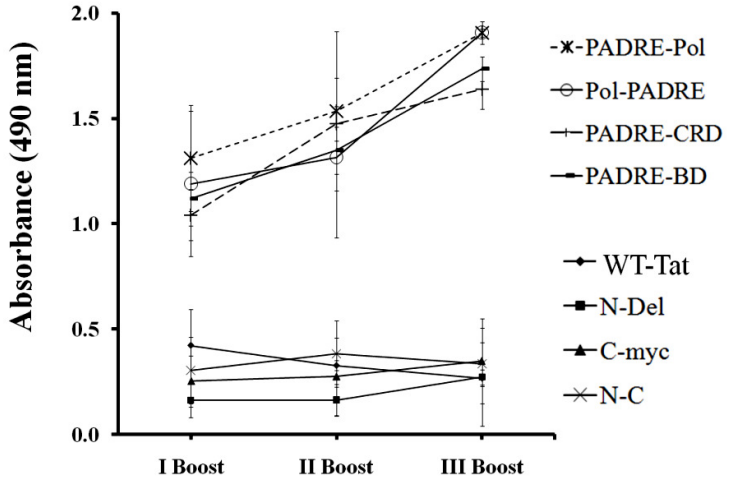


Figure 3.5: Total antibody levels in sera of immunized mice after each booster immunizations. Antisera collected 14 days after each of the booster immunization were diluted 1:1000 times and used in the assay to evaluate the total antibody levels. The x-axis corresponds to the booster immunizations and y-axis to absorbance. Mean absorbance of at least 3 mice $\pm$ 1SD have been represented.

3.2.5 HTL-epitope grafting extends immune responses to subdominant epitopes in Tat

Data presented in the previous section demonstrated enhanced magnitude of immune response to Tat after the HTL epitopes have been grafted into the viral antigen (Fig. 3.4 and 3.5). The enhanced immune response observed could be the result of reinforced immune response against the original dominant NTD epitope. Alternatively, it could be the net outcome of immune responses spread to novel epitopes in addition to the NTD epitope. To distinguish between these two possibilities, we performed a pepscan analysis using overlapping peptide sets of 20 amino acid residues spanning the length of each of the individual Tat proteins (Fig. 3.2). Overlapping peptides corresponding to the sequences generated due to the HTL-insertion (see table 3.1 for sequences) were also tested, none of these sequences eliciting antibody response (data not shown).

Peptide	Sequence
A	AKFVAAWTLKAAA
B	EKVYLAWVPAHKGIG
C	ACNNCYCKHCAKFAAWTLKAAA
D	AKFVAAWTLKAAASYHCLVCFQT
E	GLGISYGRKKAKFVAAWTLKAAA
F	AKFVAAWTLKAAARRQRRSAPPS
G	ACNNCYCKHCEKVYLAWVPAHKGIG
H	EKVYLAWVPAHKGIGSYHCLVCFQT
I	GLGISYGRKKEKVYLAWVPAHKGIG
J	EVVYLAWVPAHKGIGRRQRRSAPPS

Table 3.1: Overlapping peptides corresponding to the sequences of HTL-Tat constructs

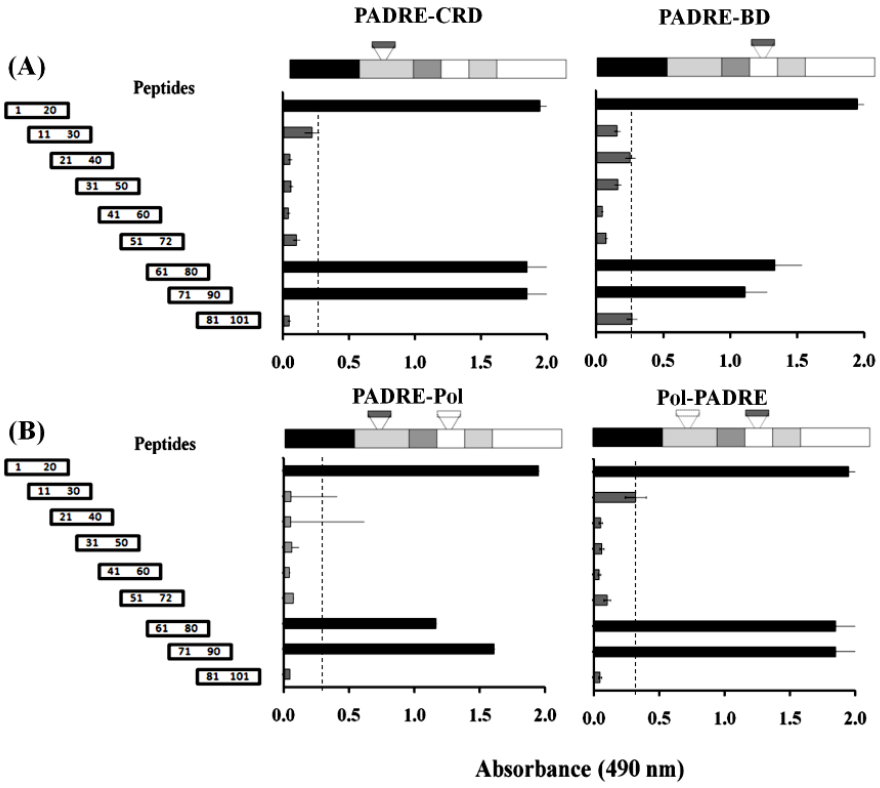


Figure 3.6: Identification of the B-cell epitopes in Tat by pepscan analysis. Antisera collected from mice 14 days after the second booster immunization with **(A)** single- or **(B)** two HTL-epitope Tat proteins were used in the analysis. Microtiter wells were coated with peptides of 20 amino acid length spanning the full-length of the specific Tat recombinant protein. Note that the peptides sets vary from protein to protein depending on the variation in the primary amino acid sequences due to HTL grafting. The schematic diagrams of Tat proteins used in the immunization were depicted above each panel and the peptide profile of Tat has been presented on the left. Antisera were tested at a dilution of 1000 fold in ELISA. The assay is expected to detect all the antibody isotypes including IgM and IgG. The dashed line corresponds to the cut-off value, calculated as mean of control peptide $\pm$ 1 SD.

Interestingly, like the wild-type Tat immunized mice, all the four HTL-Tat immunizations elicited response to the NTD epitope. However, unlike the wild-type Tat immunization that exclusively recognized the NTD epitope, all the four HTL-Tat protein immunizations recognized a novel B-cell epitope embedded within peptides 7 and 8 corresponding to amino acid residues 61 to 90 within exon-2 of Tat (Fig. 3.6). The exon-2 epitope was not recognized previously in wild-type Tat immunization in BALB/c mice. Importantly, the strength of the novel B-cell epitope appears to be not only comparable to the immunodominant NTD epitope in terms of the relative magnitude of immune response elicited. Alternatively, the new epitope being recognized was co-immunodominant with NTD epitope. Unlike in the N-del and C-myc Tat immunizations where the sub-dominant epitopes were recognized only in the absence of the NTD (Fig. 3.2), the exon-2 epitope was recognized in the presence of the NTD suggesting that the HTL-epitopes provided help to both the epitopes simultaneously. However, the HTL epitopes did not elicit immune response to the CRD and CD B-cell epitopes that were recognized in the N-del and C-myc Tat immunizations (see Fig. 3.2). Collectively, the data suggest that HTL-epitopes not only improved the magnitude of immune response to the dominant NTD epitope but also resulted in the spread immune response to a novel epitope in Tat exon-2 thus improvising both quality and the breadth of the immune response.

3.2.6 HTL-epitope grafting induces a predominantly Th2-like immune response to Tat

Given that the isotype nature of the antigen-specific antibodies could be suggestive of the Th-profile of the immune response induced, we determined the isotype

profile of the anti-Tat antibodies in the antisera raised against eight different Tat proteins in mice. IgG1 and IgG2a antibodies are broadly indicative of the Th2 and Th1 polarized immune responses, respectively, in mice (Toellner et al. 1998). To determine the titers of these antibodies we used the IgG1- and IgG2a-specific secondary antibodies in indirect ELISA as described in the materials and methods (section 3.1.5).

The data presented clearly show that Tat protein immunization broadly elicited a Th2-like immune response as represented by a relative domination of the IgG1 Tat antibody over IgG2a in each immunization (Fig. 3.7). The difference between these two Tat-specific antibody isotypes was statistically significant especially in HTL-Tat protein immunizations. Of note, although Tat protein immunization skewed the Th profile in favor of the Th2 type, the one desirable for inducing humoral immune response, there is significant magnitude of Th1 type immune response elicited in each of the immunizations as well. In other words, the nature of immune response is predominantly, but not exclusively, Th2 type. Although traditionally BALB/c mice are believed to be genetically predisposed to Th2 type of response (Le Goff et al. 2002), it is important to note the enhanced Th1 profile in HTL-constructs immunized mice. Interestingly, DNA immunization of BALB/c mice with HTL-constructs demonstrated a strong Th1 profile as analyzed by ELISPOT assay (Anand KK et al, manuscript in preparation) whereas protein immunizations reveal a Th2 type. This difference in the pattern of Th response based on the mode of immunization further emphasizes on the importance of pursuing DNA-prime protein-boosting regimen to improve response from both the wings of immune response.

