Optimization of DNA Vaccines: Molecular Strategies to Augment Immune Responses to HIV-1 Tat

A Thesis submitted in partial fulfillment
of the requirements of the degree of

Doctor of philosophy

By

Anand Kumar K

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

December 2008

Declaration

I hereby declare that this thesis entitled “Optimization of DNA Vaccines: Molecular Strategies to augment Immune responses to HIV-1 Tat” is a bonafide record of research work carried out by me under the supervision of Dr. Udaykumar Ranga at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore.

In keeping with general practice of reporting scientific observations, due acknowledgement has been made whenever work described here has been based on the findings of other investigators. Any oversight due to error of judgment is regretted.

Anand Kumar K

Certificate

Certified that the work described here has been done under my supervision at the Molecular Biology and Genetics Unit at Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, P.O., Bangalore.

Dr. Udaykumar Ranga

Acknowledgements

I am grateful to my research supervisor Dr. Ranga Uday Kumar for his constant guidance, support and understanding. I am awed by his level of commitment and I hope to emulate him as a role model. I have learned a million things under his tutelage. I wish to strive to reach up to his level of perfection.

I would like to take this opportunity to thank Prof. Tapas Kumar Kundu, for his encouragement and his being instrumental in my joining Dr. Uday's lab. I would like to thank Prof.MRS Rao, the chairman of the department and all the faculty members of Molecular Biology and Genetics Unit, JNCASR for their help, support and encouragement. I would also like to thank Dr.Vijaya, Dr. Anjali Karande, Dr.Ramana, for their guidance. A special note of thanks to Dr. N. Jayasuryan for his constant moral support and guidance.

Special thanks to Dr.Lakshmi, who had primed me into the field of immunology. A lot of good wishes and thanks to Mangai, for all the stimulating scientific discussions and the assistance rendered at various stages. Lots and lots of thanks are due to ABS, Balasiddaiah and Sundaram RC for their efforts and support. I have had the privilege of teaching and more importantly learning a lot from them. I admire the poetry and concepts of ABS and RC;s ability to cite text books of biochemistry and physiology. Special thanks to my 'summer', Preethi for teaching me a new language. I would like to thank all my lab members, past and present, for giving me their share of memorable moments. Thanks to Dr. Siddappa, Dr. Prasanth kumar Dash, Nagendran, Marathandan, Mohan, Sravan, Raghavendra, Venky, Gopinath, Siddartha, Mahesh, Kishore, Kiran, , Nagabhooshan, Imtiaz, Chincy, Rajesh, Abraham, Roshen, Narayana, Avinash, Venkatramanan, Raghunath, Venkateswaran, Roshan, Aruna, Lavanya, Lakkappa and Sridhar.

The love and affection I constantly enjoy from my parents, my sisters 'angels' nirmala and narayanee have helped me tide many a situation in life with ease. I thank myself for having treaded this path.

Table of contents

Abbreviations

ADC	AIDS Dementia Complex
AEC	3-amino 9-ethyl-carbazole
AIDS	Acquired Immunodeficiency syndrome
Bp	base pairs
BSA	Bovine serum albumin
Con A	Concanavalin A
Cpm	counts per minute
CTL assay	Cytotoxic T Lymphocyte assay
CTL	Cytotoxic T-lymphocyte
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme linked immunosorbent assay
ELISPOT assay	Enzyme linked immunospot assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HAD	HIV associated dementia
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HTL	Helper T-lymphocyte
IL-2	Interleukin 2
LAL	Limulus amoebocyte lysate
LTR	Long terminal repeat
LTR	Long terminal repeat
Mab	Monoclonal antibody
mM	milli Molar
NF-κB	Nuclear factor κB
nM	nano Molar
ONPG	O-nitro phenyl galactoside
ONPG	O-nitro phenyl galactoside
OPD	O-phenylenediamine
PADRE	Pan DR helper T cell epitope
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PHA	Phytohemaglutinin
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulfonyl fluoride
pNPP	para nitro phenyl phosphate
pv	parental vector
PVDF	Poly vinylidene difluoride

RT	Reverse transcriptase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEAP	Secretary alkaline phosphatase
Tat_{co}	Codon-optimized
Tat_{int}	codon-optimized Tat with a synthetic intron
Th1	T-helper 1
Th2	T-helper 2
wt	wild type
β-ME	β- Mercaptoethanol
µl	microlitre
^{0}C	degree Celcius

Synopsis

Thesis Title:

Optimization of DNA Vaccines: Molecular Strategies to augment Immune responses to HIV-1 Tat

Submitted by: **Anand kumar K**

Guide: Dr. Ranga Udaykumar,
Associate Professor,
Molecular Virology Lab,
Molecular Biology and Genetics Unit,
Jawaharlal Nehru Centre for Advanced Scientific Research,
Jakkur PO, Bangalore – 560 064,
Karnataka, India.

Introduction:

DNA vaccine Technology has emerged as a novel and promising mode of vaccination over the past 15 years. DNA vaccines offer the critical advantage of introducing the encoded antigens to the MHC class I pathway, which is essential for controlling viral infections. Post-translation modifications, safety and cost effectiveness are other added advantages of the DNA vaccine. In contrast to these technical merits, DNA vaccines have low adjuvanticity and are incapable of inducing strong immune responses, especially in larger animals. Optimization of DNA vaccines, by means of adapting different molecular strategies, is critical for enhanced performance of this format of vaccination.

HIV-1 Tat protein is a key viral regulatory protein that plays an essential role in viral transactivation, replication, and disease progression. Tat is secreted from infected cells and functions as a viral factor modulating several cellular pathways. In the natural infection, immune responses to Tat are negatively correlated with disease progression suggesting a protective role. Tat antigen, however, is inherently non-immunodominant

and only in a minority of the seropositive subjects anti-Tat immune responses are elicited at a low magnitude when present.

On the one hand, anti-Tat immune responses are protective although Tat itself is non-immunodominant. On the other hand, DNA vaccination fails to elicit strong immune responses. We therefore intentionally elected to use the combination of Tat and the DNA expression vector and evaluated several molecular strategies to elicit potent immune responses to Tat in the mouse model. The protective nature of the elicited immune responses was evaluated using a chimera virus challenge model in mice.

Approaches:

We evaluated three different molecular strategies including the promoter optimization, intron engineering and T-helper epitopes incorporation into Tat, to augment antigenicity of Tat-DNA vaccines.

We characterized the Elongation Factor 1 alpha (EF-1α) cellular promoter as a substitute for the cytomegalovirus (CMV) viral promoter. With an objective to minimize the length of the EF-1α promoter, without affecting the transcription efficiency, and to eliminate the putative negative regulatory element (NRE) identified in the first intron (Wakabayashi-Ito and Nagata, 1994), we undertook a progressive deletion analysis of this cellular promoter. We generated several deletion constructs of the EF-1α intron and compared those constructs with the CMV promoter, in reporter and immune assays.

In the second approach, a synthetic intron from pIRESpuro (clontech, #6031-1), that was previously shown to enhance the stability of cytoplasmic RNA (Huang and Gorman, 1990), was introduced between the two exons of Tat_{co} (codon-optimised for mammalian expression) using the primer extension strategy. The intron-containing Tat gene, $Tat_{co\text{-}int}$, was placed downstream of both the CMV and EF-1α promoters. The $Tat_{co\text{-}int}$ constructs demonstrated superior performance in reporter assays as compared to Tat_{wt} and Tat_{co}. The $Tat_{co\text{-}int}$ vectors also elicited significantly enhanced immune responses evaluated using thymidine incorporation assay and Elispot assays, thus confirming the immuno-modulatory function of the synthetic intron engineered into Tat.

In the third approach, we introduced strong universal T-helper (HTL) epitopes into Tat. One of the epitopes is a non-natural Pan HLA-DR binding epitope, PADRE

(Alexander et al., 2000;Alexander et al., 1994) derived from tetanus toxoid. The other epitope, Pol_{711}, is derived from HIV-1 polymerase (van der Burg et al., 1999). Both of these epitopes have very high binding affinity to mouse MHC class II molecules and they also bind several common Human MHC. HTL epitopes were engineered into the cysteine-rich domain (CRD) and/or basic domain (BD) of Tat singly or in combinations, and in either orientation without disrupting the known B- and CTL epitopes. Engineering HTLs served two diverse but complementary objectives, one, recruitment of efficient T-help and two, abrogation of toxic properties of Tat, thereby improving its safety profile. Disrupting the CRD and/or BD is aimed at abrogating several harmful biological functions of Tat as these two domains play significant role in governing Tat functions.

EcoHIV Challenge Model:

David J Volksy's group previously reported a chimera model of HIV-1 that could infect conventional mice for the investigation of viral replication, control and pathogenesis. To enable viral proliferation in mice, although at a restricted level, the coding region of gp120 in HIV-1/NL4-3 was replaced with that of gp80 from ecotropic murine leukemia virus, a retrovirus that infects only rodents (Potash et al., 2005). We used this virus challenge model to evaluate the efficacy of our Tat DNA vaccination protocol.

Results:

Using a wide range of assays we demonstrate that EF-1α promoter mediates high level reporter gene expression as efficiently as the CMV promoter. Importantly, the cellular promoter elicited higher immune responses at a magnitude equivalent to that of the viral promoter. Furthermore, engineering of a synthetic intron into Tat alone resulted in significantly increased protein expression and subsequently enhanced immune response in the context of both of the promoters, viral as well as the cellular. Disruption of the CRD and BD of Tat by inserting HTL into these domains resulted in the expected abrogated transactivation property of Tat. Additionally, HTL-engineered Tat proved to be a safe vaccine candidate as it failed to induce apoptosis in THP-1, human monocytic cell line, in contrast to wild type Tat. HTL-engineered Tat not only induced high level

immune responses as evaluated using ELISPOT assay and T-cell proliferation assay but also the immune responses induced are mainly of Th1 type that are believed to be more desirable, especially in the context of a viral infection. The virus challenge studies using the EcoHIV chimera virus infection of C57BL/6 mice found significant levels of protection in the preliminary experiments. Further evaluation of protection is presently in progress. Additionally, studies on the immune protection using syngeneic tumor challenge model are also in progress. We have established stable transfectants of EL4 cell lines of subtype-C Tat (full-length) in our laboratory. EL4 cells are compatible with C57BL/6 (H-2^b) mice. We have optimized tumor establishment in C57BL/6 mice with parental EL4 and Tat-EL4 cells. Using this model, we plan to study the nature of the immune responses elicited by the Tat DNA immunization.

Significance:

The limited success attained with HIV-1 env vaccines and other structural genes at the human clinical trials prompted search for alternative viral antigens that may serve as potential candidates of a multi-component HIV vaccine. The viral regulatory protein Tat offers several advantages as one of such potential candidate antigens. Most importantly, immune responses to Tat inversely correlate with disease progression. Despite the merits, attempts by various groups with Tat vaccine previously met with limited success, for they failed to pay attention to basic aspects of vaccine design. Our approaches have circumvented those potential pitfalls.

1) Most of the groups employed Tat as a protein or a toxoid in which form Tat would not have access to the MHC class I pathway critical for viral control. We optimized Tat DNA vaccine by promoter optimization and engineering of an intron, which augmented antigen expression levels with a consequential enhancement in antigen-specific immune response.

2) Another serious limitation of the reported approaches is the use of *native* Tat protein, which is inherently non-immunodominant. Our approach of introducing universal T-helper epitopes into Tat protein, led to significantly elevated Th1-biased immune response that is more desirable in controlling viral infection.

3) Third major concern of the Tat application as a potential vaccine is its safety profile. Tat as an extracellular viral factor, causes pleotropic effects on the host that are highly cytotoxic and immunosuppressive. Disruption of the important CRD and BD domains of Tat, by means of incorporating HTLs, resulted in abrogated transactivation property and importantly loss of harmful biological functions of Tat thus potentially enhancing its safety profile, a single most advancement in Tat vaccine design. This approach is likely to elevate the position of Tat as a potential vaccine candidate.

The strategies reported here, could have broader and far-reaching impact on DNA vaccine engineering.

1. INTRODUCTION

It has been twenty five years since Human Immunodeficiency Virus (HIV) has been identified as the causative agent for Acquired Immunodeficiency Syndrome (AIDS). The pandemic of HIV infection is clearly the defining medical and public health issue of our generation and ranks among the greatest infectious disease scourges in history (Fauci, 2003; Fauci, 1999). Since the time the world first became aware of AIDS, in 1981, the disease has spread in various regions around the globe. More than 60 million people worldwide have been infected with HIV-1, mostly in the developing world, and nearly half of these individuals have died (Barouch, 2008). Everyday around 6,800 persons become infected with HIV and over 5,700 persons die from AIDS mainly because of inadequate access to HIV prevention and treatment services (UNAIDS, 2007). Unfortunately, the catastrophic potential of the AIDS pandemic has not been fully realized. HIV and AIDS continue to exact a good toll through-out the world, and remain a leading cause of mortality worldwide and the primary cause of death in Sub-Saharan Africa. Presently, more accurate estimates of HIV indicate that approximately two and half million (2 million-3. 1 million) people in India are living with HIV. Although the proportion of people living with HIV is lower than previously estimated, India's epidemics continue to affect large numbers of people (UNAIDS, 2007).

1.1 Classification of HIV-1:

Based on the genetic heterogeneity, the strains of the human immunodeficiency virus type-1 (HIV-1) are classified into three main groups M (major), O (outlier) and N (non-M, non-O) (Robertson et al., 2000; Simon et al., 1998). Group M viruses are responsible for the majority of HIV-1 infections in the world and can be further subdivided into 9 primary subtypes, also called clades, consisting of A, B, C, D, F, G, H, J, K which differ from one another up to 25% to 35% in their env and gag sequences. Phylogenetic analyses of nucleotide sequences from the env gene classified the group M strains into at least 11 different envelope genetic subtypes, designated by letters from A to L (Osmanov et al., 2002). Also, a minimum of 43 circulating recombinant forms

(CRFs) and a large number of unique recombinant forms have been reported (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). The viral subtypes are unevenly distributed across the globe (Esparza and Bhamarapravati, 2000). Importantly, subtype C strains of HIV-1 are responsible for the rapidly expanding epidemics in the most populous nations such as India, China, Sub-Saharan African countries and Southern Brazil. HIV-1 subtype C is the most prevalent subtype in India with nearly 99% of the viral infections ascribed to this viral clade (Siddappa et al., 2004; Siddappa et al., 2005). Subtype C viruses are globally responsible for 56% of the HIV-1 infections (Esparza and Bhamarapravati, 2000). During the past decade, the infection incidence of subtype C in Southern Brazil reportedly increased from 3% to 30% (Soares et al., 2003) and eventually to 45% (Soares et al., 2005). Additionally, subtype C viruses have been reported from geographical regions from where they have not been identified previously such as the UK (Tatt et al., 2004) and several South American countries (Hecht et al., 1998; Perez et al., 2006).

1.2 Virion Structure:

HIV-1 and HIV-2 contain a cone-shaped capsid (CA) composed of the viral Gag protein p24 (Figure-1). Inside this capsid are two identical RNA molecules associated with the nucleocapsid (NC) protein p7. The mature virion contains pol gene products (i.e., p12 protease, reverse transcriptase (p66, p5l) and integrase (p34), as well as incomplete DNA copies of genomic RNA, believed to be products of limited DNA polymerization. The infectious particle contains vpr, a protein involved in virus replication. Myristoylated p17 provides the matrix (MA) for the viral structure. The MA is required for incorporation of the envelope proteins into the virus particle. The envelope glycoproteins (gp120 and gp4l) are derived from a 160 kD precursor (gpl60), which is cleaved by

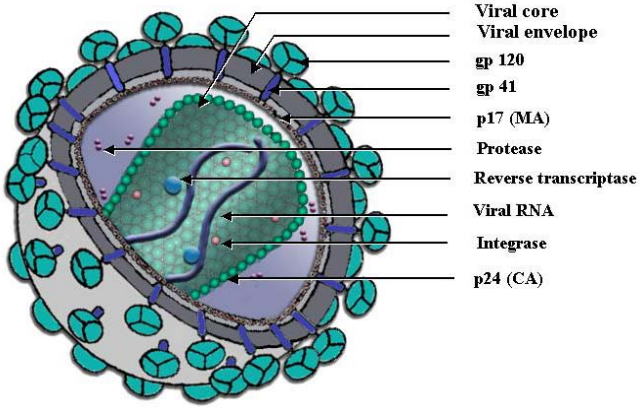


Figure-1: Structure of HIV virion

cellular enzymes in the Golgi apparatus. The viral surface contains trimers of the envelope glycoproteins.

In addition, cellular proteins are present in gradient-purified HIV-1, HIV-2, and SIV preparations. These cellular proteins include the alpha and beta chains of human lymphocyte antigen (HLA-DR), beta 2 microglobulin, HLA class I, intercellular adhesion molecule-1, and leukocyte function antigen-1 (LFA-1) (Capobianchi et al., 1994). The above observation suggests a selective incorporation of human cellular proteins in the virus particle. Some of these cellular antigens outnumber the molecules of gp 120 present in the virions and are considered to be an integral part of the virus envelope.

1.3 Life Cycle of HIV:

HIV belongs to the group retroviridae and is a member of the lentivirus genus, which includes retroviruses that possess complex genomes and exhibit cone-shaped capsid core particles. HIV's genome is encoded by RNA, which is reverse-transcribed to viral DNA by the viral reverse transcriptase (RT), which is a common feature in all retroviruses. The life cycle of the virus has two phases, early and the late phase. The early phase begins with the attachment of the virus to the receptor CD4 and a co-receptor (CCR5 or CXCR4), followed by fusion with the target cell membrane. After the entry of the virus, the viral RNA genome undergoes reverse transcription, and the proviral DNA integrates into the host chromosome. Both viral and host cell machineries are necessary for transcription of the viral genes using the integrated provirus as the template. The late phase of the virus life cycle begins with the synthesis of unspliced and spliced mRNA transcripts which are transported out of the nucleus for translation. After translation the viral proteins assemble at the cell membrane and the immature viral particle containing the RNA genome and the viral enzymes, importantly, reverse transcriptase and integrase, egress the cell. After budding, proteolytic processing of the capsid leads to structural rearrangement of the virion and generates a mature viral particle (Figure-2).

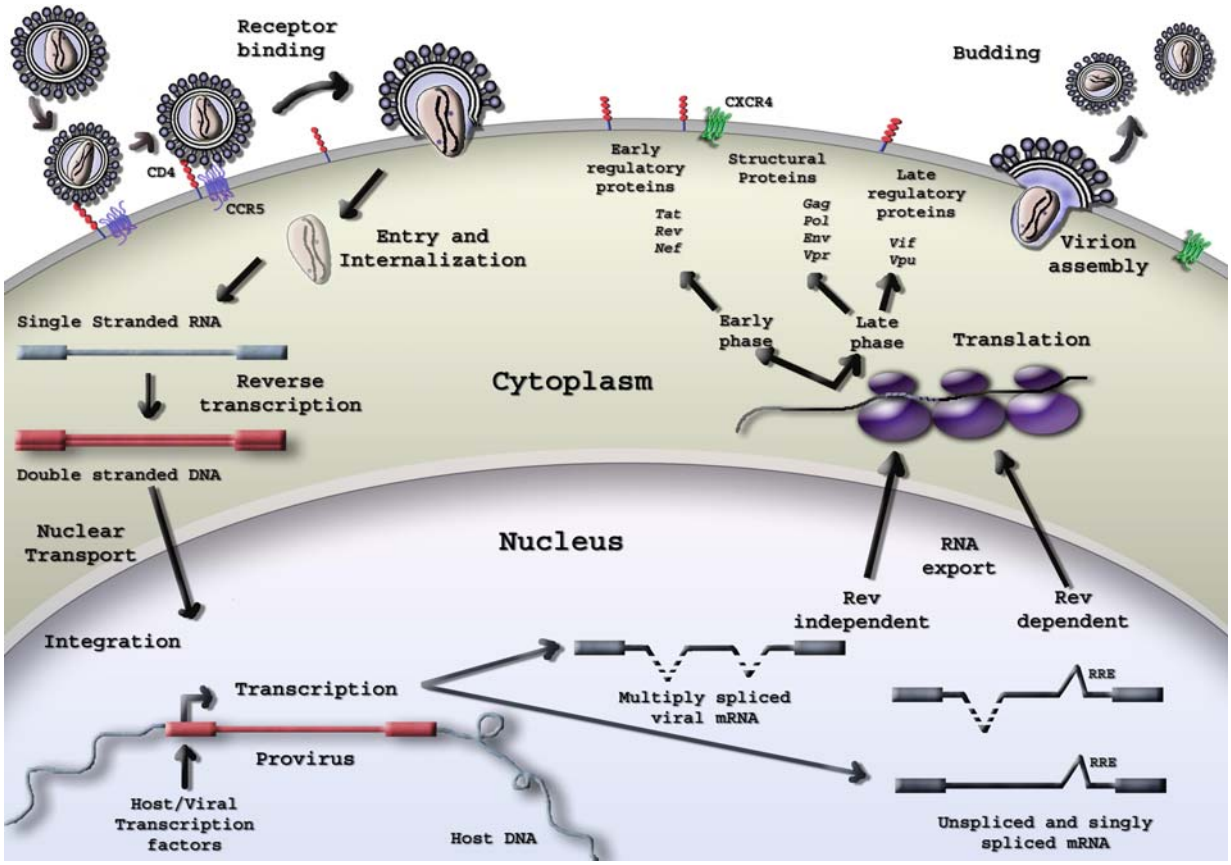


Figure-2: Life cycle of HIV

1.4 Genes of HIV:

The genome organization of primate lentiviruses is quite similar and is unique among retroviruses. A common feature among the primate lentiviruses is the abundance of open reading frames (ORFs) not found among other groups of retroviruses. The virion contains two identical copies of single-stranded RNA, about 9,200 bases long. The HIV-1 provirus as a chromosomal integrant has the characteristic retroviral features of having the long terminal repeats flanking the genomic sequence. The genome encodes several genes, which can be categorized into three groups namely,

1. Structural proteins (Gag, Pol, Env)
2. Regulatory proteins (Tat, Rev)
3. Accessory proteins (Nef, Vif, Vpr, Vpu (in HIV-1), Vpx (in HIV-2)

The different proteins are produced at various stages of the viral life cycle from a single primary transcript by different strategies: generation and proteolytic processing of precursor polyproteins, ribosomal frameshifting or suppression of translational termination, alternative splicing of the primary transcript and bicistronic mRNAs producing two proteins.

1.5 Immune responses to HIV:

The hallmark of HIV-1 infection is the progressive depletion of $CD4^+$ T-cells. Yet the extent and nature of this depletion, and the mechanisms by which it arises remain highly controversial. HIV-1 infection also induces profound qualitative changes in $CD4^+$ T-cells and in most other compartments of the immune system too. No viral infection in humans is known to cause such profound and inevitable $CD4^+$T-cell loss, like in the case of HIV-1. Immune responses elicited during the course of HIV infection are determined by several complex factors that involve host pathogen interactions. Most people infected with HIV do mount an effective immune response to the virus during the first few weeks to months after infection. However, over time, this response turns out to be insufficient or ineffective. Progressive infection of the immune system, over the course of years, leads to the destruction and fatal immunodeficiency.

The immune response mounted by the host comes in two forms: cellular and humoral. The cellular response refers to the activity of the $CD4^+$ and $CD8^+$ T-cells, while the humoral response refers to antibody production and activity by the B-cells. The course of a typical HIV infection is marked by a raise in $CD8^+$ cytotoxic T-lymphocyte (CTL) response followed by a substantial amount of neutralizing antibody response against the surface-exposed envelope proteins.

1.5.1 Cellular immune responses:

In the first few weeks after infection, the number of $CD8^+$ T-cells increases to up to 20-fold above the normal range, whilst $CD4^+$ T-cell numbers fall sharply. CTLs against HIV are the first mode of adaptive immune response established during the early phase of HIV infection followed by a rapid control of viremia. $CD8^+$ T-cells appear to act against HIV in two ways during primary infection: by killing HIV-infected cells and

by secreting antiviral cytokines. HIV-specific $CD8^+$ T-cells target at the dominant viral variant and their emergence is associated with a rapid fall in viral load, before the development of an antibody response. Most of the $CD8^+$ T-cells generated during primary infection die within a few weeks, leaving a reservoir of HIV-specific $CD8^+$ memory T-cells which will persist regardless of the presence of an antigen or $CD4^+$ helper T-cells. Several studies indicate cellular immune responses are critical in controlling HIV-1 (Sewell et al., 2000; Gandhi et al., 2002; Borrow et al., 1994b; Koup et al., 1994). Eliciting cellular immune responses has therefore, become a priority for candidate HIV vaccines. NK cells, CTLs and T-helper cells augmented by optimal co-stimulation and chemokines constitute the cellular immune responses. NK cells kill the infected cells in a MHC-independent manner and are an important defense against viral infections. Importance of NK cell activity is emphasized by the observation that anti-HIV ADCC (Antibody-dependent cellular cytotoxicity) and NK cell activity decrease with progression to AIDS.

MHC-I restricted CTL activity has been shown against several HIV proteins, with their frequency being higher in asymptomatic sero-positive individuals (10-20 CTLp/10^4 PBMCs). The initial fall in primary viremia correlates best with the appearance of anti-HIV MHC-I restricted $CD8^+$ CTLs in the peripheral blood. $CD8^+$ CTLs are also believed to be important in the immune response to HIV during the chronic phase of HIV infection for the elimination of productively infected cells and for control of the viral load. $CD8^+$ T-cells are also known to secrete a '$CD8^+$ cell anti-viral factor, CAF', which is a soluble factor blocking HIV infection. This factor has been proposed to be a group of small molecules called α-defensins (Zhang et al., 2002).

Individuals with chronic progressive HIV-1 infection have dysfunctional $CD8^+$ T-cells, a finding ascribed to impaired maturation of virus specific cells. A few patients with HIV-specific $CD8^+$ T-cells with mature "effector" memory phenotype were able to control viremia, while a few others with the same "effector "$CD8^+$ T-cells could not (Hess et al., 2004) raising the question on the cell-surface markers to designate maturation or effector function of $CD8^+$ T-cells in humans.

Several theories have attempted to account for the gradual failure of $CD8^+$ T-cells to control HIV replication. The first, 'viral escape' theory states that the cells begin to lose

the ability to recognize viral antigenic sequences due to the high level of viral turnover and mutation. HIV's ability to mutate to escape from CTL pressure is increasingly recognized (Jones et al., 2004; Sewell et al., 2000). A comparison of $CD8^+$ CTLs from HIV-infected asymptomatic individuals with those from symptomatic individuals revealed that the $CD8^+$ T-cells lose their ability to recognize and kill viral variants, while they may be responsive to normal 'wild type' viruses.

Second theory states that HIV may actually deplete off some of the $CD8^+$ T-cell repertoire. In other words, the rapid progression and death may be linked to a defect in the activation and proliferation of HIV-specific $CD8^+$ T-cells. Studies have found that HIV-specific $CD8^+$ T-cells do recognize viral variants, questioning the validity of the viral escape theory outlined above. Further evidence to support this theory has come from a publication that reported $CD8^+$ T-cells which specifically targeted 3TC (lamivudine, Epivir)-resistant virus in individuals with 3TC resistance, indicating the body's ability to adapt to viral variation even during advanced HIV disease (Schmitt et al., 2000). A poor immune response to HIV may not therefore be due to a lack of HIV-specific $CD8^+$ T-cells, but to a lack of activity by these cells.

This lack of activity by HIV-specific $CD8^+$ T-cells is possibly due to lack of help signals from $CD4^+$ T-cells which are directly disrupted by HIV. Strong $CD8^+$ T-cell responses which proliferate in response to HIV are associated with IL-2-producing HIV-specific $CD4^+$ T-cells which only seem to be present in people who can control viral load such as long-term non-progressors (Boaz et al., 2002a). A subset of $CD8^+$ T-cells called $CD8^+$ / $CD28^+$ T-cells seem to be the most important cytotoxic cells, but their number decreases during HIV infection (Weekes et al., 1999; Boaz et al., 2002b).

A study of long-term non-progressors (LTNPs) found that these subjects had relatively low levels of HIV-1-specific $CD8^+$ T-cells targeting HIV's Gag and Env proteins, but high levels of $CD8^+$ precursor cells. Three of six long-term non-progressors had high HIV-1 p24-specific $CD8^+$ T-cell responses (Greenough et al., 1999).

HIV proteins Nef and Tat could downregulate MHC-I molecules and as a consequence the immune system fails to see the infected cells. These proteins have been shown to selectively downregulate HLA-A and HLA-B, but doesn't affect HLA-C or

HLA-E, thereby protecting HIV-infected cells from Natural Killer (NK) cells (Cohen et al., 1999b).

Research with a group of long-term non-progressors has identified that the $CD8^+$ T-cells of non-progressors could divide and proliferate more readily when called up on to fight infection. In addition, they also produced higher levels of a molecule called perforin which assists in the destruction of HIV-infected cells (Migueles et al., 2008; Migueles et al., 2004; Migueles et al., 2002).

There is conflicting evidence about the impact of chemokine activity on HIV disease progression. Reports have suggested that chemokines suppress HIV replication, and that high levels of certain chemokines are associated with delayed HIV disease progression (Garzino-Demo et al., 1999). However, one group of researchers found either no consistent relationship between beta-chemokine production and HIV replication or an unexpected correlation between high beta-chemokine levels and high-level virus replication (Greco et al., 1998).

The major hallmark of AIDS includes the destruction of $CD4^+$ T-cells and subsequent loss of immune competence. Six months after infection, $CD4^+$ T-cell function improves except in relation to HIV-specific antigens (Musey et al., 1999). The few people who maintain strong HIV-specific $CD4^+$ T-cell responses have lower viral loads than people with poor responses. Importantly, those people who maintain HIV-specific $CD4^+$ T-cells which can respond by producing the cytokines IFNγ and IL-2 appear to be able to control viral loads. In contrast, most other people's HIV-specific $CD4^+$ T-cells are thought not to be functioning correctly as they can only produce interferon gamma, and this is associated with a lack of viral control (Boaz et al., 2002b; Palmer et al., 2004; Younes et al., 2003; Aiuti and Mezzaroma, 2006) .

Although multiple mechanisms contribute to the gradual T-cell decline that occurs in HIV-infected patients, programmed cell death of uninfected bystander T-lymphocytes, including $CD4^+$ and $CD8^+$ T-cells, is an important event leading to immunodeficiency (Ahr et al., 2004). $CD4^+$ T-cells are heterogeneous in phenotype and function, and it is unknown how preferential depletion of specific $CD4^+$ T-cell subsets influences disease severity. Studies have thrown light that during HIV infection, specific changes in the

fraction of $CD4^+$ T-cells expressing CD25 and/or CD127 are associated with disease progression (Dunham et al., 2008).

There is a decline in the immune functions which are governed by $CD4^+$ T-cells, and this decline sometimes leads to the appearance of infections such as Candida (thrush), herpes and *Pneumocystis pneumonia* (PCP) during seroconversion illness. Constitutional symptoms, such as fever, weight loss, night sweats and diarrhea may also develop. With the decline in $CD4^+$ T-cells to less than 200 $CD4^+$ T-cells/μl, the risks of many AIDS-defining illnesses increases and among them are several opportunistic infections and certain neoplasms.

1.5.2 Humoral responses:

Antibodies to HIV can be detected soon after acute infection, often as early as a few days after exposure to virus, but generally within 1-3 months. Seroconversion is one of the evidences for the induction of an adaptive immune response to HIV infection. Antibodies against env and p24 are seen early in infection marking seroconversion, IgG_1 being an important isotype. IgG1 is a key player in host defense at all stages of infection and helps to combat the virus with Antibody-dependent cellular cytotoxicity (ADCC), and neutralizing and blocking responses. Antibodies are chiefly targeted against free-floating virions, although some antibodies may destroy HIV-infected cells. Antibodies seen during the course of the HIV infection may be neutralizing, but this effectiveness is short-lived for most of the antibodies. Neutralizing antibodies offer some protection by limiting viral replication during the early asymptomatic stages, but their overall titers tend to remain too low to clear the HIV infection. The low titer is probably a result of the fact that the virus's envelope epitopes have a highly dynamic configuration, which changes often, depending on the state of activation and binding to cellular receptors. The low titer also favors the emergence of resistant mutants during active replication. Patients often produce many neutralizing antibodies, but these are specific to the earlier virus isolate and cannot neutralize these "escape mutants" as effectively. A very small number of people infected with HIV do not develop antibodies to the virus. A study of

six HIV-infected people found that the absence of antibodies was due to individual immune dysfunction (Ellenberger et al., 1999).

Several regions of env sensitive to antibody (Ab) neutralization have been identified. They include the V3 loop and $CD4^+$ binding domains of gp120 and a linear epitope (ELDKWA) exposed post binding on gp41 (2F5). Additionally, long-term non-progressors (LTNPs) contain neutralizing Abs against the regulatory proteins Tat, Rev and Nef leading to the supposition that such humoral responses could be viewed as correlates of protection (Richardson et al., 2003; Zagury et al., 1998b)

Certain Abs against gp120 are also shown to promote ADCC, while yet another detrimental set of Abs actually enhances HIV infectivity. Infection-enhancing antibodies bind to epitopes on gp41. Antibody-dependent enhancement involves the binding of the Fab portion of non-neutralizing Abs to the surface of the virion and the transfer of virus to a cell though the complement of Fc receptor or via opsonisation (Levy JA, 1998).

The correlates of protection in the event of HIV infection seem to be a robust-cellular immune response targeted primarily at conserved proteins like the regulatory proteins, with a predominant Th-1 cytokine profile. Antibodies against circulating Tat and conserved neutralizing epitopes present on the env protein are also important correlates. Humoral responses, when a neutralizing effect is evident, are the most clinically relevant outcome desired of a vaccine designed to raise antibodies against HIV proteins. Responses against multiple regions of HIV would be optimal and may be augmented with currently available antiretroviral drug therapy.

1.6 Current modes of HIV therapy:

Current modes of HIV therapy can be broadly classified into chemotherapy involving the use of antiretroviral compounds as drugs, gene therapy involving the modification of cells to bring about anti-viral effects and vaccines to elicit anti-viral cellular and humoral responses against various viral genes.

1.7 Antiretroviral Therapy (ART)

Second to the identification of HIV as the causative agent for AIDS, the most impressive scientific advances have occurred in the development of antiretroviral (ART) drugs for treatment of HIV infected individuals. Considerable progress made in understanding HIV and its pathogenesis led to the discovery and development of a variety of agents that might have a role in treating HIV infection. The spectrum of drug discovery for HIV centers on an appreciation of vulnerable targets in the replication cycle of the virus.

1.7.1 Nucleoside reverse transcriptase inhibitors (NRTI):

Nucleoside analogs ("nukes") are also referred to as Nucleoside reverse transcriptase inhibitors (NRTI)**.** Their target is the HIV enzyme reverse transcriptase. Acting as alternative substrates or false building bricks, they compete with physiological nucleosides, differing from them only by a minor modification in the ribose molecule. The incorporation of nucleoside analogs induces the abortion of DNA synthesis, as phosphodiester bridges can no longer be built to stabilize the double strand. AZT and d4T are thymidine analogs, while FTC and 3TC are cytidine analogs. There is a high degree of cross-resistance between nucleoside analogs. Nucleoside analogs were the first drugs to be used in HIV treatment, and therefore, most of the experience is based on them. Other NRTIs approved for treatment of HIV-1 infection include Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Lamivudine (3TC) etc. A combination therapy is being advocated rather than monotherapy to help overcome rapid accumulation of resistance by reverse transcriptase in monotherapy experienced individuals.

1.7.2 Non-nucleoside reverse transcriptase inhibitors (NNRTI):

NNRTIs bind directly and noncompetitively to the enzyme at a position in close proximity to the substrate binding site for nucleosides. The resulting complex blocks the catalyst activated binding site of the reverse transcriptase. This, in turn, can bind fewer nucleosides, slowing polymerization process significantly. The three available NNRTIs, nevirapine, delavirdine and efavirenz, were introduced between 1996 and 1998.

1.7.3 Protease inhibitors (PI):

The HIV protease processes the viral gag-pol polyprotein into its functional subunits. With the knowledge of the molecular structure of the viral protease, the first protease inhibitors (PIs) were designed in the early nineties. Since 1995, protease inhibitors have revolutionized the treatment of HIV infection. At least three large studies with clinical endpoints have demonstrated the efficacy of indinavir, ritonavir and saquinavir (Cameron et al., 1998; Hammer et al., 1997; Plosker and Scott, 2003). With growing knowledge of the mitochondrial toxicity of nucleoside analogs and through the introduction of easy-to-take PIs, this class of drugs is currently experiencing a renaissance. Even PI-only regimens are now being investigated.

1.7.4 Entry inhibitors:

There are three crucial steps for entry of HIV into the $CD4^+$ cell:

1. Binding of HIV to the $CD4^+$ receptor,
2. Binding to co-receptors, and finally
3. Fusion of virus and cell.

Every step of HIV entry can theoretically be inhibited. All three drug classes, namely attachment inhibitors, co-receptor antagonists and fusion inhibitors (FIs) are currently summarized as entry inhibitors. In 2003, Fuzeon, the first and so far only drug of this class was licensed. T-20 (Enfurvitide, Fuzeon**)** is the prototype of the FIs. Since May 2003, it has been licensed in Europe and the US for the treatment of HIV-1 infection in treatment-experienced adults and children over 6 years of age.