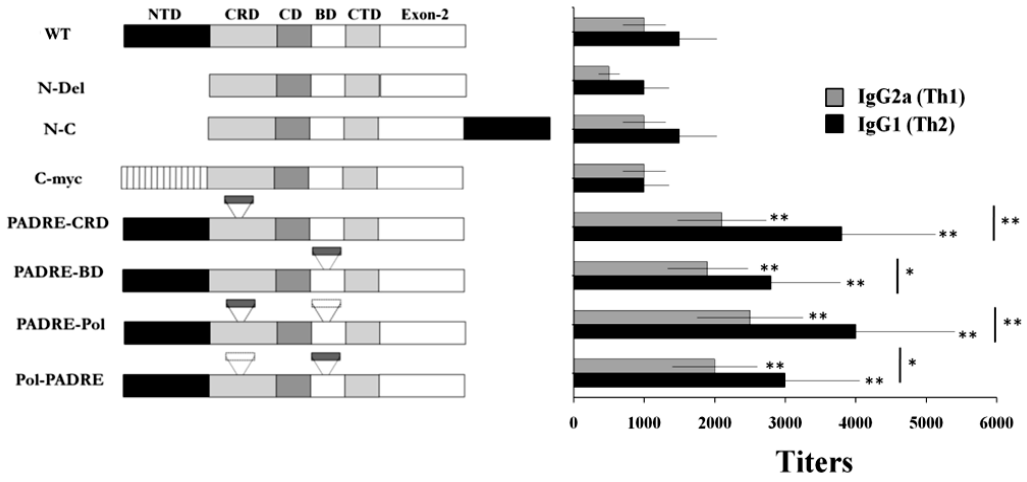


Figure 3.7: Isotype determination of the anti-Tat antibodies in immunized mice. The domain structure of the recombinant Tat proteins used in the immunization has been depicted on the left. Antisera collected from mice 14 days after the second booster immunization were diluted 1:500 and used in the ELISA. Homologous Tat proteins were coated in the microtiter wells and the IgG2a or IgG1 identity of the Tat-reactive antibodies was determined by using isotype specific secondary antibodies. The grey and dark bars correspond to IgG2a (Th1) and IgG1 (Th2) isotypes respectively. Each bar represents mean titer $\pm$1 SD of three or more mice. * $p<0.01$ and ** $p<0.001$.

3.2.7 HTL-epitopes grafted into Tat, boost T-helper response

Tat proteins with HTL epitope graft elicited a stronger humoral response as compared to those without the HTL insertion including the wild-type Tat (Fig. 3.4). We carried out a lymphoproliferation assay to evaluate and confirm that the HTL-epitopes indeed conferred a strong T-helper response to augment the humoral response. The data presented confirmed the T-helper function of the HTL conferred on Tat in the immunization (Fig. 3.8). While the SI for the wild-type Tat was 1.92$\pm$0.39, the SI for the two HTL constructs was significantly superior for the two HTL-Tat proteins evaluated 3.8$\pm$1.1 ($p<0.05$) and 2.7$\pm$0.25 ($p<0.001$) for Pol-PADRE and PADRE-pol Tat proteins respectively. Among the HTL-constructs, Pol-PADRE Tat induced significantly higher magnitude immune response as compared to the PADRE-Pol Tat protein (Fig. 3.8.B). It is

possible that the flanking amino acid context where the specific epitopes have been inserted in Tat could play a critical role in antigen processing and presentation thereby modulating the overall immunogenicity of the protein.

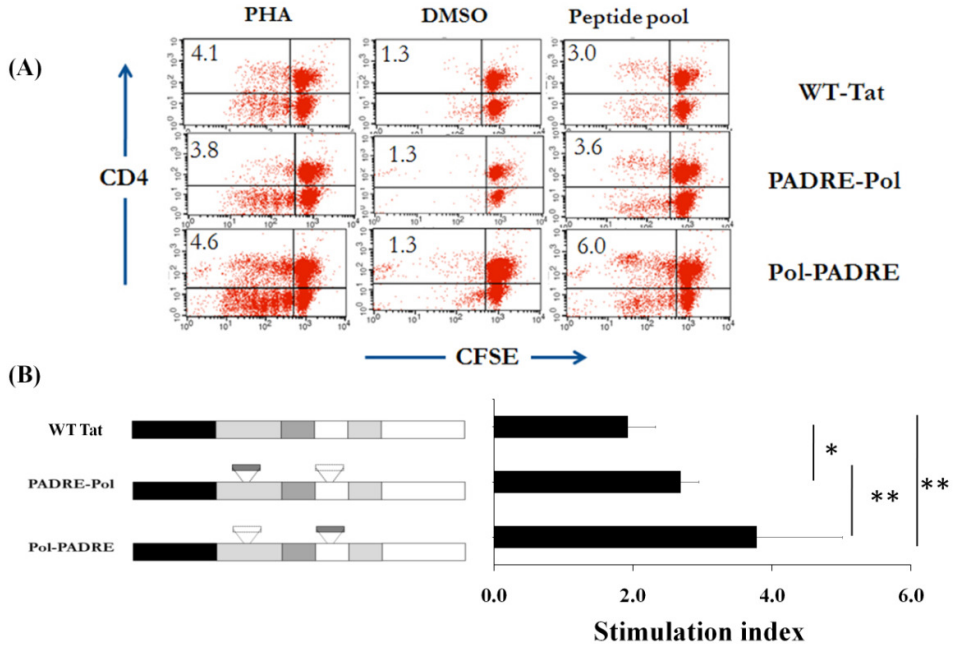


Figure 3.8: Lymphoproliferation assay to map T-helper epitopes in Tat. BALB/c mice, 6 animals per group, were immunized with one of the three Tat proteins as shown. Splenocytes isolated from immunized mice were loaded with CFSE and stimulated with a pool of Tat peptides. Note that the peptide pool varies depending on the Tat recombinant protein. The cells were stimulated *in vitro* for four days and the dilution of CFSE was analyzed by flow cytometry. Live cells were gated using on the forward and side scatter profile. **(A)** Representative data for CFSE-dilution. The upper-left quadrant corresponds to CD4+ cells proliferated and the numbers in the quadrant represent the percent of cells. **(B)** Graphical representation of the SI on the x-axis and the Tat constructs on the y-axis. The mean SI $\pm$ 1 SD of three or more mice have been shown. *p<0.05, **p<0.001

3.2.8 Anti-Tat antibodies block exogenous Tat efficiently

Data presented above showed induction of high levels of anti-Tat antibodies in mice immunized with HTL-Tat proteins. We performed two different assays, viral rescue from HLM1 cells or apoptosis induction in Jurkat T-cells, to examine if these antibodies neutralize extracellular Tat and abrogate its toxic functions. Optimized quantities of recombinant subtype-B Tat protein (B-Tat) were incubated with suitably diluted antisera prior to adding the mixture to cells to trigger Tat-mediated biological function. We used B-Tat, not subtype-C Tat (Wild-type Tat), in these assays given the intrinsically low toxic properties of C-Tat (Rao et al. 2008; Mishra et al. 2008). In the virus-rescue assay we used HLM1 cells that contain a Tat-defective virus stably integrated into the genome and produce little p24. When complemented with biologically functional extracellular Tat, elevated levels of p24 are secreted into the medium which is quantified (Fig 3.9). Using this cell-line, we assessed the Tat-neutralization potential of the antiserum antibodies. The results confirmed the neutralization potential of the anti-Tat antibodies in all the sera tested. The mean absorbance value of p24 in the absence of the antibodies 1.19 was reduced to 0.35 in the presence of anti-Wild-type Tat antiserum with the difference being statistically significant ($p<0.005$). Importantly, the antisera of all the four HTL-Tat immunizations demonstrated higher potential of Tat-neutralization as compared to immunization with the four Tat proteins devoid of the HTL epitopes where the differences were statistically significant.

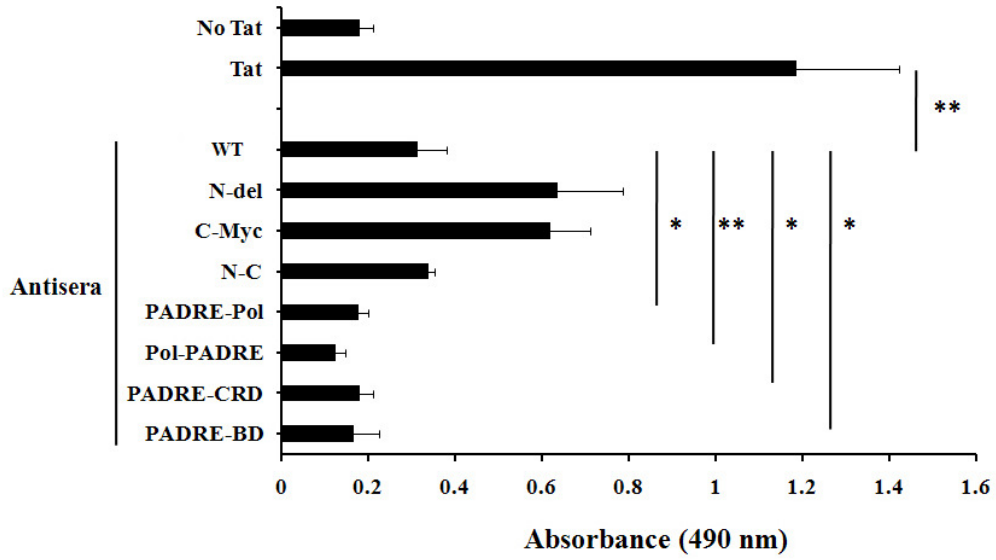


Figure 3.9: Antibody-mediated neutralization of extracellular Tat abrogates viral rescue. Tat-transactivation is effectively blocked by anti-Tat antibodies in the mouse sera. 500 ng of subtype C Tat protein was incubated for 30 with mouse anti-Tat antisera diluted 1:1000 folds. Treated sera were added to appropriate wells containing HLM1 cells in 96-well plate and the quantity of p24 released into the medium was determined after 72 h using an antigen-capture assay. Various Tat proteins used for immunization have been depicted on the left. The bars represent the mean absorbance $\pm$ 1 SD of three independent biological replicate wells. Two independent assays were performed and data of one experiment are presented. *p<0.05, **p<0.005

Tat is known to induce apoptosis in T-cells (Campbell et al. 2004). Using Jurkat T-cells, we tested whether anti-Tat antibodies in the antisera of different Tat immunizations could block the Tat-mediated apoptosis. Cells subjected to Tat treatment or none were stained with PI and Annexin V-FITC and analyzed using flow cytometry. Anti-Tat antibodies in all the antisera efficiently blocked Tat-mediated cellular apoptosis (Fig. 3.10). The neutralization of Tat-induced apoptosis was efficiently blocked by antisera from mice immunized with Tat constructs. Although a direct correlation (correlation coefficient = 0.87) was observed with the magnitude of antibodies elicited and the neutralization potential, the difference was not significant. Taken together, these observations suggest that the grafting of HTL-epitopes into Tat not only enhanced the

antigen-specific antibody levels, but also resulted in higher magnitude of Tat neutralization.

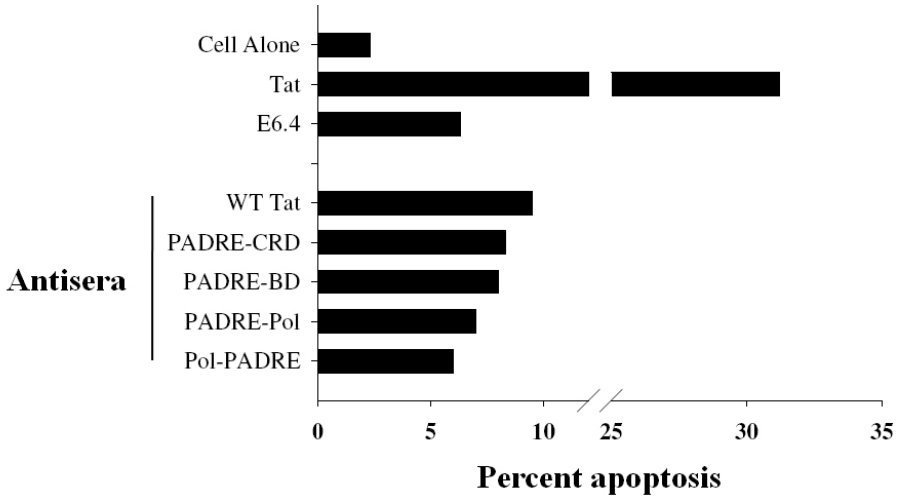


Figure 3.10: Antibody-mediated neutralization of extracellular Tat abolishes cellular apoptosis. Subtype-B Tat protein at a concentration of 2 µg/ml was incubated with the antisera diluted 1:1000 prior to addition of the mixture to 1 x 10^6 Jurkat cells seeded in a 96-well-plate. The magnitude of apoptosis was determined by staining the cells for PI and Annexin V. Tat proteins used in the immunization have been depicted on the left. E6.4, an anti-Tat monoclonal antibody that recognizes the NTD in Tat was used the positive control for neutralization.

3.3 Discussion:

Tat vaccine design is limited by several technical challenges including moderate immunogenicity of Tat and its cytotoxic properties. Importantly, the NTD of Tat is immunodominant in the natural infection and in experimental immunizations of various animal-models to the extent that immune response to other B-cell epitopes has been compromised.

Targeting several independent epitopes should be an advantage when the objective is to neutralize extracellular Tat from exerting deleterious effects on the host cell and immune system. However, the observed immunodominant nature of the NTD is recalcitrant to achieve induction of neutralizing antibodies to other epitopes in Tat. Furthermore, Tat is a moderate immunogen hence various strategies have been used to augment its immunogenicity.

In the present work we employed two different strategies to improve antigenicity of Tat when administered as a protein. In the first attempt, we tried to understand the molecular basis for the immunodominance of the NTD. Regardless of the temporal location in Tat, NTD retained immunodominance over other epitopes. Only its deletion led to the recognition of two different sub-dominant epitopes in the CRD and CD of Tat, however, at a lower magnitude. To enhance the immune response and also to boost responses to sub-dominant epitopes, we used a second strategy of grafting two different T-helper epitopes into two functionally important domains of Tat. This strategy not only improved safety profile of Tat for vaccination, by abrogating transactivation and apoptosis induction properties, but also led to the induction of strong cell-mediated immune responses to Tat in DNA immunization lowering proviral load in EcoHIV-mouse challenge model (Anand KK et al. manuscript in preparation). The HTL-epitope graft

strategy not only enhanced significantly higher magnitude of immune response to Tat but also we could elicit strong response to a second epitope in exon-2 importantly in the presence of the immunodominant NTD. Thus we could successfully break the immunodminance of the NTD and spread immune response to at least one of the sub-dominant epitopes. Whereas DNA immunization regimen predominantly elicits cell-mediated immune response with several of the epitopes co-dominantly recognized, protein immunization is necessary to induce humoral immune response needed for neutralizing extracellular Tat. It remains to be seen if a Tat immunization strategy of DNA-priming and protein-boosting will be able to elicit both the arms of the immune system to efficiently control the viral proliferation at the cellular and extracellular levels.

In summary, we have evaluated two different strategies to improve Tat as a candidate vaccine. Of these strategies, HTL-grafting improved the breadth and the magnitude of the immune response to Tat in addition to enhancing its safety profile for vaccination. Additional studies are presently in progress to test if the DNA-priming and protein-boosting strategy would enhance the desired immune responses further. HTL-Tat constructs are being tested in DNA-prime-protein-boost regimen within a goal to elicit both cellular and humoral immune responses. Importantly, adjuvants that can be administered to humans are being foreseen as possible means to enhance the immunogenicity and also to make human application possible.

Bibliography

Agadjanyan, M.G. et al., 2005. Prototype Alzheimer's disease vaccine using the immunodominant B cell epitope from beta-amyloid and promiscuous T cell epitope pan HLA DR-binding peptide. Journal of Immunology (Baltimore, Md.: 1950), 174(3), 1580-1586.

Alexander, J. et al., 1998. The optimization of helper T lymphocyte (HTL) function in vaccine development. Immunologic Research, 18(2), 79-92.

Alexander, J. et al., 2000. Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. Journal of Immunology (Baltimore, Md.: 1950), 164(3), 1625-1633.

Alexander, J. et al., 1994. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity, 1(9), 751-761.

Bangari, D.S. & Mittal, S.K., 2006. Current strategies and future directions for eluding adenoviral vector immunity. Current Gene Therapy, 6(2), 215-226.

Barouch, D.H., Letvin, N.L. & Seder, R.A., 2004. The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses. Immunological Reviews, 202, 266-274.

Bartz, S.R. & Emerman, M., 1999. Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. Journal of Virology, 73(3), 1956-1963.

Beebe, M. et al., 2007. Formulation and Characterization of a Ten-peptide Single-vial Vaccine, EP-2101, Designed to Induce Cytotoxic T-lymphocyte Responses for Cancer Immunotherapy. Human Vaccines, 4(3). Available at: http://www.ncbi.nlm.nih.gov/pubmed/18382135 [Accessed November 18, 2009].

Belliard, G. et al., 2005. Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. Vaccine, 23(11), 1399-407.

Bélot, F. et al., 2005. Synthesis of two linear PADRE conjugates bearing a deca- or pentadecasaccharide B epitope as potential synthetic vaccines against Shigella flexneri serotype 2a infection. Chemistry (Weinheim an Der Bergstrasse, Germany), 11(5), 1625-1635.

Boaz, M.J. et al., 2003. CD4 responses to conserved HIV-1 T helper epitopes show both negative and positive associations with virus load in chronically infected subjects. Clinical and Experimental Immunology, 134(3), 454-463.

Brake, D.A. et al., 1990. Characterization of murine monoclonal antibodies to the tat protein from human immunodeficiency virus type 1. Journal of Virology, 64(2), 962-965.

Cafaro, A. et al., 1999. Control of SHIV-89.6 P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. Nature medicine, 5(6), 643-650.

Campbell, G.R. et al., 2007. Tat mutations in an African cohort that do not prevent transactivation but change its immunogenic properties. Vaccine, 25(50), 8441-8447.

Campbell, G.R. et al., 2005. The C terminus of HIV-1 Tat modulates the extent of CD178-mediated apoptosis of T cells. The Journal of Biological Chemistry, 280(46), 38376-38382.

Campbell, G.R. et al., 2004. The Glutamine-rich Region of the HIV-1 Tat Protein Is Involved in T-cell Apoptosis. J. Biol. Chem., 279(46), 48197-48204.

Caputo, A. et al., 2009. Induction of humoral and enhanced cellular immune responses by novel core-shell nanosphere- and microsphere-based vaccine formulations following systemic and mucosal administration. Vaccine, 27(27), 3605-3615.

Caputo, A. et al., 2005. The presence of anti-Tat antibodies is predictive of long-term nonprogression to AIDS or severe immunodeficiency: findings in a cohort of HIV-1 seroconverters. The Journal of Infectious Diseases, 191(8), 1321-1324.

Caselli, E. et al., 1999. DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. Journal of Immunology (Baltimore, Md.: 1950), 162(9), 5631-5638.

Cohen, S.S. et al., 1999. Pronounced acute immunosuppression in vivo mediated by HIV Tat challenge. Proceedings of the National Academy of Sciences of the United States of America, 96(19), 10842-10847.

Dean, H.J., 2005. Epidermal delivery of protein and DNA vaccines. Expert Opinion on Drug Delivery, 2(2), 227-236.

Decroix, N. et al., 2002. Mucosal immunity induced by intramuscular administration of free peptides in-line with PADRE: IgA antibodies to the ELDKWA epitope of HIV gp41. Scandinavian Journal of Immunology, 56(1), 59-65.

Demirhan, I. et al., 1999. Detection of distinct patterns of anti-tat antibodies in HIV-infected individuals with or without Kaposi's sarcoma. Journal of Acquired Immune Deficiency Syndromes (1999), 22(4), 364-368.

Donnelly, J.J., Wahren, B. & Liu, M.A., 2005. DNA vaccines: progress and challenges. Journal of Immunology (Baltimore, Md.: 1950), 175(2), 633-639.

Ensoli, B. & Cafaro, A., 2000. Control of viral replication and disease onset in cynomolgus monkeys by HIV-1 TAT vaccine. Journal of Biological Regulators and Homeostatic Agents, 14(1), 22-26.

Ensoli, B. et al., 2006. Candidate HIV-1 Tat vaccine development: from basic science to clinical trials. AIDS (London, England), 20(18), 2245-2261.

Fanales-Belasio, E. et al., 2002. Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. Journal of Immunology (Baltimore, Md.: 1950), 168(1), 197-206.

Fitzmaurice, C.J. et al., 1996. The assembly and immunological properties of non-linear synthetic immunogens containing T-cell and B-cell determinants. Vaccine, 14(6), 553-560.