1.7.5 Integrase inhibitors:

Integrase, along with reverse transcriptase and protease, is one of the three key enzymes in the HIV-1 replication cycle. This enzyme, coded by the HIV pol gene, is involved in the integration of viral DNA into the host genome, and is essential for the proliferation of HIV (Nair, 2002). This fact makes it an interesting starting point for antiviral drugs. The developments of integrase inhibitors have been relatively slow. Since 2005, clinical studies have proceeded rapidly, and lately, following the first data from raltegravir (MK-0518), integrase inhibitors became the promising new drug class in HIV medicine. Further integrase is probably not present in human cells.

1.7.6 Maturation inhibitors:

Maturation inhibitors inhibit HIV replication in a late phase of the reproduction cycle, i. e., by the budding of new virions. Bevirimat (PA-457) is a derivative of betulinic acid, which is isolated as triterpene carbonic acid from birch bark. Bevirimat (manufacturer: Panacos) inhibits replication in a very late phase of the reproduction cycle, i. e. the budding or maturation of new virions. Bevirimat inhibits the transition of the capsid precursor (p25) into the mature capsid protein (p24), to produce non-infectious viruses. Bevirimat is also effective against resistant viruses.

1.7.7 Highly Active Anti-Retroviral Therapy (HAART):

HAART is a combination therapy, which includes two reverse transcriptase inhibitors, and at least one PI. HAART offers an intensive chemotherapy, which is capable of reducing the viral load of HIV-1 infected individuals. However, infected cells still persist. Efforts at flushing out latent viral reservoirs include administration of IL-2 or structured treatment interruption (STI) regimes (Allen et al., 2002a). STI, however, is often associated with a rebound of viremia and a consensus as to the optimal protocol of STI or whether it is to be used at all is yet to be reached. The combinations currently used as initial regimens consist of two nucleoside analogs plus either a PI or an NNRTI. A third nucleoside analog is only used in exceptional cases. All three strategies reduce the risk of AIDS approximately equally. There are various strategies of primary therapy. These include:

1. Two NRTIs plus a NNRTI
2. Two NRTIs plus a protease inhibitor
3. Three NRTIs (triple nuke)
4. Once-daily combinations
5. Experimental combinations (nuke sparing, intensive approaches) etc.

Antiretroviral therapy is frequently modified. This occurs in up to 50% of patients within the first year of therapy. The most important reasons are discussed below. These are:

1. Acute side effects
2. Long-term toxicity (or concern regarding this)
3. Virological treatment failure

The major shortcoming of chemotherapy is the cost factor involved. New generation high potency drugs are being introduced in the market with escalating costs. In the third world countries where the prevalence of infection is highest, chemotherapy cannot reach majority of the affected populations. Non-compliance to treatment regimes is a major contributor to acquiring resistance along with rapid selection against drug selection pressure owing to the error prone reverse transcriptase, which ultimately leads to failure of ART. The demerits notwithstanding, chemotherapy is still a feasible and the only mode of therapy that can help reduce viral loads and improve the quality of life of people living with HIV/AIDS.

1.8 Microbicides:

Apart from the therapeutic ART, use of certain microbicides by high-risk behavior groups has been shown to reduce the risk of acquiring infection. Microbicides are chemical substances, which are usually applied topically as vaginal gels, in order to kill or immobilize HIV and other germs. Currently, very heterogeneous mechanisms are being investigated. These include inactivated substances, which disrupt the viral structures, as well as those which bind to the target-cell and inhibit them or antiretroviral drugs such as tenofovir or the nucleoside analog stampidine (Stone and Jiang, 2006). Ideally, microbicides would also be active against sexually transmitted diseases, as these significantly increase the risk of HIV transmission. It should not be forgotten that so far, no microbicide has demonstrated a protective effect in clinical studies.

1.9 Immunotherapy:

In addition to conventional ART, immunomodulatory treatment strategies have been investigated. All of these therapies still lack proof of clinical benefit. Some approaches have been addressed briefly here.

Interleukin-2 (IL-2) is a cytokine that is produced by activated T-cells and which induces proliferation and cytokine production in T-, B and NK cells. It is licensed in Europe for the treatment of metastatic renal cell carcinoma. At the beginning of the 90s, IL-2 was already used intravenously in HIV-infected patients (Wood et al., 1993), but it is now administered subcutaneously. The most important effect is the increase in $CD4^+$ and $CD8^+$ cells (Kovacs et al., 2005). Memory cells initially increase, followed by naïve

-T-cells. The increasing $CD4^+$ cells under IL-2 probably have the same qualities as normal $CD4^+$ cells. The source of the $CD4^+$ cell increases is also a subject of some discussion. Some authors suspect that the increase is more due to peripheral expansion than to increased thymus output, while others have assigned greater importance to the thymus. Newer studies suggest that the effect of IL-2 is above all based on a reduced T-cell turnover or cell death (Kovacs et al., 2005).

Interleukin-12 stimulates T lymphocytes and NK cells to generate a Th1-type immune response. In a randomized Phase I study with 100 ng per kg, twice weekly/bi-weekly, the drug was well tolerated but had no effect on lymphocyte subpopulations, antigen-specific immune response or viral load. Further development is therefore uncertain. The same would appear to be true for **interleukin-10** (Angel et al., 2000) or **interleukin-15** (Ahmad et al., 2005).

Interleukin-7 seems to be more promising. This cytokine plays a fundamental role in T-cell homeostasis and influences amongst other things the formation and maturation of $CD4^+$ cells. In two pilot studies, 6 and 16 HIV patients received different doses injected subcutaneously. In both trials, good $CD4^+$ increases were observed together with good tolerability. The IL-2-type side effects were not observed. If these results can be confirmed in large studies, interleukin-7 could become an option for those patients in whom immune reconstitution remains low despite good viral suppression

1.10 Gene therapy:

Highly active antiretroviral therapy prolongs the life of HIV-infected individuals, but it requires lifelong treatment and results in cumulative toxicities and viral-escape mutants. Gene therapy offers the promise of preventing progressive HIV infection by sustained interference with viral replication in the absence of chronic chemotherapy. Gene-targeting strategies are being developed with RNA-based agents, such as ribozymes, antisense, RNA aptamers and small interfering RNA, and protein-based agents, such as the mutant HIV Rev protein M10, fusion inhibitors and zinc-finger nucleases. Recent advances in T-cell–based strategies include gene-modified HIV-resistant T-cells, lentiviral gene delivery, CD8+ T-cells, T bodies and engineered T-cell receptors. Some of the gene therapy approaches are discussed below.

1.10.1 RNA-based gene therapy:

RNA-based gene therapy approaches for HIV include the use of antisense RNA, ribozymes, RNA decoys and RNAi. Unlike foreign proteins, RNA-based gene therapeutic approaches are unlikely to serve as targets for undesirable immune responses.

Antisense RNA: Short and long antisense RNA transgenes that simply pair with HIV transcripts to form nonfunctional duplexes have also proven to be effective in blocking HIV replication in hematopoietic cells. Antisense RNA molecules act by hybridization to the target RNA molecules leading to the degradation of duplexed RNA. They also inhibit mRNA transport and translation. Antisense RNA molecules are either synthesized chemically comprising of a phosphorothioate backbone to enhance the intracellular stability or expressed in the target-cell itself following transduction. Antisense molecules targeting regulatory components of HIV like Tat, Rev and their interacting motifs TAR and RRE have shown to be effective in reducing the production of infectious particles. Sub genomic antisense RNAs for multiple genes are employed to circumvent the possibility of generation of resistant mutants. Several of these approaches have been tested in human clinical trials where retrovirally expressed molecules were delivered to hematopoietic stem cells and CD4 cells. The first demonstration of this principle came from studies using adeno-associated virus to deliver a short anti–U5-region antisense RNA. More recently, a clinical trial using an HIV LTR–expressed anti-*env* antisense has been reported (Rossi et al., 2007). Although the actual mechanism by which these antisense transcripts inhibit HIV replication is not clear, it may involve triggering extensive adenosine deamination of the HIV-antisense duplex, resulting in nuclear retention of transcripts or the generation of multiple viral-disabling mutations.

***RNA Aptamers*:** Another group of RNA molecules, RNA aptamers, have been evolved *in vitro* to bind targeted ligands with high affinity. Although aptamers against HIV show promise, thus far there have been no clinical trials using anti-HIV aptamers. One potential problem is that aptamers selected *in vitro* may not form the required tertiary structure in cells to effectively bind target proteins.

Ribozymes: Ribozymes are catalytic antisense molecules designed to specifically cleave a target RNA sequence. Two kinds of ribozymes have been studied for anti-viral activity namely hammerhead and hairpin ribozymes, which may be expressed from RNA

polymerase III promoters. They are trans-cleaving small molecules spanning about 40 – 60 nucleotides. Ribozymes act by binding a minimal 8 – 12 bp long specific sequence followed by a cleavage step. They have certain advantages over antisense RNA molecules in that, they are smaller in size and can be more easily expressed. They also afford the advantage of expressing multiple ribozymes by a single vector and are also believed to turnover several times. Ribozymes targeted at various HIV sequences have been retrovirally transduced into hematopoietic stem cells, T-cell lines and primary $CD4^+$ cells and assayed for anti-viral activity. Retrovirally expressed anti-HIV ribozymes are shown to confer a selective survival advantage on $CD4^+$ T-cells *in vitro* and may perform synergistically with antiretroviral drugs (Khan and Lal, 2003).

Decoy RNAs: RNA decoys are molecules that mimic the conformational structure of HIV RNA and thereby compete with the transcriptional machinery for factors that bind to the HIV RNA (Khan and Lal, 2003). The motifs used are either RRE or TAR decoys targeting the action of Rev and Tat, respectively. Viral mutations might occur which would render the antisense or ribozyme construct ineffective. An RNA decoy might be less liable to the effect of viral mutation as it would be expected to compete with the natural ligand for protein binding or sequester the essential interacting protein. Both RRE and TAR decoys have shown anti-viral activity (Yamamoto et al., 2000; Yamamoto et al., 1997).

RNAi: RNAi is a regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA molecules as triggers to direct homology-dependent control of gene activity. Known as small interfering RNAs (siRNAs), these ~21- to 22–base pair (bp) double-stranded RNA molecules have characteristic two-nucleotide 3′overhangs that allow them to be recognized by the host RNAi enzymatic machinery, leading to homology-dependent degradation of the target mRNA. RNAi triggers can be produced by expressing short hairpin (shRNA) precursors that partly resemble endogenous microRNA precursors, allowing them to be exported to the cytoplasm and processed by the RNAi machinery. Expressing short hairpin precursors encoding siRNAs targeting viral or cellular sequences can be readily accomplished from the backbone of viral vectors used in gene therapy.

HIV-1 was one of the first infectious agents targeted by RNAi as a result of the virus' well-understood life cycle and pattern of gene expression. Virtually all the HIV-encoded RNAs—including *tat*, *rev*, *gag*, *pol*, *nef*, *vif*, *env*, *vpr* and the LTR—are susceptible to RNAi downregulation in cell lines. A substantial challenge for clinical applications of RNAi triggers is the high viral mutation rate of HIV, which generates mutants that escape being targeted.

siRNAs can also be produced from shRNA precursors expressed from retroviral or lentiviral vector backbones by transcription from either Pol III or Pol II promoters. Because the transcription units are short in both cases, shRNAs can readily be multiplexed in various combinations. Using multiple shRNAs to target separate conserved sites in HIV—akin to the HAART approach- should prevent cross-resistance among different RNAi effectors or among RNAi effectors and conventional pharmaceuticals. Multiple RNAi effectors would thus have the advantage of limiting escape and targeting a range of sequences as is found in different viral genotypes or quasi species. Viruses that escape the antiviral effects of RNAi can be reinhibited by targeting different sequences. Thus, a multiple inhibitory approach should aim to target distinct genomic regions of HIV-1 or, alternatively, target host-derived factors that contribute to viral replication. A potential drawback of using multiple shRNAs is that expressed hairpins and the siRNAs processed from them can compete with endogenous microRNAs for nuclear-to-cytoplasmic export and incorporation into the RNA silencing machinery. The expression levels of shRNAs can be a critical determinant of whether they are toxic, so caution is necessary in using expressed shRNAs for gene therapy.

1.10.2 Protein based gene therapy:

Protein-based inhibitors: Similar to the RNA-based inhibitors of HIV, proteins can be directed to inhibit either cellular or viral targets. The majority of the protein inhibitors have been expressed from the viral vector LTRs, but in several instances they were produced from strong constitutive promoters inserted within the bodies of the viral vectors. The first protein used in an HIV gene therapy trial is a mutant form of the HIV Rev protein called M10. Rev M10 is believed to work by blocking the export of singly spliced and unspliced HIV RNA from the nucleus to the cytoplasm, thereby preventing packaging and subsequent transmission. This mutant protein is one of the most potent

inhibitors of HIV replication. Intracellular antibodies and intrakines have also proven to be very potent inhibitors of HIV replication. These proteins work by binding to viral or cellular target proteins, most often resulting in targeting of the proteins to the proteasome for degradation. Of all these approaches, thus far only the M10 dominant-negative protein has been tested in human clinical trials.

Transdominant Tat: Tat, the transactivator protein of HIV, is being extensively studied as a possible candidate target for anti-HIV therapy. Three models have been proposed to explain the mechanism of action of various transdominant Tat proteins that have been studied. One possible mode envisions the competition of the transdominant and wild type proteins for binding to TAR RNA. Second, as Tat is known to multimerize *in vitro*, certain transdominant Tat proteins may form mixed complexes with wild type Tat, trapping it in the cytoplasm and preventing its function. Third, as Tat acts in synergy with several cellular factors to bring about transactivation, some transdominant proteins might act by sequestering a critical cellular factor required for function - a phenomenon termed squelching (Caputo et al., 1996; Orsini and Debouck, 1996; Betti et al., 2001).

New entrant in the pool of protein-based agents is fusion inhibitors, which bind to HIV gp41 at the cell surface and block viral entry (Egelhofer et al., 2004; Perez et al., 2005). 'As with the other protein-based inhibitors, these entry-blocking proteins can be expressed constitutively from the backbone of retroviral or lentiviral vectors, making them suitable for use in gene therapy' (Rossi et al., 2007). A different protein-based approach uses zinc-finger nuclease (ZFN) fusion proteins (Mani et al., 2005). ZFN proteins can be engineered to bind with exquisite selectivity to specific sequence motifs in the genome, and the associated nuclease cleaves the targeted DNA. When these double-stranded breaks are repaired, high-frequency deletions and insertions are introduced at the site of cleavage. The CCR5 gene is a target for *ex vivo* gene therapy of HS cells. Disruption of the coding sequence of this gene will generate nonfunctional CCR5 mutants, rendering the cells resistant to CCR5-tropic HIV. The challenge with this approach is to transiently introduce the ZFN protein or a genetic transcription unit into primary hematopoietic cells for stable expression of the ZFN may cause genotoxicity. The goal is to have a single hit of mutagenesis and eliminate the nuclease from the cells after that hit. The efficiency of ZFN-mediated gene modification must be high to achieve

bi-allelic CCR5 gene knockout. Despite these challenges, this is a particularly exciting approach in that the *ex vivo*–modified cells should have a selective growth advantage in HIV-infected individuals. Furthermore, the recent approval of CCR5 inhibitor maraviroc for advanced HIV infection increases enthusiasm for strategies that target this co-receptor. That said, the emergence of dual-tropic or CXCR4-tropic virus would abrogate this advantage. In addition, potential genotoxicities of ZFNs, from chromosomal breakage, such as translocations, or effects of 'off-target' DNA cleavage, must also be determined (Rossi et al., 2007).

Single-chain antibodies: Single-chain Variable fragments (scFv) targeted against structural and regulatory proteins of HIV have been studied. Termed intrabodies, these molecules are genetically engineered antibody fragments in which the variable domain of the heavy chain is joined to the light chain through a peptide linker, preserving the affinity of the original antibody. Intrabodies act by sequestering viral proteins in an inappropriate sub-cellular compartment leading to blocking of HIV replication or maturation (Mhashilkar et al., 1995b; Marasco et al., 1999; Valvatne et al., 1996; Hust et al., 2007). Env, Gag, Tat and Rev have been targeted in various studies some of which have reached clinical trials (Mhashilkar et al., 1995b; Hust et al., 2007).

Both anti retroviral chemotherapy and gene therapeutic approaches against HIV come with in built deterrents for use in a wide populace especially in the light of the fact that most infections are reported from the third world countries. Chemotherapy still remains the treatment of choice, but with escalating costs, alternatives are the need of the hour. Gene therapy is not practical in a global setting, as it requires a massive mobilization of resources with the added disadvantage that the therapy has to be custom made – an unthinkable option considering the teeming millions already infected and an equal number on the threshold. Traditional and novel vaccination strategies offer a viable alternative in this scenario in that, a vaccine, prophylactic or therapeutic could have a global application whilst keeping the production cost low and a wider spectrum of affected individuals to cover.

1.11 HIV vaccines:

The search for an AIDS vaccine began with great optimism and high expectations. Early after the discovery of HIV as the cause of AIDS, researchers predicted that a preventative vaccine would follow close behind. It soon became clear that that task is a daunting one and despite a large concerted effort for almost 25 years, the problem has proven more difficult than anticipated and the progress has not matched the initial hopes. The search for an effective vaccine still continues.

HIV has been studied in great detail, producing insights into viral immunopathogenesis that initially provided encouragement for the rapid development of a vaccine. Despite the initial enthusiasm, several factors have deterred the development of a HIV vaccine. First, the extraordinary genetic diversity of the virus, became evident. Factors such as high mutation rate, RNA recombination and immune selection combined to produce viral strains with considerable genetic heterogeneity, making it necessary to develop a vaccine not just for a single virus, but for millions of variants that have evolved around the globe. Second, as the virus spread through the population, it began to develop mechanisms for evasion of host immune response. There has been no documented cause of long-term immunity to HIV infection based on a cellular or humoral immune response that eradicates the virus.

Historically speaking, the successful vaccines in the past have been developed by identifying examples of immunity to infection in nature and generating vaccines that elicit the most crucial aspects of the protective immune response. For instance, the classic 'Jenner's milkmaids' represented a protected population, an experiment of nature that provided a clear direction for the small pox vaccine. In the case of HIV/ AIDS, there are no such examples of protected population from nature, no immune correlates of protection and hence no well-charted path to an effective vaccine. It had taken several decades to develop effective vaccines for various viral infections, such as measles, mumps, rubella and chicken pox etc, it would take even longer in the case of HIV/AIDS. In the absence of a well-charted path of natural immunity, vaccine development for HIV must rely on rational vaccine design, including use of the latest advances in viral and host

molecular genetics, structural biology, immunology and modern vaccine production technologies.

The lack of immune correlates remains one of the most compelling challenges for the development of an AIDS vaccine. Although, no history of sterilizing immunity (where infected people have completely cleared the virus) has been documented, not all hope is lost, for there are a few exceptions. In fact, at one end of the spectrum of natural infection are some individuals, defined as long-term non-progressors (LTNPs), who are able to mount effective antiviral responses capable of controlling viral replication and progression to disease. These individuals remain clinically healthy for longer period of time with almost undetectable plasma viremia and with a minimal loss of $CD4^+$ T-cells, even in the absence of any ART. Second group of people, defined as multiply exposed individuals (MEUs), who have remained seronegative for years, despite repeated exposure to the virus, suggesting that resistance to infection may occur in the natural setting. Studying these individuals and understanding the mechanisms are of critical importance for the development of vaccines to HIV.

Progress in HIV vaccine research also suffers from the lack of well-suited animal models. Mice and rats are the most ideal animals for laboratory experiments, but they are not permissive to HIV replication and several attempts in the past to circumvent this resistance haven't been successful(Titti et al., 2007). The modified approaches currently being used are: a) Development of hu-SCID mouse model, b) establishment of HIV-2 infection through modifications of genetic background of mice and rats (Borkow, 2005; Reid et al., 2001), c) generation of pseudoviruses containing HIV-1 genes capable of replicating efficiently in conventional mice or rats (Chan et al., 2006; Potash et al., 2005).

The human transgenic (CD4/CCR5) rabbit should represent a better model as HIV-1 infection in rabbit recapitulates some aspects of HIV-1 infection in humans (Speck et al., 1998). Adaptation to a heterologous host has its own drawbacks. Although the small animal models aren't ideal for HIV/AIDS, they contribute to a better understanding of safety, immunogenicity and efficacy of vaccine approaches, as well as the effects of antiviral drugs.

Non-human primates prove to be an unusually good animal model for viral vaccine development, although with several limitations. Chimpanzees can be infected with HIV and some pathogenic effects were observable in vivo. However, the high cost, low number of available animals, international restrictions and ethical sensitivity discourage researchers from using them. African green monkeys and Sooty mangabeys, which are naturally susceptible to a simian equivalent of HIV (SIV or Simian Immunodeficiency Virus), have led to their extensive use as animal models of HIV. To overcome the issue of low sequence homology, chimeric simian/human immunodeficiency viruses (SHIV) have been generated by inserting HIV genes into the genome of SIV. The relevance of SIV and SHIV models is under considerable debate because cell-free viruses are used in these studies, also at a much higher dosage than estimated to be transmitted in the course of natural infection with HIV, possibly hampering recognition of potentially protective vaccines (Gray et al., 2001; McDermott et al., 2004). Also these primates seem to be largely resistant to the CCR5-tropic primary isolates responsible for infection of most of the humans globally, suggesting species differences in the host response to virus. The characteristics of molecular clones and laboratory-adapted viruses also differ from naturally infectious virus. Thus, despite the attractive features of the primate model, it is evident that human clinical studies will be needed for the development of effective vaccines. Such trials would require the production of clinical-grade vaccines and also warrant safety and toxicity studies, which pose further challenges in the development of HIV vaccines.

1.11.1 Requirements of an effective HIV vaccine:

In the absence of known immune correlates of protection, it would be most prudent to develop a vaccine that stimulates multiple components of the immune system. The logical conclusion from the extensive literature on immune protection in lentiviral infection is that a combination of long-lived memory T-cells, both $CD8^+$ CTLs and $CD4^+$ memory helper T-cells, will probably be needed for a highly effective AIDS vaccine. At the same time, a strategy to induce broadly neutralizing antibodies will be required for highly effective, long-lasting immunity. An effective vaccine for HIV should entail most, if not all of the mentioned attributes.

1.11.2 Cellular immune responses:

Virus-specific T lymphocyte responses are believed to have a critical role in controlling HIV replication and hence it has become a priority for HIV vaccine candidates. Early studies showed that virus-specific $CD8^+$ T-cell responses emerge during acute infection coincident with the initial control of primary viremia (Borrow et al., 1994a). Potent cellular immune responses have also been reported in long-term non-progressors (Gea-Banacloche et al., 2000), and specific HLA alleles and the breadth of Gag-specific T lymphocyte responses have been correlated with control of viral replication in HIV-1 infected individuals (Pereyra et al., 2008). Concordant with these observations, experimental depletion of $CD8^+$ T-cells have been shown to abrogate immune control of SIV replication (Schmitz et al., 1999).

Despite the importance of CTLs in containing the viral replication, concerns do remain as to whether cell-mediated immunity would alone be necessary and sufficient for an effective vaccine. A limitation of CTL responses is the propensity of the virus to accumulate mutations in the epitopes and to evade cellular immune control (Allen et al., 2000; Cao et al., 2003; Barouch et al., 2002; Barouch et al., 2005a). Hence, it becomes critical to maximize immunologic coverage of HIV-1 diversity and in parallel minimize the potential for viral escape.

HIV also has evolved mechanisms to disrupt or downregulate the MHC proteins that act as windows for the effector T-cells to monitor the infection of the cells. One of these mechanisms involve the HIV gene products Tat and Nef, selectively downregulating HLA-A and HLA-B but do not significantly affect HLA-C or HLA-E (Cohen et al., 1999b). The selective downregulation of Class I MHC proteins by HIV-1 protects HIV-infected cells from NK cells.

Elicitation of strong cellular immune responses against multiple regions of HIV (Borrow et al., 1994a) especially the regulatory genes like Tat, Rev and Nef is desirable of a vaccine. The responses should be long-lasting with memory establishment ideally for the period of perceivable risk in an individual's lifetime (in most cases, the period for which an individual remains sexually active).

Sufficient T-cell help should be generated to augment the responses. HIV-1 preferentially infects HIV-1 specific $CD4^+$ T-cells and rapidly depletes most memory $CD4^+$ T-cells in gut-associated lymphoid tissue within the first 4-10 days of infection. This sets the stage for progressive immunodeficiency as well as for chronic immune activation. Given the time required for the vaccine-induced $CD8^+$ T-cell responses to expand after infection, it may be difficult to prevent these early immuno-pathologic events completely.

1.11.3 Humoral immune responses:

As previously stated, virus-specific neutralizing antibody titers represent key immune correlates of protection for most licensed viral vaccines, and thus early studies focused on developing HIV-1 Env subunit immunogens. Advances in our understanding of Env structure and function have begun to elucidate why generating broadly reactive neutralizing antibodies to HIV-1 by vaccination may be so difficult. The HIV-1 Env glycoprotein is a trimer on the virion surface with extensive N-linked glycosylation that effectively shields many conserved epitopes from antibody recognition (Quinones-Kochs et al., 2002; Bolmstedt et al., 1996). Highly immunogenic variable loops also elicit type-specific antibodies that may redirect humoral responses away from conserved regions. In addition, key conserved regions, such as the binding site of the chemokine co-receptor, are only formed after Env binds its cellular receptor CD4 and undergoes an extensive conformational change (Humbert and Dietrich, 2006). The development of mutations in N-linked glycans has also been shown to lead to rapid evasion of host neutralizing antibody responses.

The development of immunogens that induce broadly reactive neutralizing antibodies is perhaps the most important priority for the HIV-1 vaccine field (Srivastava et al., 2004; Srivastava et al., 2005). Proof-of-concept passive transfer studies in non-human primates have shown that administration of high doses of broadly reactive monoclonal antibodies can afford sterilizing protection from infection, thus demonstrating the potential of virus-specific humoral immunity (Parren et al., 2001; Nishimura et al., 2003; Mascola et al., 2000). However, it has not been possible to induce such broadly reactive neutralizing antibodies by vaccination so far. Although there has

been substantial progress in our understanding of Env structure and function, there are currently no vaccine candidates that are aimed at eliciting broadly reactive Env specific neutralizing antibodies in clinical trials.

Generation of neutralizing antibody responses against env would theoretically impede the virus at the portals of entry (Lewis et al., 1993). Antibody responses against circulating pathogenic proteins, especially Tat, would help prevent activation of latent reservoirs and other neurotoxic effects associated with Tat (Re et al., 2001a).

In summary, although the immune correlates of protection remain unknown, there is evidence that cell-mediated immunity controls viral replication. At the same time, evidence from other successful vaccine approaches has indicated that long-term B-cell memory, through the antibody response, is crucial in immune protection. The challenge is to develop a vaccine that can elicit a broadly reactive T-cell response that is long lasting, and to identify antigens that will elicit the 'correct' (broadly neutralizing) antibody response.

1.11.4 Current HIV-1 vaccine strategies:

1.11.4a Traditional strategies:

Vaccine strategies for HIV-1 could be divided into traditional and novel approaches. Traditional vaccine technologies include live attenuated viruses, whole killed viruses and protein subunits.

1.11.4b Live attenuated viruses: Many viruses can be attenuated through in vitro passage, a process that generates several spontaneous mutations, a consequence of which the viruses lose their pathogenic potential but retain infectivity. A successful application of this strategy led to the development of potential vaccines against small pox, measles and polio. Preliminary studies using the SIV/macaque model demonstrated that prior infection with such attenuated viruses prevents subsequent infection with pathogenic wild-type viruses (Daniel et al., 1992). While these findings raised hopes in the HIV vaccine community, extending this work to the SIV model led to disappointing results when new-born or adult monkeys infected with attenuated SIV strains developed AIDS after prolonged incubation periods (Baba et al., 1995). Similar observations were made in a clinical cohort of recipients who received blood contaminated with an HIV strain

containing large deletions in Nef who eventually progressed to AIDS although with a significant delay in disease progression (Learmont et al., 1999). Given the high prospects of attenuated strains reverting to virulent forms, the vaccine strategy of attenuated HIV never gained the required support from the research community (Andino et al., 1994).

1.11.4c Whole killed viruses: This modality has been successful in preventing infections of influenza and Polio viruses in human beings. However, the results from the SIV/macaque model have been disappointing. The protection provided by the whole killed vaccines of HIV/SIV is restricted in the breadth of immune responses elicited and by the enormous genetic diversity of the viral isolates. The antibody response generated by the inactivated viral vaccines failed to neutralize diverse viral isolates. In the absence of de novo protein synthesis, the killed vaccines also failed to induce cell-mediated immune responses. Needless to say inactivated HIV vaccines evaluated in a limited number of early human clinical trials met with only failure and disappointing results (Levine et al., 1996).

1.11.4d Recombinant protein or protein subunit vaccines: Based on the impressive successes of protein vaccines in eliciting neutralizing antibodies against potential pathogens including the Hepatitis B virus, the HIV vaccine community was quite optimistic of developing a similar vaccine quite rapidly. The idea was to adopt a similar strategy that worked efficiently against other viral infections. A good deal of effort had been invested into the recombinant envelope glycoprotein gp120 vaccine. Immunized animals demonstrated modest levels of protection against homologous, but not heterologous, viral challenge (Berman et al., 1990). Inspite of this set back, early-phase human clinical trials have been carried out. VaxGen, a San Francisco-based company, initiated the first phase III clinical trial in 1998 using its gp120 subunit vaccine known as AIDS VAX. Though the vaccine was found safe and well tolerated, it didn't prove effective in human trials in the USA and Europe. The percentage of individuals who became infected was comparable between the vaccinated and placebo groups, suggesting that the vaccine was not protective (http://iavi.org/press/2003/n20030224.htm).

Furthermore, data from two recently concluded efficacy trials of the HIV-1 vaccines in the USA and Thailand showed no evidence of protection against the viral infection (Cohen, 2003).

1.11.5 Novel strategies:

The traditional vaccine strategies not rising to the occasion, researchers began to explore a plethora of novel vaccine designs.

1.11.5a Synthetic Peptide vaccines: Synthetic peptides comprising of small epitopes of HIV proteins offer the advantage of targeting specific conserved epitopes of the virus. Several reports have shown that the immune system does mount a response to short peptides of a protein antigen when presented appropriately to the immune system. The limitation of this approach is that peptides administered without an adjuvant are unlikely to be immunogenic. The renewed interest in peptides vaccines is due to the recent advances in the delivery, stability and design of peptides. Peptide vaccines also offer a great flexibility of including multiple epitopes from several viral subtypes or even multiple pathogens so as to increase the breadth of the vaccine-induced response (Brander et al., 1996).

1.11.5b Virus like particles (VLPs) and pseudovirions: These are replication incompetent viruses produced in mammalian cells that contain viral proteins required for viral assembly, but not the viral genome, thus making them non-infectious. VLPs are stable and more immunogenic than unassembled purified antigens. Their particulate nature provides the advantage of presenting multiple epitopes to the immune system. Hepatitis B virus surface and core antigens have been engineered and evaluated in preclinical studies to present HIV antigens. This mode elicits potent humoral immune responses. The fusion of HIV-1 p17 and p24 gag proteins to C-terminus of the p1 protein of yeast *(Saccharomyces cerevisiae)* resulted in assembling of 50 nm virus particles (Aldovini and Young, 1990) and the testing of these constructs is expected to begin soon.

1.11.5c Live vector vaccines: Genes of HIV can be inserted by molecular approaches into live, replication-competent microorganisms (vectors) e.g., into a bacteria or virus that are harmless to humans. Infection with these recombinant microorganisms will elicit

responses to the vector and to the product of the HIV gene encoded by that vector, these include live attenuated bacterial vectors such as *Bacille Calmette-Guerin* (BCG) and *Salmonella.* These recombinant vectors are safe and can establish infection via a mucosal route and can elicit strong mucosal immune responses.

New viral vectors include the well studied vaccine constructs of the pox family of viruses. Canary pox vectors (e.g., ALVAC) and Modified Vaccinia Ankara (MVA) are infectious and immunogenic but highly attenuated viruses. MVA vaccines in non-human primates elicit potent CTL responses. NYVAC, another attenuated vaccinia strain, elicited immune responses comparable to that of MVA. Canary pox vaccines are presently being evaluated in human clinical trials. Other live vectors expressing HIV antigens that are currently being evaluated include adenoviruses, adeno-associated viruses, polio virus, influenza virus, Semliki Forest virus, Venezuelan equine encephalitis virus and parvovirus.

1.11.6 The STEP study:

Studies with recombinant adenovirus vectors showed great promise. Especially Adenovirus serotype 5 (Shiver and Emini, 2004) proved to be highly immunogenic as a vector for HIV gene products in small laboratory animals and non-human primates (Shiver et al., 2002). A trivalent mixture of rAD5 vectors expressing HIV-1 clade B Gag, Pol and Nef was formulated by Merck. This HIV-1 candidate vaccine was well tolerated and was immunogenic in most of the volunteers in the phase I clinical trials (Priddy et al., 2008). The Merck Ad5 virus candidate efficacy testing was initiated by Merck and the National Institutes of Health to determine HIV-1 specific cellular immunity and to evaluate if the immune responses would prevent HIV-1 infection or reduce viral load. This 'STEP' trial or NIAID's HIV Vaccine Initiative Trials Network (HVTN 502), was a 3000-subject study in the Americas, the Caribbean and Australia. A parallel study termed 'Phambilis' enrolling 3000-subject in South Africa was also planned.

The HVTN 502 vaccine neither prevented the viral infection nor reduced the viral load in the vaccinated individuals. There were a higher number of viral infections in the vaccine recipients compared with those given placebo, who had pre-existing Ad5 immunity from prior exposure to the virus. The apparent increased risk of HIV-1

acquisition in vaccines with pre-existing Ad5-specific neutralizing antibodies was unexpected. The biological basis for this observation remains unclear.

On 18th September 2007, HVTN 502 was terminated at the first planned interim analysis by the Data and Safety Monitoring Board (DSMB). The trial HVTN 503 was also terminated.

Based on these results, the NIAID announced that it will not move forward with the PAVE-100 HIV vaccine trial. The NIH's Vaccine Research Centre's (VRC) vaccine candidate PAVE-100 is similar to the Merck vaccine in that both stimulate $CD4^+$ T-cells against HIV and both contain the cold virus adenovirus 5.

1.12 DNA vaccines:

DNA vaccines emerged as a novel mode of vaccination in the early 1990s. Since the discovery over a decade and a half ago, that genetically engineered DNA can be delivered in vaccine form and elicit an immune response, there has been much progress in understanding the basic biology of this platform. The demonstration that plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models has created considerable excitement.

A DNA vaccine is a 'naked DNA' construct encoding an antigenic gene/ epitope(s) in a non-replicating plasmid format which when injected into a host elicits immune responses against the encoded cassette.

The historical basis for DNA vaccines rests on the observation that direct in vitro and in vivo gene transfer of recombinant DNA by a variety of techniques resulted in the expression of the protein. In the seminal study by Wolff et al., vaccination with plasmid DNA encoding reporter genes could induce protein expression within muscle cells (Wolff et al., 1990). This observation was taken further ahead when the delivery of DNA into the skin of mice using a 'gene gun', in an attempt to deliver human growth hormone as a gene therapy was described (Tang et al., 1992), thereby heralding the era of the 'DNA vaccine'. More studies began to appear reporting the use of DNA vectors to drive both humoral and cellular immune responses against influenza virus antigens in mice, (Ulmer et al., 1993) HIV antigens or tumor antigens. This new approach also afforded the flexibility to include protective epitopes whilst excluding the inhibitory or cross-reactive epitopes in a format that could be manipulated in many unexpected ways.

1.12.1 Mechanism of action:

The mechanisms by which DNA vaccines produce antigen-specific immunity in vivo are under intense investigation, with an idealized model presented in Figure-3. The gene sequence, usually codon-optimized is delivered to the skin (intradermally), subcutaneum or muscle (intramuscularly). The plasmid enters the nucleus of transfected local cells (such as myocytes or keratinocytes); including resident antigen presenting cells (APCs).The expression of plasmid-encoded genes takes place and results in the production of protein (foreign antigen). The antigens are presented in the context of both

MHC Class I and Class II molecules of the APCs. The antigen-loaded APCs, move to the draining lymph nodes, where they present antigen-peptide-MHC complexes, along with co-stimulatory molecules to the naïve T-cells. This interaction leads to initiation of an immune response, viz., activation and expansion of T-cells or B cells. Thus, both cellular and humoral immune responses are elicited by the DNA vaccines (Figure-3).

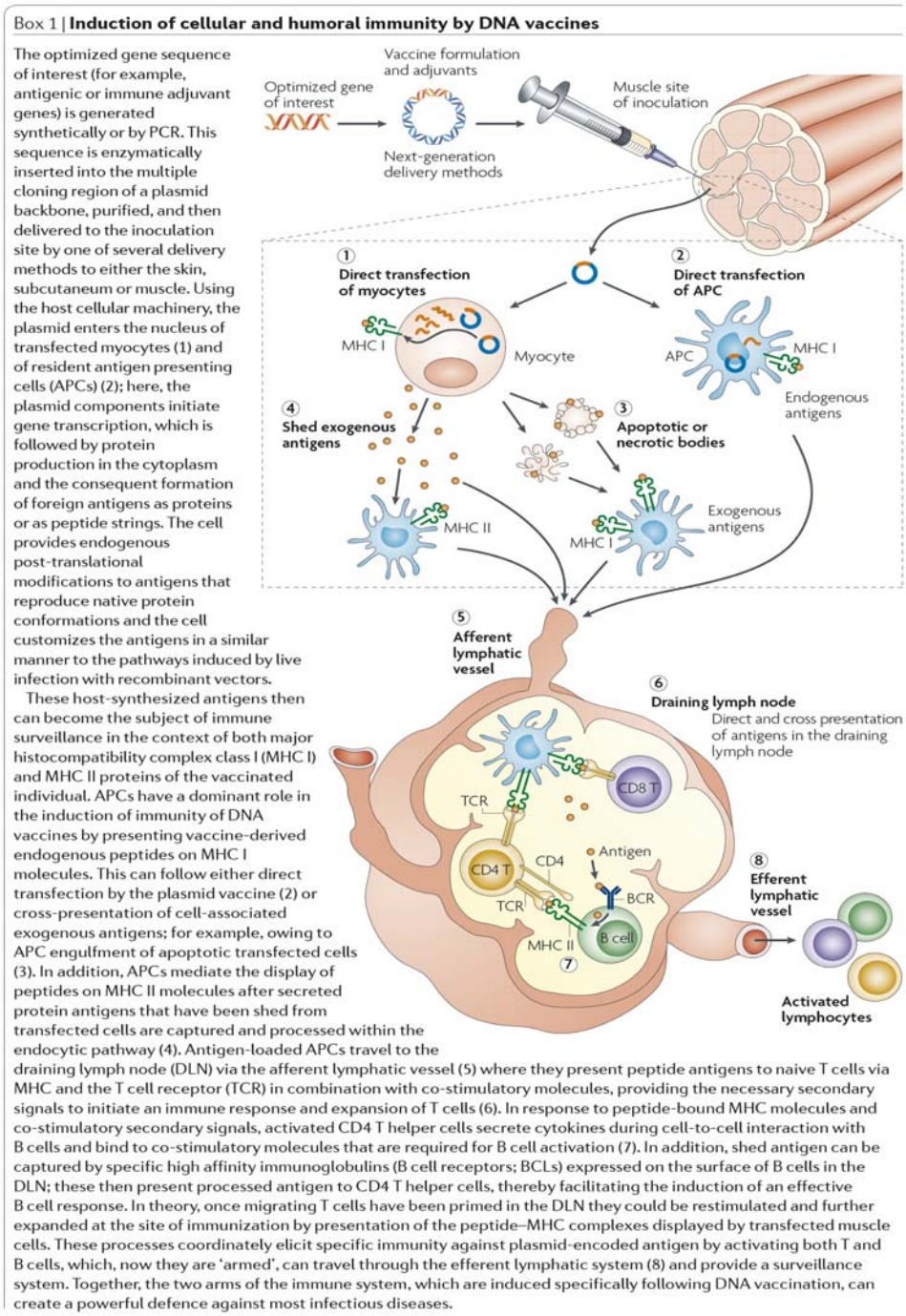


Figure-3: Induction of cellular and humoral immunity by DNA vaccine. Figure adapted from (Kutzler and Weiner, 2008)

1.12.2 Advantages of DNA vaccines:

- DNA vaccines, like viruses, encode antigens intra-cellularly. The antigens are presented through the MHC Class I pathway, inducing $CD8^+$ CTL response which is critical for containing and controlling viral proliferation. This is one of the strongest merits of the DNA vaccines.
- Induction of antigen-specific T and B cell responses similar to those elicited by live attenuated platforms. Since DNA vaccines mediate synthesis of the encoded antigens by the host protein synthesis machinery, post-translation modifications are expected to be natural and authentic, a critical aspect for inducing relevant immune response.
- Synthetic and PCR methods allow with great ease engineering and design modifications like optimization of codon, grafting HTL (Helper T Lymphocyte) epitopes etc.
- Cross-priming of the encoded antigens is possible by DNA vaccines when professional APCs take up antigen or peptides from somatic cells that have been transfected with DNA, thereby augmenting the breadth of immune response (Corr et al., 1999).
- Rapid production, formulation, reproducible large-scale production and isolation in a relatively cost effective manner.
- Safety is a big plus point for DNA vaccines. No significant adverse events in any of the clinical trial have been noticed. Many thousands of individuals have been vaccinated to date.
- DNA vaccines are more temperature-stable than conventional vaccines and have a long shelf life and hence can be stored and transported with relative ease and it also eliminates the need for a ‘cold chain’. This is of importance when the vaccines are to be transported to remote corners of the world.
- DNA vaccines also bypass concerns that adventitial agents might be transferred from tissue-culture lines to the vaccinated individual.

1.12.3 Potential Concerns:

There are three main concerns with regard to the safety of DNA vaccines namely, potential to integrate into cellular DNA, the development of autoimmunity and the possibility of antibiotic resistance.

- DNA vaccines that are currently being tested do not show relevant levels of integration into host cellular DNA (Kurth, 1995; Sheets et al., 2006). The concern is that an integrated vaccine might cause insertional mutagenesis through the activation of oncogenes or the inactivation of tumor suppressor genes. However, none of these concerns have been witnessed in the preclinical or clinical evaluation of DNA products.
- With regards to the development of autoimmunity induced by DNA vaccination, preclinical studies in mice, nonhuman primates and early studies in humans did not detect increases in anti-nuclear or anti-DNA antibodies. To date, there has been no evidence of DNA vaccine –associated autoimmunity (MacGregor et al., 1998; Klinman et al., 2000).
- Part of production process of plasmids involves selection of bacterial cells carrying the plasmid. This selection is accomplished by culturing the cells in the presence of an antibiotic to which resistance is conferred by a gene in the plasmid. There is a concern that these antibiotic resistances might be introduced to the participants. This is unlikely because the antibiotic resistance genes are driven by a bacterial origin of replication and therefore expressed only in bacteria and not host cells. Also, these antibiotics are not used to treat human infections.

The FDA have developed specific advice on safety testing of DNA vaccines <http://www.fda.gov/CbER/gdlns/plasdnavac.pdf>) as has the European Union (Robertson and Cichutek, 2000).

1.12.4 DNA vaccines against HIV:

Immunization with DNA vaccine expressing HIV-1 gp 160 and rev, in HIV-infected chimpanzees led to enhanced virus specific immune responses (Novembre et al., 1997). These early studies led to testing DNA vaccines in humans. The first human trial of DNA vaccine was initiated almost 15 years ago. The study was an open-label dose-

escalating trial of DNA vaccine to demonstrate the safety and tolerability of DNA vaccine and to explore its immunogenicity in humans. The study used HIV-1_{MN} env gp160 and rev genes (MacGregor et al., 1998)**.** The vaccine was well-tolerated by the asymptomatic HIV-infected, ART-naïve individuals, but there was no change in $CD4^+$ T-cell count or plasma viral load. However, a marginal increase in anti-gp120 $CD8^+$ activity was reported in a few individuals.

The study was followed by up a double-blind placebo controlled dose-ranging clinical study (MacGregor et al., 2005) on asymptomatic HIV-1 infected patients on HAART, with a combination of env/rev DNA vaccine and a similar one expressing HIV-1MN gag/pol genes. There was no change noticed in the T-cell counts with vaccination in this study, while it reported a significant control of viral blips, in vaccinated individuals, compared to placebo group.

Several studies have now been documented the use of DNA vaccines against HIV. Virtually every gene of HIV has been tested either individually or as a part of DNA vaccine or along with other formats of vaccines. Numerous studies have now documented that DNA vaccine platform is safe and well-tolerated as no adverse effects have been reported as yet.

The Th profile of the immune response induced by DNA vaccine is influenced by multiple factors, including the mode of the route of immunization and the nature of the encoded antigen. Cellular and humoral responses are generally elicited by using intramuscular, intradermal or subcutaneous routes. Eliciting mucosal immune response involves priming the host through intranasal, intrarectal or intravaginal routes. The induction of the Th1 type of responses after intradermal and intramuscular DNA vaccination has been attributed to the presence of immunostimulatory sequences containing CpG motifs in the plasmid DNA. Gene gun mediated delivery, in which the DNA coated onto gold beads are propelled into the epidermis, seems to bias the immune response towards a Th2 profile and overrides the Th1 inducing property of the CpG motifs.

DNA vaccine field has been given a boost through the so-called “Prime-boost strategies” which involve priming with DNA vaccines and boosting by administration of recombinant protein or attenuated recombinant viral vectors expressing similar antigens.

The commonly used viruses to construct recombinant vectors (live and infectious) include adenovirus (AdV), adeno-associated viruses (AAV) and the pox viruses (Canary pox, Fowl pox (FPV), vaccinia and Modified vaccinia Ankara (MVA), etc. These approaches have led to great increase in neutralizing antibodies and have also boosted DNA primed CTL responses in preclinical animal models and in some cases, afforded protection against infectious challenge.

1.12.5 DNA vaccine platform: room for improvement:

The principal issue regarding the future of DNA vaccines concerns improving their immunogenicity in larger animals and in humans. The DNA vaccine platform has driven significantly weaker immune responses in non-human primates and in humans compared with mice. It seems to be less immunogenic compared with recombinant viral vectors such as adenoviral vectors or recombinant protein for induction of antibody responses.

***1.12.5a Optimization of transcriptional elements*:**

- For most vaccine plasmids, the human CMV promoter is a common choice because it promotes high-level constitutive expression in a wide range of mammalian cells. Expression from the CMV promoter, like from other viral regulatory elements, nevertheless, is downregulated in several physiological contexts either through the interferon-mediated (Gribaudo et al., 1993) or DNA methylation (Prosch et al., 1996) pathways. Gene expression form the CMV promoter may not only be transient (Yew et al., 1997) but also be irregular often resulting in the failure of establishing stable cell lines (Tokushige et al., 1997; Teschendorf et al., 2002)
- 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 (Ferrari et al., 2002).
- The use of host tissue-specific promoters avoids constitutive expression of antigens in inappropriate tissues. For example, the use of the promoter of the muscle creatine kinase gene leads to the induction of antibody and T-cell

responses, although levels were at least tenfold lower than plasmids that contain the CMV promoter (Cazeaux et al., 2002; Bojak et al., 2002). Data from the above mentioned studies suggest that the use of host-cell promoters limits expression and, ultimately, immunogenicity, which might be an advantage for gene delivery. Moreover, promoters with significant homology to host-cell sequences might need to be optimized for improved human clinical outcome.

- The promoter element of the Elongation factor-1α, (EF-1α) gene has been shown to perform as efficiently as the CMV promoter, or in a few cases even better (Tokushige et al., 1997; Song et al., 1998; Teschendorf et al., 2002). Like in the case of the CMV promoter, gene expression form the EF-1α promoter is ubiquitous and not restricted to any cell lineage (Kim et al., 1990). In parallel studies, EF-1α promoter was demonstrated to be far superior to the CMV promoter in establishing stable cell lines (Tokushige et al., 1997). Importantly, unlike the CMV promoter, EF-1α promoter was not subjected to gene silencing through the IFN-mediated and CpG methylation pathways (Teschendorf et al., 2002).
- Many DNA vaccines use the bovine growth hormone terminator sequence or endogenous terminators that are downstream from the ORF of the gene of interest to ensure proper transcriptional termination (Cazeaux et al., 2002; Montgomery et al., 1993; Manthorpe et al., 2005). It remains to be seen whether modifications to the polyadenylation and termination signals influence gene expression.
- Both enhancer elements and transcriptional transactivators can enhance promoter activity when placed either upstream or downstream of the ORF. The regulatory R region from the 5′ long terminal repeat (LTR) of human T-cell leukemia virus type 1 acts as a transcriptional and post-transcriptional enhancer; the resulting CMV–R DNA vaccines elicit substantially higher specific cellular immune responses to HIV-1 compared with the analogous parental DNA vaccines in both mice and cynomolgus monkeys (Barouch et al., 2005b).
- The use of high-efficiency origins of bacterial replication relevant for the bacterial strains used for production can also markedly improve the quantity of plasmid product.

1.12.5b Enhancing Protein expression:

- Optimization of the initiation start site for protein synthesis is another aspect for consideration, because endogenous sites of viruses and bacteria might not be optimal for expression in mammalian cells. Modification of the Kozak consensus sequence (Kozak, 1997) is a focus for this purpose. Along the same vein, ensuring correct termination of the protein is also important.
- One of the most effective ways to increase protein production is through the use of codon optimization. This procedure results in increased protein production, leading to enhanced T-cell (zur et al., 2000; Nagata et al., 1999; Uchijima et al., 1998; Liu et al., 2002; Gao et al., 2003; Ramakrishna et al., 2004b; Garmory et al., 2003; Ramakrishna et al., 2004a; Steinberg et al., 2005) and antibody (Deml et al., 2001; Liu et al., 2004; Smith et al., 2004) responses.
- RNA optimization can also lead to more efficient translation through several important modifications, including removal of instability elements that lower expression.
- High antigenic expression rates and prolonged mRNA stability are not only crucial for heterologous mammalian expression, but are important for the generation of effective DNA vaccines (Yan et al., 2007).
- It has been well documented that intron-containing and intron-less versions of identical genes have dramatically different expression profiles (Chapman et al., 1991; Duncker et al., 1997; Kim et al., 2002; Le Hir et al., 2003; Nasim et al., 2002; Nott et al., 2003). Introns and its act of removal by the spliceosome affects the gene expression in a variety of ways (Black, 2003) by having its effect on the rate of transcription (Furger et al., 2002), polyadenylation (Nott et al., 2003; Zhao et al., 1999),mRNA transport, translational efficiency (Matsumoto et al., 1998) and rate of mRNA decay (Nott et al., 2003).
- Enhanced expression of plasmid antigens is also observed using the efficient leader from the IgE gene (Wang et al., 2006).
- 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 (Barouch et al., 2004; Garmory et al.,

2003). In the cancer model, for example, tumor-specific antigen epitopes have been linked to fragments of tetanus toxoid to improve helper T-cell responses (Stevenson FK et al., 2004).

- It has been seen that the potency of this strategy will be improved by new approaches to link epitopes, by the inclusion of helper T-cell sequences, and by new epitope selection technologies — particularly when coupled with new delivery strategies.

1.12.5c Augmenting immunogenicity of DNA vaccines:

- 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 (Ferrari et al., 2002; Dean et al., 2005). Liposome vehicles can protect DNA from degradation by serum proteins during transfer of DNA across membranes and after the release of genetic material following fusion with endosomes (Gao and Huang, 1995). Liposomes can be prepared with significant structural versatility, including vesicle surface charge (both cationic and anionic liposomes can be made), size, lipid content and co-delivery with other adjuvants, they offer considerable flexibility towards vaccine optimization and have been shown to induce cellular and humoral immunity (Yamano et al., 2006; Wang et al., 2007).

- Co-injection of plasmids encoding cytokines, chemokines or co-stimulatory molecules can have a substantial effect on the immune response to plasmid-encoded antigen; For example, in non-human primates interleukin-12 is a potent DNA vaccine adjuvant for cellular immunity. Many other classes of immune modulatory molecule exist including target death receptors, growth factors, adhesion molecules, cytokines and chemokines as well as Toll-receptor ligands, synthetic oligodeoxynucleotides containing unmethylated CpG motifs have all been explored to various extents.

1.12.5d Next-generation delivery strategies:

- The most common route of immunization used in DNA vaccine studies is the intramuscular route. However, several studies have demonstrated the importance of direct transfection of APCs (Porgador et al., 1998; Gaffal et al., 2007). More recent delivery methods, including the transcutaneous microneedle, the use of low-frequency ultrasound as a potent physical adjuvant for successful transcutaneous immunization has been developed (Tezel et al., 2005).
  - Another topical application method includes 'painting' DNA vaccine with cytokine-expression plasmids onto the skin of mice after elimination of the keratinocyte layers; this method induced marked immune responses, both cellular and humoral, against the HIV-1 env protein (Liu et al., 2001).
  - New improvements in particle-mediated epidermal delivery (PMED) technology and vector design, including co-formulation of PMED DNA vaccines with adjuvants, are in progress to further enhance the potency of particle mediated DNA vaccines (Fuller et al., 2006).
  - Electroporation technique is among the most impressive preclinical delivery strategy that has been in use for almost two decades, as a method to improve delivery of chemotherapy drugs to kill specific tumor cells (Giardino et al., 2006; Sersa et al., 2008). It has also been studied in dogs, pigs, cattle and non-human primates to deliver a variety of therapeutic genes (Hirao et al., 2008). The greatest potential of this technique is, it improves *in vivo* expression levels several folds over plasmid injection alone (Babiuk et al., 2006; Tsang et al., 2007).

Summary:

A lot of interest has been engendered since the appearance of DNA vaccines on the scene of vaccine community. Many studies in animal models have been very promising but these haven't been replicated in humans. Many trials are ongoing and will soon provide the experts with the needed insights regarding the safety and the immune responses required for protection in humans. The development of novel DNA delivery platforms would eventually move the DNA vaccines into drugs of choice for the treatment of complex disorders. The hope and scope of this mode of vaccine can be

understood by the fact that, this vaccine platform represents almost one quarter of all gene therapy vector systems under clinical evaluation (Kutzler and Weiner, 2008). It is clear that the advancement of the DNA platform will continue to be an exciting and highly productive adventure and may represent an important component of the next generation of vaccines that are efficient, and more importantly, economically accessible to majority of the people.

1.13 Tat – a multifaceted viral protein:

Tat, the transactivator of HIV gene expression is an essential viral regulatory factor. Tat is expressed early in the viral life cycle and is functionally important for the infectivity and pathogenicity of the virus (Huang et al., 1994; Huang et al., 1998a). HIV-1 Tat is a 14-kDa protein encoded by two exons. Exon I encodes for amino acids 1-72 and the exon 2 for 73-86 or 73-101 amino acids depending on the viral strain. Analysis of HIV-1 tat protein in several clinical isolates showed that more than 90% of isolates maintain the 101 amino acids Tat form (Meyers et al., 1996).

Tat localizes primarily in the nucleolus/ nucleus as seen by immunolocalization studies (Cao et al., 2006; Efthymiadis et al., 1998; Modesti et al., 1991; Ruben et al., 1989; Siomi et al., 1990). HIV-1 Tat increases transcriptional initiation and stabilizes elongation (Laspia et al., 1989). It is also believed to act as an anti-terminator permitting elongation of transcripts (Kao et al., 1987; Peterlin and Price, 2006).

The protein acts by binding to the nascent TAR RNA element (-18 and +83 relative to the transcription start site) and activates transcription from the viral LTR. Tat recruits chromatin-remodeling complexes, including SWI/SNF, p300/CREB-binding protein (CBP), p300/CBP-associated factor (PCAF) etc. Tat binds to the positive transcription elongation factor b (P-TEFb), composed of Cyclin T1 (CycT1) and cyclin-dependent kinase 9 (CDK9). This complex binds to the transactivation response RNA (TAR). Tat recruits the TATA-binding protein (TBP), TFIIB and P-TEFB to the promoter to form an active preinitiation complex. CDK9 hyperphosphorylates the C-terminal domain (CTD) of the RNA polymerase II (RNAPII) and the modified polymerase then clears the promoter and transcribes the viral genome.

1.13.1 Domain architecture of Tat:

According to the nature of the amino acids, Tat can be divided into six domains (Kuppuswamy et al., 1989; Jeang et al., 1999; Campbell et al., 2004) with various functional and pathogenic characteristics (Figure-1).

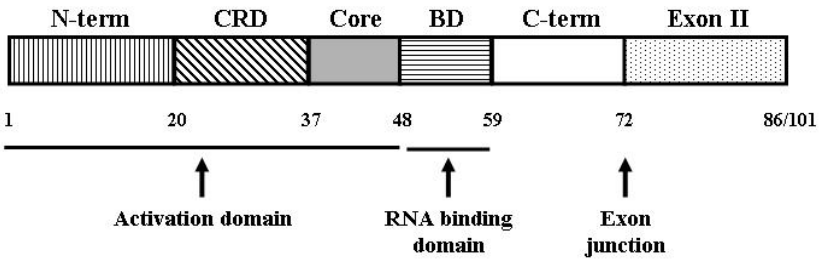


Figure-4: Domain structure of the Tat protein.

The first acidic N-terminal domain (aa 1-20) contains several acidic amino acids and is predicted to form an α-helix structure sandwiched between the glutamine-rich and core regions (Bayer et al., 1995). This domain has been shown to bind to the T-cell activation marker CD26 and inhibit its dipeptidyl peptidase IV activity (Wrenger et al., 1997), which is necessary for the regulation of immune responses (Reinhold et al., 1999; Kahne et al., 1999). Thus, the amino terminus may be responsible for the reported Tat mediated inhibition of antigen- and mitogen-induced proliferation of PBMC and T-cell clones (Chirmule et al., 1995; Zagury et al., 1998a) as well as for the detected impairment of T-cell functions in HIV-infected individuals (Mhashilkar et al., 1995a). Also, the amino terminus enhances viral reverse transcription (Ulich et al., 1999). Mutations in this domain do not modify transactivation to a large extent. The N-terminal portion of Tat binds cell surface CD26 with high affinity and is believed to be responsible for CD26-mediated immunosuppressive activity (Wrenger et al., 1997).

Domain II is termed the Cysteine-rich domain (aa 21-37) contains highly conserved cysteine molecules, most of which are essential for viral replication (Sadaie et al., 1990). This region is responsible for the intramolecular disulphide bond formation (Koken et al., 1994) and induces HIV replication. Tat binds to cell surface receptors on monocytes and induces chemotaxis (Albini et al., 1998b; Albini et al., 1998a). Tat protein from majority of the subtypes have the CC chemokine motif at positions 30, 31, with an exception of subtype C, where it is a CS motif. This mutation has lead to C-Tat being a defective chemokine (Ranga et al., 2004). The CRD binds divalent cations Zn and Cd.

Precise structural configuration of the CRD, a Zinc-finger motif, is critical for immobilization of Zinc ions that is required for Tat transactivation (Frankel et al., 1988; Huang and Wang, 1996). Evidence suggest that cellular activation by Tat involves the CRD and Tat 21-40 aa is able to efficiently induce both NF-κB mediated HIV replication and TAR dependent transactivation of HIV-LTR. This domain also possesses potent angiogenic activity and plays a significant role in HIV pathogenesis (Boykins et al., 1999).

The core domain (aa 38-48),a conserved and rigid α-helical structure shown to enhance Tat-TAR interaction (Bayer et al., 1995), is essential for viral transactivation. A single change at K41 abolishes Tat activity. The core region along with the CRD is postulated to confer chemotactic activity on monocytes (Albini et al., 1998a). Tat has shown to be a potent chemoattractant and promotes migration of several cell types including monocytes, endothelial cells and dendritic cells (DCs), thereby favoring the spread of HIV-1 infection (Izmailova E et al., 2003). A highly conserved dicysteine motif of Tat is critical for the monocyte-chemotactic property of the viral protein (Albini et al., 1998a). We have previously identified a natural variation at position 31 of subtype C Tat and demonstrated loss of monocyte chemotactic activity for subtype C Tat without loss in transactivation property (Ranga et al., 2004). Although the evidence was not conclusive, we hypothesized that the Tat variation could be one of the factors for the low incidence of HIV-1 encephalitis and HAD in India.

The first three domains, the N-terminal, cysteine-rich and the core domain (aa 1-48) together constitute the 'minimal Activation Domain' of Tat (Carroll et al., 1991) and it functions as a transactivator when linked to domain IV or to a heterologous RNA/DNA-binding domain.

The basic domain (aa 49-59) is also highly conserved and contains an RKKRRQRRR motif needed for TAR RNA binding (Dingwall et al., 1989; Dingwall et al., 1990) and nuclear localization through a novel import pathway (Hauber et al., 1989). The TAR-binding activity of Tat has been localized in an Arg-rich basic domain located between residues 49 and 57 (Subramanian et al., 1991). This arginine-rich domain functions as NLS, nuclear localization signal and involves importin β (Truant and

Cullen, 1999). In addition, this domain can act as a chemo-attractant for dendritic cells and monocytes (Benelli et al., 1998), and is involved in the uptake of protein from extracellular space. This region is also implicated in the neurotoxic effects of Tat and angiogenesis leading to Kaposi's sarcoma (Weeks et al., 1995). Tat has also been shown to bind the histone chaperone hNAP-1 both in vitro and in vivo and shows that this interaction participates in the regulation of Tat-mediated activation of viral gene expression. The integrity of basic domain is necessary for this interaction (Vardabasso et al., 2008).

The fifth domain, the glutamine rich domain, (as 60-72), contains several regularly spaced glutamine residues. Tat 1-66 aa is sufficient to mediate full transactivation (Kuppuswamy et al., 1989). The glutamine-rich region of Tat has been implicated in Tat-mediated apoptosis of T-cells (Campbell et al., 2004).

Domain VI, the C-terminal region, (aa 59-72) contains an RGD motif able to recognize the integrinic receptor on cell membrane, and has been shown to act as a chemo-attractant for dendritic cells and monocytes. It is also involved in MHC-I downregulation and Tat-induced apoptosis in T-cell lines and primary CD4 T-cells (Bartz and Emerman, 1999b; Zocchi et al., 1997).

Second exon of Tat (73-101 aa) contributes to viral infectivity and to other Tat functions (Gatignol and Jeang, 2000; Rana and Jeang, 1999). It is also implicated in the downregulation of MHC-I (Verhoef et al., 1998) on the cell surface thereby compromising immune surveillance. Tat is a potent repressor of major histocompatibility complex (MHC) class I transcription (Brown et al., 1998). While the repressor activity is dependent on the C-terminal sequences, the transactivation activity is carried out by the N-terminal sequences; both functions require core sequence, especially the K41 residue.

Tat has also been shown to have potent nucleic acid-chaperoning activities, using the standard DNA annealing, DNA and RNA strand exchange, RNA ribozyme cleavage and trans-splicing assays. Tat (44-61 aa) peptide has been identified as the smallest known sequence with DNA/RNA chaperoning properties (Kuciak et al., 2008).

Tat, in addition to its role in HIV-1 transcription, has a more extensive role in the pathogenesis of HIV-1 infection. Tat affects several important cell biological activities acting on cellular signals and gene activation in infected and on uninfected cells too.

Several groups have demonstrated that Tat protein can be actively secreted by HIV-1 infected cells (also by tat-transfected cells) and can be taken up by several cell types both in vivo and in vitro. Tat is reported to be present in significant quantities in circulation, termed extracellular Tat (eTat) or soluble Tat (sTat) (Ensoli et al., 1993).

Tat released by infected cells is able to enter other cells through interactions with heparan sulphate proteoglycans on the cell surface and translocates to the nucleus by the nuclear localization sequence of its basic domain. Extracellular Tat is able to enter latently infected cells and activate the transcription of the latent viral genome, which leads to the burst of HIV replication by several folds.

Soluble Tat, at concentrations in the picomolar range which is close to that observed in sera from HIV-1 infected individuals, upregulates the expression of chemokine receptors CXCR4 and CCR5, co-receptors of HIV-1 in infected PBMC (Huang et al., 1998b; Secchiero et al., 1999).

Tat can also trigger intracellular signaling cascades by activating various kinases. Different isoforms of Protein kinase C (PKC) are selectively induced in Tat treated serum-starved rat pheochromocytoma, PC12 cells. sTat induced PKC at a range of 0.1 – 1 nm (Borgatti et al., 1998). Other kinases that are activated by Tat include Src kinase, MAP kinase etc (Kumar et al., 1998; Borgatti et al., 1998; Ganju et al., 1998). sTat stimulates FasL on purified murine macrophages thereby imposing a state of acute immunosuppression in vivo (Cohen et al., 1999c). Tat also induces functional unresponsiveness in T-cells by a phospholipase Cγ1 independent pathway as seen by the failure to induce proliferation in CD4 cells on specific antigenic induction (Chirmule et al., 1995; Zocchi et al., 1997).

Tat also modulates the production of several cytokines in different cell lines (Westendorp et al., 1994). Tat can activate heterologous promoters like that of TNF, which in turn control the production of inflammatory cytokines like IL-1 and IL-6 (Buonaguro et al., 1992; Nath et al., 1999b; Garza, Jr. et al., 1996), the production of which leads to increased HIV-1 gene expression and affect the immune, vascular and

central nervous systems. Tat-induced cytokines persist in time and are more likely to be driven by a positive feedback loop mediated by NFkappB (Nath et al., 1999a).

Tat is also known to stimulate the growth of cells derived from Kaposi's sarcoma lesions the effect of which can be inhibited by anti-Tat antibodies. This stimulation is brought about by a synergistic effect between Tat and basic fibroblast growth factor (Ensoli et al., 1990). Studies have also shown that Tat elicits inflammatory cytokine activated-endothelial cells proliferation and survival accelerating tumorigenesis (Barillari and Ensoli, 2002).

Tat protein has been reported to be implicated in induction of apoptosis. The CD95 ligand (FasL) expression is upregulated by Tat, indicating Tat accelerates CD95-mediated activation-induced T-cell apoptosis (Westendorp et al., 1995). Tat mediated dysregulation of caspase-8 pathway, by inhibition of expression of manganese dependent superoxide dismutase, activation of cyclin-dependent kinases and upregulation of FLICE/Caspase-8 thereby rendering neurons and T-cells sensitive to apoptosis (Bartz and Emerman, 1999a) .The second exon is also implicated for this function. Another effect attributed to the second exon of Tat includes the induction of immune hyperactivation seen in HIV-1 infection, which is mediated by IL-2 superinduction through the CD28 responsive element in the IL-2 promoter (Ott et al., 1997). Similar effect of Tat protein on superinduction of IL-8 has been reported to be dependent on NF-κB factors (Ott et al., 1998). Second exon of Tat is also implicated in the downregulation of MHC-I on the cell surface thereby compromising immune surveillance. HIV-1 proteins Tat and Nef could selectively downregulate HLA-A and HLA-B, but doesn't significantly affect HLA-C or HLA-E (Cohen et al., 1999a), thereby protecting HIV-infected cells from Natural Killer (NK) cells.

1.13.2 Tat - a potential vaccine candidate

1.13.2a Tat remains a potential vaccine candidate: Most of the HIV vaccine strategies focused mainly on the envelope (env) gene of the virus. Although env vaccines conferred protection against autologous viral strains, antigenic variation is a challenge for vaccine design (Osmanov et al., 1996). A need for developing multi-component vaccines is being increasingly realized, which can broaden immune responses against the viral infection (Ho and Huang, 2002). Extensive work from various laboratories has identified the viral structural proteins, gag and pol, and viral regulatory proteins Nef, Tat and Rev, as potential candidates for vaccine development (Calarota et al., 1999; Evans et al., 1999; Putkonen et al., 1998). Of these non-env candidates, Tat occupies a special place for several reasons.

First of all, the functional importance of this viral antigen to the infectivity of the virus (Gallo, 1999; Jeang et al., 1999; Rubartelli et al., 1998; Rusnati and Presta, 2002), and the existence of an inverse correlation between immune responses to Tat and disease progression (Allen et al., 2000; Re et al., 1995; Re et al., 2001b; Reiss et al., 1990; Zagury et al., 1998b; van Baalen et al., 1997) make Tat an important candidate vaccine. Several studies showed that immune responses to Tat, humoral or cellular, appear to have protected against disease progression although a few studies demonstrated absence of such effect.

Tat is expressed early in the viral life cycle and is functionally important for its infectivity and pathogenicity (Jeang et al., 1999). In addition to regulating viral gene expression, Tat modulates expression of various genes of the host. Further, Tat is secreted extracellularly and the extracellular Tat governs viral latency and contributes to disease progression (Noonan and Albini, 2000). The small size of the antigen consisting of only 101 aa permits genetic manipulation less strenuous. Most important of all, in spite of the functional importance of Tat for the virus, this antigen is not immunodominant either in natural infection (Lamhamedi-Cherradi et al., 1992; Lieberman et al., 1997; Reiss et al., 1990; Krone et al., 1988; Wieland et al., 1990) or in experimental immunization, especially when delivered as a genetic vaccine

(Billaut-Mulot et al., 2001; Calarota et al., 1999). Inducing cellular as well as humoral immune responses against Tat is critical owing to its early expression in the viral life cycle and to its extracellular secretion (Goldstein, 1996; Rusnati and Presta, 2002). Lastly, as a consequence of its pleiotropic biologic functions, a variety of functional assays are available for Tat, to study the inhibitory effect of immune components on its biological functions.

1.13.2b Application of Tat as a potential vaccine candidate in primates and human beings: Induction of Tat-specific cell-mediated (Allen et al., 2000) (Cao et al., 2003) and humoral immune responses (Krone et al., 1988; Zagury et al., 1998b; Reiss et al., 1990) is essential for restricting the viral infection. However, the potential value of Tat as a vaccine candidate is controversial. Several approaches have been used to immunize experimental animals with Tat peptides (Belliard et al., 2005; Goldstein et al., 2000), biologically active protein (Ensoli and Cafaro, 2000; Caselli et al., 1999; Agwale et al., 2002; Cafaro et al., 1999; Dominici et al., 2003), chemically modified toxoid (Pauza et al., 2000; Gringeri et al., 1999; Tikhonov et al., 2003), recombinant viruses (Osterhaus et al., 1999), genetic vaccines (Caselli et al., 1999; Cafaro et al., 2001; Billaut-Mulot et al., 2001; Caputo et al., 1996) or prime-boost strategies employing naked DNA priming followed by protein (Billaut-Mulot et al., 2001; Putkonen et al., 1998) or recombinant vaccinia boosters (Allen et al., 2002b; Hel et al., 2002; Matano et al., 2003). Mixed results have been obtained in primate immunizations followed up by virus challenge experiments. Regardless of the nature of the Tat presentation, in some studies attenuation of the viral infection was seen and the immunized animals were partially or completely protected (Pauza et al., 2000; Cafaro et al., 2001; Agwale et al., 2002; Cafaro et al., 1999; Osterhaus et al., 1999; Stittelaar et al., 2002; Ensoli and Cafaro, 2000; Goldstein et al., 2000; Pauza et al., 2000). In contrast, several other studies failed to show such protection regardless of immunization status of the animals (Allen et al., 2002b; Putkonen et al., 1998)}(Richardson et al., 2002; Silvera et al., 2002; Cafaro et al., 1999). A direct comparative evaluation of these results is difficult, as several variables have been incorporated into the experimental design. Further, most of the studies evaluated

protective immune responses using a viral strain, 89.6p, that doesn't represent the natural viral infection thus raising doubts on the validity of the data (Feinberg and Moore, 2002). Importantly, of all the variables, the biological activity of the Tat protein appears to be critical for the nature of the immune responses generated. While the use of biologically active Tat protein elicited a broad range humoral and cellular immune responses (Ensoli and Cafaro, 2000; Cafaro et al., 1999), use of Tat toxoid, in contrast, generated immune responses against restricted epitopes that were not cross-reactive across viral subtypes (Tikhonov et al., 2003). A recent publication chemically modified Tat in vitro by adding sulfated sugars to reduce toxicity and augment humoral immune responses (Lecoq et al., 2008).

A small number of studies tested Tat DNA vaccine in human experimental vaccination and the immune responses elicited were evaluated. The first immunization attempt of humans with Tat and other regulatory protein DNA expression vectors, demonstrated the elimination of virus-infected cells by antigen-specific CTL (Calarota et al., 1998). Other studies identified Tat-specific immune responses that were broader when delivered in combination with other regulatory proteins rev and nef (Calarota et al., 1999; Calarota et al., 2001; Hejdeman et al., 2004).

1.14 References

Agwale SM, Shata MT et al, A Tat subunit vaccine confers protective immunity against the immune-modulating activity of the human immunodeficiency virus type-1 Tat protein in mice. *Proc Natl Acad Sci U S A*, **99**, 10037-10041 (2002).

Ahmad A, Ahmad R et al, IL-15 and HIV infection: lessons for immunotherapy and vaccination. *Curr HIV Res 2005 Jul ;3(3):261 -70*, **3**, 261-270 (2005).

Ahr B, Robert-Hebmann V, Devaux C and Biard-Piechaczyk M: Apoptosis of uninfected cells induced by HIV envelope glycoproteins. *Retrovirology*, **1**, 12(2004).

Aiuti F and Mezzaroma I: Failure to reconstitute CD4+ T-cells despite suppression of HIV replication under HAART. *AIDS Rev 2006 Apr-Jun ;8 (2):88 -97*, **8**, 88-97 (2006).

Albini A, Benelli R et al, Identification of a novel domain of HIV tat involved in monocyte chemotaxis. *J Biol Chem*, **273**, 15895-15900 (1998a).

Albini A, Ferrini S et al, HIV-1 Tat protein mimicry of chemokines. *Proc Natl Acad Sci U S A*, **95**, 13153-13158 (1998b).