Franke, E.D. et al., 1999. Pan DR binding sequence provides T-cell help for induction of protective antibodies against Plasmodium yoelii sporozoites. Vaccine, 17(9-10), 1201-1205.

Gao, W. et al., 2004. Construction of exogenous multiple epitopes of helper T lymphocytes and DNA immunization of its chimeric plasmid with HBV pre-S2/S gene. World Journal of Gastroenterology: WJG, 10(20), 2979-2983.

Garmory, H., Brown, K. & Titball, R., 2003. DNA vaccines: improving expression of antigens. Genetic Vaccines and Therapy, 1(1), 2.

Gavioli, R. et al., 2004. HIV-1 tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity. Journal of Immunology (Baltimore, Md.: 1950), 173(6), 3838-3843.

Goldstein, G., Tribbick, G. & Manson, K., 2001. Two B cell epitopes of HIV-1 Tat protein have limited antigenic polymorphism in geographically diverse HIV-1 strains. Vaccine, 19(13-14), 1738-46.

Gorse, G.J. et al., 2008. Safety and immunogenicity of cytotoxic T-lymphocyte poly-epitope, DNA plasmid (EP HIV-1090) vaccine in healthy, human immunodeficiency virus type 1 (HIV-1)-uninfected adults. Vaccine, 26(2), 215-223.

Greenstein, J.L. et al., 1992. A universal T cell epitope-containing peptide from hepatitis B surface antigen can enhance antibody specific for HIV gp120. Journal of Immunology (Baltimore, Md.: 1950), 148(12), 3970-3977.

Gringeri, A. et al., 1998. Antibodies to the HIV-1 Tat protein correlated with nonprogression to AIDS: a rationale for the use of Tat toxoid as an HIV-1 vaccine. Journal of Human Virology, 1(4), 282-292.

Haas, G. et al., 1991. Identification of a major human immunodeficiency virus-1 reverse transcriptase epitope recognized by mouse CD4+ T lymphocytes. European Journal of Immunology, 21(6), 1371-1377.

Hsu, S.C. et al., 1999. Synergistic effect of immunization with a peptide cocktail inducing antibody, helper and cytotoxic T-cell responses on protection against respiratory syncytial virus. The Journal of General Virology, 80 (Pt 6), 1401-1405.

Hung, C. et al., 2007. DNA vaccines encoding Ii-PADRE generates potent PADRE-specific CD4+ T-cell immune responses and enhances vaccine potency. Molecular Therapy: The Journal of the American Society of Gene Therapy, 15(6), 1211-1219.

Kashi, V.P. et al., 2009. HIV-1 Tat-specific IgG antibodies in high-responders target a B-cell epitope in the cysteine-rich domain and block extracellular Tat efficiently. Vaccine. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19744585 [Accessed September 22, 2009].

Kim, D.T. et al., 1997. Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. Journal of Immunology (Baltimore, Md.: 1950), 159(4), 1666-1668.

Le Buanec, H. et al., 2001. Induction in mice of anti-Tat mucosal immunity by the intranasal and oral routes. Biomedicine & Pharmacotherapy = Biomédecine & Pharmacothérapie, 55(6), 316-320.

Le Goff, L. et al., 2002. IL-4 is required to prevent filarial nematode development in resistant but not susceptible strains of mice. International Journal for Parasitology, 32(10), 1277-1284.

Lecoq, A. et al., 2008. Increasing the humoral immunogenic properties of the HIV-1 Tat protein using a ligand-stabilizing strategy. Vaccine, 26(21), 2615-26.

Livingston, B. et al., 2002. A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. Journal of Immunology (Baltimore, Md.: 1950), 168(11), 5499-5506.

Longo, O. et al., 2009. Phase I therapeutic trial of the HIV-1 Tat protein and long term follow-up. Vaccine, 27(25-26), 3306-3312.

Mansour, M. et al., 2007. Therapy of established B16-F10 melanoma tumors by a single vaccination of CTL/T helper peptides in VacciMax. Journal of Translational Medicine, 5, 20.

Mascarell, L. et al., 2006. Delivery of the HIV-1 Tat protein to dendritic cells by the CyaA vector induces specific Th1 responses and high affinity neutralizing antibodies in non human primates. Vaccine, 24(17), 3490-9.

Mascarell, L. et al., 2005. Induction of neutralizing antibodies and Th1-polarized and CD4-independent CD8+ T-cell responses following delivery of human immunodeficiency virus type 1 Tat protein by recombinant adenylate cyclase of Bordetella pertussis. Journal of virology, 79(15), 9872-84.

Mayol, K. et al., 2007. Design and characterization of an HIV-1 Tat mutant: inactivation of viral and cellular functions but not antigenicity. Vaccine, 25(32), 6047-6060.

Mishra, M. et al., 2008. Clade-specific differences in neurotoxicity of human immunodeficiency virus-1 B and C Tat of human neurons: significance of dicysteine C30C31 motif. Annals of Neurology, 63(3), 366-376.

Moreau, E. et al., 2004. Generation and characterization of neutralizing human monoclonal antibodies against human immunodeficiency virus type 1 Tat antigen. Journal of virology, 78(7), 3792-6.

Newman, M.J. et al., 2002. T-lymphocyte epitope identification and their use in vaccine development for HIV-1. Frontiers in Bioscience: A Journal and Virtual Library, 7, d1503-1515.

Noonan, D.M. et al., 2003. Identification of immunodominant epitopes in inactivated Tat-vaccinated healthy and HIV-1-infected volunteers. Journal of acquired immune deficiency syndromes (1999), 33(1), 47-55.

Pauza, C.D. et al., 2000. Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. Proceedings of the National Academy of Sciences of the United States of America, 97(7), 3515-3519.

Pauza, C.D. et al., 2004. Sequence Variation within the Dominant Amino Terminus Epitope Affects Antibody Binding and Neutralization of Human Immunodeficiency Virus Type 1 Tat Protein. J. Virol., 78(23), 13190-13196.

Quah, B.J.C., Warren, H.S. & Parish, C.R., 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. Nature Protocols, 2(9), 2049-2056.

Ramakrishna, L. et al., 2004. Codon optimization and ubiquitin conjugation of human immunodeficiency virus-1 Tat lead to enhanced cell-mediated immune responses. Vaccine, 22(20), 2586-2598.

Ranga, U. et al., 2004. Tat protein of human immunodeficiency virus type 1 subtype C strains is a defective chemokine. Journal of Virology, 78(5), 2586-2590.

Rao, V.R. et al., 2008. HIV-1 clade-specific differences in the induction of neuropathogenesis. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 28(40), 10010-10016.

Re, M.C. et al., 1996. Antibody against human immunodeficiency virus type 1 (HIV-1) Tat protein may have influenced the progression of AIDS in HIV-1-infected hemophiliac patients. Clinical and diagnostic laboratory immunology, 3(2), 230-2.

Remoli, A.L. et al., 2006. Intracellular HIV-1 Tat protein represses constitutive LMP2 transcription increasing proteasome activity by interfering with the binding of IRF-1 to STAT1. The Biochemical Journal, 396(2), 371-380.

Rice, J. et al., 2004. DNA fusion vaccines induce targeted epitope-specific CTLs against minor histocompatibility antigens from a normal or tolerized repertoire. Journal of Immunology (Baltimore, Md.: 1950), 173(7), 4492-4499.

Richardson, M.W. et al., 2003. Antibodies to Tat and Vpr in the GRIV cohort: differential association with maintenance of long-term non-progression status in HIV-1 infection. Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie, 57(1), 4-14.

Richardson, M.W. et al., 2002. Immunogenicity of HIV-1 IIIB and SHIV 89.6P Tat and Tat toxoids in rhesus macaques: induction of humoral and cellular immune responses. DNA and Cell Biology, 21(9), 637-651.

Rosa, D.S. et al., 2004. The pan HLA DR-binding epitope improves adjuvant-assisted immunization with a recombinant protein containing a malaria vaccine candidate. Immunology Letters, 92(3), 259-268.

Ruckwardt, T.J. et al., 2004. Sequence variation within the dominant amino terminus epitope affects antibody binding and neutralization of human immunodeficiency virus type 1 Tat protein. Journal of virology, 78(23), 13190-6.

Siddappa, N.B. et al., 2006. Transactivation and signaling functions of Tat are not correlated: biological and immunological characterization of HIV-1 subtype-C Tat protein. Retrovirology, 3, 53.

Tikhonov, I. et al., 2003. Tat-neutralizing antibodies in vaccinated macaques. Journal of virology, 77(5), 3157-66.

Toellner, K.M. et al., 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. The Journal of Experimental Medicine, 187(8), 1193-1204.

Uyttenhove, C. et al., 2004. Development of an anti-IL-12 p40 auto-vaccine: protection in experimental autoimmune encephalomyelitis at the expense of increased sensitivity to infection. European Journal of Immunology, 34(12), 3572-3581.

Wieland, U. et al., 1990. Antibodies to recombinant HIV-1 vif, tat, and nef proteins in human sera. Medical microbiology and immunology, 179(1), 1-11.

Yezid, H. et al., 2009. Mechanism for HIV-1 TAT insertion into the endosome membrane. The Journal of Biological Chemistry. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19549783 [Accessed June 26, 2009].

4. Generation and characterization of Tat-specific monoclonal antibodies

The importance of monoclonal antibodies as a research tool has been established beyond doubt. Currently both *in vitro* and *in vivo* methods are being applied to generate monoclonal antibodies. The conventional method of using immunized host immune system to generate high-affinity and antigen-specific monoclonal antibodies (MAbs) is still widely practiced. In our laboratory we have generated a panel of monoclonal antibodies against Tat and have characterized some of these antibodies for binding affinities, epitopes targeted and their Tat-neutralization potential in virus rescue and apoptosis assays. These antibodies can be of significant importance in unraveling the pathogenic effects of Tat in animal models. In the process of generating panels of anti-Tat antibodies, we have encountered the phenomenon of immune interference that precluded identification of hybridomas against any B-cell epitope in Tat other than the N-terminal domain (NTD). Despite several attempts, we failed to generate antibodies to other B-cell epitopes when mice were immunized with Tat protein. For the second set of immunizations, we generated a recombinant Tat protein, N-del Tat, where the NTD was deleted. We could generate several hybridomas against the core domain (CD) of Tat using N-del Tat immunizations.

4.1 Materials and methods:

4.1.1 The immunization protocol: Batches of eight-week old BALB/c mice were administered one primary, one booster and one tail-vein immunization. The primary immunization was administered in Freund's complete adjuvant and the booster immunizations in incomplete adjuvant on 21 days after the primary immunization. Three weeks after the booster immunization, the mice were tested for anti-Tat antibodies in the sera. Two Tat constructs with varying quantities of proteins were used to generate antibodies to different epitopes of Tat. In the first regimen (coded E), each mouse received 10 μg of recombinant subtype C Tat (C-Tat) protein consisting of exon-1 immunization in the primary followed by 6 μg in the booster immunizations. In the second regimen (coded I), mice immunized with N-del Tat, received 25 and 15 μg of the antigen in the primary and booster immunizations, respectively. The subsequent steps were identical for both the immunization regimens. Six to eight weeks after the booster immunization, antisera were screened for antibody titers in ELISA and the mice containing the highest titers were selected for fusion. Three days prior to the fusion, a final booster of 100 μg of protein in PBS was administered through the tail-vein in both the sets of mice. On the day of fusion, splenocytes were collected from the mice and fused with SP2/O cells as per protocols described below.

4.1.2 Generation of Tat hybridomas: All the reagents were from Sigma-Aldrich unless specified otherwise. The SP2/O parental cell line ($HGPRT^{-ve}$, TK^{-ve}, IgG non-secretor) was used in all the cell-fusion experiments. Cells were maintained in Ivosky Modified Dulbecco medium (IMDM) containing 20% FCS, 2 mM L-glutamine and 10,000 U of penicillin-streptomycin per ml. A feeder cell suspension of BALB/c peritoneal macrophages was seeded (0.5×10^6 in 100 μl) in a 96-well plate on the day prior to the

fusion. Cells were fused at a ratio of 1:10 myeloma:splenocytes using 50% of polyethylene glycol (PEG 4000, Gas chromatography grade, Fluka). The fused cells were washed, resuspended in IMDM supplemented with 20% FCS and 1× HAT (1×10^{-4} M hypoxanthine, 4×10^{-1} M aminopterin, 1.6×10^{-5} M thymidine) and seeded in 96-well plates. The plates were left undisturbed for a week in a CO_2 incubator. The growth of the hybridomas was visually monitored during this period. On day eight, half of the medium from each well was replaced with fresh IMDM supplemented with 20% FCS and 1x HT (1×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine). Medium was replaced every 2 to 3 days through second week. The wells positive for Tat were identified in indirect ELISA. Selected clones were expanded, limit diluted and frozen in liquid nitrogen or subjected to limit dilution cloning procedure.

4.1.3 Purification of monoclonal antibodies: Tat hybridomas that secreted high concentration of anti-Tat antibodies were expanded to a large scale in IMDM medium supplemented with 10% FCS. The immunoglobulin fractions were concentrated 10 to 15 fold by ammonium sulfate precipitation at 50% saturation. The precipitates were resuspended in PBS and subjected to affinity purification using Protein-G Sepharose columns (Montage antibody purification kit, Millipore). The antibody fractions of different hybridoma clones were dialyzed against PBS. The aliquots of purified antibodies were stored at -70^0C.

4.1.4 ELISA: Please refer to section 3.1.5 for detailed description of the procedure.

4.1.5 Pepscan analysis: The protocol is essentially as described in section 3.1.6.

4.1.6 Affinity evaluation of the Tat monoclonal antibodies: The affinity determination of antibodies was carried out at Reametrix, Bangalore. The dissociation constant values

(Kd) of anti-Tat monoclonal antibodies were evaluated using serial dilutions of affinity purified IgG fractions in indirect ELISA. The wells were coated with 50 µl/well of Tat protein at a concentration of 1 µg/ml in a 96-well plate. After overnight incubation, the wells were washed thrice with PBS Tween solution (PBS, pH 7.4 with 0.05% Tween 20), followed by three washes with MilliQ® water. The antigen coated wells were then blocked with 200 µl of 0.5% BSA in PBS for 2 h at room temperature. The plates were then washed thrice with PBS supplemented with 0.05% Tween 20 and twice with MilliQ water. Monoclonal antibodies, diluted to different concentrations (0.05 nM – 1 µM) were dispensed 50 µl/well and incubated at 37^0C for 2 h. Following this, the plates were washed thrice with PBS Tween solution and twice with MilliQ water. To each well 50 µl of 20,000 fold diluted goat anti-mouse-HRP conjugate (1 mg/ml stock solution, ReaMetrix India Private Ltd, Bangalore, India) was added and the plates were incubated at 37^0C for 2 h. The plates were washed thrice with PBS Tween solution and twice with MilliQ water. To each well 100 µl of 1X HRP/TMB substrate was added and the plates were incubated for 5-10 minutes till an intense green color developed. The reaction was stopped with 1 N H_2SO_4 and the plates were read on an ELISA reader at an absorbance of 450 nm. Three different negative controls were used for each antibody clone- antigen coated wells without the primary antibody, antigen coated wells without the secondary antibody and uncoated wells with primary and secondary antibodies. The OD values were plotted against log concentrations of antibodies as a hyperbolic curve to calculate the Kd values of the antibodies. A three parameter generalized hill slope model was fit to the antibody concentration versus optical density curve, to determine the affinity of the different antibodies using GraphPad Prism 4 software and the following formula.

$Y = B + (T-B)/(1+10^{(Log(EC_{50})-X)})$

Where:

Y = Fluorescence or absorbance of Antigen-Ab-Ab complex

B = Parameter for bottom of the sigmoidal curve

T = Top or saturation value of the sigmoidal curve

EC50 = K_d = affinity of the primary antibody

X = primary antibody concentration converted to logarhythmic units

4.1.7 Tat neutralization assays: The protocols for Tat-induced transactivation in HLM1 cells and cellular apoptosis in Jurkat T-cells have been described previously in sections 2.1.8 and 3.1.9, respectively.

4.1.8 SDS-PAGE and Western blot analysis: Recombinant Tat protein was boiled for 5 min in the gel loading buffer (250 mM Tris-HCl, pH 6.8, with 4% SDS, 20% glycerol, 0.01% bromophenol blue and 10% β-mercaptoethanol), resolved on a 15% SDS-PAGE gel and transferred to an Immobilon-P membrane (IPVH00010, Millipore, Massachusetts). The membrane was blocked with 3% BSA and probed with the monoclonal antibodies, at a concentration of 1 µg/ml in PBS; at room temperature for 2 h. Membranes were washed with PBS containing 0.1% Tween 20 and incubated with anti-mouse secondary antibody conjugated to horseradish peroxidase (Calbiochem). The blot was developed by incubating the membrane in the substrate solution containing 3 µl/ml H_2O_2 and 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma) 10 mM Tris (pH 8.0).

4.2 Results:

4.2.1 Mice immunized with Tat exon-1 and N-del Tat generate antibodies of distinct properties

A total of 18 hybridoma pools were generated which were subsequently limit diluted to establish 12 clones from mice immunized with Tat exon-1. All of the antibodies of the E series recognized only the NTD (Fig. 4.1, left panel) but no other domain in Tat. We were unable to identify antibodies against any other domain when mice were immunized with the Tat protein confirming the immunodominant nature of the NTD in Tat (reviewed in chapter 3). The immunodominance of NTD probably precluded antibodies against other epitopes in Tat.

Since DNA immunizations tend to elicit antibody response to a broader spectrum of epitopes in Tat, although at a lower magnitude (Ramakrishna et al. 2004), we attempted a DNA-prime- protein-boost regimen to enhance antibody response to a broader spectrum of epitopes. We immunized mice intramuscularly with 100 µg of codon optimized Tat expression plasmid (described in Ramakrishna et al. 2004) followed by booster immunization with Tat exon-1 protein. Eight clones were established from these mice (coded F); however, even in this attempt all the monoclonal antibodies recognized the linear epitope in NTD (data not shown).

Deletion of dominant epitopes often reassorted immune responses to subdominant epitopes (Riedl et al. 2009). Using this strategy, we generated N-del Tat construct where the N-terminal 15 amino acids have been deleted and used this protein for mouse immunization. A higher amount of protein was used in these immunizations, 25 and 15 µg in the primary and the booster immunizations, respectively, given that Tat is inherently a moderate immunogen and additionally, the deletion of the NTD lowered its

immunogenicity further (see Fig. 3.1). We could establish five limit diluted hybridomas from N-del Tat immunization. Interestingly, all the 5 monoclonal antibodies recognized the core domain (CD) in Tat (peptide 4, Fig 4.1, right panel). Of note, the CD epitope was naturally recognized by the antisera of mice immunized with N-del previously (see Fig 3.2, upper panel).

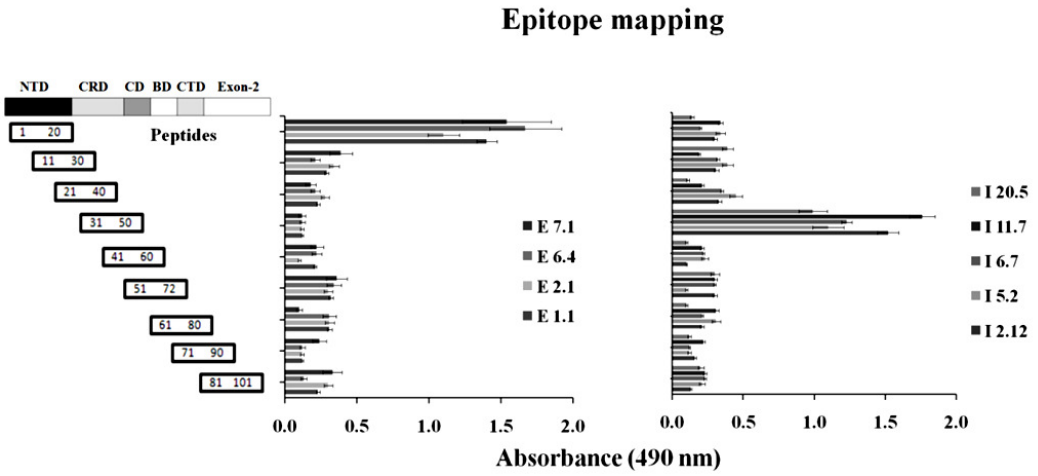


Figure 4.1: Epitope analysis of two different panels of Tat-specific monoclonal antibodies. Left and right panels represent hybridomas of E and I series that were raised against Tat exon-1 and N-del Tat, respectively. A series of peptides of 20 amino acid length with 10 residue overlap between peptides and spanning the full-length of C-Tat was used in the ELISA. Culture supernatant of four hybridomas from E series and five from I series were used directly in the assay. Mean absorbance $\pm$ 1 SD from two independent experiments was used to plot the graphs.