Aldovini A and Young RA: Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. *J Virol*, **64**, 1920-1926 (1990).

Allen TM, Kelleher AD, Zaunders J and Walker BD: STI and beyond: the prospects of boosting anti-HIV immune responses. *Trends Immunol 2002 Sep ;23(9):456 -60*, **23**, 456-460 (2002a).

Allen TM, Mortara L et al, Tat-Vaccinated Macaques Do Not Control Simian Immunodeficiency Virus SIVmac239 Replication. *J Virol*, **76**, 4108-4112 (2002b).

Allen TM, O'Connor DH et al, Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature*, **407**, 386-390 (2000).

Andino R, Silvera D et al, Engineering poliovirus as a vaccine vector for the expression of diverse antigens. *Science Sep 2;265 (5177):1448 -51*,(1994).

Angel JB, Jacobson MA et al, A multicenter, randomized, double-blind, placebo-controlled trial of recombinant human interleukin-10 in HIV-infected subjects. *AIDS 2000 Nov 10;14(16):2503 -8*, **14**, 2503-2508 (2000).

Baba TW, Jeong YS et al, Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science Mar 24 ;267 (5205):1820 -5*,(1995).

Babiuk S, van Drunen Littel-van den Hurk and Babiuk LA: Delivery of DNA vaccines using electroporation. *Methods Mol Med 2006 ;127 :73 -82*, **127**, 73-82 (2006).

Barillari G and Ensoli B: Angiogenic effects of extracellular human immunodeficiency virus type 1 Tat protein and its role in the pathogenesis of AIDS-associated Kaposi's sarcoma. *Clin Microbiol Rev*, **15**, 310-326 (2002).

Barouch DH: Challenges in the development of an HIV-1 vaccine. *Nature Oct 2;455 (7213):613 -9*,(2008).

Barouch DH, Kunstman J et al, Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. *Nature*, **415**, 335-339 (2002).

Barouch DH, Letvin NL and Seder RA: The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses. *Immunol Rev*, **202**, 266-274 (2004).

Barouch DH, Powers J et al, Dynamic immune responses maintain cytotoxic T lymphocyte epitope mutations in transmitted simian immunodeficiency virus variants. *Nat Immunol*, **6**, 247-252 (2005a).

Barouch DH, Yang ZY et al, A human T-cell leukemia virus type 1 regulatory element enhances the immunogenicity of human immunodeficiency virus type 1 DNA vaccines in mice and nonhuman primates. *J Virol 2005 Jul ;79 (14):8828 -34*, **79**, 8828-8834 (2005b).

Bartz SR and Emerman M: Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. *J Virol Mar ;73 (3):1956 -63*,(1999b).

Bartz SR and Emerman M: Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8. *J Virol*, **73**, 1956-1963 (1999a).

Bayer P, Kraft M et al, Structural studies of HIV-1 Tat protein. *J Mol Biol*, **247**, 529-535 (1995).

Belliard G, Hurtrel B et al, Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. *Vaccine*, **23**, 1399-1407 (2005).

Benelli R, Mortarini R et al, Monocyte-derived dendritic cells and monocytes migrate to HIV-Tat RGD and basic peptides. *AIDS*, **12**, 261-268 (1998).

Berman PW, Gregory TJ et al, Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature Jun 14;345 (6276):622 -5*,(1990).

Betti M, Voltan R et al, Characterization of HIV-1 Tat proteins mutated in the transactivation domain for prophylactic and therapeutic application. *Vaccine*, **19**, 3408-3419 (2001).

Billaut-Mulot O, Idziorek T et al, Modulation of cellular and humoral immune responses to a multiepitopic HIV-1 DNA vaccine by interleukin-18 DNA immunization/viral protein boost. *Vaccine*, **19**, 2803-2811 (2001).

Black DL: Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem*, **72**, 291-336 (2003).

Boaz MJ, Waters A et al, Presence of HIV-1 Gag-Specific IFN-gamma(+)IL-2(+) and CD28(+)IL-2(+) CD4 T Cell Responses Is Associated with Nonprogression in HIV-1 Infection. *J Immunol*, **169**, 6376-6385 (2002a).

Boaz MJ, Waters A et al, Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. *J Immunol 2002 Dec 1;169 (11):6376 -85*, **169**, 6376-6385 (2002b).

Bojak A, Hammer D, Wolf H and Wagner R: Muscle specific versus ubiquitous expression of Gag based HIV-1 DNA vaccines: a comparative analysis. *Vaccine 2002 May 6 ;20 (15):1975 -9*, **20**, 1975-1979 (2002).

Bolmstedt A, Sjolander S et al, Influence of N-linked glycans in V4-V5 region of human immunodeficiency virus type 1 glycoprotein gp160 on induction of a virus-neutralizing humoral response. *J Acquir Immune Defic Syndr Hum Retrovirol*, **12**, 213-220 (1996).

Borgatti P, Zauli G, Cantley LC and Capitani S: Extracellular HIV-1 Tat protein induces a rapid and selective activation of protein kinase C (PKC)-alpha, and -epsilon and -zeta isoforms in PC12 cells. *Biochem Biophys Res Commun*, **242**, 332-337 (1998).

Borkow G: Mouse models for HIV-1 infection. *IUBMB Life 2005 Dec ;57 (12):819 -23*, **57**, 819-823 (2005).

Borrow P, Lewicki H et al, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*, **68**, 6103-6110 (1994a).

Borrow P, Lewicki H et al, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*, **68**, 6103-6110 (1994b).

Boykins RA, Mahieux R et al, A short polypeptide domain of HIV-1-Tat protein mediates pathogenesis. *J Immunol*, **163**, 15-20 (1999).

Brander C, Corradin G, Hasler T and Pichler WJ: Peptide immunization in humans: a combined CD8+/CD4+ T cell-targeted vaccine restimulates the memory CD4 T cell response but fails to induce cytotoxic T lymphocytes (CTL). *Clin Exp Immunol Jul ;105 (1):18 -25*,(1996).

Brown JA, Howcroft TK and Singer DS: HIV Tat protein requirements for transactivation and repression of transcription are separable. *J Acquir Immune Defic Syndr Hum Retrovirol*, **17**, 9-16 (1998).

Buonaguro L, Barillari G et al, Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. *J Virol*, **66**, 7159-7167 (1992).

Cafaro A, Caputo A et al, Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. *Nat Med*, **5**, 643-650 (1999).

Cafaro A, Titti F et al, 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**, 2862-2877 (2001).

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

Calarota SA, Kjerrstrom A, Islam KB and Wahren B: Gene combination raises broad human immunodeficiency virus-specific cytotoxicity. *Hum Gene Ther*, **12**, 1623-1637 (2001).

Calarota SA, Leandersson AC et al, Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *J Immunol*, **163**, 2330-2338 (1999).

Cameron DW, Heath-Chiozzi M et al, Randomised placebo-controlled trial of ritonavir in advanced HIV-1 disease. The Advanced HIV Disease Ritonavir Study Group. *Lancet*, **351**, 543-549 (1998).

Campbell GR, Pasquier E et al, The glutamine-rich region of the HIV-1 Tat protein is involved in T-cell apoptosis. *J Biol Chem*, **279**, 48197-48204 (2004).

Cao J, McNevin J, Malhotra U and McElrath MJ: Evolution of CD8(+) T Cell Immunity and Viral Escape Following Acute HIV-1 Infection. *J Immunol*, **171**, 3837-3846 (2003).

Cao L, Si J et al, Intracellular localization and sustained prodrug cell killing activity of TAT-HSVTK fusion protein in hepatocelullar carcinoma cells. *Mol Cells*, **21**, 104-111 (2006).

Capobianchi MR, Fais S et al, A simple and reliable method to detect cell membrane proteins on infectious human immunodeficiency virus type 1 particles. *J Infect Dis*, **169**, 886-889 (1994).

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

Carroll R, Martarano L and Derse D: Identification of lentivirus tat functional domains through generation of equine infectious anemia virus/human immunodeficiency virus type 1 tat gene chimeras. *J Virol Jul ;65 (7):3460 -7*,(1991).

Caselli E, Betti M et al, DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. *J Immunol*, **162**, 5631-5638 (1999).

Cazeaux N, Bennasser Y et al, Comparative study of immune responses induced after immunization with plasmids encoding the HIV-1 Nef protein under the control of the CMV-IE or the muscle-specific desmin promoter. *Vaccine 2002 Sep 10;20 (27 -28):3322 -31*, **20**, 3322-3331 (2002).

Chan E, Heilek-Snyder G et al, Development of a Moloney murine leukemia virus-based pseudotype anti-HIV assay suitable for accurate and rapid evaluation of HIV entry inhibitors. *J Biomol Screen 2006 Sep ;11(6):652 -63 Epub 2006 Jul 14*, **11**, 652-663 (2006).

Chapman BS, Thayer RM, Vincent KA and Haigwood NL: Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res*, **19**, 3979-3986 (1991).

Chirmule N, Than S, Khan SA and Pahwa S: Human immunodeficiency virus Tat induces functional unresponsiveness in T cells. *J Virol Jan ;69 (1):492 -8*,(1995).

Cohen GB, Gandhi RT et al, The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity Jun ;10(6):661 -71*,(1999a).

Cohen GB, Gandhi RT et al, The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity*, **10**, 661-671 (1999b).

Cohen J: Public health. AIDS vaccine trial produces disappointment and confusion. *Science Feb 28 ;299 (5611):1290 -1*,(2003).

Cohen SS, Li C et al, Pronounced acute immunosuppression in vivo mediated by HIV Tat challenge. *Proc Natl Acad Sci U S A*, **96**, 10842-10847 (1999c).

Corr M, von Damm A, Lee DJ and Tighe H: In vivo priming by DNA injection occurs predominantly by antigen transfer. *J Immunol*, **163**, 4721-4727 (1999).

Daniel MD, Kirchhoff F et al, Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science Dec 18 ;258 (5090):1938 -41*,(1992).

Dean DA, Strong DD and Zimmer WE: Nuclear entry of nonviral vectors. *Gene Ther*, **12**, 881-890 (2005).

Deml L, Bojak A et al, Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. *J Virol*, **75**, 10991-11001 (2001).

Dingwall C, Ernberg I et al, HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. *EMBO J*, **9**, 4145-4153 (1990).

Dingwall C, Ernberg I et al, Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. *Proc Natl Acad Sci U S A*, **86**, 6925-6929 (1989).

Dominici S, Laguardia ME et al, Red blood cell-mediated delivery of recombinant HIV-1 Tat protein in mice induces anti-Tat neutralizing antibodies and CTL. *Vaccine*, **21**, 2073-2081 (2003).

Duncker BP, Davies PL and Walker VK: Introns boost transgene expression in Drosophila melanogaster. *Mol Gen Genet*, **254**, 291-296 (1997).

Dunham RM, Cervasi B et al, CD127 and CD25 Expression Defines CD4+ T Cell Subsets That Are Differentially Depleted during HIV Infection. *J Immunol*, **180**, 5582-5592 (2008).

Efthymiadis A, Briggs LJ and Jans DA: The HIV-1 Tat nuclear localization sequence confers novel nuclear import properties. *J Biol Chem*, **273**, 1623-1628 (1998).

Egelhofer M, Brandenburg G et al, Inhibition of human immunodeficiency virus type 1 entry in cells expressing gp41-derived peptides. *J Virol 2004 Jan ;78 (2):568 -75*, **78**, 568-575 (2004).

Ellenberger DL, Sullivan PS et al, Viral and immunologic examination of human immunodeficiency virus type 1-infected, persistently seronegative persons. *J Infect Dis*, **180**, 1033-1042 (1999).

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

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

Ensoli B and Cafaro A: Control of viral replication and disease onset in cynomolgus monkeys by HIV-1 TAT vaccine. *J Biol Regul Homeost Agents*, **14**, 22-26 (2000).

Esparza J and Bhamarapravati N: Accelerating the development and future availability of HIV-1 vaccines: why, when, where, and how? *Lancet*, **355**, 2061-2066 (2000).

Evans TG, Keefer MC et al, A Canarypox Vaccine Expressing Multiple Human Immunodeficiency Virus Type 1 Genes Given Alone or with Rgp120 Elicits Broad and Durable CD8+ Cytotoxic T Lymphocyte Responses in Seronegative Volunteers. *J Infect Dis*, **180**, 290-298 (1999).

Fauci AS: The AIDS epidemic--considerations for the 21st century. *N Engl J Med Sep 30 ;341 (14):1046 -50*,(1999).

Fauci AS: HIV and AIDS: 20 years of science. *Nat Med Jul ;9 (7):839 -43*,(2003).

Feinberg MB and Moore JP: AIDS vaccine models: challenging challenge viruses. *Nat Med*, **8**, 207-210 (2002).

Ferrari S, Geddes DM and Alton EW: Barriers to and new approaches for gene therapy and gene delivery in cystic fibrosis. *Adv Drug Deliv Rev*, **54**, 1373-1393 (2002).

Frankel AD, Bredt DS and Pabo CO: Tat protein from human immunodeficiency virus forms a metal-linked dimer. *Science*, **240**, 70-73 (1988).

Fuller DH, Loudon P and Schmaljohn C: Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods 2006 Sep ;40 (1):86 -97*, **40**, 86-97 (2006).

Furger A, O'Sullivan JM et al, Promoter proximal splice sites enhance transcription. *Genes Dev*, **16**, 2792-2799 (2002).

Gaffal E, Schweichel D et al, Comparative evaluation of CD8+CTL responses following gene gun immunization targeting the skin with intracutaneous injection of antigen-transduced dendritic cells. *Eur J Cell Biol 2007 Dec ;86 (11-12):817 -26 Epub 2006 Aug 22*, **86**, 817-826 (2007).

Gallo RC: Tat as one key to HIV-induced immune pathogenesis and Tat toxoid as an important component of a vaccine. *Proc Natl Acad Sci U S A*, **96**, 8324-8326 (1999).

Gandhi RT, Walker BD et al, Immunologic control of HIV-1
Proliferative responses to human immunodeficiency virus type 1 (HIV-1) antigens in HIV-1-infected patients with immune reconstitution
The impact of highly active antiretroviral therapy on HIV-specific immune function
Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection

Discordance between frequency of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-producing CD4(+) T cells and HIV-1-specific lymphoproliferation in HIV-1-infected subjects with active viral replication

Mitogens for curative therapy of HIV-1 infections; an update. *Cancer Biother Radiopharm*, **53**, 149-172 (2002).

Ganju RK, Munshi N et al, Human immunodeficiency virus tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases, and components of focal adhesion in Kaposi's sarcoma cells. *J Virol*, **72**, 6131-6137 (1998).

Gao F, Li Y et al, Codon Usage Optimization of HIV Type 1 Subtype C gag, pol, env, and nef Genes: In Vitro Expression and Immune Responses in DNA-Vaccinated Mice. *AIDS Res Hum Retroviruses*, **19**, 817-823 (2003).

Gao X and Huang L: Cationic liposome-mediated gene transfer. *Gene Ther*, **2**, 710-722 (1995).

Garmory HS, Brown KA and Titball RW: DNA vaccines: improving expression of antigens. *Genet Vaccines Ther*, **1**, 2(2003).

Garza HH, Jr., Prakash O and Carr DJ: Aberrant regulation of cytokines in HIV-1 TAT72-transgenic mice. *J Immunol*, **156**, 3631-3637 (1996).

Garzino-Demo A, Moss RB et al, Spontaneous and antigen-induced production of HIV-inhibitory beta-chemokines are associated with AIDS-free status. *Proc Natl Acad Sci U S A*, **96**, 11986-11991 (1999).

Gatignol A and Jeang KT: Tat as a transcriptional activator and a potential therapeutic target for HIV-1. *Adv Pharmacol 2000 ;48 :209 -27*, **48**, 209-227 (2000).

Gea-Banacloche JC, Migueles SA et al, Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. *J Immunol*, **165**, 1082-1092 (2000).

Giardino R, Fini M et al, Electrochemotherapy a novel approach to the treatment of metastatic nodules on the skin and subcutaneous tissues. *Biomed Pharmacother 2006 Sep ;60 (8):458 -62 Epub 2006 Aug 14*, **60**, 458-462 (2006).

Goldstein G: HIV-1 Tat protein as a potential AIDS vaccine. *Nat Med*, **2**, 960-964 (1996).

Goldstein G, Manson K, Tribbick G and Smith R: Minimization of chronic plasma viremia in rhesus macaques immunized with synthetic HIV-1 Tat peptides and infected with a chimeric simian/human immunodeficiency virus (SHIV(33)). *Vaccine*, **18**, 2789-2795 (2000).

Gray RH, Wawer MJ et al, Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet 2001 Apr 14;357 (9263):1149 -53*, **357**, 1149-1153 (2001).

Greco G, Barker E and Levy JA: Differences in HIV replication in CD4+ lymphocytes are not related to beta-chemokine production. *AIDS Res Hum Retroviruses*, **14**, 1407-1411 (1998).

Greenough TC, Brettler DB et al, Long-term nonprogressive infection with human immunodeficiency virus type 1 in a hemophilia cohort. *J Infect Dis*, **180**, 1790-1802 (1999).

Gribaudo G, Ravaglia S et al, Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription. *Virology*, **197**, 303-311 (1993).

Gringeri A, Santagostino E et al, Tat toxoid as a component of a preventive vaccine in seronegative subjects. *J Acquir Immune Defic Syndr Hum Retrovirol*, **20**, 371-375 (1999).

Hammer SM, Squires KE et al, A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med*, **337**, 725-733 (1997).

Hauber J, Malim MH and Cullen BR: Mutational analysis of the conserved basic domain of human immunodeficiency virus tat protein. *J Virol*, **63**, 1181-1187 (1989).

Hecht FM, Grant RM et al, Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. *N Engl J Med*, **339**, 307-311 (1998).

Hejdeman B, Bostrom AC et al, DNA Immunization with HIV Early Genes in HIV Type 1-Infected Patients on Highly Active Antiretroviral Therapy. *AIDS Res Hum Retroviruses*, **20**, 860-870 (2004).

Hel Z, Johnson J et al, A novel chimeric Rev, Tat, and Nef (Retanef) antigen as a component of an SIV/HIV vaccine. *Vaccine*, **20**, 3171-3186 (2002).

Hess C, Altfeld M et al, HIV-1 specific CD8+ T cells with an effector phenotype and control of viral replication. *Lancet*, **363**, 863-866 (2004).

Hirao LA, Wu L et al, Intradermal/subcutaneous immunization by electroporation improves plasmid vaccine delivery and potency in pigs and rhesus macaques. *Vaccine 2008 Jan 17;26 (3):440 -8 Epub 2007 Nov 8*, **26**, 440-448 (2008).

Ho DD and Huang Y: The HIV-1 vaccine race. *Cell*, **110**, 135-138 (2002).

Huang HW and Wang KT: Structural characterization of the metal binding site in the cysteine-rich region of HIV-1 Tat protein. *Biochem Biophys Res Commun*, **227**, 615-621 (1996).

Huang L, Bosch I et al, Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. *J Virol*, **72**, 8952-8960 (1998a).

Huang L, Bosch I et al, Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. *J Virol*, **72**, 8952-8960 (1998b).

Huang LM, Joshi A et al, Human immunodeficiency viruses regulated by alternative trans-activators: genetic evidence for a novel non-transcriptional function of Tat in virion infectivity. *EMBO J*, **13**, 2886-2896 (1994).

Humbert M and Dietrich U: The role of neutralizing antibodies in HIV infection. *AIDS Rev*, **8**, 51-59 (2006).

Hust M, Jostock T et al, Single chain Fab (scFab) fragment. *BMC Biotechnol*, **7**, 14(2007).

Jeang KT, Xiao H and Rich EA: Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem*, **274**, 28837-28840 (1999).

Jones NA, Wei X et al, Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. *J Exp Med*, **200**, 1243-1256 (2004).

Kahne T, Lendeckel U et al, Dipeptidyl peptidase IV: a cell surface peptidase involved in regulating T cell growth (review). *Int J Mol Med Jul ;4(1):3-15*,(1999).

Kao SY, Calman AF, Luciw PA and Peterlin BM: Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature*, **330**, 489-493 (1987).

Khan AU and Lal SK: Ribozymes: a modern tool in medicine. *J Biomed Sci*, **10**, 457-467 (2003).

Kim DW, Uetsuki T et al, Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. *Gene*, **91**, 217-223 (1990).

Kim SY, Lee JH et al, The human elongation factor 1 alpha (EF-1 alpha) first intron highly enhances expression of foreign genes from the murine cytomegalovirus promoter. *J Biotechnol*, **93**, 183-187 (2002).

Klinman DM, Takeshita F et al, DNA vaccines: capacity to induce auto-immunity and tolerance. *Dev Biol (Basel) 2000 ;104 :45 -51*, **104**, 45-51 (2000).

Koken SE, Greijer AE et al, Intracellular analysis of in vitro modified HIV Tat protein. *J Biol Chem Mar 18 ;269 (11):8366 -75*,(1994).

Koup RA, Safrit JT et al, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol*, **68**, 4650-4655 (1994).

Kovacs JA, Lempicki RA et al, Induction of prolonged survival of CD4+ T lymphocytes by intermittent IL-2 therapy in HIV-infected patients. *J Clin Invest 2005 Aug ;115 (8):2139 -48 Epub 2005 Jul 14*, **115**, 2139-2148 (2005).

Kozak M: Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. *EMBO J*, **16**, 2482-2492 (1997).

Krone WJ, Debouck C et al, Natural antibodies to HIV-tat epitopes and expression of HIV-1 genes in vivo. *J Med Virol*, **26**, 261-270 (1988).

Kuciak M, Gabus C et al, The HIV-1 transcriptional activator Tat has potent nucleic acid chaperoning activities in vitro. *Nucleic Acids Res Jun ;36 (10):3389 -400 Epub 2008 Apr 28*,Epub(2008).

Kumar A, Manna SK, Dhawan S and Aggarwal BB: HIV-Tat protein activates c-Jun N-terminal kinase and activator protein-1. *J Immunol*, **161**, 776-781 (1998).

Kuppuswamy M, Subramanian T, Srinivasan A and Chinnadurai G: Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. *Nucleic Acids Res*, **17**, 3551-3561 (1989).

Kurth R: Risk potential of the chromosomal insertion of foreign DNA. *Ann N Y Acad Sci*, **772**, 140-151 (1995).

Kutzler MA and Weiner DB: DNA vaccines: ready for prime time? *Nat Rev Genet Oct ;9 (10):776 -88*,(2008).

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

Laspia MF, Rice AP and Mathews MB: HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell Oct 20 ;59 (2):283 -92*,(1989).

Le Hir H, Nott A and Moore MJ: How introns influence and enhance eukaryotic gene expression. *Trends Biochem Sci*, **28**, 215-220 (2003).

Learmont JC, Geczy AF et al, 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. *N Engl J Med Jun 3;340 (22):1715 -22*,(1999).

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

Levine AM, Groshen S et al, Initial studies on active immunization of HIV-infected subjects using a gp120-depleted HIV-1 Immunogen: long-term follow-up. *J Acquir Immune Defic Syndr Hum Retrovirol Apr 1;11(4):351 -64*,(1996).

Lewis MG, Elkins WR et al, Passively transferred antibodies directed against conserved regions of SIV envelope protect macaques from SIV infection. *Vaccine*, **11**, 1347-1355 (1993).

Lieberman J, Fabry JA, Fong DM and Parkerson GR: Recognition of a small number of diverse epitopes dominates the cytotoxic T lymphocytes response to HIV type 1 in an infected individual. *AIDS Res Hum Retroviruses*, **13**, 383-392 (1997).

Liu F, Mboudjeka I et al, Independent but not synergistic enhancement to the immunogenicity of DNA vaccine expressing HIV-1 gp120 glycoprotein by codon optimization and C3d fusion in a mouse model. *Vaccine*, **22**, 1764-1772 (2004).

Liu LJ, Watabe S et al, Topical application of HIV DNA vaccine with cytokine-expression plasmids induces strong antigen-specific immune responses. *Vaccine 2001 Oct 12 ;20 (1-2):42 -8*, **20**, 42-48 (2001).

Liu W, Gao F et al, Codon Modified Human Papillomavirus Type 16 E7 DNA Vaccine Enhances Cytotoxic T-Lymphocyte Induction and Anti-tumour Activity. *Virology*, **301**, 43-52 (2002).

MacGregor RR, Boyer JD et al, First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis*, **178**, 92-100 (1998).

MacGregor RR, Boyer JD et al, Plasmid vaccination of stable HIV-positive subjects on antiviral treatment results in enhanced CD8 T-cell immunity and increased control of viral "blips". *Vaccine 2005 Mar 18 ;23(17-18):2066 -73*, **23**, 2066-2073 (2005).

Mani M, Kandavelou K et al, Design, engineering, and characterization of zinc finger nucleases. *Biochem Biophys Res Commun 2005 Sep 23;335 (2):447 -57*, **335**, 447-457 (2005).

Manthorpe M, Hobart P et al, Plasmid vaccines and therapeutics: from design to applications. *Adv Biochem Eng Biotechnol 2005 ;99 :41 -92*, **99**, 41-92 (2005).

Marasco WA, LaVecchio J and Winkler A: Human anti-HIV-1 tat sFv intrabodies for gene therapy of advanced HIV-1-infection and AIDS. *J Immunol Methods*, **231**, 223-238 (1999).

Mascola JR, Stiegler G et al, Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med 2000 Feb ;6 (2):207 -10*, **6**, 207-210 (2000).

Matano T, Kano M et al, No significant enhancement of protection by Tat-expressing Sendai viral vector-booster in a macaque AIDS model. *AIDS*, **17**, 1392-1394 (2003).

Matsumoto K, Wassarman KM and Wolffe AP: Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J*, **17**, 2107-2121 (1998).

McDermott AB, Mitchen J et al, Repeated low-dose mucosal simian immunodeficiency virus SIVmac239 challenge results in the same viral and immunological kinetics as high-dose challenge: a model for the evaluation of vaccine efficacy in nonhuman primates. *J Virol 2004 Mar ;78 (6):3140 -4*, **78**, 3140-3144 (2004).

Mhashilkar AM, Bagley J et al, Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies. *EMBO J*, **14**, 1542-1551 (1995b).

Mhashilkar AM, Bagley J et al, Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies. *Embo J Apr 3;14(7):1542 -51*,(1995a).

Migueles SA, Laborico AC et al, HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol 2002 Nov ;3(11):1061 -8 Epub 2002 Oct 7*, **3**, 1061-1068 (2002).

Migueles SA, Osborne CM et al, Lytic Granule Loading of CD8(+) T Cells Is Required for HIV-Infected Cell Elimination Associated with Immune Control. *Immunity 2008 Dec 3*,(2008).

Migueles SA, Tilton JC and Connors M: Advances in understanding immunologic control of HIV infection. *Curr HIV /AIDS Rep 2004 Apr;1(1):12 -7*, **1**, 12-17 (2004).

Modesti N, Garcia J et al, Trans-dominant Tat mutants with alterations in the basic domain inhibit HIV-1 gene expression. *New Biol*, **3**, 759-768 (1991).

Montgomery DL, Shiver JW et al, Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol*, **12**, 777-783 (1993).

Musey LK, Krieger JN et al, Early and persistent human immunodeficiency virus type 1 (HIV-1)-specific T helper dysfunction in blood and lymph nodes following acute HIV-1 infection. *J Infect Dis*, **180**, 278-284 (1999).

Nagata T, Uchijima M et al, Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem Biophys Res Commun*, **261**, 445-451 (1999).

Nair V: HIV integrase as a target for antiviral chemotherapy. *Rev Med Virol 2002 May - Jun ;12 (3):179 -93*, **12**, 179-193 (2002).

Nasim MT, Chowdhury HM and Eperon IC: A double reporter assay for detecting changes in the ratio of spliced and unspliced mRNA in mammalian cells. *Nucleic Acids Res*, **30**, e109(2002).

Nath A, Conant K et al, Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J Biol Chem*, **274**, 17098-17102 (1999b).

Nath A, Conant K et al, Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J Biol Chem*, **274**, 17098-17102 (1999a).

Nishimura Y, Igarashi T et al, Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. *Proc Natl Acad Sci U S A 2003 Dec 9 ;100 (25):15131 -6 Epub 2003 Nov*, **100**, 15131-15136 (2003).

Noonan D and Albini A: From the outside in: extracellular activities of HIV Tat. *Adv Pharmacol*, **48**, 229-250 (2000).

Nott A, Meislin SH and Moore MJ: A quantitative analysis of intron effects on mammalian gene expression. *RNA*, **9**, 607-617 (2003).

Novembre FJ, Saucier M et al, Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. *J Virol*, **71**, 4086-4091 (1997).

Orsini MJ and Debouck CM: Inhibition of human immunodeficiency virus type 1 and type 2 Tat function by transdominant Tat protein localized to both the nucleus and cytoplasm. *J Virol*, **70**, 8055-8063 (1996).

Osmanov S, Heyward WL and Esparza J: HIV-1 genetic variability: implications for the development of HIV vaccines. *Antibiot Chemother*, **48**, 30-38 (1996).

Osmanov S, Pattou C et al, Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. *J Acquir Immune Defic Syndr*, **29**, 184-190 (2002).

Osterhaus AD, van Baalen CA et al, Vaccination with Rev and Tat against AIDS. *Vaccine*, **17**, 2713-2714 (1999).

Ott M, Emiliani S et al, Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the CD28 pathway. *Science Mar 7 ;275 (5305):1481 -5*,(1997).

Ott M, Lovett JL, Mueller L and Verdin E: Superinduction of IL-8 in T cells by HIV-1 Tat protein is mediated through NF-kappaB factors. *J Immunol*, **160**, 2872-2880 (1998).

Palmer BE, Boritz E and Wilson CC: Effects of sustained HIV-1 plasma viremia on HIV-1 Gag-specific CD4+ T cell maturation and function. *J Immunol 2004 Mar 1;172 (5):3337 -47*, **172**, 3337-3347 (2004).

Parren PW, Marx PA et al, Antibody Protects Macaques against Vaginal Challenge with a Pathogenic R5 Simian/Human Immunodeficiency Virus at Serum Levels Giving Complete Neutralization In Vitro. *J Virol*, **75**, 8340-8347 (2001).

Pauza CD, Trivedi P et al, Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. *Proc Natl Acad Sci U S A*, **97**, 3515-3519 (2000).

Pereyra F, Addo MM et al, Genetic and Immunologic Heterogeneity among Persons Who Control HIV Infection in the Absence of Therapy. *J Infect Dis*, **197**, 563-571 (2008).

Perez EE, Riley JL et al, Suppression of HIV-1 infection in primary CD4 T cells transduced with a self-inactivating lentiviral vector encoding a membrane expressed gp41-derived fusion inhibitor. *Clin Immunol 2005 Apr;115 (1):26 -32*, **115**, 26-32 (2005).

Perez L, Thomson MM et al, HIV Type 1 Molecular Epidemiology in Cuba: High Genetic Diversity, Frequent Mosaicism, and Recent Expansion of BG Intersubtype Recombinant Forms. *AIDS Res Hum Retroviruses*, **22**, 724-733 (2006).

Peterlin BM and Price DH: Controlling the elongation phase of transcription with P-TEFb. *Mol Cell Aug 4;23(3):297 -305*,(2006).

Plosker GL and Scott LJ: Saquinavir: a review of its use in boosted regimens for treating HIV infection. *Drugs 2003 ;63 (12):1299 -324*, **63**, 1299-1324 (2003).

Porgador A, Irvine KR et al, Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med*, **188**, 1075-1082 (1998).

Potash MJ, Chao W et al, A mouse model for study of systemic HIV-1 infection, antiviral immune responses, and neuroinvasiveness. *Proc Natl Acad Sci U S A*, **102**, 3760-3765 (2005).

Priddy FH, Brown D et al, Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. *Clin Infect Dis Jun 1;46 (11):1769 -81*,(2008).

Prosch S, Stein J et al, Inactivation of the very strong HCMV immediate early promoter by DNA CpG methylation in vitro. *Biol Chem Hoppe Seyler*, **377**, 195-201 (1996).

Putkonen P, Quesada-Rolander M et al, Immune responses but no protection against SHIV by gene-gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. *Virology*, **250**, 293-301 (1998).

Quinones-Kochs MI, Buonocore L and Rose JK: Role of N-linked glycans in a human immunodeficiency virus envelope glycoprotein: effects on protein function and the neutralizing antibody response. *J Virol*, **76**, 4199-4211 (2002).

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

Ramakrishna L, Anand KK, Mohankumar KM and Ranga U: Codon Optimization of the Tat Antigen of Human Immunodeficiency Virus Type 1 Generates Strong Immune Responses in Mice following Genetic Immunization. *J Virol*, **78**, 9174-9189 (2004b).

Rana TM and Jeang KT: Biochemical and functional interactions between HIV-1 Tat protein and TAR RNA. *Arch Biochem Biophys*, **365**, 175-185 (1999).

Ranga U, Shankarappa R et al, Tat Protein of Human Immunodeficiency Virus Type 1 Subtype C Strains Is a Defective Chemokine. *J Virol*, **78**, 2586-2590 (2004).

Re MC, Furlini G et al, Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. *J Acquir Immune Defic Syndr Hum Retrovirol*, **10**, 408-416 (1995).

Re MC, Gibellini D, Vitone F and La Placa M: Antibody to HIV-1 Tat protein, a key molecule in HIV-1 pathogenesis. A brief review. *New Microbiol 2001 Apr;24 (2):197 -205*, **24**, 197-205 (2001a).

Re MC, Vignoli M et al, 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. *J Clin Virol*, **21**, 81-89 (2001b).

Reid W, Sadowska M et al, An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction. *Proc Natl Acad Sci U S A 2001 Jul 31 ;98 (16):9271 -6*, **98**, 9271-9276 (2001).

Reinhold D, Wrenger S, Kahne T and Ansorge S: HIV-1 Tat: immunosuppression via TGF-beta1 induction. *Immunol Today*, **20**, 384-385 (1999).

Reiss P, Lange JM et al, Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. *J Med Virol*, **30**, 163-168 (1990).

Richardson MW, Mirchandani J et al, Antibodies to Tat and Vpr in the GRIV cohort: differential association with maintenance of long-term non-progression status in HIV-1 infection. *Biomed Pharmacother*, **57**, 4-14 (2003).

Richardson MW, Mirchandani J et al, Immunogenicity of HIV-1 IIIB and SHIV 89.6P Tat and Tat toxoids in rhesus macaques: induction of humoral and cellular immune responses. *DNA Cell Biol*, **21**, 637-651 (2002).

Robertson DL, Anderson JP et al, HIV-1 nomenclature proposal. *Science 2000 Apr 7 ;288 (5463):55 -6*, **288**, 55-56 (2000).

Robertson JS and Cichutek K: European Union guidance on the quality, safety and efficacy of DNA vaccines and regulatory requirements. *Dev Biol (Basel) 2000 ;104 :53 -6*, **104**, 53-56 (2000).

Rossi JJ, June CH and Kohn DB: Genetic therapies against HIV. *Nat Biotechnol 2007 Dec ;25 (12):1444 -54*, **25**, 1444-1454 (2007).

Rubartelli A, Poggi A, Sitia R and Zocchi MR: HIV-I Tat: a polypeptide for all seasons. *Immunol Today*, **19**, 543-545 (1998).

Ruben S, Perkins A et al, Structural and functional characterization of human immunodeficiency virus tat protein. *J Virol*, **63**, 1-8 (1989).

Rusnati M and Presta M: HIV-1 Tat protein: a target for the development of anti-AIDS therapies. *Drugs Fut*, **27**, 481-493 (2002).

Sadaie MR, Mukhopadhyaya R et al, Conservative mutations in the putative metal-binding region of human immunodeficiency virus tat disrupt virus replication. *AIDS Res Hum Retroviruses Nov ;6 (11):1257 -63*,(1990).

Schmitt M, Harrer E et al, Specific recognition of lamivudine-resistant HIV-1 by cytotoxic T lymphocytes. *AIDS 2000 Apr 14;14(6):653 -8*, **14**, 653-658 (2000).

Schmitz JE, Kuroda MJ et al, Control of viremia in simian immunodeficiency virus infection by CD8(+) lymphocytes. *Science*, **283**, 857-860 (1999).

Secchiero P, Zella D et al, Extracellular HIV-1 tat protein up-regulates the expression of surface CXC-chemokine receptor 4 in resting CD4+ T cells. *J Immunol*, **162**, 2427-2431 (1999).

Sersa G, Miklavcic D et al, Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol 2008 Feb ;34 (2):232 -40 Epub 2007 Jul 5*, **34**, 232-240 (2008).

Sewell AK, Price DA et al, Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. *Stem Cells*, **18**, 230-244 (2000).

Sheets RL, Stein J et al, Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. *Toxicol Sci 2006 Jun ;91 (2):610 -9 Epub 2006 Mar 28*, **91**, 610-619 (2006).

Shiver JW and Emini EA: Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu Rev Med %2004 ;55 :355 -72*,(2004).

Shiver JW, Fu TM et al, Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature Jan 17;415 (6869):331 -5*,(2002).

Siddappa NB, Dash PK et al, Identification of unique B/C recombinant strains of HIV-1 in the southern state of Karnataka, India. *AIDS*, **19**, 1426-1429 (2005).