Using isotype-specific secondary antibodies, we determined the isotype profile of anti-Tat monoclonal antibodies of the E and I series. This analysis revealed a distinct isotypic profile of the antibodies as function of the Tat antigen used for the immunization. Out of eighteen monoclonal antibodies of the E-series (Tat exon-1), with the exception of two, all others were found to be IgG1 (Fig 4.2). Monoclonal antibodies E8 and E13 alone were found to be IgM and IgA, respectively (data not shown). All the 5 antibodies of the I-series (N-del Tat) were of the IgG2b class (Fig. 4.2, right panel).

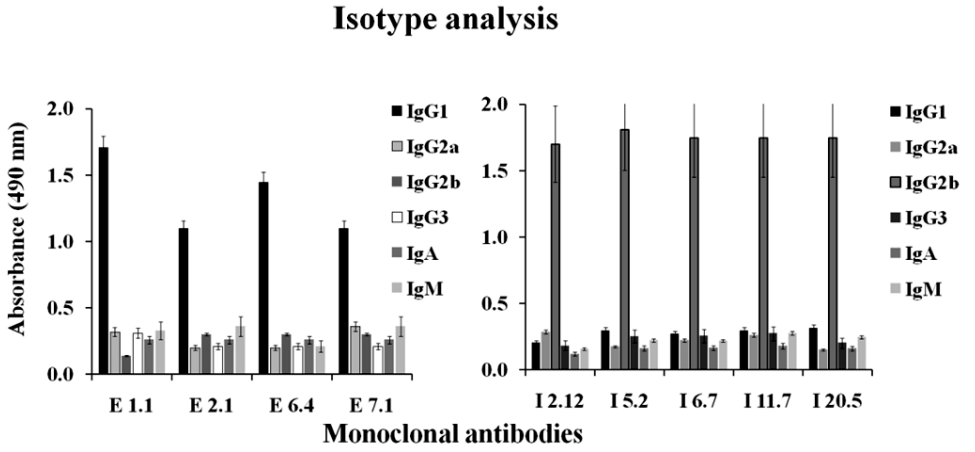


Figure 4.2: Isotype analysis of the monoclonal antibodies. The analysis was carried out using isotype-specific secondary antibodies in an indirect ELISA format. The culture supernatants were used directly in the assay. The left panel corresponds to the E-series and the right panel to the I-series of antibodies. The mean absorbance value of experimental triplicates $\pm$1SD has been plotted.

4.2.2 Tat-specific monoclonal antibodies generated are of high affinity

The binding affinity of the individual monoclonal antibodies (MAb) for Tat was determined using a constant quantity of Tat exon-1 protein for both E and I series of antibodies, respectively, and varying concentrations of the antibodies in indirect ELISA. A three parameter generalized hill slope model was fit to the antibody concentration versus optical density curve, to determine the affinity of the different antibodies. Affinity analysis was carried out for four antibodies recognizing the NTD and four recognizing the CD. The hyperbolic curve generated by taking the log concentration of the antibody versus that absorbance for the MAbs is as shown (Figure 4.3). Using these curves, we determined the affinity constant values of the antibodies. As depicted, all the monoclonal antibodies tested bound the cognitive antigen with affinities in the nano-molar range. The immune properties of some of the anti-Tat MAbs have been summarized (Table 4.1).

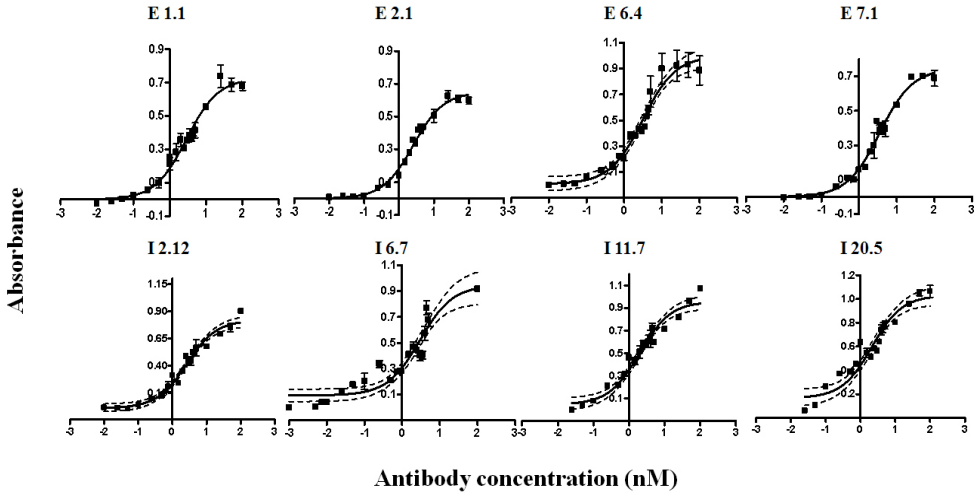


Figure 4.3: Affinity determination of the Tat monoclonal antibodies: Indirect ELISA was used to estimate binding affinities of the anti-Tat monoclonal antibodies of the E (Tat exon-1) and I (N-del Tat) series. The ELISA was performed with a fixed quantity of the cognitive Tat protein and variable concentrations of the affinity purified MAbs. Each antibody concentration was used in triplicate wells for the assay. The OD values are plotted against log concentrations of antibodies as a hyperbolic curve using GraphPad Prism Software and the K_d values were calculated. The y-axis corresponds to the absorbance and x-axis to the concentration of antibodies.

Table: 4.1 Immunologic properties of Tat-specific monoclonal antibodies

Clone no.	Mean Kd Value (nM)	Isotype	Epitope (amino acid)
E1.1	1.9495	IgG1	1-20
E2.1	3.309	IgG1	1-20
E6.4	3.93	IgG1	1-20
E7.1	3.092	IgG1	1-20
I6.7	3.39	IgG2b	30-50
I11.7	1.9075	IgG2b	30-50
I20.5	1.739	IgG2b	30-50
I2.12	3.179	IgG2b	30-50

4.2.3 Anti-Tat monoclonal antibodies efficiently neutralize extracellular-Tat across clades

The neutralization potential of the monoclonal antibodies was evaluated in a viral rescue assay as described before (section 2.1.8). The specificity of neutralization was evaluated by using different concentrations of MAb in virus rescue assay. The monoclonal antibody E1.1 was incubated with 500 ng of Tat at concentrations 6, 12, 25 and 50 μg/ml demonstrated dose dependant neutralization. The transactivation property was almost completely abrogated at 50 μg/ml and hence subsequent neutralization assays were carried out at this concentration of antibody (Fig. 4.4.A). Further a control antibody at 50 μg/ml did not neutralize Tat protein (data not shown) and hence confirming the specificity of the assay.

Tat protein is conserved to a greater extent at the amino acid level across diverse viral clades although clade-specific amino acid residues have been reported. Given the cytotoxic properties of Tat, cross-clade and efficient neutralization of the extracellular Tat is an important and desirable property of the antibodies. Sequence variation at the critical positions within a B-cell epitope could significantly affect Tat-neutralization by antibodies especially if clade-specific variations correlate with B-cell epitope specifications. For instance, the N-terminal B-cell epitope of Tat is characterized by differences at two locations, residues 7 and 12, between subtype-B and non-B sequences including subtype C. Since the E series of the anti-Tat monoclonal antibodies were generated using subtype C Tat antigen, and these antibodies target the N-terminal epitope, we examined if these monoclonal antibodies could efficiently neutralize Tat proteins of subtypes B and C. Tat proteins were pre-incubated with monoclonal antibodies at before adding the Tat mixture to HLM1 cells that contain a Tat defective provirus which secretes viral p24 antigen when induced with functional Tat.

Tat monoclonal antibodies belonging to E- (Tat exon-1) or the I-series (N-del Tat) neutralized the transactivation property of both B- and C-Tat proteins efficiently (Fig. 4.4.B). The antibodies blocked Tat transactivation to 90-95% of the control cells without antibody. The inhibition of Tat-mediated transactivation and the consequential reduction in the levels of p24 secretion into the medium was monitored as a function of time. We also tested the cross-reactivity of the antibodies to Tat protein from various clades in western blotting assay. All the antibodies tested cross-reacted with the Tat from different clades. Data for two antibodies namely E6.4 and I5.2 have been presented (Fig. 4.4.B. inset).

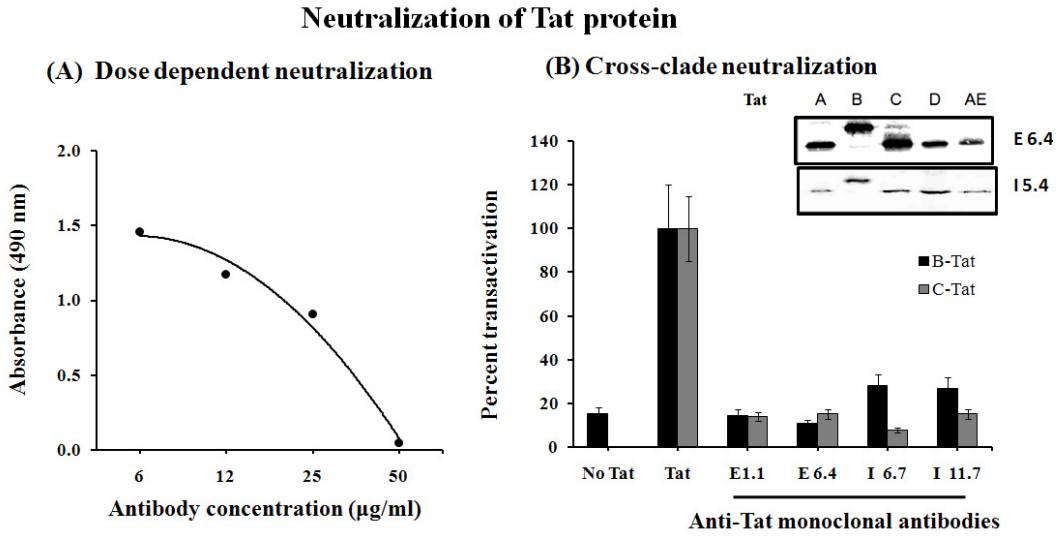


Figure 4.4: Efficient neutralization of Tat by the monoclonal antibodies. (A) 500 ng C-Tat recombinant proteins were incubated with different concentration of E1.1 for 30 min, prior to addition of the sample mix to HLM1 cells seeded in 96-well plates. The quantities of the p24 released into the medium after 72 h of incubation were assayed by using a commercial antigen-capture assay. **(B)** Cross-clade neutralization of monoclonal antibodies. 500 ng of B-and C-Tat proteins were used in this assay. Data for two each MAb representing the 'E' or 'I' series of hybridomas were presented and identical data were obtained for other MAbs in each series. The bars correspond to the mean value of three independent experiments and the error bars represent $\pm$ 1 SD. The dark bars correspond to B-Tat while the grey bars to C-Tat. **(Inset)** Western blot analysis to demonstrate cross-reactivity. Tat-specific monoclonal antibodies E 6.4 amd I 5.2 were used. Exon-1 Tat was used for all the subtypes except subtype-B where 86 amino acid long protein from HXB2 was used.

Furthermore, we also examined the neutralization potential of the monoclonal antibodies in abrogating Tat-induced apoptosis in Jurkat T-cells. Given that B-Tat protein, owing to the presence of the RGD motif in the exon-2, is believed to be more apoptotic than C-Tat (Sood et al. 2008), we used only B-Tat protein in the apoptosis assay. Jurkat cells in a 96-well plate, 1×10^6 cells/well, were incubated in the presence or absence of 2 µg/ml of Tat protein for 8 h, following which the cells were stained with PI and Annexin V-FITC and analyzed using flow cytometry. Exposure to Tat induced significant levels of apoptosis in Jurkat cells. While approximately 16% of untreated cells were positive for both PI and Annexin V (Fig. 4.5, upper right quadrant, left most panel) thus representing the late apoptotic cells, Tat treatment increased the cell death to 32.8% (second panel from the left, $p<0.005$). Tat-induced apoptosis, however, was reduced to normal levels of the control cells when the Tat protein was pre-incubated with the monoclonal antibodies. Pre-incubation of Tat with monoclonal antibodies E1.1 and I 6.7 reduced the incidence of apoptosis to 19.1 and 21%, respectively, a magnitude same as that of the untreated cells suggesting efficient Tat neutralization (Fig. 4.5, right panels). A similar magnitude of Tat neutralization was observed with all the other monoclonal antibodies from the both of the panels (data not shown). These observations suggest that antibodies against NTD as well as CD can be equally neutralizing. The MAbs were able to block the apoptotic property of B-Tat efficiently, hence demonstrating not only the strong neutralizing potential but more importantly, cross-clade neutralizing ability.

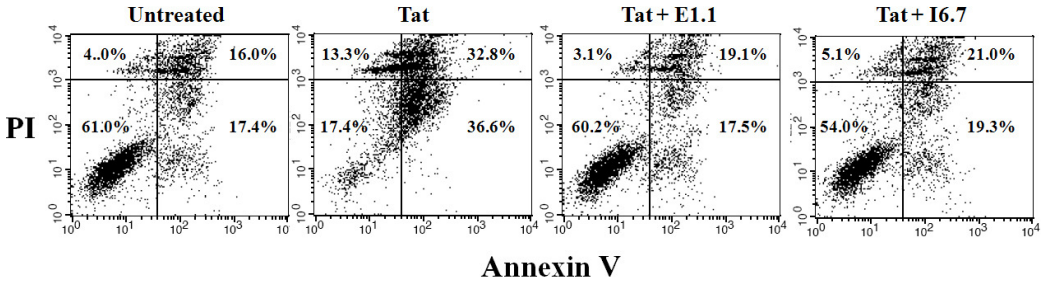


Figure 4.5: Neutralization of Tat-induced apoptosis in Jurkat T-cells. MAbs block the apoptotic activity of B-Tat efficiently. 1 x 10^6 Jurkat cells were treated with 2 µg/ml of Tat protein with or without prior incubation with the MAbs. The cells were stained with PI and Annexin V-FITC and analyzed using flow-cytometry. X-axis corresponds to Annexin V-FITC intensity and y-axis to PI. The numbers in the quadrant represent the percent cells in the respective quadrants. Lower right corresponds to Annexin V positive cells and upper right corresponds to PI and Annexin V double positive cells.

4.3 Discussion:

Our goal was to generate a panel of well-characterized monoclonal antibodies to HIV-1 subtype C Tat protein. Monoclonal antibodies to clade B Tat have been generated previously (Brake et al. 1990). Given that multiple and clade-specific amino acid variations have been reported in Tat and such variations have been shown to modulate important biological properties of this viral factor, we attempted to generate monoclonal antibodies to C-Tat. None of the monoclonal antibodies generated in our laboratory differentially recognized Tat proteins of different clades. Instead, all of them cross reacted with Tat proteins of different viral clades thus demonstrating broader reactivity. One of the original objectives of the present work is also to generate monoclonal antibodies to diverse epitopes of Tat. *In vivo*, Tat is believed to be secreted into the body fluid and a large spectrum of biological properties has been ascribed to the extracellular Tat. High quality monoclonal antibodies targeting non-overlapping epitopes are necessary to develop sensitive antigen-capture assay to detect the presence of Tat in body fluids and to measure its quantity if present. Unexpectedly, many of our initial attempts at generating anti-Tat monoclonal antibody generation ended up with antibodies that exclusively recognized the NTD of Tat but no other domain. Only deletion of the first 15 amino acid residues of Tat permitted raising antibodies to a different epitope in the core domain of Tat. In addition to the observed immunodominant nature of the NTD over other domains of Tat, the isotype profile of the antibodies obtained through the two immunizations is also of interest. While the E-series of monoclonal antibodies, immunization with Tat exon-1 protein, all were predominantly IgG1, the I-series of antibodies, immunization with N-del Tat, were all found to be IgG2b. The reasons underlying the unique isotype nature of these sets of antibodies are not clear. It is not known if absence of the Trp11

residue known to play an important role in membrane fusion and endosome trafficking in N-del Tat could be partly responsible for isotype polarity.

The affinities of the antibodies were in the nano molar range suggesting that these MAbs can bind the antigen with high affinity. Consistent with the high-affinities all the monoclonal antibodies regardless of the epitope specificity, whether recognizing NTD and CD, neutralized the trans-activation and apoptotic properties of Tat efficiently. Importantly, the neutralizing ability was similar for both B- and C-Tat protein and hence demonstrating strong cross-clade neutralization potential. Well-characterized MAbs are valuable tools in unraveling pathogenic properties of Tat protein *in vitro* and *in vivo*. E1.1 was used in a recently reported study on the differential neuropathogenic property of Tat protein from clade B and C. E1.1 was used to neutralize Tat proteins in a monocyte migration assay and abrogated the Tat-induced migration (Rao et al. 2008).

Bibliography

Brake, D.A. et al., 1990. Characterization of murine monoclonal antibodies to the tat protein from human immunodeficiency virus type 1. *Journal of Virology*, 64(2), 962-965.

Ramakrishna, L. et al., 2004. Codon optimization of the tat antigen of human immunodeficiency virus type 1 generates strong immune responses in mice following genetic immunization. *Journal of Virology*, 78(17), 9174-9189.

Rao, V.R. et al., 2008. HIV-1 clade-specific differences in the induction of neuropathogenesis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 28(40), 10010-10016.

Riedl, P. et al., 2009. Elimination of immunodominant epitopes from multispecific DNA-based vaccines allows induction of CD8 T cells that have a striking antiviral potential. *Journal of Immunology (Baltimore, Md.: 1950)*, 183(1), 370-380.

Sood, V., Ranjan, R. & Banerjea, A.C., 2008. Functional analysis of HIV-1 subtypes B and C HIV-1 Tat exons and RGD/QGD motifs with respect to Tat-mediated transactivation and apoptosis. *AIDS (London, England)*, 22(13), 1683-5.

5. Conclusions

HIV/AIDS has been the worst pandemic faced by mankind with more than 60 million individuals infected globally. All the concerted and enormous efforts to develop a vaccine have met with disappointing out come. Only recently has a vaccine clinical trial provided encouraging results where nearly a third of the participants seem to have been protected against HIV infection, thus providing vital boost for the continued efforts at vaccine development (Rerks-Ngarm et al. 2009).