Siddappa NB, Dash PK et al, 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. *J Clin Microbiol*, **42**, 2742-2751 (2004).

Silvera P, Richardson MW et al, Outcome of simian-human immunodeficiency virus strain 89.6p challenge following vaccination of rhesus macaques with human immunodeficiency virus Tat protein. *J Virol*, **76**, 3800-3809 (2002).

Simon F, Mauclere P et al, Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat Med*, **4**, 1032-1037 (1998).

Siomi H, Shida H, Maki M and Hatanaka M: Effects of a highly basic region of human immunodeficiency virus Tat protein on nucleolar localization. *J Virol*, **64**, 1803-1807 (1990).

Smith JM, Amara RR et al, DNA/MVA vaccine for HIV type 1: effects of codon-optimization and the expression of aggregates or virus-like particles on the immunogenicity of the DNA prime. *AIDS Res Hum Retroviruses 2004 Dec ;20 (12):1335 -47*, **20**, 1335-1347 (2004).

Soares EA, Martinez AM et al, HIV-1 subtype C dissemination in southern Brazil. *AIDS*, **19 Suppl 4**, S81-S86(2005).

Soares MA, De Oliveira T et al, A specific subtype C of human immunodeficiency virus type 1 circulates in Brazil. *AIDS*, **17**, 11-21 (2003).

Song S, Morgan M et al, Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. *Proc Natl Acad Sci U S A*, **95**, 14384-14388 (1998).

Speck RF, Penn ML et al, Rabbit cells expressing human CD4 and human CCR5 are highly permissive for human immunodeficiency virus type 1 infection. *J Virol*, **72**, 5728-5734 (1998).

Srivastava IK, Ulmer JB and Barnett SW: Neutralizing antibody responses to HIV: role in protective immunity and challenges for vaccine design. *Expert Rev Vaccines 2004 Aug ;3(4 Suppl):S33 -52*, **3**, S33-S52(2004).

Srivastava IK, Ulmer JB and Barnett SW: Role of neutralizing antibodies in protective immunity against HIV. *Hum Vaccin 2005 Mar -Apr;1(2):45 -60 Epub 2005 Mar 17*, **1**, 45-60 (2005).

Steinberg T, Ohlschlager P et al, Modification of HPV 16 E7 genes: correlation between the level of protein expression and CTL response after immunization of C57BL/6 mice. *Vaccine*, **23**, 1149-1157 (2005).

Stittelaar KJ, Gruters RA et al, Comparison of the efficacy of early versus late viral proteins in vaccination against SIV. *Vaccine*, **20**, 2921-2927 (2002).

Stone A and Jiang S: Microbicides: stopping HIV at the gate. *Lancet 2006 Aug 5;368 (9534):431 -3*, **368**, 431-433 (2006).

Subramanian T, Govindarajan R and Chinnadurai G: Heterologous basic domain substitutions in the HIV-1 Tat protein reveal an arginine-rich motif required for transactivation. *EMBO J*, **10**, 2311-2318 (1991).

Tang DC, DeVit M and Johnston SA: Genetic immunization is a simple method for eliciting an immune response. *Nature*, **356**, 152-154 (1992).

Tatt ID, Barlow KL et al, Surveillance of HIV-1 Subtypes Among Heterosexuals in England and Wales, 1997-2000. *J Acquir Immune Defic Syndr*, **36**, 1092-1099 (2004).

Teschendorf C, Warrington KH, Jr., Siemann DW and Muzyczka N: Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. *Anticancer Res*, **22**, 3325-3330 (2002).

Tezel A, Paliwal S, Shen Z and Mitragotri S: Low-frequency ultrasound as a transcutaneous immunization adjuvant. *Vaccine 2005 May 31 ;23(29):3800 -7 Epub 2005 Mar 16*, **23**, 3800-3807 (2005).

Tikhonov I, Ruckwardt TJ, Hatfield GS and Pauza CD: Tat-neutralizing antibodies in vaccinated macaques. *J Virol*, **77**, 3157-3166 (2003).

Titti F, Cafaro A et al, Problems and emerging approaches in HIV/AIDS vaccine development. *Expert Opin Emerg Drugs 2007 Mar ;12 (1):23-48*, **12**, 23-48 (2007).

Tokushige K, Moradpour D et al, Comparison between cytomegalovirus promoter and elongation factor-1 alpha promoter-driven constructs in the establishment of cell lines expressing hepatitis C virus core protein. *J Virol Methods*, **64**, 73-80 (1997).

Truant R and Cullen BR: The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol Cell Biol*, **19**, 1210-1217 (1999).

Tsang C, Babiuk S et al, A single DNA immunization in combination with electroporation prolongs the primary immune response and maintains immune memory for six months. *Vaccine 2007 Jul 26 ;25 (30):5485 -94 Epub 2007 Mar 22*, **25**, 5485-5494 (2007).

Uchijima M, Yoshida A, Nagata T and Koide Y: Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *J Immunol*, **161**, 5594-5599 (1998).

Ulich C, Dunne A et al, Functional Domains of Tat Required for Efficient Human Immunodeficiency Virus Type 1 Reverse Transcription. *J Virol*, **73**, 2499-2508 (1999).

Ulmer JB, Donnelly JJ et al, Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*, **259**, 1745-1749 (1993).

Valvatne H, Szilvay AM et al, A monoclonal antibody defines a novel HIV type 1 Tat domain involved in trans-cellular trans-activation Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies Expression and purification of recombinant HIV-1 tat protein using HIV-1-tat specific monoclonal antibodies Intercellular traffic of human immunodeficiency virus type 1 transactivator protein defined by monoclonal antibodies. *AIDS Res Hum Retroviruses*, **12**, 611-619 (1996).

van Baalen CA, Pontesilli O et al, Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. *J Gen Virol*, **78**, 1913-1918 (1997).

Vardabasso C, Manganaro L et al, The histone chaperone protein Nucleosome Assembly Protein-1 (hNAP-1) binds HIV-1 Tat and promotes viral transcription. *Retrovirology Jan 28 ;5:8*,(2008).

Verhoef K, Bauer M, Meyerhans A and Berkhout B: On the role of the second coding exon of the HIV-1 Tat protein in virus replication and MHC class I downregulation. *AIDS Res Hum Retroviruses*, **14**, 1553-1559 (1998).

Wang J, Hu JH et al, Strong cellular and humoral immune responses induced by transcutaneous immunization with HBsAg DNA-cationic deformable liposome complex. *Exp Dermatol 2007 Sep ;16 (9):724 -9*, **16**, 724-729 (2007).

Wang S, Farfan-Arribas DJ et al, Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. *Vaccine 2006 May 22 ;24 (21):4531 -40 Epub 2005 Aug 18*, **24**, 4531-4540 (2006).

Weekes MP, Carmichael AJ et al, Human CD28-CD8+ T cells contain greatly expanded functional virus-specific memory CTL clones. *J Immunol*, **162**, 7569-7577 (1999).

Weeks BS, Lieberman DM et al, Neurotoxicity of the human immunodeficiency virus type 1 tat transactivator to PC12 cells requires the Tat amino acid 49-58 basic domain. *J Neurosci Res Sep 1;42 (1):34 -40*,(1995).

Westendorp MO, Frank R et al, Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature*, **375**, 497-500 (1995).

Westendorp MO, Li-Weber M, Frank RW and Krammer PH: Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. *J Virol Jul ;68 (7):4177 -85*,(1994).

Wieland U, Kuhn JE et al, Antibodies to recombinant HIV-1 vif, tat, and nef proteins in human sera. *Med Microbiol Immunol (Berl)*, **179**, 1-11 (1990).

Wolff JA, Malone RW et al, Direct gene transfer into mouse muscle in vivo. *Science*, **247**, 1465-1468 (1990).

Wood R, Montoya JG et al, Safety and efficacy of polyethylene glycol-modified interleukin-2 and zidovudine in human immunodeficiency virus type 1 infection: a phase I/II study. *J Infect Dis*, **167**, 519-525 (1993).

Wrenger S, Hoffmann T et al, The N-terminal structure of HIV-1 Tat is required for suppression of CD26-dependent T cell growth. *J Biol Chem Nov 28 ;272 (48):30283 -8*,(1997).

Yamamoto R, Katahira M et al, A novel RNA motif that binds efficiently and specifically to the Ttat protein of HIV and inhibits the trans-activation by Tat of transcription in vitro and in vivo. *Genes Cells*, **5**, 371-388 (2000).

Yamamoto R, Koseki S et al, Inhibition of transcription by the TAR RNA of HIV-1 in a nuclear extract of HeLa cells. *Nucleic Acids Res*, **25**, 3445-3450 (1997).

Yamano T, Kaneda Y et al, Enhancement of immunity by a DNA melanoma vaccine against TRP2 with CCL21 as an adjuvant. *Mol Ther 2006 Jan ;13(1):194 -202 Epub 2005 Aug 22*, **13**, 194-202 (2006).

Yan J, Yoon H et al, Enhanced cellular immune responses elicited by an engineered HIV-1 subtype B consensus-based envelope DNA vaccine. *Mol Ther 2007 Feb ;15 (2):411 -21*, **15**, 411-421 (2007).

Yew NS, Wysokenski DM et al, Optimization of plasmid vectors for high-level expression in lung epithelial cells. *Hum Gene Ther*, **8**, 575-584 (1997).

Younes SA, Yassine-Diab B et al, HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med 2003 Dec 15 ;198 (12):1909 -22*, **198**, 1909-1922 (2003).

Zagury D, Lachgar A et al, Interferon alpha and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. *Proc Natl Acad Sci U S A Mar 31 ;95 (7):3851 -6*,(1998a).

Zagury JF, Sill A et al, 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. *J Hum Virol*, **1**, 282-292 (1998b).

Zhang L, Yu W et al, Contribution of Human {alpha}-Defensin-1, -2 and -3 to the Anti-HIV-1 Activity of CD8 Antiviral Factor. *Science*,(2002).

Zhao J, Hyman L and Moore C: Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol Mol Biol Rev*, **63**, 405-445 (1999).

Zocchi MR, Poggi A and Rubartelli A: The RGD-containing domain of exogenous HIV-1 Tat inhibits the engulfment of apoptotic bodies by dendritic cells. *Aids Aug ;11(10):1227 -35*,(1997).

zur MJ, Chen MC et al, Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. *J Virol*, **74**, 2628-2635 (2000).

2. Materials and methods

2.1 Modified alkaline lysis method (Triton-silica method or TSM) for plasmid miniprep:

Plasmid DNA for routine molecular applications was prepared at a smaller scale using an inexpensive protocol described previously from our laboratory (Lakshmi et al., 1999). Briefly, bacteria were grown overnight in terrific broth and 1.5 to 3 ml of the culture was harvested at 5,000 rpm for 5 min at room temperature. The bacterial pellet was resuspended in 100 µl of solution-1 (Glucose-Tris-EDTA, pH 8.0) and 200 µl of solution-2 was added to each vial. Solution-2 was modified to contain Triton X – 100 (# 22686, Amersham), instead of SDS, to a final concentration of 4% along with 0.2 N NaOH. This was followed by the addition of 150 µl of solution-3 (3 M potassium acetate, pH 5.2) by gentle mixing and spinning at 10,000 rpm for 10 min at room temperature. The supernatant was transferred to a fresh vial and an equal volume of 5 N NaCl and 15 µl of silica suspension was added to each vial. The plasmid DNA was allowed to bind to the silica matrix for 15 min at room temperature with gentle agitation. The silica-DNA complex was pelleted by spinning the vials at 10,000 rpm for 1 min and the supernatant decanted. The pellet was washed thrice with wash solution containing 60% ethanol at 10,000 rpm for 1 min at room temperature. Vials were subjected to a quick spin, residual wash solution was removed with a microtip and the pellet was partially dried at 56^0C for 2 min. DNA was eluted from the silica particles by adding 50 µl of sterile distilled water which contained 6.6 µg/ml RNase A and incubated at 56^0C for 20 min. Vials were centrifuged at 10,000 rpm for 1 min and the DNA solution was transferred to fresh vials. A typical yield of plasmid DNA was around 0.1 to 0.2 µg/µl.

We used *E. coli* strain DH5α, for cloning. Competent cells were prepared according to the Inoue method for preparation and transformation of competent *E. coli* (Inoue et al., 1990) . Transformation of competent cells with plasmid DNA was performed using a heat-shock protocol according to Sambrook and Russell (1989).

Commercial kits were used for the isolation of plasmid DNA for sensitive applications like sequencing and transfections.

2.1.1 Preparation of silica suspension for TSM of plasmid miniprep:

Six g of silica (# S5631, Sigma) were suspended in sterile distilled water and allowed to stand undisturbed for 24 h at room temperature in a 50 ml plastic tube. Forty-three ml of unsedimented fine silica suspension were carefully decanted. The volume was made up to 50 ml with sterile distilled water and the silica particles were allowed to sediment by gravity for 5 h at room temperature. Forty three ml of unsedimented fine silica suspension were decanted. To reduce the pH of the silica suspension and to destroy contaminating DNA by depurination, 60 µl of concentrated HCl was added to the processed silica and the volume was made up to 50 ml. The silica suspension was autoclaved, aliquoted and stored at 4^0C. The silica suspension is stable for more than a year under these conditions of storage.

2.2 Generation of expression vectors:

2.2.1 HIV-1 subtype C Tat wild type and codon-optimized expression vectors:

The cloning of full-length wild type subtype C Tat vector was reported previously from our laboratory (Siddappa et al., 2007). Construction of the codon-optimized HIV-1 Tat gene, corresponding to the first exon of the consensus HIV-1 subtype C sequence, pDV2-Tat_{co}, has also been described previously (Ramakrishna et al., 2004). This vector, however, lacked exon II. Using the overlap PCR approach, we assembled the full-length Tat expression vector, by adding 30 aa of subtype C consensus sequence of the exon II to pDV2-Tat_{co}, in which all the codons have been optimized for the mammalian expression. Exon I was amplified using the forward primer N113 (5'-TAGAATTCGCCGCCGCCATGGAGCCAGTAGATCCTAACCTA-3') and reverse primer N458 (5'-

GTCGCCCTGGGTCCTAGGCAGGGGCTGCTTTGATATAAGATTTT-3'). The reverse primer was designed to contain a 20 bp overlap with exon I and a 24 bp overlap with exon II. The plasmid pDV2-Tat_{co} (containing only exon I of Tat) served as template for the amplification. Amplification conditions were as follows: 94°C for 1 min, 54°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 12 cycles. The amplicon was gel purified using a commercial kit (Qiagen, Hilden, Germany). Codon optimized consensus sequence of the second exon was synthesized as a single primer of 87 bases (N456) (5'-CCCCTGCCTAGGACCCAGGGCGACCCCACAGGCAGCGAGGAGAGCAAGAAG AAGGTGGAGAGCAAGACAGAGACAGACCCCTTCGAC-3'). Exon II amplicon of ~100 bp was obtained using the primers N456 and N457 (5'-AGAGTCTAGACTAGTCGAAGGGGTCTGTCTCTGTCTT-3') in a non-templated PCR using the amplification conditions 94°C for 1 min, 54°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 12 cycles. The exon I and exon II amplicons were gel purified and an overlap PCR was carried out, using the primers N113 and N457, which contained the enzymes sites EcoRI and XbaI, respectively. The final product of 350 bp was gel purified and directionally cloned between the EcoRI and XbaI sites, downstream of the CMV promoter on the vector pcDNA3.1 (+), generating the pCMV-Tat_{co} that contains full-length Tat with optimized codons.

2.2.2 Intron engineering into Tat_{co}:

We employed the overlap PCR approach to amplify a synthetic intron of 230 bp derived from vector pIRESpuro (# 6031-1, Clontech), and cloned it between the two exons of codon-optimized Tat on vector pcDNA3.1 (+) (Figure-5). The intron was first added to exon I of Tat followed by the addition of exon II thus generating full-length Tat with engineered intron between the exons. Amplification of the synthetic intron was performed using the primer pair

N521 (5'-GACCACCAAAATCTTATATCAAAGCAGGTGAGTACTCCCTCTC-3') and N522 (5'-TCGCCCGGGTCCTAGGCAGGGGCTGTGGAGAGAAAGGC-3') while 100 ng of the plasmid pIRESpuro (#6031-1, Clontech) served as the template for the reaction. The amplification conditions were as follows: 94°C for 1 min, 42°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 12 cycles. The amplicon of ~300 bp was gel purified and used as a mega-primer for the next round of PCR, where the plasmid pCMV-Tat_{co} served as the template. The initial 3 cycles of amplification was carried out in the absence of the primers. The amplification conditions were as follows: 94°C for 1 min, 42°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 12 cycles. The cycler was paused for a few seconds after the third cycle and the primer mix consisting of

N113 (5'-TAGAATTCGCCGCCGCCATGGAGCCAGTAGATCCTAACCTA-3') and N522 (5'-TCGCCCGGGTCCTAGGCAGGGGCTGTGGAGAGAAAGGC-3'), the forward and reverse primers respectively, was added to the vial. We obtained amplification of a 480 bp fragment from this PCR. The product comprising the first exon and the synthetic intron was gel eluted and mixed with the second exon amplicon of Tat, obtained as described above. The cycler was paused after the third cycle and the primer mix comprising of N113 (5'-TAGAATTCGCCGCCGCCATGGAGCCAGTAGATCCTAACCTA-3') and N457 (5'-AGAGTCTAGACTAGTCGAAGGGGTCTGTCTCTGTCTT-3') containing the restriction enzyme sites EcoRI and XbaI, respectively, was added to the vial and the amplification was continued for an additional 15 cycles. The amplicon of 560 bp comprising of exon I-synthetic intron-exon II, was gel eluted and directionally cloned into two different vectors under the control of the CMV or the EF-1α promoters, on the pcDNA3.1 (+) backbone to obtain pCMV-Tat_{int} and pEF-1α Tat_{int} constructs.

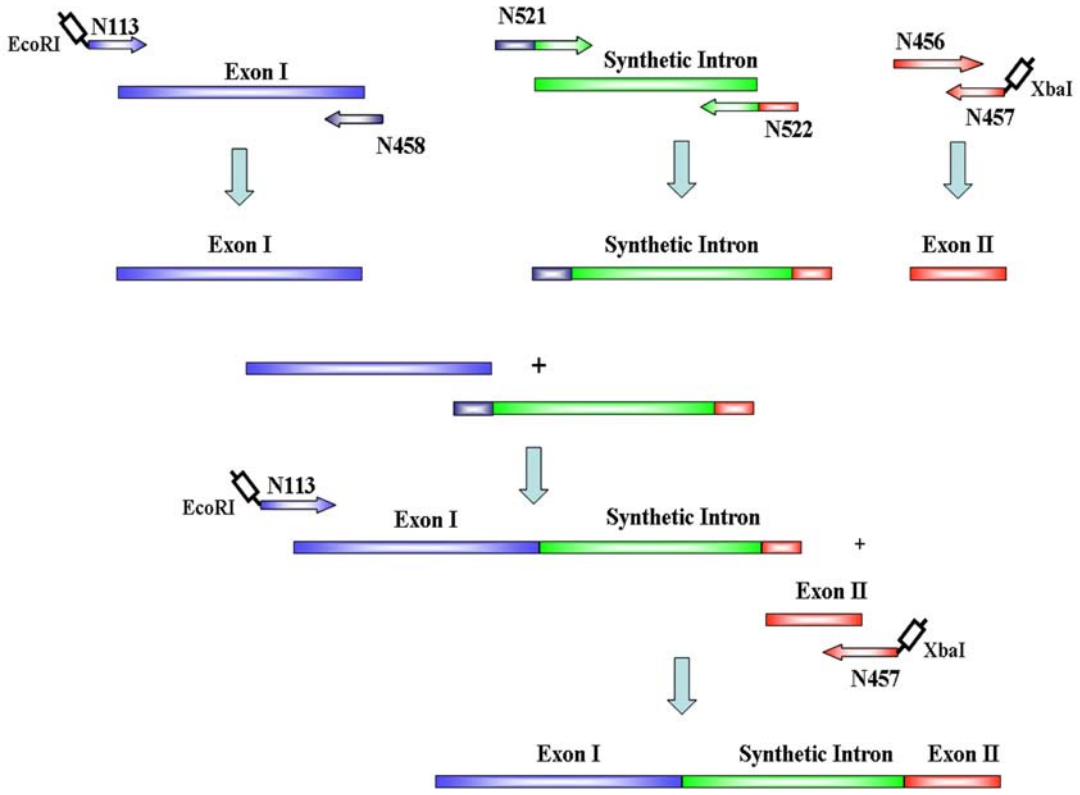


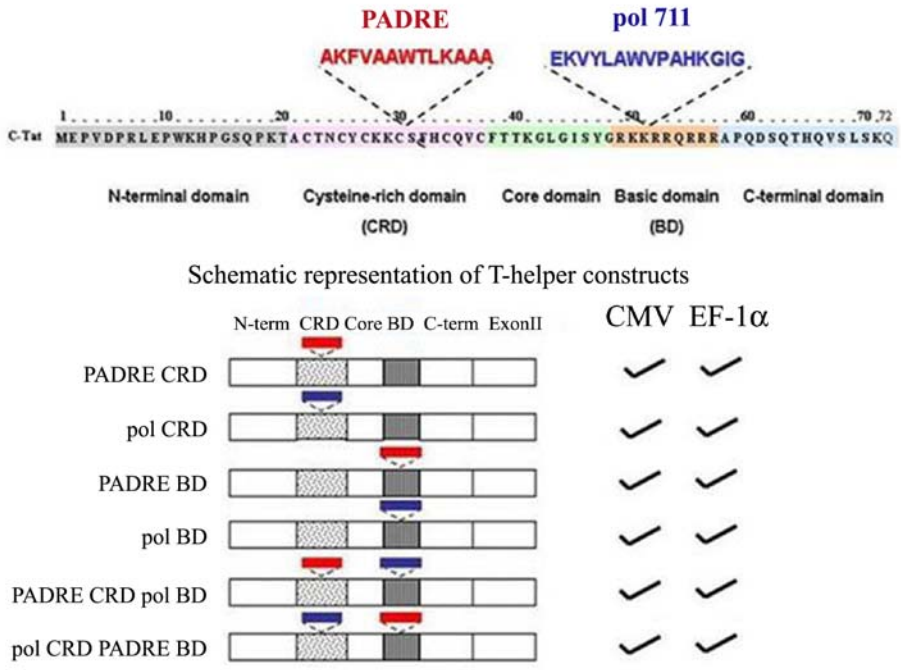
Figure-5: Schematic representation of inserting a synthetic intron between the two exons of Tat. Exon I, synthetic intron and Exon II were individually amplified as illustrated above. Overlap of Exon I and the synthetic intron was performed to generate a product of 500 bp. In the subsequent PCR final Tat expression construct was generated which was directionally cloned downstream of CMV and EF-1α promoter on the pcDNA3.1 (+) backbone.

2.2.3 Grafting T-helper epitopes HTL into Tat_{co}:

We used the overlap PCR approach, to graft the HTL epitopes into Tat_{co} constructs. Towards this, we chose two different epitopes the 'PADRE' epitope (Pan DR helper T-cell epitope, AKFVAAWTLKAAA) originally identified in the tetanus toxin that has been subjected to additional modification to bind most common HLA-DR molecules with high affinity (Alexander et al., 2000;Alexander et al., 1994b) and the pol 711 epitope (EKVYLAWVPAHKGIG) derived from HIV-1 RT polymerase, which has been reported to bind a large number of different HLA-DR (Wilson C et al., 2001) as

well as several murine class II molecules. As depicted in Figure-6, the HTL epitopes were engineered into the cysteine-rich domain (CRD, between residues C^{30} and S^{31}) and/or the basic domain (BD, between residues K^{52} and R^{53}), singly or in combination in both of the orientations.

(A)



(B)

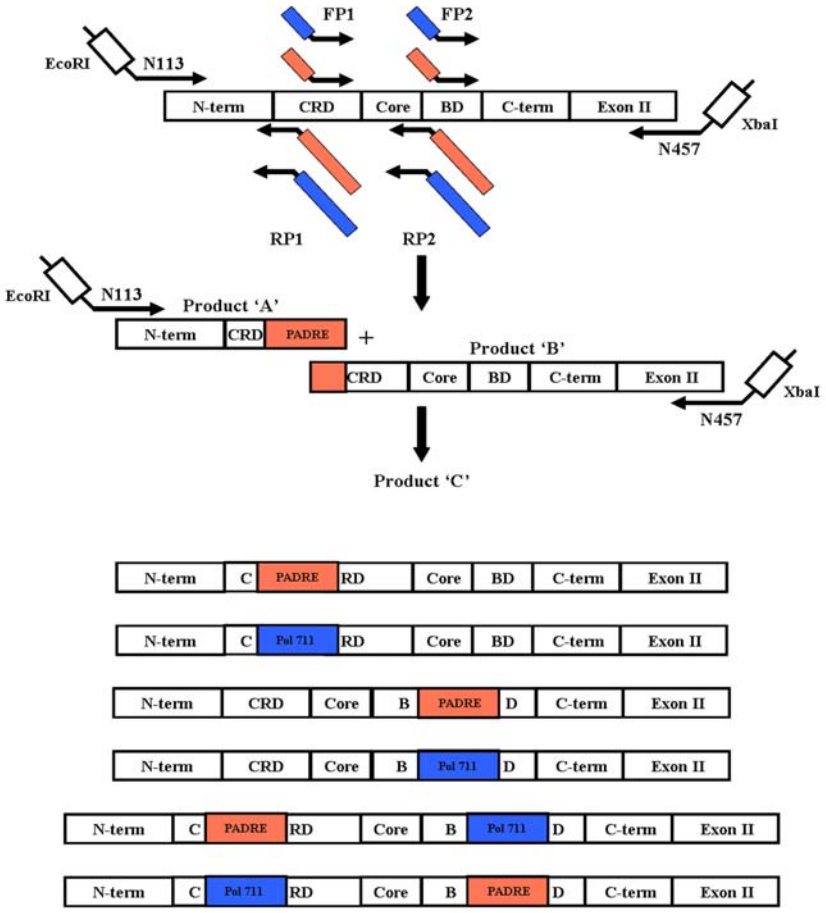


Figure-6: **(A)** Schematic representation of T-helper epitope grafting into Tat. Using overlap PCR approach, the PADRE and pol 711 epitopes have been grafted into the CRD and/or BD singly or in combination in both the orientations without disrupting the previously identified B- and CTL epitopes (Figure-23, p 119). Two independent panels of Tat expression vectors under the control of CMV or EF-1a promoters were constructed. The HTL epitope Tat constructs were also cloned into the bacterial expression vector pET21b. **(B)** Schematic representation of the overlap PCR approach used to graft two different HTL epitopes (PADRE and pol 711) into two different domains (CRD and BD) of Tat. The line diagram at the top depicts Tat domain structure. N113 and N457 are the forward and reverse primers common for all the constructs containing EcoRI and XbaI restriction enzyme sites for cloning of the expression cassettes, respectively. Black arrows depict the other primers and their orientation. These primers contain partial sequences of the HTL epitopes color coded (red and blue representing PADRE and pol 711 epitopes, respectively). The middle panel illustrates the extent of overlap between PCR-A and -B products. The

bottom panel presents the panel of Tat expression cassettes with the reconstituted HTL epitopes. The final Tat expression cassettes were directionally cloned into pcDNA3.1 (+) vector under the control of CMV or EF-1α promoters. The primer sequences have been presented in Table-1.

2.2.4 Table-1: Primers used to graft HTL epitopes into Tat:

Target Tat Domain	HTL Epitope Engineered	Primer	Sequence (5' – 3')
-	-	N113	TAGAATTCGCCGCCGCCATGGAGCCAGTAGATCCTAACCTA
-	-	N457	AGAGTCTAGACTAGTCGAAGGGGTCTGTCTCTGTCTT
CRD	PADRE	N648	CTGAAGGCTGCTGCC * AGCTACCACTGCCTGGTG
		N649	GGCAGCAGCCTTCAGCGTCCAGGCAGCGACAAACTTGGC * GCAGTGCTTGCAGTAGC
	Pol 711	N650	CACAAGGGCATTGGC * AGCTACCACTGCCTGGTG
		N651	GCCAATGCCCTTGTGGGCAGGCACCCATGCGAGGTACACCTTCTC * GCAGTGCTTGCAGTAGC
BD	PADRE	N679	CTGAAGGCTGCTGCC * CGGCGCCAGCGCCGGAGC
		N680	GGCAGCAGCCTTCAGGCTCCAGGCAGCGACAAACTTGGC * CTTCTTCCGGCCGTAGC
	Pol 711	N652	CCACAAGGGCATTGGC * CGGCGCCAGCGCCGGAGC
		N653	GCCAATGCCCTTGTGGGCAGGCACCCATGCGAGGTACACCTTCTC * CTTCTTCCGGCCGTAGC

Note: Top and bottom primers in a pair represent forward and reverse primers respectively. The reverse primers are presented as reverse complement. The asterisk represents the junction between two adjacent domains. Restriction enzymes have been highlighted by underlining.

The amplification conditions were as follows: 94°C for 1 min, 42°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 12 cycles for 12 cycles, for the first round of PCR, which yielded Product 'A' and Product 'B', and the amplification conditions for the second round of PCR that yielded product 'C' were 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 15 cycles. Product 'C' was directionally cloned between EcoRI and XbaI sites downstream of the CMV or the EF-1α promoter on the pcDNA3.1 (+) backbone.

2.2.5 Cloning of the full-length EF-1α promoter:

We procured the vector, pEF-BOS, containing the 1.2 kb upstream element of the EF-1α gene, as a gift from Dr. Shigekazu Nagata (Mizushima and Nagata, 1990). Placing the EF1-α promoter upstream of the Tat vector was achieved in two successive steps (Figure-12, p100) . In the first step, a larger fragment of the promoter (800 bp fragment) was directionally transferred upstream of Tat between AflII and EcoRI RE sites of the pcDNA3.1 (+) vector. In the second step, the reminder 400 bp 5' part of the EF-1α promoter was amplified using the forward primer, N570 (5'-ATAGACGCGTGTGAGGCTCAGGTCGCCGTCAGTGGGC-3') and reverse primer, N573 (5'-GGGCTTAAGCGCAAGGCGTCG-3'), which contained the RE sites MluI and AflII, respectively. The amplification conditions were as follows: 94°C for 1 min, 48°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 65°C for 30 sec, and 72°C for 1 min for 12 cycles. 100 ng of pEF-BOS plasmid was used as template for the amplification. The ~400 bp fragment was directionally cloned between the MluI and AflII sites, thus assembling the full-length EF-1α promoter on the pcDNA3.1 (+) backbone, in the place of the original CMV promoter.

2.2.6 Construction of the deletion mutants of the EF-1α promoter:

We undertook deletion analysis of the EF-1α intron I to reduce the overall length of the promoter on the one hand and to evaluate the suppressive role of the putative 'negative regulatory element' (NRE) or any other cis-acting regulatory element on the other hand. To generate deletion mutants within the first intron, we transferred the whole EF-1α promoter to pUC19 vector from pcDNA3.1 (+) using NruI and EcoRI sites. Diverse restriction enzymes, either singly or in combination, were used to generate a series of deletion mutations in the first intron as schematically shown in (Figure-14, p102). Each of the EF-1α promoter deletion mutants was subsequently returned to pcDNA3.1 (+) using MluI and EcoRI sites thus replacing the original CMV promoter in CMV-Tat_{co} and placing Tat_{co} under the control of the mutant EF-1α promoter. The

constructs were labeled after the RE used for the deletion. The constructs have been labeled as: ApaI-SacI, SacII, BglII-XhoI, PstI, PstI-SacII, and SacII-EcoRI (Int-less). The final construct lacks the intron completely.

2.2.7 Cloning of pCMV-DsRed2-Cyto:

pDsRed2-Nuc plasmid (#6981-1, Clontech) is a mammalian expression vector that encodes *Discosoma sp.* red fluorescent protein (DsRed2) fused with three copies of the nuclear localization signal (NLS) of the simian virus 49 large T-antigen. Using primers N668 (5'-CTAGTAGGTACCATGGCCTCCTCCGAGAACGTC-3') and reverse primer N669 (5'-AGCCGTTCTAGACTACAGGAACAGGTGGTGGCG-3'), we amplified the DsRed2 from pDsRed2-Nuc and cloned the ORF into pIRESpuro and thus placed the RFP expression under the control of the CMV promoter. Of note, a stop codon was introduced following the RFP ORF through the reverse primer. The amplification conditions were as follows: 94°C for 1 min, 42°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 15 cycles. The ~770 bp amplicon was Klenow-filled, digested with XbaI restriction enzyme and cloned directionally downstream of the CMV promoter on the plasmid pIRESpuro. After the cloning, the IVS, IRES and puromycin cassettes of the pIRESpuro plasmid were replaced by the DsRed2-Cyto. This RFP reporter plasmid served as internal control for monitoring the transfection efficiency in several experiments.

2.2.8 Expression of recombinant Tat proteins:

Cloning of B- and C-Tat bacterial expression vectors was previously reported from our laboratory (Siddappa et al., 2006). Using the protocol described in the publication, various recombinant Tat proteins have been expressed and purified. Briefly, individual bacterial colonies (*E. coli* BL21 (DE3)) were grown to 1 liter cultures in LB medium supplemented with 100 μg/ml ampicillin. Protein synthesis was induced by adding IPTG to a final concentration of 1 mM to the cultures at 0.4 OD and the cultures were incubated for additional 3 h. Under these experimental conditions, most of the

recombinant Tat protein is known to be present in the soluble fraction. Cells were harvested by high-speed centrifugation and resuspended in 20 ml of lysis buffer (20 mM Tris-HCl, pH 7.9; 10% glycerol; 0.4 mM, EDTA; 300 mM KCl; 0.1% IGEPAL; 10 mM imidazole; 0.2 mM PMSF and 1 mM DTT). Cells were lysed by sonication at 20 pulses at 3 min interval for 25 times (Branson Sonifier 450). The bacterial lysate was centrifuged at 16,000 rpm for 30 min at 4°C to remove cell debris. The lysate was stored frozen at -70°C until subsequent protein purification. Tat proteins were subsequently purified essentially as described (Siddappa et al., 2006).

2.2.9 Cloning of the murine GAPDH gene:

Total RNA was extracted from the peripheral blood lymphocytes using the Trizol reagent (Sigma, St. Louis, MO). Reverse transcription was carried out using oligo-dT and the following conditions: 30 min at 42°C and 5 min at 94°C. PCR reaction was carried out using the forward primer
N1042 (5'-AGTGCTGAATTCATGGTGAAGGTCGGTGTGAACGG-3') and the reverse primer N1043 (5'-GACATGTCTAGATTACTCCTTGGAGGCCATGTAGG-3'). The amplification conditions were: 94°C for 1 min, 54°C for 30 sec, and 72°C for 1 min for 3 cycles and 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min for 15 cycles. The amplicon of ~1 kb was gel eluted, digested with EcoRI and XbaI and cloned directionally into pcDNA3.1 (+), downstream of the CMV promoter. The plasmid was used as an internal standard in real-time PCR for the viral load assay.

2.3 Functional analyses of Tat:

2.3.1 Transfection of the mammalian cells:

HEK 293 cells, the primary human embryonic kidney cells (Graham FL et al., 1977), were transfected with mammalian expression vectors using the standard $CaCl_2$ method (Jordan et al., 1996). Cells were seeded at low confluency in 100 mm dishes one

day prior to the transfection. At the time of transfection, the cells were at 30 – 40% confluency. Ten µg of total plasmid DNA (#12381, Qiagen-EndoFree Plasmid Mega Kit) were mixed with 50 µl of 2.5 M $CaCl_2$ and the volume was made up to 500 µl with sterile distilled water. To the calcium-DNA mix an equal volume of 2X HBS was added. This solution was mixed by pipetting up and down a few times and immediately, the calcium phosphate-DNA suspension was sprinkled onto the cell monolayer using a micropipette. The plate was rocked gently to ensure even spreading of the suspension over the monolayer. Six to eight hours following the transfection, the culture medium was removed by aspiration, fresh growth medium was added to the wells and the dishes were returned to the incubator for 48 to 72 h of incubation or until the cells were harvested.

2.4 Reporter gene assays:

2.4.1 β-Galactosidase assay:

pCMVβ is a mammalian reporter vector that expresses β-galactosidase under the control of the human cytomegalovirus immediate early gene promoter (# 631719, Clontech). This reporter plasmid was co-transfected with Tat expression vectors for transfection normalization in some of the experiments. Cells from 100 mm dishes were harvested using a cell scraper, washed once and resuspended in 300 µl of PBS. For the β-gal assay, one third volume of the cell suspension was used. The cells were pelleted and lysed in 500 µl of lysis buffer (see Appendix) by incubating at 37^0C for 15 min. The cell lysate was centrifuged at 10,000 rpm for 10 min at room temperature to remove cell debris. The lysate was incubated at 50^0C for 45 min to inactivate the endogenous enzyme. The reaction buffer of a final volume of 300 µl contained 66 µl 1X ONPG, 3 µl 100X Mg^{2+} solution,(see Appendix) 201 µl of 0.1 M Sodium phosphate (pH 7.5) and 30 µl of the lysate. The reaction was incubated for 30 min at 37^0C. On development of a faint yellow color, the reaction was stopped by adding 500 µl of 1 M Na_2CO_3. The absorbance was recorded at 415 nm (Sambrook J and Russell DW, 2001).

2.4.2 Green fluorescent protein (GFP) analysis:

GFP expression was visualized 24-72 h after transfection under UV and images were captured at 20X magnification using a digital camera (DFC320, Leica, Heerbrugg, Germany). The images were processed with Image Manager software (IM50, Leica).

2.4.3 Secreted alkaline phosphatase (SEAP) analysis:

HIV-1 Tat constructs were co-transfected with a dual reporter plasmid, HIV-LTR-SEAP-IRES-GFP, that was previously described from our laboratory (Siddappa et al., 2007). HEK 293 cells were transfected using the $CaCl_2$ method (Jordan et al., 1996). The culture supernatant (200 µl) was sampled at regular intervals and stored at $–20^0$C until use. The reporter SEAP activity was measured using pNPP as the colorimetric substrate at 405 nm as described previously (Cullen BR and Malim MH, 1992). Briefly, 10 µl of the samples were incubated with 100 µl of 2X SEAP buffer (see Appendix) in a final volume of 200 µl, at 37 ^{0}C for 10 min. Twenty µl of pNPP substrate solution (1 mg/ml pNPP in 1XSEAP buffer) was added to the reaction mix, incubated at 37^0C (inside the ELISA reader, Molecular Dynamics) and read at 405 nm following 15 min of incubation.

2.4.4 Western blot analysis:

HEK 293 cells were transiently transfected with Tat expression vectors in 100 mm plates. Culture medium was aspirated and the plates were washed once with sterile PBS. The cells were scrapped and collected into a 1.5 ml vial. The cells were lysed by adding 250 µl of ice-cold TNN buffer (see Appendix) and incubated on ice for 30 min. The cell lysate was sonicated using a waterbath sonicator (Model D80, Ultrasonic Cleaner, MRC), three times with pulses lasting for 3 min and spaced apart by 2 min. The lysate was centrifuged at 10,000 rpm for 2 min at room temperature and the supernatant was transferred to a fresh vial. The protein concentration of the lysate was estimated at 280 nM (Nanodrop, ND 1000 Spectrophotometer, Thermo Scientific). The samples were boiled after adding appropriate amount of SDS loading buffer containing β-

mercaptoethanol and stored at –20^{0}C till further use. Up to 40-50 µl of the lysate was loaded per well and resolved on a 15% SDS polyacrylamide gel at a constant voltage of 150 V for 3 h at room temperature. The resolved proteins were blotted onto a PVDF membrane (# 162-0177, Immun-Blot PVDF, BIORAD) using a semi-dry transfer apparatus (#80-6211-86, Hoefer TE77, Semi-dry transfer unit, Amersham Biosciences) at constant current of 400 mA, for 1 h. All the transfections were internally controlled for experimental variation by including a CMVβ expression vector. β-galactosidase enzyme activity of the cell lysates was assayed by a chromogenic reaction as previously described. The blots were independently probed for β-actin expression using a commercial antibody (# A1978, Sigma, as per the manufacturer's instructions) to serve as the loading control.

HIV-1 Tat was probed using monoclonal antibodies (Mab) E6.4 or H-2 made in our laboratory. E6.4 and H-2 recognize the N-term 15 amino acids and exon II of Tat, respectively. The membranes were incubated at 4^{0}C for 3 h with the Mab diluted 12,500 times in 1% BSA in 1X PBS supplemented with 0.1% Tween-20. The membranes were then washed three times with 1X PBS containing 0.05% Tween-20. Following this, the membranes were incubated with anti-mouse HRP conjugate (Cat # 401253, CalBiochem) diluted 1:10,000 in 1XPBS supplemented with 0.05% Tween-20 for 3 h at 4^{0}C. The blots were developed using DAB (# D8001, Sigma) as the substrate.

2.5 Virus-related assays:

2.5.1 Preparation of the EcoHIV viral stocks:

HEK 293T cells were transfected with the 10 µg of EcoHIV plasmid, a gift from Dr. David J Volsky (Potash et al., 2005), using the $CaCl_2$ method (Jordan et al., 1996). The supernatant was harvested 48 h post-transfection. The supernatant was spun at 1,500 rpm for 10 min at room temperature, and then filtered through a 0.45 µm membrane filter and stored frozen at -80°C until use. The concentration of p24 in the supernatant was

quantified using a commercial ELISA kit (Cat # NEK050, Perkin Elmer, Inc). The virus was pelleted using high-speed centrifugation (SS34 rotor, 50,000 x g for 3 h), washed once and resuspended in saline. One hundred ng of p24 was used when the virus was injected through the tail vein for establishing the infection in mice (Potash et al., 2005). For viral challenge experiment, following immunization, 5 μg, of virus was administered through the intraperitoneal route (Saini et al., 2007).

2.5.2 Complement-mediated depletion of the $CD8^{+ve}$ T-cell subset:

Splenocytes (40 x 10^6 cells) from EcoHIV infected mice (BALB/c or C57BL/6) were incubated in 0.5 ml culture supernatant of anti-mouse CD8 (clone 3.155, generated in our laboratory) hybridoma for 1 h at 4^0C. The low temperature incubation was to minimize internalization of the receptors. The antibody-coated cells were incubated with rabbit serum (4-week old) as the source of complement at 1:20 dilution for 30 min at 37^0C. The cells were washed with RPMI-1640 containing 5% FBS. Viable lymphocytes were isolated by passing the cells through a ficoll gradient (#1083-1, Histopaque-1083, Sigma) according to the manufacturer's recommendations. The cells were collected into 15 ml tubes, washed three times with 5 ml RPMI-5% FBS. Cell number was determined and the cells were washed once with 1X PBS prior to flow cytometry.

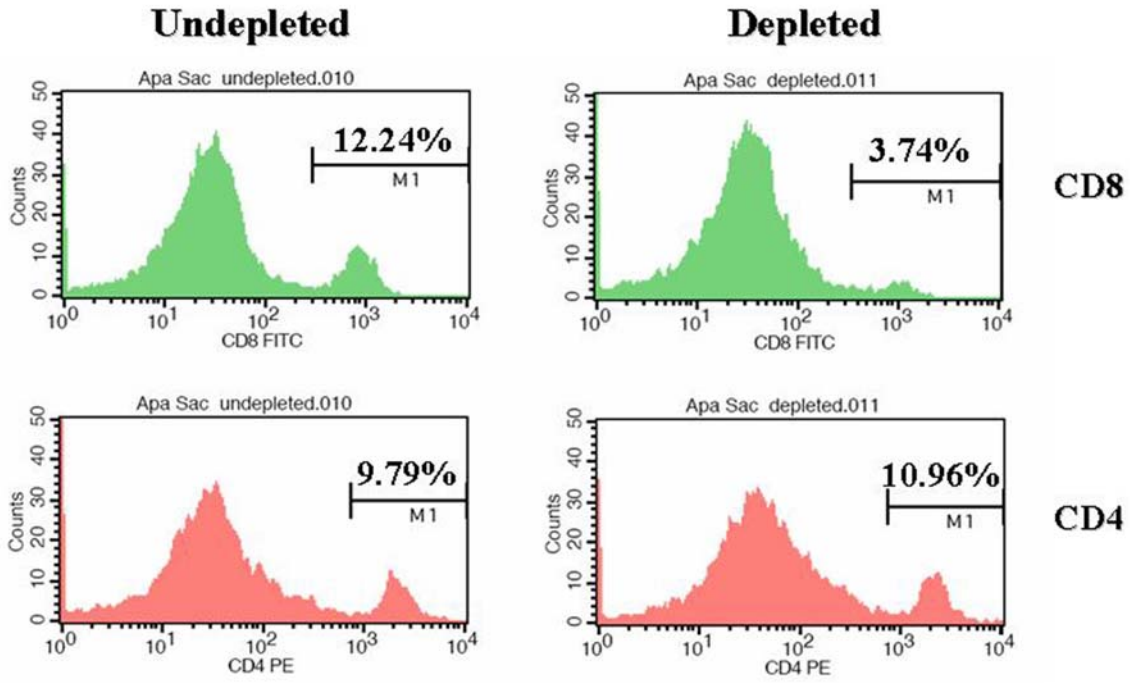


Figure-7: Flow cytometry analysis of the T-cell subsets. Control and depleted splenocytes were stained for the T-cell surface markers CD4 or CD8. $CD8^+$ T cells show marked depletion following the treatment while the $CD4^+$ T-cell population remains unaffected, thus ascertaining the efficiency of the depletion protocol.

2.5.3 p24 antigen capture ELISA:

HLM1 cells contain a Tat-deficient provirus integrated in the genome and produce small quantities of virus under normal culture conditions. However, when the functional Tat protein is supplemented extraneously, HLM1 cells generate large quantities of the virus. Tat-responsive nature of these cells offers a convenient way to measure the functional integrity of Tat-expression vectors. HIV-1 Tat constructs were transfected into HLM1 cells using a cationic reagent (#T201015, GenePORTER, Gene Therapy Systems, Inc) as per the manufacturer's instructions. The culture supernatant was sampled at regular intervals and stored at -20^0C until use. Empigen was added to each sample to a final concentration of 1% and the samples were incubated at 56°C for 30

min to inactivate the virus and to release the p24 antigen from the viral particles. A commercial HIV-1 p24 ELISA kit (# NEK050, Perkin Elmer Inc.) was used to quantify p24 in the culture supernatants, as per the manufacturer's instructions. The absorbance was measured at 490 nm using an ELISA reader (Molecular Dynamics). The transfection experiments were always performed in triplicate while HLM1 cells without Tat transfection served as the baseline control.

2.5.4 Confocal microscopy for intracellular p24:

Splenocytes, harvested from EcoHIV infected or control mice were subjected to complement-mediated depletion of the $CD8^+$ T cell subset as described above. The cells were washed once and fixed for 20 min in 2% paraformaldehyde in PBS, at room temperature. Fixed cells were permeabilized in PBS supplemented with 0.5% BSA and 0.5% saponin for 20 min. Cells were incubated for 20 min with a mouse anti-p24 monoclonal antibody G8.3, generated in our laboratory and tagged to Atto 488 (ReaMetrix, Bangalore, India). This was followed by 3 washes with PBS containing saponin (0.05%). Nuclei were stained with DAPI (100 µg/ml). The cells were then mounted with glycerol for image acquisition using a confocal microscope (LSM 510 Meta, Carl Zeiss, Germany).

2.6 Immunological assays

2.6.1 DNA immunization:

The immunization quality plasmid DNA was prepared using (#12381, Qiagen EndoFree Plasmid Mega kit) as per the manufacturer's instructions. The DNA was resuspended in endofree PBS (Manukirti, endotoxin <0.06 EU) and the endotoxin amounts were analyzed using a standard LAL assay (QCL-1000, Biowhittaker) and found to be within recommended limits (<0.1 EU/ µg DNA). 100 µg of the DNA was injected into the *tibialis anterior* muscle of mice that were 8-12 week old. Each immunization consisted of four or five mice per group. The immunization schedule involved one

primary immunization followed by a single booster or three boosters. Animals were housed and maintained in a facility adhering to the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of India and the Institutional Animal Ethics Committee (IAEC) of JNCASR.

2.6.2 Lymphoproliferation assay:

The DNA primed mice were sacrificed by cervical dislocation and the spleens were collected aseptically into a 60 mm sterile dish containing 2 ml of complete medium. To release splenocytes into the medium, the organ was crushed by using the hub of a 2 ml disposable syringe and by applying gentle pressure. The cells were collected into 15 ml screw-cap tubes (Corning) containing 5 ml of RPMI medium supplemented with 10% FBS. The cell debris was allowed to settle by gravity for 2 min and the upper layer containing the cells was carefully transferred to fresh tubes. Viable cells were counted using trypan blue exclusion technique. We typically recover 50-100 x 10^6 cells per spleen. Splenocytes were cultured in triplicate wells in a flat-bottom 96-well microplate at $2x10^5$ cells per well. Cells were activated in the presence of a mitogen, antigen or peptide pool for four days. In some experiments, the in vitro peptide activation was precluded and the cells were directly characterized for the immune function. A pool of six overlapping 20 mer peptides, with a 10 residue overlap between peptides, spanning the exon I of Tat, was used at 2 μg/ml concentration for cell activation as reported previously (Ramakrishna et al., 2004). Conconavalin A was used as positive control, for cell proliferation at a final concentration of 5 μg/ml. After incubation, the extent of cell proliferation was measured by adding [^{3}H] Thymidine, (10 μCi/ml) to the wells and cultures were incubated for additional 4 h at 37^0C for incorporation of the label. Plates were harvested using a cell harvester (Skatron, Norway). The filters were dried and radioactive counts were determined using a β-scintillation counter (Wallac, 1409).

2.6.3 ELISPOT assay:

ELISPOT Assay was performed for the Th1 cytokine IFNγ (mouse IFNγ ELISPOT, BD pharmingen) and the Th2 cytokine IL-4 (mouse IL-4 ELISPOT, BD pharmingen) before invitro stimulation. Briefly, the IFNγ-specific capture antibody (5 μg /ml) was adsorbed onto the PVDF-backed 96-well plates by incubating the antibody solution overnight at 4°C. The plates were blocked with complete medium for 2 h at room temperature, and primed splenocytes ($0.2x10^6$ cells) were added to each well. Antigen, peptide pool or a suitable mitogen was added at appropriate concentration to labeled wells and the cells in a final volume of 200 μl medium were incubated for 24 h. The cells were decanted by inverting the plate. To each well 200 μl of sterile distilled water was added and the plates were incubated in 4° C for 5 min to osmotically lyse the cells. Cell debris was removed by washing the wells three times with 1X PBS (200 μl per wash) and the wells were incubated with a biotinylated anti-IFNγ antibody (0.5 μg /ml) for 2 h. The plates were washed three times with 1X PBS containing 0.05% Tween-20, and the wells were incubated with HRP-conjugated avidin (0.25 μg/ml) for 1 h. Spots were developed using the substrate 3-amino-9-ethylcarbazole substrate solution (# 551951, BD Biosciences) and incubating the plates for 20 min at room temperature. A combination of phorbol myristate acetate (1 μg/ml) and ionomycin (0.5 μg/ml) was used as a positive control for cell stimulation. The spots were enumerated using the KS ELISPOT system (Carl Zeiss, Germany)**.**

2.6.4 Statistical analysis:

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 paired *t*-test. Differences were considered significant at $P < 0.05$.

2.7 Challenge models

2.7.1 EcoHIV challenge model:

David J Volksy's group reported an HIV-1 chimera virus that can replicate in the conventional mice, although in a restricted manner, therefore offering a powerful tool of investigation of viral replication, control and pathogenesis (Potash et al., 2005). The sequence encoding gp120 of subtype B NL4-3 (Adachi A et al., 1986) was replaced with that of the gp80, the envelope of ecotropic murine leukemia virus (MLV), a retrovirus that infects only rodents. The resulting chimeric virus construct, EcoHIV, productively infected murine, but not human, lymphocytes in culture. Adult, immunocompetent mice were readily susceptible to infection by a single inoculation of EcoHIV as shown by the detection of the virus in spleenic lymphocytes, peritoneal macrophages, and the brain (Hadas et al., 2007; Saini et al., 2007). The virus produced in animals was infectious as shown by passage in culture, and immunogenic as shown by induction of antibodies to HIV-1 Gag and Tat. EcoHIV challenge model offers a cost effective and simple alternative for the primate models to evaluate vaccine efficacy especially in a resource-poor setting. Importantly, a significant reduction in viral load could be suggestive of elicitation of protective immune response by the vaccine candidates.

2.7.1a Qualitative analysis of infection of mice by EcoHIV:

We elected to use this simple model to test the efficacy of our Tat vaccine constructs. We obtained the EcoHIV plasmid as a kind gift from Dr. David J. Volsky. We standardized the infection by EcoHIV in two different strains of mice, BALB/c and C57BL/6, essentially following the protocol outlined in their paper (Potash et al., 2005). Briefly, 293T cells transfected with the EcoHIV plasmid, served as the producer cell line, to obtain the viral stock. Cell-free viral stock was prepared using high-speed centrifugation. BALB/c or C57BL/6 mice were inoculated by an i.v. injection (tail vein)

of 0.1μg p24 EcoHIV. Six weeks after infection, or mock-infection, mice were euthanized and splenocytes were collected for analysis. The splenocytes are depleted of the $CD8^+$ T-cell subset using the complement mediated lysis procedure as described above. The $CD8^+$ -depleted splenocytes were fixed, permeabilized, stained for intra-cellular p24 and analyzed by confocal microscopy as described. Immunofluorescence staining for intracellular p24 revealed the presence of several brightly stained cells in infected mice, ascertaining efficient and progressive viral replication (Figure-8).

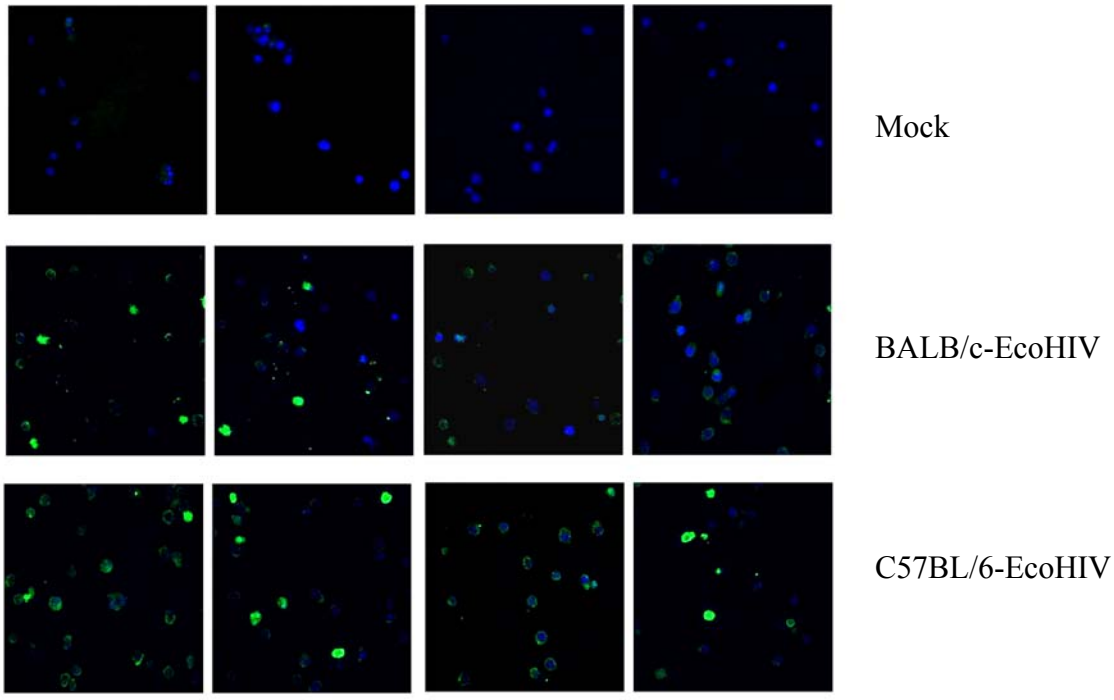


Figure-8: Direct immunofluorescence of mouse splenocytes for intracellular p24. Six weeks following the viral or mock infection, mouse splenocytes were collected, fixed, permeabilized and stained for HIV-1 p24 antigen (green fluorescence) with anti p24 monoclonal antibody G8.3. Nuclei were stained with DAPI (blue color). Each panel presents representative snaps of the same spleen from a single animal.

2.7.1b Viral load determination in real-time PCR:

For the EcoHIV viral challenge experiments, mice were infected with 5 μg p24 of EcoHIV/NL4-3 by intraperitoneal injection of cell-free virus, as previously described (Saini et al., 2007). One week after the challenge, mice were euthanized by cervical

dislocation and spleen and peritoneal macrophages were collected. DNA was isolated from spleen using a commercial column (# G10N0, Sigma) and the proviral load was determined using real-time PCR.

2.7.1c Standardization of the real-time PCRs for EcoHIV and GAPDH:

We standardized the real-time PCR for EcoHIV and glyceraldehydes phosphate dehydrogenase (GAPDH) using plasmid standards (Figure: 9). GAPDH amplification was used as the internal control and to normalize the proviral load. The primers N909 (5'-GGCCAAACCCCGTTCTG-3') and N910 (5'-ACTTAACAGGTTTGGGCTTGGA-3') used for the viral load PCR were located on gp80 of EcoHIV and amplify a 56 bp fragment between 7116-7172 (Potash et al., 2005). The amplification conditions were: 94°C for 1 min, 56°C for 30 sec, and 72°C for 30 sec for 40 cycles. Primers N1040 (5'-GAGCTGAACGGGAAGCTCACT-3') and N1041 (5'-CACGTCAGATCCACGACGGACACATTG -3') were employed for GAPDH amplification using the following reaction conditions: 94°C for 1 min, 66°C for 30 sec, and 72°C for 30 sec for 40 cycles. The amplicon obtained was 120 bp in length. The optimal annealing temperatures for these two pairs of primers were identified using a gradient PCR (MyCyler Thermal cycler, Biorad). The real-time PCR was performed using a commercial kit (#62345, Bio-Rad) and using Rotor-Gene 6000 (Corbett life Sciences, Australia) and the data were analyzed using Rotor-Gene 1.7.28 software.

A) EcoHIV real-time PCR standardization **B) GAPDH real-time PCR standardization**

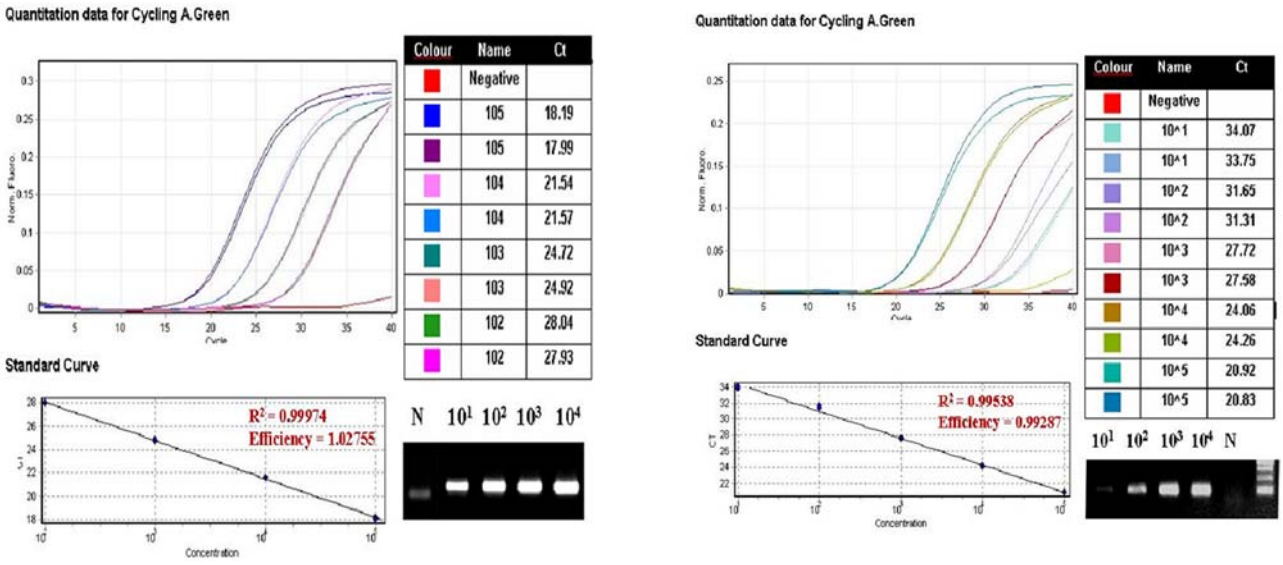


Figure-9: Standardization of real-time PCR for EcoHIV and GAPDH using plasmid templates. A dilution series of plasmids EcoHIV (left panel) and pCMV-GAPDH (right panel) were prepared as shown, in a dilution solution containing 50 µg/ml of salmon sperm genomic DNA. Each plasmid dilution was amplified in duplicate. Standard curves were constructed by plotting the threshold cycle (Ct) number on the *Y*-axis and copy number on the *X*-axis. The PCRs are highly reproducible and characterized by a high correlation coefficient values as shown. The equation describing this relationship extrapolates the gene copy number in experimental samples. The agarose gel electrophoresis profiles of the amplified products have also been shown.

2.7.2 The Tumor -challenge model

2.7.2a Tat-transfected stable cell lines:

To evaluate the quality of immune response, we plan to use autologous tumor challenge model in Tat-immunized mice. We established stable cell lines for Tat expression in two different lineages, P815 cells syngenic for BALB/c (H-2^d) and EL4 cells for C57BL/6 (H-2^b). Mammalian expression vector expressing Tat_{co} under the

control of the EF-1α promoter was transfected into the two syngenic cell lines, and selected against G418 drug selection marker. Five million cells were transfected with 10 μg of PvuI-linearized Tat vector by electroporation (Genepulser, BIORAD @ 950 μF and 240 V). Cells were seeded in DMEM supplemented with 10% FBS and incubated for 48 h without imposing the drug selection. Selection pressure was imposed two days after the transfection starting at 200 μg/ml concentration of G418 (see Appendix) which was gradually increased to 1 mg/ml in subsequent passages. The transfected cells, but not the parental cells, were stable at 1 mg/ml G418 concentration. Expression of Tat in the stably transfected cells was confirmed by western blot analysis (Figure-10A).

2.7.2b Establishment of tumors in mice:

Parental EL-4 or Tat-expressing EL-4 cells (EL4-Tat), $1X10^6$ cells per injection, were inoculated intradermally into the hind flanks of C57BL/6 mice. The growth of the tumor was measured using a Vernier calipers. Both the parental and Tat-expressing cells efficiently induced tumors in the mice (Figure-10B). At present, evaluation of this tumor challenge model is in progress in Tat-immunized mice. A schematic representation of the tumor challenge model is depicted in Figure-11.

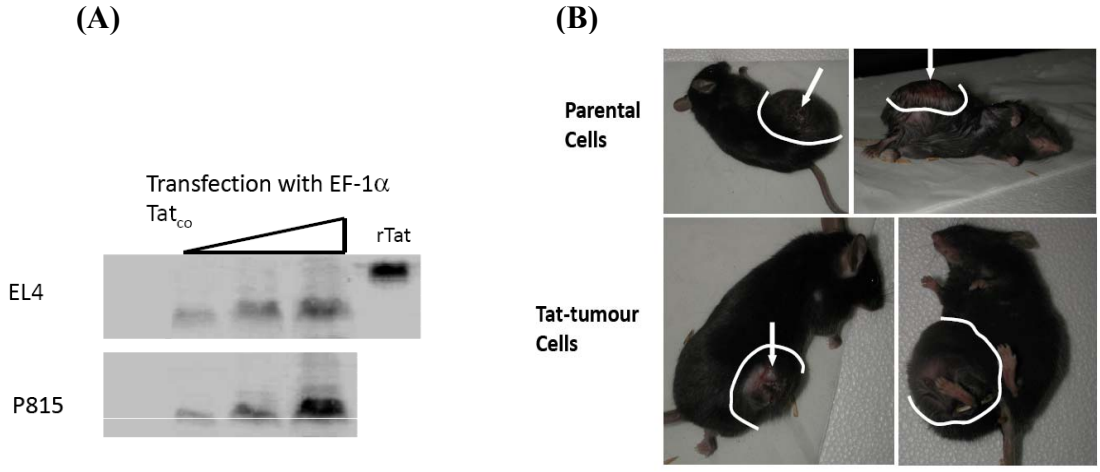


Figure-10: Tat expression from stably transfected cells. **(A)** Two mouse cell lines, P815 (syngenic for BALB/c) and EL4 (syngenic for C57BL/6) were established for stable Tat expression. Western blot analysis was performed using Mab E6.4 to confirm Tat expression. **(B)** Establishment of tumors in

C57BL/6 mice. 1x 10^6 EL4 or EL4-Tat cells were injected into the hind flanks of the mice intradermally. Tumor induction was seen in both the cases.

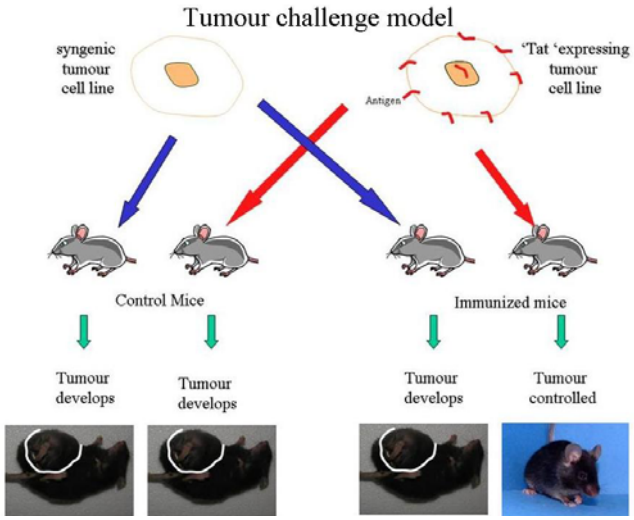


Figure-11: Schematic representation of the tumor challenge model**.** In control mice, both parental and the Tat-expressing tumor cells are expected to develop tumors. In contrast, in Tat-immunized mice, only the parental but not Tat-expressing tumor cells can establish tumors provided the immune response elicited is protective in nature.

2.8 References

Cullen BR and Malim MH: Secreted placental alkaline phosphatase as a eukaryotic reporter gene. *Methods Enzymol*, **216**, 362-368 (1992).

Graham FL, Smiley J, Russell WC and Nairn R: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*, **36**, 59-74 (1977).

Hadas E, Borjabad A et al, Testing antiretroviral drug efficacy in conventional mice infected with chimeric HIV-1. *AIDS*, **21**, 905-909 (2007).

Inoue H, Nojima H and Okayama H: High efficiency transformation of Escherichia coli with plasmids. *Gene Nov 30 ;96 (1):23-8*,(1990).

Jordan M, Schallhorn A and Wurm FM: Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res*, **24**, 596-601 (1996).

Lakshmi R, Baskar V and Ranga U: Extraction of superior-quality plasmid DNA by a combination of modified alkaline lysis and silica matrix. *Anal Biochem*, **272**, 109-112 (1999).

Mizushima S and Nagata S: pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res*, **18**, 5322(1990).

Potash MJ, Chao W et al, A mouse model for study of systemic HIV-1 infection, antiviral immune responses, and neuroinvasiveness. *Proc Natl Acad Sci U S A*, **102**, 3760-3765 (2005).

Ramakrishna L, Anand KK, Mohankumar KM and Ranga U: Codon Optimization of the Tat Antigen of Human Immunodeficiency Virus Type 1 Generates Strong Immune Responses in Mice following Genetic Immunization. *J Virol*, **78**, 9174-9189 (2004).

Saini M, Hadas E, Volsky DJ and Potash MJ: Vaccine-induced protection from infection of mice by chimeric human immunodeficiency virus type 1, EcoHIV/NL4-3. *Vaccine*, (2007).

Sambrook J and Russell DW: Molecular Cloning. A laboratory manual (Cold Spring Harbor Laboratory Press, New York) 17.48-17.51(2001).

Siddappa NB, Kashi VP et al, Gene expression analysis from Human Immunodeficiency Virus type-1 subtype C promoter and construction of bicistronic reporter vectors. *AIDS Res Hum Retroviruses*, **23**, 1268-1278 (2007).

Siddappa NB, Venkatramanan M et al, Transactivation and signaling functions of Tat are not correlated: biological and immunological characterization of HIV-1 subtype-C Tat protein. *Retrovirology*, **3**, 53(2006).

3. Characterization of the Elongation Factor-1α (EF-1α) promoter

3.1 EF-1α promoter has not been evaluated adequately in genetic immunization:

The human cytomegalovirus major immediate early (CMV) promoter/enhancer is one of the strongest promoters known. This promoter is most frequently used in gene therapeutic applications, as it is highly functional in cell lines and tissues of diverse origin. Expression from the CMV promoter, like from other viral regulatory elements, nevertheless, is downregulated in several physiological contexts either through the interferon-mediated (Gribaudo et al., 1993), DNA methylation (Prosch et al., 1996) pathways or mechanisms yet to be defined. Given its viral origin, it is possible that host cells could have evolved molecular strategies to silence such elements. Gene expression form the CMV promoter often is not only transient (Yew et al., 1997) but also irregular resulting in the failure to establish stable cell lines (Tokushige et al., 1997; Teschendorf et al., 2002). Inconsistency of gene expression from the CMV promoter made it necessary to search for an alternative promoters with improved expression properties. The promoter element of the Elongation factor-1α (EF-1α) gene has been shown to perform as efficiently as the CMV promoter, or even superior in a few cases (Tokushige et al., 1997; Song et al., 1998; Teschendorf et al., 2002). Similar to the CMV promoter, gene expression form the EF-1α promoter is ubiquitous and not restricted to any particular cell lineage (Kim et al., 1990; Chung et al., 2002; Gill et al., 2001; Nakai et al., 2005). In parallel studies, establishing stable cell lines was found to be superior with the EF-1α promoter as compared to the CMV promoter (Tokushige et al., 1997). Importantly, unlike the CMV promoter, EF-1α promoter was not subjected to gene silencing through the IFN-mediated or CpG methylation pathways (Teschendorf et al., 2002). Given the ubiquitous and consistent pattern of gene expression, EF-1α promoter has been proposed to be the promoter of choice for gene therapeutic and targeting strategies. Paradoxically, except for a single publication, (Nishimura et al., 1999), the potential of EF-1α has not been evaluated for genetic immunization, although this promoter has been used in gene therapeutic approaches as mentioned above. With this objective in mind, we constructed DNA expression vectors containing the EF-1α promoter and compared its performance with that of the CMV promoter. The objectives of this analysis are two-fold, to delineate

important regulatory elements of the EF-1α promoter on the one hand and to evaluate its performance in immunologically important cell lines such as the T-cells, monocytes and myoblasts on the other hand. A previous publication identified a negative regulatory element (NRE) in the intron of the EF-1α promoter in HeLa cells (Wakabayashi-Ito and Nagata, 1994b). Performing a similar analysis in immunologically relevant cells is important which will be attempted through the present study.

3.2 Construction of Tat DNA-expression vectors:

Two identical mammalian Tat-expression vectors were constructed under the control of the CMV promoter or the full-length EF-1α promoter. Construction of the codon-optimized HIV-1 Tat gene corresponding to the first exon of the consensus HIV-1 subtype C, has been described previously (Ramakrishna et al., 2004). Using the overlap PCR approach, we assembled the full-length Tat expression vector, in which all the codons have been optimized for mammalian expression. The amplified product was directionally cloned between EcoRI and XbaI sites on the pcDNA3.1 (+) backbone downstream of the CMV promoter, thus generating the CMV-Tat_{co} expression vector used in this study.

We generated the expression vector EF-1α Tat_{co} as follows. We procured the vector, pEF-BOS, containing the 1.2 kb upstream element of the EF-1α gene, as a gift from Dr. Shigekazu Nagata (Mizushima and Nagata, 1990). The promoter region of EF-1α gene consists of the 5' upstream region (203 bp), the first exon (33 bp), the first intron (943 bp) and 10 bp of the part of the second exon located 20 bp upstream of the ATG initiation codon. Our first objective was to move the EF-1α promoter cassette to a Tat-expression vector on the pcDNA3.1 (+) vector backbone. Placing the EF-1α promoter upstream of the Tat vector was achieved in two successive steps. In the first step, a larger fragment of the promoter (800 bp fragment) was directionally transferred upstream of Tat between AflII and EcoRI enzyme sites replacing the original CMV promoter on the vector. In the second step, the reminder 400 bp 5' part of the EF-1α promoter was amplified and the PCR fragment was directionally cloned between the MluI and AflII restriction sites, thus generating the intact promoter (Figure-12). In the final format, HIV-1 subtype C Tat was placed under the control of CMV or EF-1α promoter to permit

comparison of the performance of these regulatory elements under identical experimental conditions (Figure-13A).