Traditionally, the envelope of viruses has been the primary target for virus neutralization. However, in case of HIV, this traditional approach has not been proved successful for several technical reasons including the enormous magnitude of sequence variation and glycosylation of the envelope. In view of this failure, regulatory proteins such as Tat have been projected as potential vaccine candidates. Tat protein plays an important role in viral infectivity and pathogenicity. In addition to regulating viral gene expression, Tat modulates expression of various genes of the host thus contributing to the overall viral pathogenicity. Furthermore, Tat is believed to be secreted extracellularly and the extracellular Tat governs viral latency and contributes to disease progression. However, unlike the structural proteins, Tat is moderately immunogenic. Importantly, presence of humoral as well as cell-mediated immune responses is believed to be negatively correlated with disease progression. In this study we show that 14% of seropositive individuals from two different southern Indian cohorts harbor antibodies to Tat. We observed that 4.6% of the subjects (high-Tat group) demonstrated a class-switch to IgG while the rest harbored IgM antibodies. We also show that the CRD of Tat is the immunodominant epitope in HIV positive individuals more so in those with isotype switch to Tat. Delineating the underlying factors leading to a strong immune response to

Tat in the high-Tat group can have a significant bearing in vaccine design. Various factors including host genetics factors such as the MHC-haplotype are known to influence the magnitude of immune responses. Additionally, the sequence variation of the antigens, in this case Tat, may also contribute significantly to immune induction. Further, it is important to understand if the presence of antibodies to Tat has a protective role and could retard the rate of disease progression. While many studies demonstrated a negative correlation between immune responses to Tat and disease progression, of, a few groups either showed no correlation or a positive correlation (Tähtinen et al. 1997; Senkaali et al. 2008). The reasons underlying this discrepancy are not adequately understood, with the nature of the viral subtype and host genetic factors probably making significant contributions. In our study, given the paucity of clinical information on viral loads and CD4+ counts, we were unable to resolve this important question of the existence of a possible correlation between disease progression and Tat immune response in the Indian clinical cohorts. To address this important question, a collaborative effort with YRG Care, Chennai, India, has now been initiated. Cell-mediated immune response and viral loads along with antibody response will be evaluated in a defined clinical cohort to understand if immune response to Tat could be a prognostic marker for slow disease progression.

Various attempts have been made to develop a vaccine based on Tat. Animal experiments as well as clinical trials have reached the desired end points. The moderate immunogenicity of Tat protein compounded by its toxic properties, however, still remains a concern. Safety concerns have been raised on the use of the intact Tat protein in human vaccine trials due to the toxic properties of this viral antigen. Although many strategies have been devised to augment the immunogenicity of Tat and also abrogate its toxicity, not many of these studies attempted to address both of these problems collectively. In our

laboratory, a strategy has been developed to address these two concerns collectively. Grafting of universal T-helper epitopes into the CRD and/or the BD not only augmented the cell-mediated immune response to Tat, but also abrogated its toxic properties in DNA immunization (Anand KK et al. manuscript in preparation). DNA vaccines, however, are limited by the low quality humoral responses elicited and by the large quantities of DNA required for immunization. This caveat probably could be overcome by the application of the DNA-prime-protein-boost regimen. In our attempts to evaluate the immunogenicity of the Tat protein in mice, we encountered the problem of epitope interference with the NTD blocking immune recognition of the other B-cell epitopes in Tat. The skewed humoral immune response to the NTD precluding recognition of other epitopes was undesirable. To elicit a response to a broader spectrum of epitopes, several recombinant Tat proteins were generated and tested using the protein-prime boost regimen in BALB/c mice. Using a NTD-deleted Tat protein, we could successfully spread the immune response to other epitopes in Tat although the elicited immune responses were of lower magnitude. A different strategy of grafting of universal T-helper epitopes into Tat not only improved the magnitude of the immune response but also directed the humoral response to multiple epitopes in Tat, importantly in the presence of the immunodominant NTD epitope. The neutralization efficiency of these antibodies targeting multiple epitopes of Tat was significantly superior to WT-Tat immunization thus validating the use of HTL-engineering of the protein vaccines. Inadvertently, HTL grafting into the CRD disturbed a B-cell epitope that was identified to be immunodominant in the Indian cohorts. Presently, a new set of Tat HTL-constructs which retain this newly characterized B-cell epitope is being evaluated in both DNA and protein immunization formats. We further intend to test the efficiency of these Tat constructs in an experimental mouse model which can support

the proliferation of a chimera HIV-1 with the end point being the control of viral proliferation *in vivo.*

In summary, our work demonstrated for the first time that although Tat is considered to be non-immunodominant and even immunosuppressive, it could serve as a strongly immunodominant antigen in a small but significant number of natural infections. Further studies delineating the factors underlying the immunodominant nature of Tat in this small subset of people are necessary. We also demonstrate a molecular strategy to spread immunogenicity to subdominant epitopes in Tat in experimental immunization. Further, we have generated a panel of Tat-specific monoclonal antibodies which is of considerable utility in understanding Tat-induced pathogenesis. The present study is not only the first evaluation of host immune response to Tat in the Indian clinical cohorts but our findings have direct implications for HIV-1 Tat vaccine design.

Appendix 1

Patient Code	Age	Sex	CD4
M14159	29	F	220
M14463	38	F	134
M14484	26	F	284
M12199	30	F	456
HG0455	26	F	235
M14422	23	F	375
HG0428	24	F	573
M14245	20	F	731
HG0199	19	F	290
G86191	30	F	530
M14363	21	F	415
M13528	18	F	319
M14230	25	F	784
M14347	23	F	349
HG0431	30	F	362
HG0425	28	F	348
HG0447	45	F	352
G86201	30	F	173
G86202	37	F	248
HT0716	44	F	302
HG0439	27	F	196
M14229	23	F	546
G86145	33	F	818
HG0437	48	F	304
M14222	27	F	416
G86126	22	F	341
G84692	25	F	112
HG0432	27	F	552
M14203	23	F	1550
HG0429	38	F	662
M14181	27	F	1123
M14164	25	F	772
M14173	30	F	936
M14150	25	F	387
HT0730	28	F	220
M14153	31	F	199
M14116	25	F	680
M14111	28	F	182
M14101	25	F	417

Patient Code	Age	Sex	CD4
M14113	22	F	144
M14061	30	F	84
HT0710	27	F	391
HT0726	36	F	191
M13909	25	F	442
M13833	20	F	427
M13843	33	F	108
HG0426	45	F	314
HG0136	34	F	255
M14040	26	F	525
HT0723	32	F	624
HG0423	23	F	508
M13948	24	F	594
HG0456	28	F	717
M14534	28	F	149
M14544	30	F	616
M14545	30	F	575
M14540	39	F	179
HG0385	26	F	416
M14462	29	F	673
M14557	36	F	540
M14557	36	F	540
M14555	26	F	831
M14592	40	F	178
M14574	31	F	246
M14562	28	F	418
M14512	23	F	119
HG0465	35	F	279
HG0470	28	F	566
M14403	28	F	1017
HG0453	21	F	422
G86081	24	F	884
M14147	25	F	647
G85151	33	F	323
HT0743	28	F	1119
M13948	24	F	594
M13950	27	F	140
M13739	32	F	234
HT0714	48	F	81

Patient Code	Age	Sex	CD4
M13959	40	F	420
HT0700	20	F	300
M13972	25	F	1125
M13129	40	F	798
HG0414	26	F	234
M14008	27	F	281
M13996	33	F	190
M13940	25	F	670
HG0406	26	F	360
HG0402	30	F	575
HG0410	31	F	234
M13790	20	F	405
M14749	30	F	410
M13712	30	F	312
G85648	24	F	120
HG0403	30	F	1247
M13635	34	F	696
M13739	32	F	234
M13872	33	F	240
M13843	33	F	108
HG0394	40	F	50
M13712	30	F	312
M13731	26	F	720
M13697	19	F	187
M13766	27	F	187
HG0359	28	F	90
M13740	42	F	44
G85512	26	F	644
M13732	20	F	768
M13685	48	F	468
M13700	28	F	285
HG0372	27	F	153
HG0397	38	F	65
HG0388	24	F	462
HT0704	31	F	30
G85376	35	F	288
HG0362	27	F	108
M13683	21	F	152
HG0389	25	F	540
HG0386	38	F	936
M13625	35	F	363
M14597	22	F	836

Patient Code	Age	Sex	CD4
HG0458	27	F	265
HG0472	22	F	211
M14626	28	F	281
M14595	30	F	538
M13750	30	F	253
M13807	26	F	377
HG0475	37	F	309
M14637	42	F	515
M14411	37	M	554
G85670	40	M	49
HT0753	42	M	179
M14479	60	M	130
M14426	40	M	49
M14396	29	M	185
HT0746	38	M	81
HG0451	45	M	164
HT0750	46	M	382
M14421	30	M	52
M14387	37	M	22
HG0449	36	M	186
M14361	47	M	155
M14362	25	M	362
HG0446	46	M	395
M14218	40	M	415
M14273	46	M	647
M14267	28	M	283
M14213	35	M	186
G86125	31	M	343
M14216	40	M	386
M14241	33	M	475
HT0732	46	M	207
HG0430	29	M	653
M14143	32	M	475
M14207	50	M	339
M14160	37	M	431
M13896	31	M	291
M14192	27	M	274
G86092	45	M	88
G86087	38	M	232
M14138	30	M	175
HG0401	42	M	160
HT0729	34	M	69
M14075	32	M	305

Patient Code	Age	Sex	CD4
HG0424	34	M	300
M14072	36	M	172
M14007	45	M	172
HG0421	38	M	258
HG0415	38	M	288
G85875	38	M	288
M14518	44	M	275
HG0442	42	M	60
HG0457	49	M	720
HG0462	31	M	96
HG0461	35	M	810
HT0720	40	M	173
M14599	45	M	420
M13958	28	M	240
HT0718	40	M	80
G85876	35	M	242
HT0715	32	M	273
HG0369	45	M	72
HG0409	34	M	168
HG0418	33	M	900
M13970	32	M	322
M13788	33	M	555
M13965	36	M	416
M13998	24	M	400
M14003	48	M	355
M13941	35	M	200
M13958	28	M	240
G85813	32	M	140
HT0715	32	M	273
M13857	35	M	168
M13859	36	M	247
M13832	29	M	421
G85554	44	M	105
HG0395	32	M	338
M13729	33	M	84
HG0387	30	M	224
HT0689	30	M	36
HG0121	33	M	105
HG0396	39	M	144
G85558	34	M	273
G85443	47	M	217
M13722	37	M	75
M13654	23	M	729
G85472	35	M	280
HT0757	36	M	304
HG0474	33	M	636
M14603	29	M	626
M14609	40	M	149
M13759	23	M	357