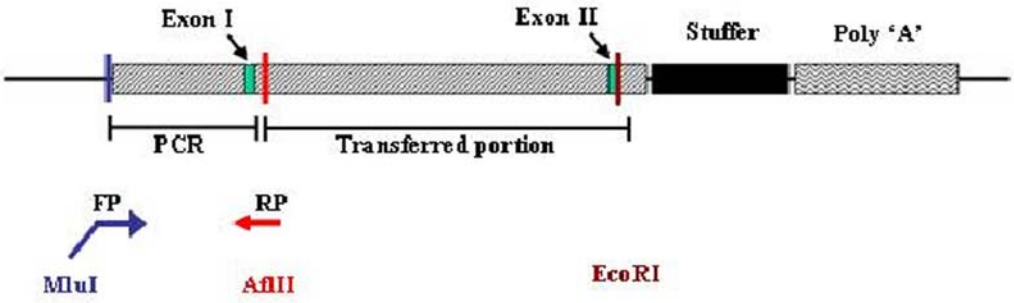
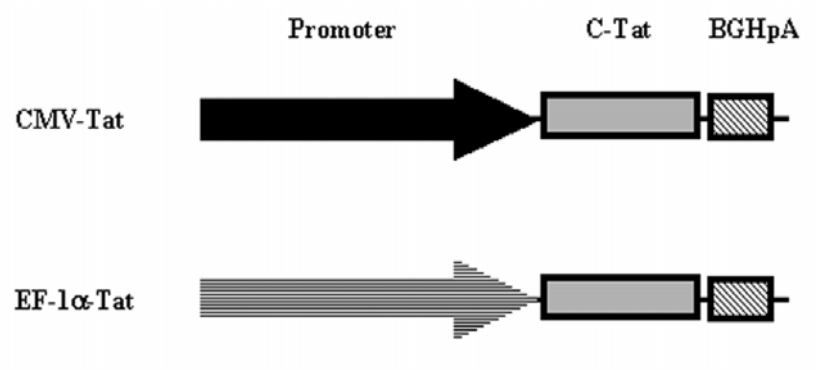


Figure-12: Sub-cloning of the EF-1α promoter. Schematic representation of the EF-1α promoter in the parental vector pEF-BOS. The full-length EF-1α promoter spanning up to the EcoRI site was transferred in two sequential steps in a Tat-expression vector to replace the CMV promoter (see Figure-13A). First, the 3' part of the promoter was transferred using restriction enzymes AflII and EcoRI to substitute for the CMV promoter. Second, the 5' part of the promoter was amplified with primers (FP with an MluI site overhang and RP spanning over the AflII site) and cloned directionally to reconstitute the full-length EF-1α promoter on pcDNA3.1 (+) backbone to drive the expression of Tat (see Figure-13A) FP: forward primer RP: reverse primer.

The functional integrity of the EF-1α promoter was confirmed in transient transfection assays. A dual-reporter expression vector HIV-LTR-SEAP-IRES-GFP expressing simultaneously two independent reporter genes, GFP and SEAP, in response to Tat, was reported previously from our laboratory (Siddappa et al., 2007). This vector was used in the reporter assays throughout. HEK 293 cells were cotransfected with the reporter vector and one of the two Tat-expression vectors, CMV-Tat or EF-1α Tat or the parental vector, using the $CaCl_2$ transfection protocol (Jordan et al., 1996). Expression of GFP (Figure-13B, left panel) and Secreted Alkaline Phosphatase (Figure-13B, right panel) was monitored 48 h after the transfection using fluorescent microscopy and a colorimetric assay (Cullen and Malim, 1992), respectively. GFP expression was also quantitatively determined using flow cytometry when required. EF-1α promoter was not only functional in both the assays but also performed as efficiently as the CMV promoter (Figure- 13B).

(A) Schematic representation of the expression vectors



(B) Transactivation assays for Tat expression

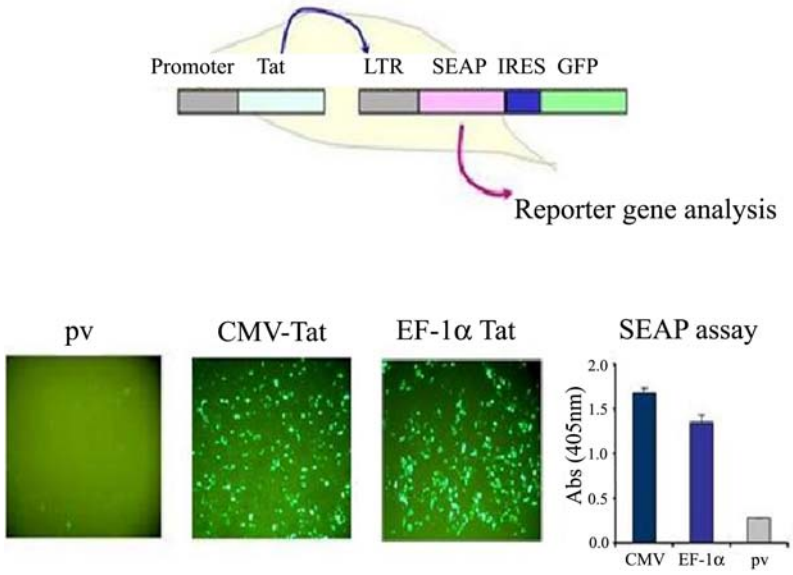


Figure-13: Schematic representation of the expression vectors. Expression of Tat from CMV or EF-1α promoter could be qualitatively evaluated by transactivation of one of the two reporter genes that are expressed simultaneously from the reporter vector. The expression of GFP was monitored using a fluorescent microscope and that of SEAP by colorimetry. pv: parental vector.

3.3 Construction of deletion mutants of the EF-1α promoter:

A previous publication identified a negative regulatory element (NRE) in the first intron of the EF-1α promoter using HeLa cells (Wakabayashi-Ito and Nagata, 1994a). Confirmation of such a negative regulatory element in immunologically relevant cells and subsequent elimination of this element, if confirmed, could be critical for optimization of this cellular promoter for gene expression.

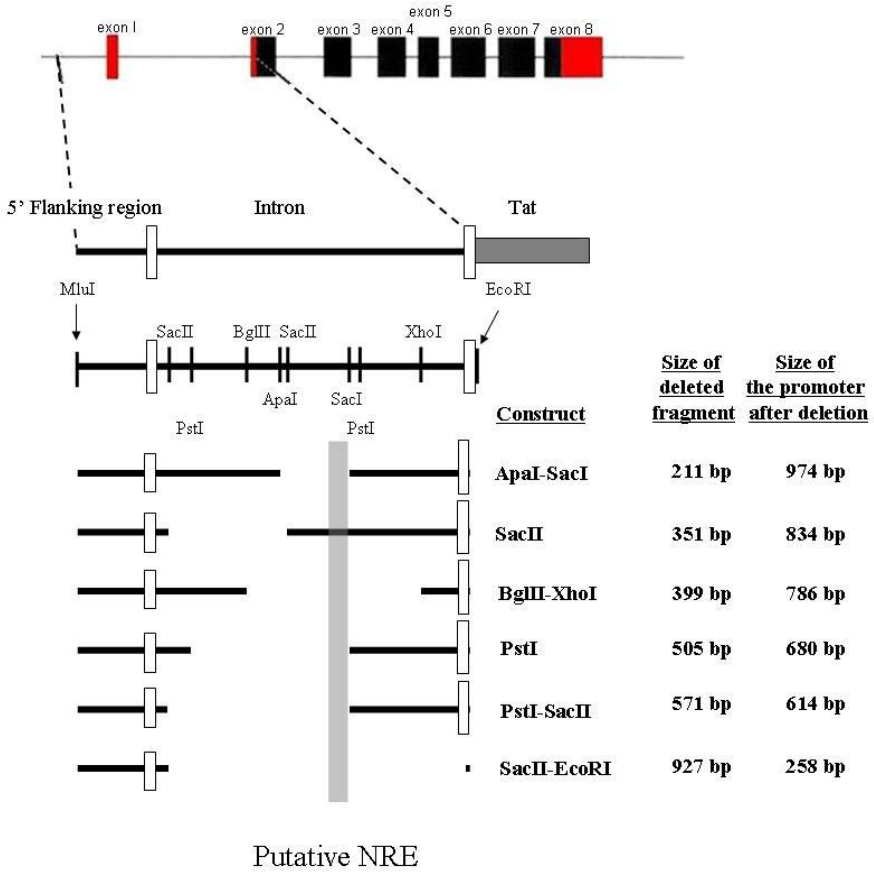


Figure-14: Schematic representation of the EF-1α promoter deletion constructs. The top panel represents the chromosomal organization of human EF-1α gene. The fragment indicated by the dotted lines represents the part of the promoter subcloned and subjected to the deletion analysis. Open boxes represent the exons. The approximate location of the restriction enzymes have been indicated. In the bottom panel, various deletion construct of the promoter have been shown. The combination of RE used, names of the deletion mutants, the size of the fragments and the size of the final promoter constructs have been indicated. The putative NRE has been highlighted by shading.

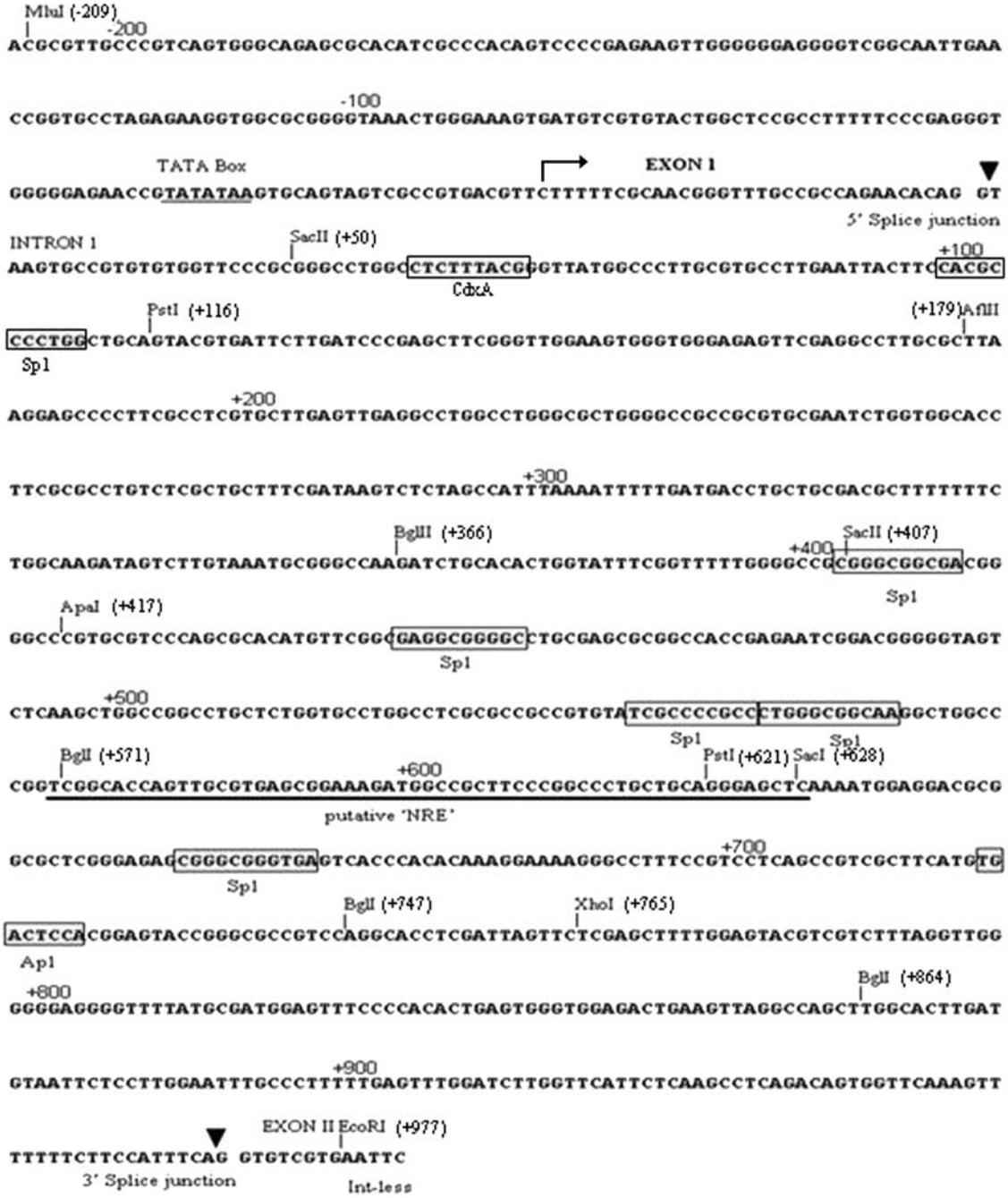


Figure-15: EF-1α promoter sequence and the TFBS. The sequence represents the full-length EF-1α promoter spanning between -203 to +977 bases (Uetsuki et al., 1989; Wakabayashi-Ito and Nagata, 1994b). The sequence coordinates have been presented with respect to the transcription start site (shown using an arrow). Filled arrow heads represent the splice donor and acceptor sites. TATA box has been underlined.

Important TFBS are shown in boxes. Restriction sites used for the deletion analysis and their coordinates have been shown in parentheses. The putative NRE has been underlined.

We undertook deletion analysis of the intron of the EF-1α promoter to reduce the overall length of the promoter on the one hand and to evaluate the suppressive role of the putative NRE if confirmed or any other cis-acting regulatory element on the other hand. To generate deletion mutants within the first intron, we transferred the EF-1α promoter to pUC19 vector from pcDNA3.1 (+) using NruI and EcoRI. Combinations of restriction enzymes were used to generate a series of deletion mutations in the first intron as schematically shown in Figure-14. The promoter sequence, transcription factor binding sites (TFBS), and restriction sites have been schematically depicted in Figure-15. Each of the EF-1α promoter deletion mutants was subsequently returned to pcDNA 3.1 (+) using MluI and EcoRI sites thus replacing the original CMV promoter in CMV-Tat and placing Tat under the control of the mutant EF-1α promoter (Figure-14). Expression of Tat from all the EF-1α deletion constructs was confirmed in a transient reporter assay (Figure-16A and C).

3.4 Functional evaluation of EF-1α promoter deletion constructs:

We constructed a panel of six deletion mutant promoters each driving the expression of Tat. We tested the expression of EGFP and SEAP from this panel and compared expression levels with the parental EF-1α promoter and the CMV promoter, in HEK293 cells, in a transient transfection assay (Figure-16). CMV-DsRed2 (RFP) reporter plasmid was used as an internal standard for normalizing the transfection efficiency.

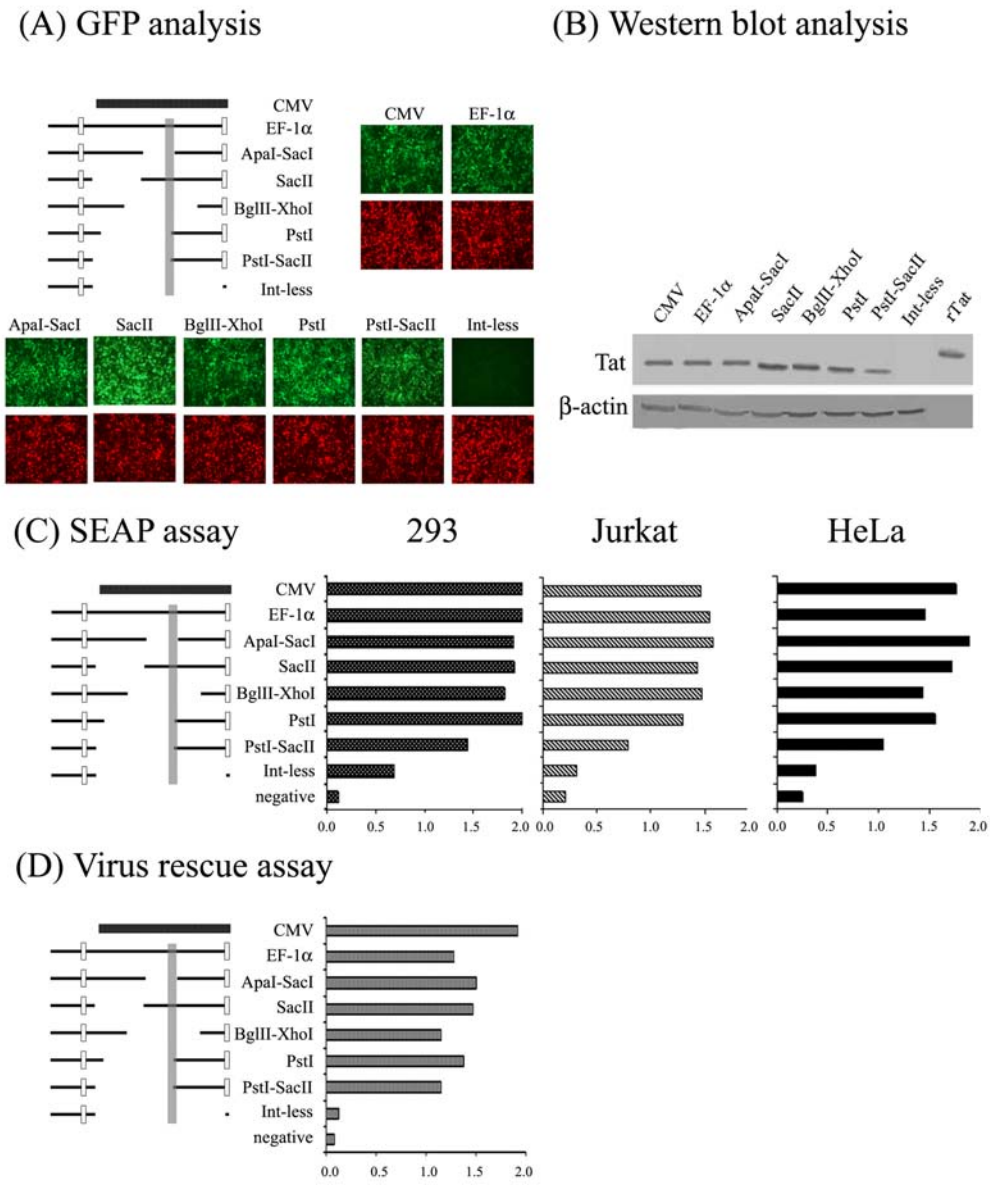


Figure-16: Transient expression of the reporter genes by the EF-1α deletion promoter constructs. **(A)** GFP analysis: HEK 293 cells were co-transfected with the dual reporter plasmid, LTR-SEAP-IRES-GFP, and one of the various Tat-expression constructs driven by the CMV or EF-1α promoter, or the deletion constructs of the EF-1α promoter. CMV promoter driving DsRed2 (RFP) was used as internal control for transfection. **(B)** Western blot detection of Tat. Tat-specific monoclonal antibody E6.4 was used for the detection of Tat. rTat, recombinant Tat protein used as a positive control that contains HIS-Tag at the C-terminal end hence migrates slower. Beta-actin expression was used as the loading control. **(C)** SEAP assay in three different mammalian cells: Expression of SEAP was monitored at 24, 48 and 72 h and the data for

the 48 h time point have been presented. Identical results were obtained at other time points. **(D)** Virus complementation assay. HLM-1 cells, containing a single copy of a Tat-defective provirus, were transfected with 1 μg of various Tat-expression vectors using the $CaCl_2$ method. Levels of p24 secreted into the medium were monitored using a commercial kit.

In the transient reporter assay, we found that all the constructs, with the exception of the intron-less promoter, regulated expression of the reporter gene GFP (Figure-16A). Although marginal differences were observed between promoters, these differences did not appear to be significant. Given the nature of high level gene expression from HEK 293 cells, it is possible that minor variations may not be apparent in this expression system. A western blot analysis on the cell extracts confirmed Tat expression from all the vectors, at broadly comparable levels, whereas Tat expression was absent from the intron-less promoter confirming the reporter gene analysis (Figure-16B). For a quantitative analysis, we monitored the levels of SEAP secreted into the medium at different periods from transfected HEK 293 cells. There was a dose response in SEAP expression proportionate to the quantity of Tat used (400 and 1,200 ng) (Figure-16C, left panel shows data for 48 h at 1,200 ng). Both CMV and EF-1α promoters expressed SEAP at comparable levels at all the time points tested. All the promoter deletion constructs, with the exception of the intron-less promoter, regulated expression of SEAP at levels comparable to the full-length EF-1α promoter whereas no gene expression was observed from the intron-less promoter. The SacII-promoter which contains the 'NRE', expressed as much SEAP as other mutants that lack this putative 'negative' element. The PstI-SacII-promoter which is the smallest in the panel, expressed the reporter gene at levels below the full-length EF-1α or the CMV promoter. To examine the effect of the host cell variation on gene expression, we evaluated the promoter panel in two other cell lines, Jurkat human T-lymphocytes and HeLa cells (Figure-16C), middle and right panels, respectively). The EF-1α promoter was originally evaluated in the HeLa cells (Wakabayashi-Ito and Nagata, 1994b). In both of these cell lines, the pattern of reporter gene expression remained identical to that of 293 cells. Promoters with smaller deletions (ApaI-SacI, SacII, and BglII-XhoI) expressed SEAP at levels comparable to the full-length EF-1α promoter. The BglII-XhoI promoter did not show significant reduction in SEAP although this promoter contained the putative 'NRE'. Only the PstI-promoter and

the PstI-SacII promoter expressed progressively decreasing quantities of SEAP as compared to the full-length EF-1α promoter in these two cell lines suggesting deletion of important TFBS. The previous study identified several Sp1 and Ap1 binding sites in the EF-1α promoter although occupancy of several of these predicted TFBS was not experimentally confirmed (Wakabayashi-Ito and Nagata, 1994b). Of note, PstI deletion (+116 to +621) in our strategy should have deleted five putative Sp1 binding sites in the EF-1α promoter thus significantly reducing gene expression from the mutant promoter even if some of these Sp1 sites are functional. Likewise, PstI-SacII deletion (+50 to +621) should have deleted an additional sequence (+50 to +116) from the promoter further compromising the strength of the promoter. We did an analysis to identify any potential TFBS in this sequence (+50 to +116) using TFSEARCH ver 1.3, and found one each Sp1 and CdxA sites at this location. CdxA is an important transcription factor known to positively regulate gene expression from several cellular promoters (Margalit et al., 1993). The promoter deletion analysis thus identified an important TFBS EF-1α promoter at +60 position. The functional significance of the CdxA and the new Sp1 site at +100 position remains to be confirmed.

Wakabayashi-Ito and Nagata subjected the EF-1α promoter to extensive deletion analysis and measured chloramphenicol acetyl transferase (CAT) reporter gene expression as a measure of promoter function in HeLa cells. This analysis identified several putative TFBS including 4 Sp1 sites and 1 Ap1 site as well as a putative negative regulatory element. We generated a different deletion promoter series and evaluated the mutant promoter in HeLa cells and two additional cell lines. Our analysis did not support the characterization of the sequence spanning +570 to +629 as a NRE. Deletion mutant promoters without the NRE (ApaI-SacI, BglII-XhoI, PstI) did not express the reporter genes at levels significantly different from the promoters with the NRE (the full-length EF-1α promoter and ApaI-SacI construct) in all the three different cell lines tested. For a more precise evaluation of the NRE, a promoter with specific deletion only in the NRE sequence is needed. Unfortunately, neither the series constructed by Wakabayashi-Ito and Nagata nor the one constructed by us contains such a construct. Given that our analysis comprises of three different cell lines, and that regardless of the presence or absence of the putative NRE sequence, the promoters broadly function at comparable levels, we

believe that the sequence spanning +570 to +629 cannot be defined as a negative regulatory element. Subsequent immunization analysis in the present work also supports our premise (Figur 17, p110). Furthermore, experiments are presently in progress to evaluate our promoter deletion series in cells of immunological significance including myocytes, monocytes, THP1 cells etc.

3.5 Virus complementation assay:

We took advantage of the EF-1α promoter deletion series driving expression of Tat by transactivating the Tat-defective provirus in HLM-1 cells (Sadaie and Hager, 1994) to evaluate gene expression from the promoters. Unlike the reporter gene expression analysis carried out above, Tat-mediated transactivation of the viral LTR of a provirus is experimentally more relevant and resembles the natural context of viral activation. Proviral activation monitored by measuring p24 in the culture medium at different time points confirmed the observations with reporter genes above (Figure-16D). With the exception of the intron-less promoter, all other promoters including the shortest PstI-SacII construct generated p24 at comparable levels. The minor variations seen in the p24 levels were not statistically significant. Put together, our reporter gene expression and provirus transactivation analyses suggest that probably no sequence in the intron of the EF-1α promoter modulates gene expression negatively. Additionally, the various TFBS in the intron may not contribute significantly for the transactivation from the upstream elements of the promoter since even the largest deletion of the intron (PstI-SacII construct) did not affect gene expression from the promoter significantly although most of the TFBS have been eliminated. Thus it appears that the other functions of the intron are more important for the transactivation from the promoter including the transcript splicing and stabilization. The intron-less promoter was inactive in most of the experiments suggesting the functional significance of the intron primarily remains in the transcript splicing. This observation, however, must be confirmed in primary cells and such experiments are presently in progress.

3.6 Genetic immunization to evaluate the promoter panel:

Each construct in the EF-1α promoter panel regulates the expression of the full-length HIV-1 subtype C Tat. Endotoxin-free plasmid DNA (100 μg) was injected into the tibialis anterior muscle of mice in the absence of additional immune-modulators like bupivacaine. Each immunization consisted of four or five female C57BL/6 mice per group, 8 to 12 weeks old (immunization in BALB/c presently in progress). The immunization schedule involved one primary immunization followed by one or three booster immunizations spaced two weeks apart. Two weeks after the final booster, mice were euthanized and the splenocytes were subjected to ELISPOT analysis to evaluate peptide- or antigen-induced cytokine responses. To examine if the elicited immune responses are protective in nature, we used a virus-challenge model in mice. A chimera HIV-1, EcoHIV, containing gp80 of ecotropic murine leukemia virus substituted for the env of HIV-1 can proliferate in normal mice although in a highly restricted manner (Potash et al., 2005; Saini et al., 2007). We optimized a real-time PCR to quantitatively determine the viral load in the splenocytes of with or without genetic immunization.

3.7 Genetic immunization induces predominantly Th1 type immune response to Tat:

We sought to evaluate the nature of the immune response generated, in the context of Th1 v/s Th2, in the immunized mice. Immune responses skewed towards the Th1 type are considered to be protective in several infections including HIV/AIDS. We used the ELISPOT technique to evaluate IFN-γ and IL-4 production, which are signature cytokines for Th1 and Th2 type of responses, respectively. Single cell suspensions of splenocytes were incubated with Tat peptide pool, in 96-well ELISPOT plates that were previously coated with cytokine-specific antibodies. The number of spots developed was enumerated as described in materials and methods (Figure-17). The single-priming-single-booster regimen induced predominantly a Th1 type immune response in the absence of measureable Th2 type. All the promoters, with the exception of the intron-less promoter, induced IFN-γ spots at broadly comparable levels. The native CMV and EF-1α promoters performed at comparable magnitude. Although the ApaI-SacI promoter, that lacked the putative NRE, induced marginally more IFN-γ spots than the full-length EF-1α promoter or the SacII construct, both of which contain the putative NRE, the difference

was not statistically significant. The promoters with larger deletions in the intron (BglII-XhoI, PstI and PstI-SacII) elicited less magnitude immune response suggesting that the TFBS in the intron that have been deleted in these mutant promoters could have contributed for gene expression. These differences, however, were not found to be statistically significant. Identical results were obtained when two additional booster immunizations were delivered (data not shown). Additional experiments are presently in progress to evaluate which lymphocyte subsets are responsible for the IFN-γ production. We plan to use intracellular cytokine staining with surface staining for CD4 or CD8 to characterize the IFN-γ secreting cells further.

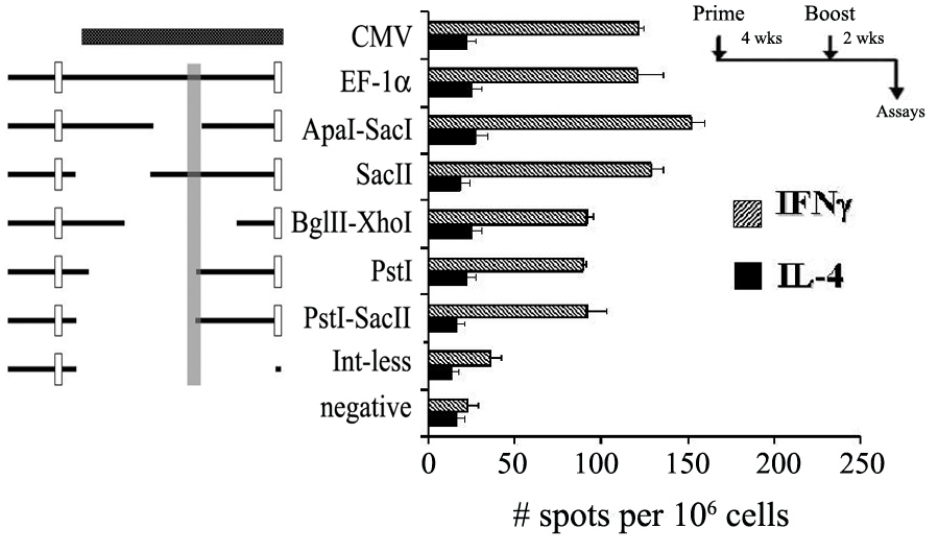


Figure-17: ELISPOT response in C57BL/6 mice genetically immunized with a panel of Tat-expression vectors. Mice (four or five animals per group) were immunized with 100 μg of plasmid DNA according to the schedule shown in the line diagram. The promoter panel, each driving expression of Tat has been depicted on the left side. The assay was performed directly without in vitro cell stimulation. Each assay consisted of 0.2 x 10^6 splenocytes incubated with Tat peptide pool, and the assay was performed as described in materials and methods. Each bar represents the mean of three individual wells ± standard deviation (error bar). Grey bars represent IFN-γ and filled bars IL-4 secreting cells. The number of spots per million cells has been presented on the x-axis.

3.8 Tat-specific immune response controls the viral load:

We used the EcoHIV virus challenge model to examine the protective nature of the induced immune responses to Tat. Based on the previous results, we selected a subset of the Tat-expression vectors for this analysis. Adult female C57BL/6 mice, four animals per group, were immunized by two intramuscular injections of Tat-expression constructs or the control plasmid. Two weeks after the final booster immunization, mice were infected with 5 μg of p24 EcoHIV by intraperitoneal injection of cell-free virus, prepared as described in materials and methods (Saini et al., 2007; Potash et al., 2005). Two weeks following the viral challenge, mice were euthanized by cervical dislocation and blood, spleen, and peritoneal macrophages were collected. DNA was isolated from the splenocytes or macrophages on the day of euthanasia using commercial columns (#G1N170, Sigma). Viral load in the genomic DNA was quantitatively determined using an optimized real-time PCR as described in materials and methods. The viral titers have been normalized against the amplification of the cellular gene glyceraldehyde phosphate dehydrogenase which was used as the internal control. Data have been presented as number of viral DNA copies per million cells. The virus challenge assay was performed a single time each with the single-booster (Figure-18) and three-booster (Figure-19) immunization regimens. Overall, Tat immunization appears to reduce viral load significantly in this challenge system although inconsistencies have been observed with certain Tat-expression vectors. Viral load suppression is more pronounced when three booster immunizations were delivered as compared to a single booster immunization (Figure-20). In the three-booster immunization, viral loads were reduced to significantly low magnitude with all the Tat-expression vectors as compared to the parental vector immunization (Figure-19). These data must be corroborated with additional experiments and such experiments are presently in progress. Furthermore, we have also optimized syngenic tumor challenge model, with tumor cell lines stably expressing Tat, as an alternative model to study the protective nature of the immune response. Experiments with this model are also in progress presently.

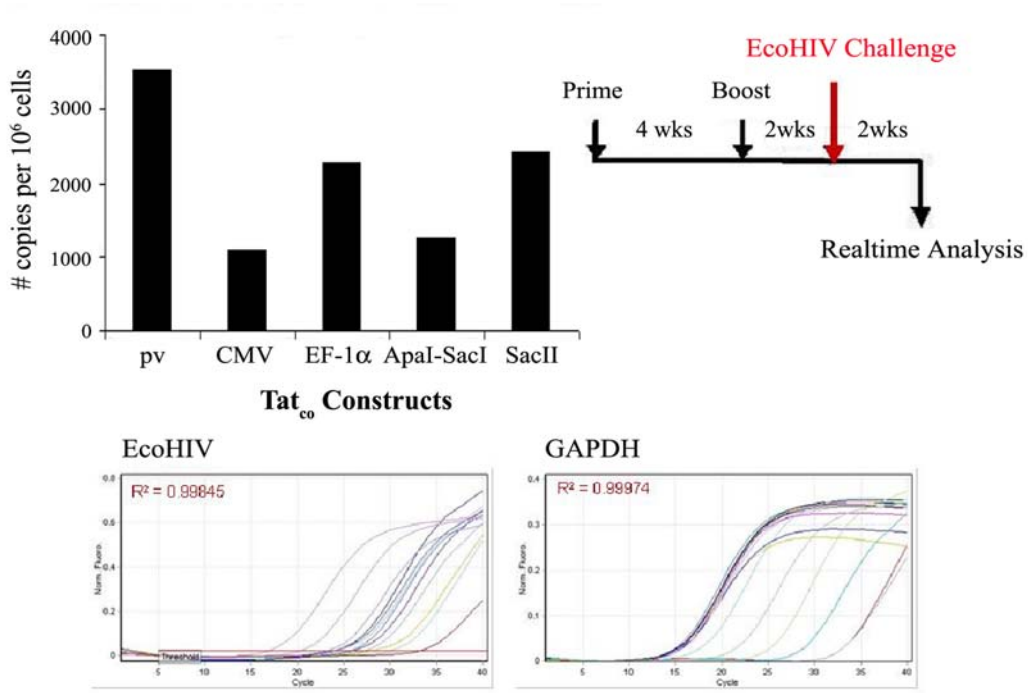


Figure-18: EcoHIV viral load in the splenocytes of mice immunized in a prime-one-booster regimen. Mice were immunized, four adult C57BL/6 female mice per group, with a panel of Tat-expression vectors. Mice were infected with 5 μg of cell-free EcoHIV virus through the intraperitoneal route. Viral load was determined in splenocytes using an optimized real-time PCR. The line diagram depicts the immunization (black arrows) and virus challenge (red arrow) schemes. The upper panel shows the proviral load against the Tat-expression vectors as shown on the x-axis. The lower panel illustrates the real-time PCR profile of the viral load with different Tat immunizations alongside the plasmid standards. The viral DNA copies were normalized against the cellular gene, GAPDH.

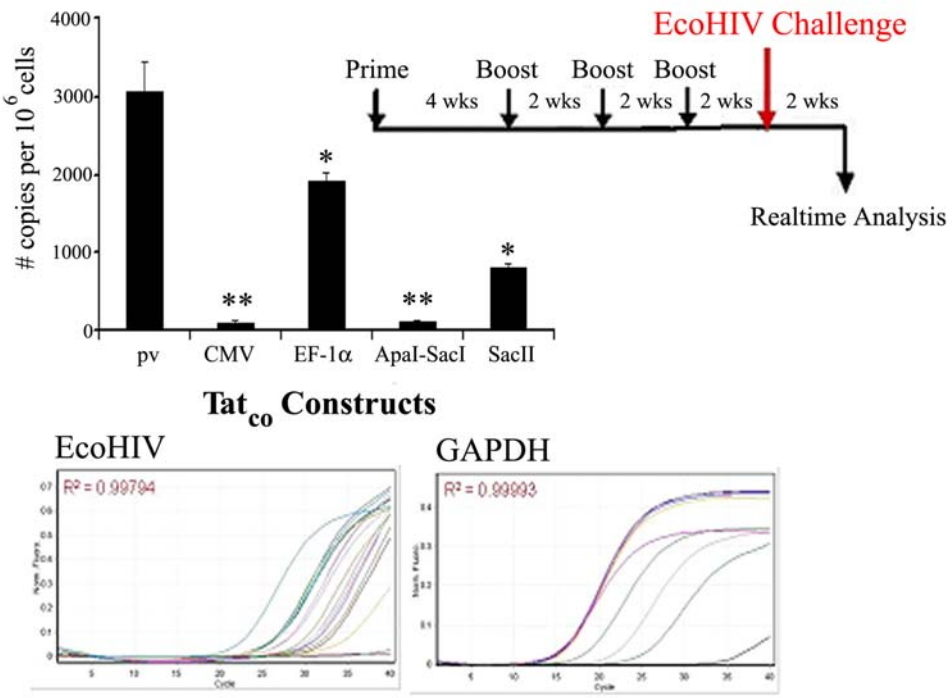


Figure-19: Protection against EcoHIV infection in mice immunized with Tat DNA vaccine. **(A)** The upper panel shows the proviral load obtained by real-time PCR of spleen cells from mice immunized with the constructs mentioned on the x-axis. Mice were infected with 5 μg of cell-free EcoHIV virus through the intraperitoneal route. The lower panel shows the real-time PCR profile obtained for the various constructs alongside the plasmid standards. The viral DNA copies were normalized by amplification of the cellular gene, GAPDH. Asterisks represent statistically significant difference in comparison with the PBS control. The differences in viral load are statistically significant by Student's t test. * , $P < 0.005$; ** $P < 0.0005$.

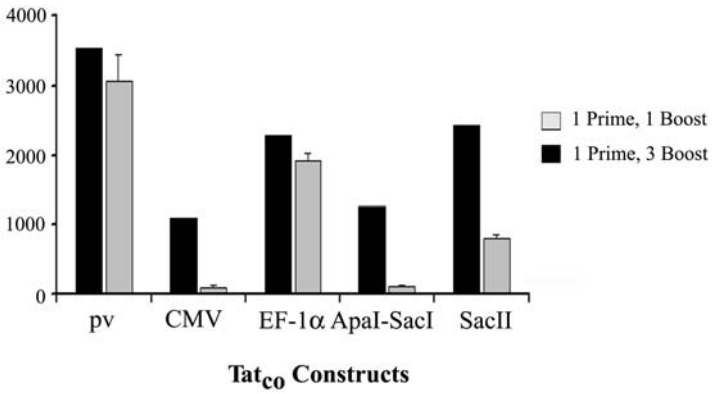


Figure-20: Comparison of the proviral loads between the one-booster and three-booster regimens.

3.9 References

Chung S, Andersson T et al, Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. *Stem Cells*, **20**, 139-145 (2002).

Gill DR, Smyth SE et al, Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. *Gene Ther*, **8**, 1539-1546 (2001).

Gribaudo G, Ravaglia S et al, Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription. *Virology*, **197**, 303-311 (1993).

Jordan M, Schallhorn A and Wurm FM: Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res*, **24**, 596-601 (1996).

Kim DW, Uetsuki T et al, Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. *Gene*, **91**, 217-223 (1990).

Margalit Y, Yarus S et al, Isolation and characterization of target sequences of the chicken CdxA homeobox gene. *Nucleic Acids Res*, **21**, 4915-4922 (1993).

Mizushima S and Nagata S: pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res*, **18**, 5322(1990).

Nakai H, Fuess S et al, Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J Virol*, **79**, 214-224 (2005).

Nishimura Y, Kamei A et al, A single immunization with a plasmid encoding hepatitis C virus (HCV) structural proteins under the elongation factor 1-alpha promoter elicits HCV-specific cytotoxic T-lymphocytes (CTL). *Vaccine*, **18**, 675-680 (1999).

Potash MJ, Chao W et al, A mouse model for study of systemic HIV-1 infection, antiviral immune responses, and neuroinvasiveness. *Proc Natl Acad Sci U S A*, **102**, 3760-3765 (2005).

Prosch S, Stein J et al, Inactivation of the very strong HCMV immediate early promoter by DNA CpG methylation in vitro. *Biol Chem Hoppe Seyler*, **377**, 195-201 (1996).

Ramakrishna L, Anand KK, Mohankumar KM and Ranga U: Codon Optimization of the Tat Antigen of Human Immunodeficiency Virus Type 1 Generates Strong Immune Responses in Mice following Genetic Immunization. *J Virol*, **78**, 9174-9189 (2004).

Sadaie MR and Hager GL: Induction of developmentally programmed cell death and activation of HIV by sodium butyrate. *Virology*, **202**, 513-518 (1994).

Saini M, Hadas E, Volsky DJ and Potash MJ: Vaccine-induced protection from infection of mice by chimeric human immunodeficiency virus type 1, EcoHIV/NL4-3. *Vaccine*,(2007).

Siddappa NB, Kashi VP et al, Gene expression analysis from Human Immunodeficiency Virus type-1 subtype C promoter and construction of bicistronic reporter vectors. *AIDS Res Hum Retroviruses*, **23**, 1268-1278 (2007).

Song S, Morgan M et al, Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. *Proc Natl Acad Sci U S A*, **95**, 14384-14388 (1998).

Teschendorf C, Warrington KH, Jr., Siemann DW and Muzyczka N: Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. *Anticancer Res*, **22**, 3325-3330 (2002).

Tokushige K, Moradpour D et al, Comparison between cytomegalovirus promoter and elongation factor-1 alpha promoter-driven constructs in the establishment of cell lines expressing hepatitis C virus core protein. *J Virol Methods*, **64**, 73-80 (1997).

Uetsuki T, Naito A, Nagata S and Kaziro Y: Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. *J Biol Chem Apr 5;264 (10):5791 -8*,(1989).

Wakabayashi-Ito N and Nagata S: Characterization of the regulatory elements in the promoter of the human elongation factor-1 alpha gene. *J Biol Chem*, **269**, 29831-29837 (1994a).

Wakabayashi-Ito N and Nagata S: Characterization of the regulatory elements in the promoter of the human elongation factor-1 alpha gene. *J Biol Chem Nov 25 ;269 (47):29831 -7*,(1994b).

Yew NS, Wysokenski DM et al, Optimization of plasmid vectors for high-level expression in lung epithelial cells. *Hum Gene Ther*, **8**, 575-584 (1997).

4. Molecular strategies to augment Tat immunogenicity

Genes directly cloned from pathogenic organisms may not be efficiently translated in a heterologous expression system such as *E. coli* as a consequence of codon bias. We previously demonstrated that codon-optimization of Tat (substituting codons that are optimally used in the mammalian system), led to increased levels of protein expression (Ramakrishna et al., 2004a; Ramakrishna et al., 2004b). The optimized Tat induced qualitatively and quantitatively superior immune responses as measured in a T-cell proliferation assay, ELISPOT and chromium release assay in the mouse model, following DNA vaccination (Ramakrishna et al., 2004b). In the present study, we devised and evaluated two additional molecular strategies to enhance protein expression further. In the first strategy, we engineered a synthetic intron between the two exons of Tat in a way simulating the natural context under which Tat is expressed from the virus and in the second, we grafted heterologous and universal T-helper epitopes into Tat to recruit T-helper function.

4.1 Engineering a synthetic intron into Tat

It has been well documented that intron-containing and intron-less versions of identical genes have dramatically different expression profile (Chapman et al., 1991; Duncker et al., 1997; Kim et al., 2002; Le Hir et al., 2003; Nasim et al., 2002; Nott et al., 2003). The act of removal of the intron by the spliceosome complex affects the gene expression in a variety of ways (Black, 2003) including modulating the rate of transcription (Furger et al., 2002), polyadenylation (Nott et al., 2003; Zhao et al., 1999), mRNA transport, translational efficiency (Matsumoto et al., 1998) and rate of mRNA decay (Nott et al., 2003).

A synthetic intron of small size (230 bp) from pIRES-puro (Clontech, # 6031-1) previously shown to enhance the stability of cytoplasmic RNA (Huang and Gorman, 1990), has been used in the present study. Using the primer extension strategy, we introduced the intron between the two exons of Tat_{co}. The intron-containing Tat gene,

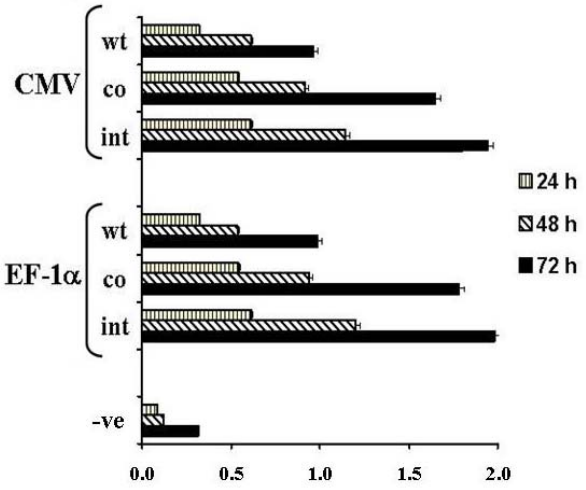
Tat_{int}, was placed downstream of both of the CMV and EF-1α promoters on the pcDNA3.1 (+) backbone.

4.2 Functional analysis of Tat_{int} constructs:

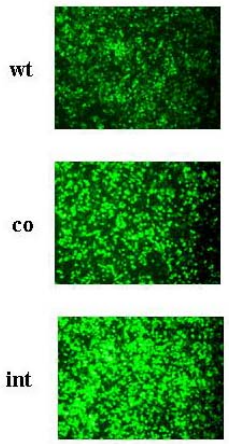
Tat_{int} construct was compared with Tat_{wt} and Tat_{co} for the transactivation property using reporter assays. Briefly, Tat_{wt}, Tat_{co} and Tat_{int} constructs driven by CMV or EF-1α promoter, were cotransfected with the dual reporter plasmid, HIV-LTR-SEAP-IRES-GFP, in HEK 293 cells. Following transfection, gene expression was monitored every 24 h. In all the transfections, CMV-betagalactosidase reporter plasmid was used as an internal standard for normalizing the transfection efficiency.

At all the time points of analysis and from both the promoters, Tat_{int} produced significantly higher levels of the reporter genes as compared to Tat_{wt} or Tat_{co} suggesting that the presence of intron indeed improved gene expression as expected (Figure-21).

(A) SEAP assay



(B) GFP analysis



(C) Western blot analysis

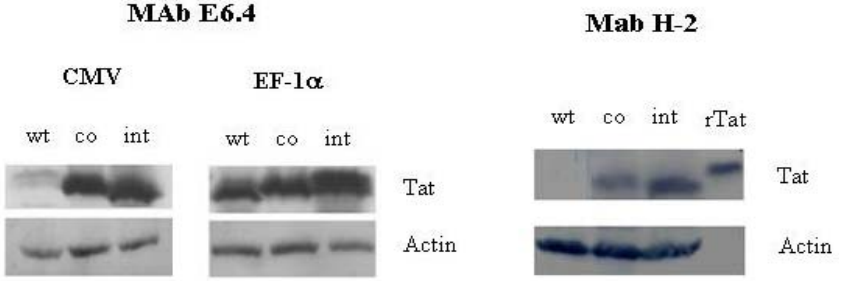


Figure-21: Transactivation assay for Tat-expression vectors. HEK 293 cells were transfected with a dual reporter plasmid and one of the Tat-expression vectors as labeled. Expression of **(A)** SEAP was measured by colorimentry every 24 h and that of **(B)** GFP was monitored using a fluorescent microscope. **(C)** Western blot analysis of Tat constructs using two different monoclonal antibodies. Beta-actin was used as the internal control. wt: wild type Tat, co: codon-optimized Tat, int: codon-optimized and intron-inserted Tat, rTat: recombinant Tat protein, which is larger due to additional tag added and migrates slower on the gel.

To validate the reporter gene analysis which is an indirect manifestation of Tat gene expression, we performed Western blot analysis on the cell lysate of the transfected cells using two different Tat-specific monoclonal antibodies raised in our laboratory (Venkatesh PK et al, Manuscript in preparation). E6.4 Mab recognizes the N-terminal region of Tat protein and H-2 recognizes the second exon of Tat. Western blot analysis confirmed an evident increase in the expression levels from that Tat_{int} construct compared to the wild type and the codon-optimized constructs (Figure-21C). Furthermore, this analysis also revealed that the intron is spliced out and full-length Tat protein is expressed efficiently from the Tat_{int} constructs.

4.3 Grafting T-Helper epitopes into Tat

4.4 Domain structure of Tat:

HIV-1 Tat exists in two forms, a 72 amino acid single-exon protein and, a 86-101 amino acid-long two-exon protein. Depending on the structural properties and its amino acid sequence, Tat protein could contain six functional domains with diverse biological characteristics (Figure-22) (Kuppuswamy et al., 1989; Jeang et al., 1999; Campbell et al., 2004).

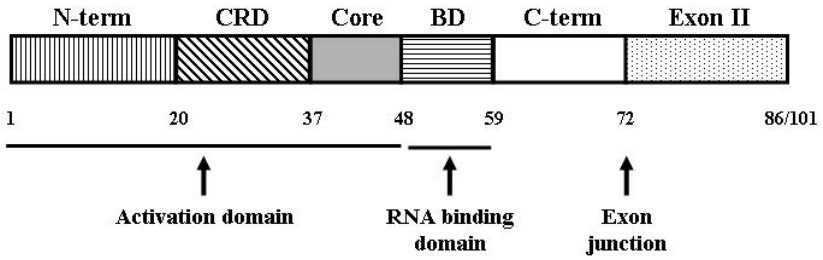


Figure-22: Domain structure of the Tat protein.

4.5 Tat is non-immunodominant:

Immune responses to Tat in natural infection and in animal models reveal the inherently non-immunodominant nature of this viral antigen (Lamhamedi-Cherradi et al., 1992; Blazevic et al., 1993; Ramakrishna et al., 2004b; Ranki et al., 1997; Silvera et al., 2002). Several studies have mapped T-helper, B-cell and CTL epitopes on Tat protein in natural infection, vaccinated individuals or experimental animals. The epitope maps are accessible using the following links.

(http://www.hiv.lanl.gov/content/immunology/maps/helper/Tat.html)

(http://www.hiv.lanl.gov/content/immunology/maps/ab/Tat.html)

(http://www.hiv.lanl.gov/content/immunology/maps/ctl/Tat.html)

A schematic representation of the mapped epitopes has been presented in Figure-23. Despite the wide spread of the epitopes, especially the T-helper epitopes, Tat paradoxically appears to be non-immunodominant. Several biological properties of Tat could possibly underlie the non-immunodominant nature of Tat including the small size, intracellular localization, limited secretion into biological fluids and importantly the quality of the T-helper epitopes present.

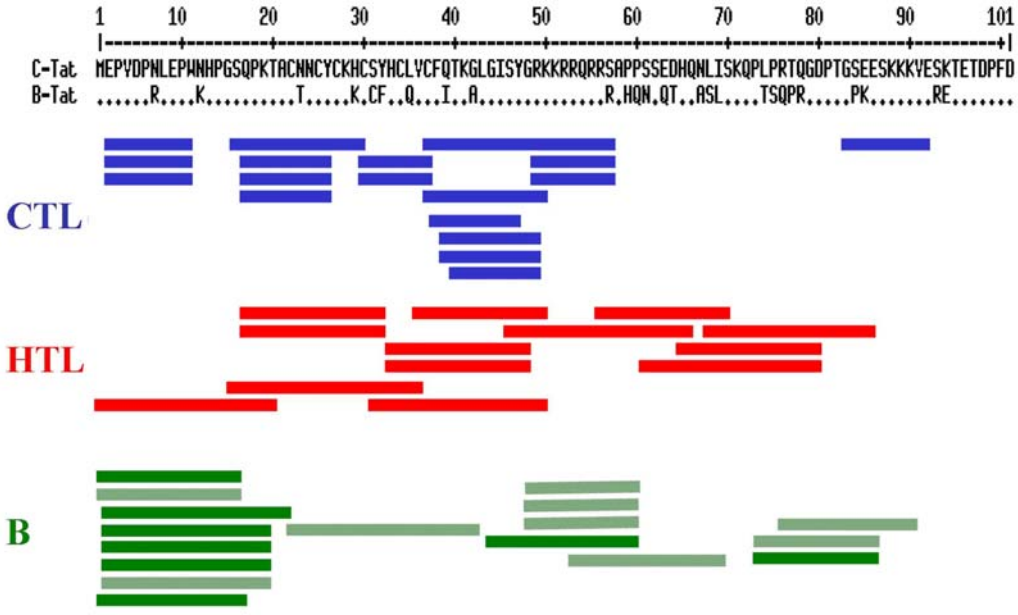


Figure-23: Epitope profile of the Tat protein. The consensus amino acid sequence of the full-length C- and B-Tat proteins has been presented in single letter code. Dots represent sequence homology. The Tat proteins share 76% sequences identity. CTL, HTL and B-cell epitopes have been color coded and drawn to

scale. The epitopes compiled here have been taken from various published reports. The dark-green filling represents epitopes recognized by monoclonal antibodies and the light-green filling by polyclonal antibodies**.**

Humoral immune responses are primarily elicited by the N-terminal, cysteine-rich or the core domains of Tat (Caselli et al., 1999). A B-cell epitope has also been mapped to the second exon (Goldstein et al., 2001). Humoral response is focused predominantly on the N-terminal domain when the antigen is delivered as a protein, whereas a broader epitope-spreading is recognized when Tat is delivered as a DNA vaccine, suggesting a codominant recognition of multiple epitopes by genetic vaccination (Caselli et al., 1999; Hinkula et al., 1997; Ramakrishna et al., 2004b). Only a fraction, 10-15% of the seropositive subjects makes anti-Tat humoral immune response (Krone et al., 1988; Reiss et al., 1990; Wieland et al., 1990), and only a minority of these subjects shows isotype switching to IgG indicating lack of efficient T-help (Venkatesh PK et al, manuscript in preparation).

Likewise, cell-mediated immune responses to Tat have also been shown to be limiting in natural infection (Borrow et al., 1994; Goulder et al., 2001; Lieberman et al., 1997; Masemola et al., 2004; Lamhamedi-Cherradi et al., 1992). The non-immunodominant nature of Tat appears to be an intrinsic property of this viral antigen given that in experimental immunization of primates (Putkonen et al., 1998; Belliard et al., 2005; Pauza et al., 2000) or human (Calarota et al., 1999; Hejdeman et al., 2004) also strong immune responses were not seen. A previous study documented the order of cumulative T-cell responses against HIV proteins as follows: Nef > Gag > Pol > Env > Vif > Rev > Vpr > Tat > Vpu (Masemola et al., 2004).

4.6 Grafting universal T-helper epitopes into Tat:

It is evident from the above discussion that molecular strategies are required to enhance immunogenicity of Tat should this antigen ever become a candidate vaccine. Nearly all the previous reports ignored this critical issue and used Tat as a protein, toxoid or DNA without means to enhance immunogenicity.

Helper T-Lymphocytes (HTLs or $CD4^+$ T-cells) play an important role in the induction and maintenance of specific T cell immunity (Boaz MJ et al., 2003).

Indications for a protective role of $CD4^+$ T cells against HIV-1 infection were found in subjects who were able to control HIV-1 viremia as well as in highly HIV-1 exposed, yet seronegative individuals (Sjoerd HV et al., 1994). The induction of $CD4^+$ T-cells (HTL) is a crucial component for both humoral and cellular immune responses. HTL secrete cytokines, such as IL-2, that play a fundamental role in the induction and differentiation of B cell precursors into antibody-forming cells (Alexander et al., 1998a) and in the differentiation of CTL (Livingston et al., 2002).

Grafting T-helper epitopes into heterologous antigens remains a powerful strategy to enhance immunogenicity of antigens. Grafted helper epitopes are expected to recruit T-help for molecularly associated B- and CTL epitopes by means of 'linked recognition' (Cassell and Forman, 1988; Snow et al., 1983; Davies et al., 1996). Of the various helper epitopes used in vaccine design, a specific helper epitope 'PADRE' (Pan DR helper T cell epitope) remains the most commonly used one. This helper epitope, originally identified in the tetanus toxin, has been modified further to bind most common HLA-DR molecules with high affinity (Alexander et al., 2000;Alexander et al., 1994b).

The PADRE epitope of 13 amino acid length could improve conjugate vaccines due to its immunogenicity in both humans and mice. The PADRE epitope (AKFVAAWTLKAAA) is characterized by a very broad pattern of MHC binding and potent antigenicity for human and murine T cells. The PADRE HTL epitope has also been shown to augment the potency of vaccines designed to stimulate cellular immune responses. Using a HBV transgenic murine model, it was found that CTL tolerances was broken by PADRE-CTL epitope lipopeptide, but not by a similar constructs containing a conventional HTL epitope (Alexander et al., 1998b). Optimization of HTL functions by use of synthetic epitopes such as PADRE or pathogen-derived, broadly cross reactive epitopes holds promise for a new generation of highly efficacious vaccines (Alexander et al., 1994).

Furthermore, a different universal T-helper epitope has been recognized in the reverse transcriptase (RT) of HIV-1. Peptides corresponding to the C-terminal region (residues 528-560) of the RT were recognized to augment T-helper cells in mice primed with RT (Haas et al., 1991). Further studies led to the identification of the pol 711 epitope in this region (corresponding to 529-543 residues in HXB2 (van der Burg et al., 1999).

This pol 711 epitope (EKVYLAWVPAHKGIG) has been reported be bind a large number of different HLA-DR (Wilson C et al., 2001) as well as several murine class II molecules (H-2^b, H-2^d, H-2^f, H-2^k) with high affinity (Boaz M et al., 2003;Haas et al., 1991;Loleit M et al., 1996;Livingston B et al., 2002) suggesting that this sequence could be a universal helper epitope.

To augment the immunogenicity of Tat, we chose to insert these two strong universal T-helper epitopes, PADRE and/or pol 711, into this protein. As depicted in Figure-24, the HTL epitopes were grafted into the cysteine-rich domain (CRD) between the C^{30} and S^{31} residues and/or basic domain (BD) between the K^{52} and R^{53} residues, singly or in combination, in both of the orientations of Tat that was codon-optimized for mammalian expression. Both of these epitopes have high binding affinity for mouse MHC class II molecules hence the immune responses could be evaluated in the murine model. Since the helper epitopes also bind several Human MHC, the inferences drawn from the mouse immunizations could probably be extrapolated to the human immunizations.

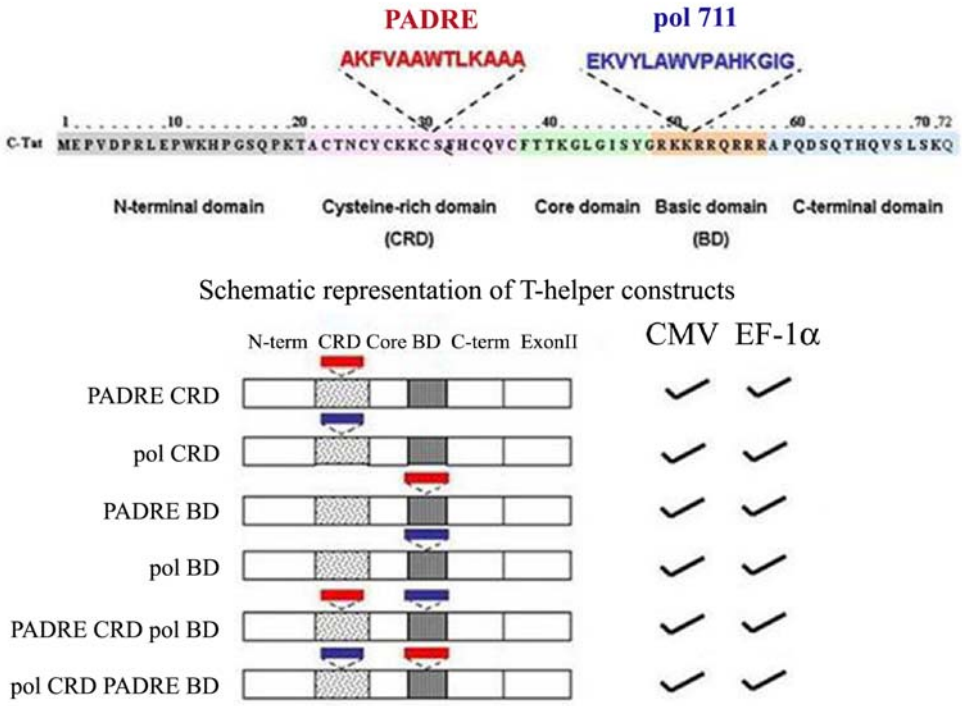


Figure-24: Schematic representation of T-helper epitope grafting into Tat. Using the overlap PCR approach, the PADRE and pol 711 epitopes have been grafted into the CRD and/or BD singly or in

combination in both the orientations. Two independent panels of Tat expression vectors under the control of CMV or EF-1a promoters were constructed.

Importantly, in addition to augmenting Tat immunogenicity, T-helper epitope grafting is aimed at abrogating toxic properties of Tat thereby improving its safety profile. Given that several harmful biological properties of Tat are governed by the two important domains of Tat, CRD and BD, grafting heterologous amino acid sequences into these domains is expected to disrupt the structural configuration of these domains and attenuate Tat safe enough for human immunization. Precise structural configuration of the CRD, a Zinc-finger motif, is critical for immobilization of Zinc ions required for Tat transactivation (Frankel et al., 1988; Huang and Wang, 1996). Additionally, an intact CRD could also mediate monocyte chemotaxis, induction of apoptosis, angiogenesis and many other properties of Tat that are expected to be abrogated following T-helper epitope grafting into this domain. Likewise, intact BD is responsible for Tat binding to TAR, membrane translocation, nuclear localization, transactivation and many other functions of Tat. These functions must be perturbed following domain disruption consequently attenuating Tat. Of note, we took precautions to cause minimum disruption to the known B- and CTL epitopes in Tat while engineering T-helper epitopes to recapitulate faithful immune responses following immunization. Importantly, the single Tat vaccine candidate permitted to be evaluated in the human clinical phase II trial in Italy got encompassed in a serious ethical controversy due to the toxic properties of intact Tat that is being used in these studies. As is evident here, we have paid attention to two of the most serious limitations of Tat vaccine in our vaccine design. Two different but essential objectives have been targeted by T-helper epitope grafting into Tat, one immune potentiation and two detoxification of Tat.

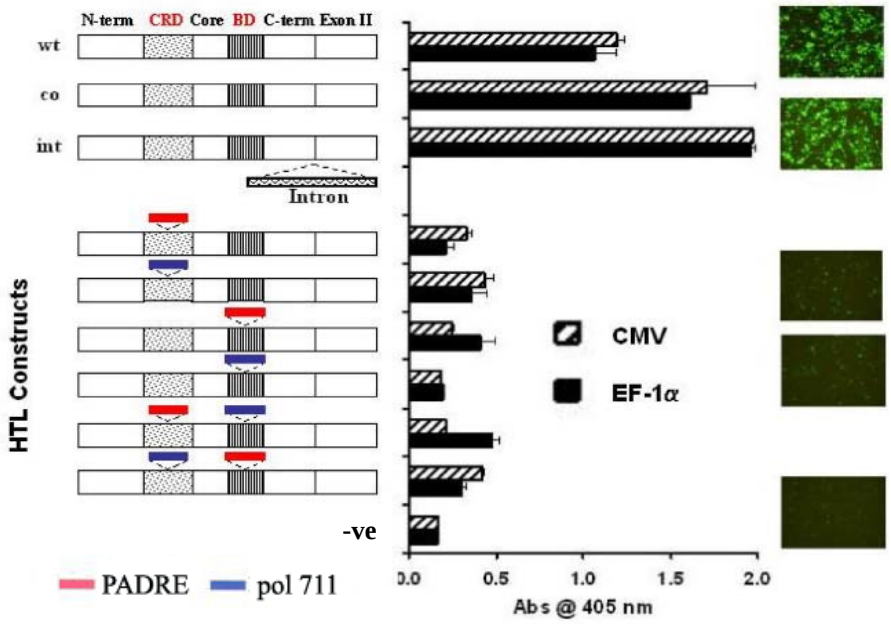
4.7 Loss of Tat transactivation following T-helper epitope grafting:

Using a transient reporter assay in HEK 293 cells, we compared the transactivation property of the codon-optimized Tat with T-helper grafted Tat expression vectors. Further, wild type Tat vector and codon-optimized Tat with engineered intron have also been used in the assay. Both of the CMV and EF-1α Tat expression panels have

been used in the assay. Cells have been cotransfected with one of the Tat-expression vectors and a dual reporter vector, LTR-SEAP-IRES-GFP, that expresses secreted alkaline phosphatase (SEAP) and GFP independently in response to Tat. Following transfection, culture supernatant was sampled at regular intervals for the expression of SEAP or GFP.

As is evident form Figure-25A, all the three vectors with intact Tat induced significant levels of SEAP or GFP as compared to the control cells that received only the empty vector. In contrast, all the HTL Tat vectors, with either one or both of the domains disrupted, failed to express the reporter genes above the background levels suggesting loss of transactivation property of Tat in these constructs, as expected. Importantly, disruption of CRD or BD alone by either of the HTLs was sufficient to knockout transactivation function of Tat. Western blot analysis confirmed the production of Tat proteins from all the Tat vectors at comparable levels (Figure-25B) ruling out the possibility that engineered Tat proteins were unstable.

(A) Tat transactivation **SEAP assay** **GFP**



(B) Western blot analysis

Figure-25: Loss of Tat transactivation in HTL engineered constructs. (A) Reporter gene analysis: HEK293 cells were co-transfected with the dual reporter plasmid, LTR-SEAP-IRES-GFP, and one of the various Tat expression constructs driven by CMV or EF-1α promoter. Reporter gene expression was monitored every 24 h following transfection and data at 72 h have been presented here. Identical data were observed at earlier time points. The experiment was repeated for three times with identical observation. **(B) Western blot analysis**. Tat-specific monoclonal antibody E6.4 was used for detecting Tat. Beta-actin expression was used as the loading control.

4.8 Tat with disrupted domains fails to cause apoptosis in THP-1 cells:

Even transient exposure to Tat can induce significant levels of apoptosis in cells of diverse lineage. Subtype C Tat, the antigen used in all our constructs, however, can cause only low level apoptosis in T-cells, monocytes and primary neurons as compared to subtype B Tat (Siddappa et al., 2006; Mishra et al., 2007). The cysteine-rich domain which plays a significant role in Tat-induced apoptosis has been disrupted in some of the Tat constructs that we generated. To examine if domain disruption of Tat abrogated the apoptotis function of Tat, we transiently transfected THP-1 cells with Tat_{wt}, Tat_{co} and with Tat in which both of the CRD and BD disrupted by HTL grafting. Twenty-four hours after the transfection, cells were stained for Annexin V-FITC in a buffer containing propidium iodide and analyzed by flow cytometry using FACSCalibur (BD Biosciences). While the wild type and codon-optimized Tat DNA induced significantly higher levels of apoptosis, 48% and 51% cells positive respectively, domain-disrupted Tat induced low level apoptosis indistinguishable from the mock (empty vector) control (Figure-26).

The difference between intact Tat constructs and the domain-disrupted Tat was explicit when mean fluorescence intensity (MFI) values were compared suggesting that domain disruption led to profound attenuation of this viral transactivator thus making it safe for human application. We are currently evaluating the induction of apoptosis by the various HTL constructs in T-cells, primary monocytes and PBMCs. Additional Tat constructs, especially Tat with single domain disruption, are being tested.

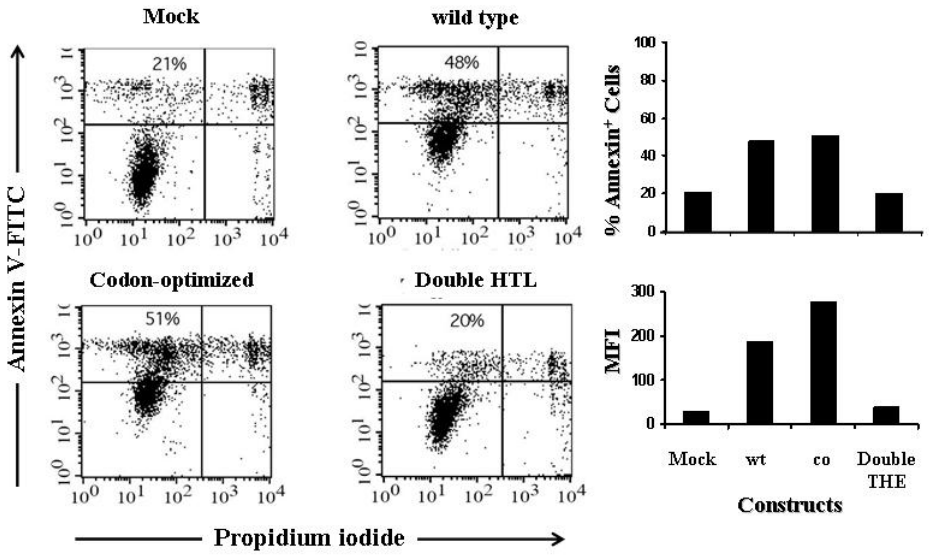


Figure-26: Domain disrupted Tat fails to induce apoptosis in THP-1 cells: THP-1 cells were transiently transfected with 1 μg of Tat DNA vectors (Nucleofector, Amaxa). Twenty four h following the transfection, the cells were stained for Annexin V-FITC and propidium iodide and analyzed by flow cytometry. The percent cells positive for Annexin V and the mean fluorescence intensity values have been presented graphically on the right panel of the Figure.

4.9 DNA immunization:

Endotoxin-free plasmid DNA (100 μg) was injected into the *tibialis anterior* muscle of mice that were 8 to 12 week old. Each immunization consisted of four or five mice per group. The immunization schedule involved one primary immunization followed by one booster immunization spaced 2 weeks apart.

4.10 Tat_{int} induces stronger immune responses:

We compared Tat_{int} with Tat_{wt} or Tat_{co} vectors in mouse immunization. Mice were immunized with 100 μg of the plasmid DNA per injection, of the CMV and EF-1α panels.Two weeks after the booster immunization, harvested splenocytes were tested in lympho-proliferation assay or ELISPOT for prototype cytokines to delineate the type of immune response elicited.

For the lympho-proliferation assay, splenocytes were cultured in triplicate wells in a flat-bottom 96-well microplate at $2x10^5$ per well. Tat peptide pool, spanning exon I, comprising of 6 overlapping 20-mer peptides with 10-mer overlap, was added to the wells at a concentration of 2 μg/ml and the cells were incubated for four days. Conconavalin A (ConA) was used as positive control for cell proliferation at a final concentration of 5 μg/ml. After incubation, the extent of cell proliferation was measured by adding [^{3}H] Thymidine, (10 μCi/ml) to the wells and cultures were incubated for an additional four h at 37^0C. Plates were harvested using a cell harvester (Skatron, Norway). The filters were dried and radioactive counts were determined using a β-scintillation counter (Wallac, 1409). Lymphoproliferation is a simple assay that serves as a useful indicator to measure cellular immune responses. This assay revealed that immunizations with Tat_{int} induced higher magnitude immune response as compared to the wild type and codon-optimized Tat constructs, under both the cellular and the viral promoters (Figure-27A). These differences, however, were not found to be statistically significant. Immunization with the empty vector did not elicit antigen-specific immune response.

ELISPOT analysis not only measures the strength of immune response but also the Th-type could be delineated by measuring the prototype cytokine profile, IFN-γ vs IL-4 production. Splenocytes harvested from mice after a single booster immunization were incubated with the Tat peptide pool, in 96-well ELISPOT plates that were previously coated with - IFN-γ specific antibodies. In this preliminary experiment we used only the IFN-γ specific antibodies. The number of spots developed was enumerated as described in materials and methods. As in the case of lymphoprolifeartion, Tat_{int} vector induced higher level immune response as compared to the other two Tat vectors. The differences was found to be statistically significant..

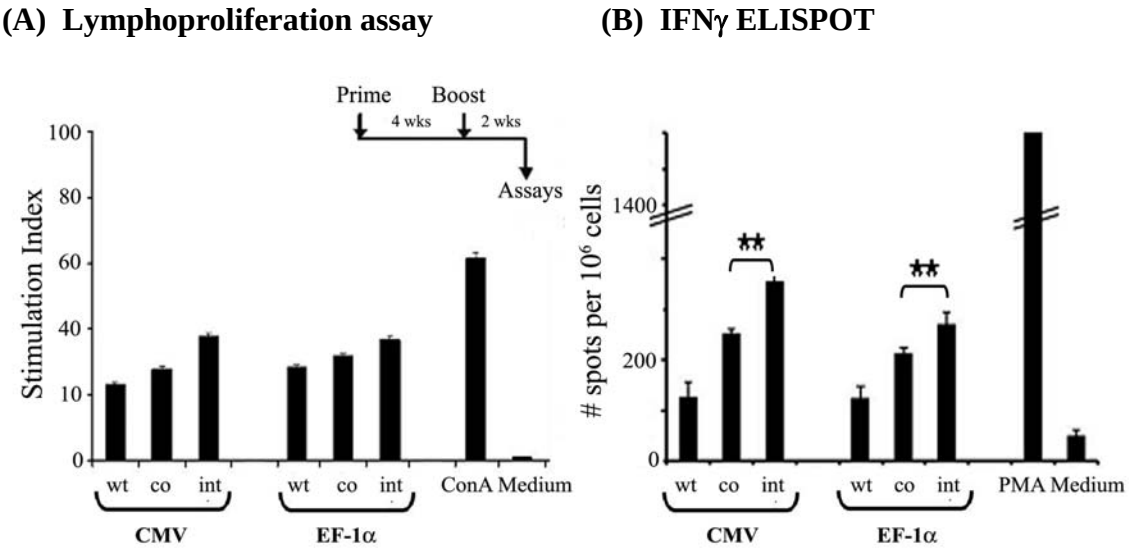


Figure-27: Tat_{int} induces stronger immune responses: Mice, 4 or 5 per group, were immunized with the immunization profile shown in the line diagram. Mice, (A) C57BL/6 or (B) BALB/c, were administered 100 µg of plasmid DNA, Tat_{wt}, Tat_{co} or Tat_{int}, under the control of CMV or EF-1α promoter, per animal per injection. Splenocytes were harvested 2 weeks after the booster immunization and immune responses were measured using (A) lympho-proliferative assay or (B) cytokine profile in ELISPOT assay were measured. A stimulation index above 3 was considered a positive response in the lymphoproliferation assay. The ELISPOT assay was performed without in vitro antigen stimulation. Each assay consisted of 0.2 x 10^6 splenocytes incubated with Tat peptide pool, and the assay was performed as described in materials and methods. Results have been as the mean values of three replicate wells ± standard deviation. The data are representative of two independent experiments. Concanavalin-A or PMA were used as positive control for cell stimulation while cells without stimulation served as negative control. An asterisks represent statistically significant difference ($P < 0.001$ to 0.01) when codon-optimized Tat was compared with the intron construst using Student's t-test. wt: wildtype, co: codon-optimized, int: codon-optimized Tat with a synthetic intron.

4.11 Highly augmented and Th1 biased immune response by the HTL-Tat constructs:

Endotoxin-free plasmid DNA (100 µg) was injected into the *tibialis anterior* muscle of C57BL/6 or BALB/c female mice that were 8 to 12 weeks old. Tat DNA vectors used for immunization were the same panels as shown in Figure-25A consisting

of all the three plasmids expressing uninterrupted Tat, and the various HTL-Tat vectors under the control of the CMV- or EF-1α promoter. Each immunization consisted of four or five mice per group. The immunization schedule involved one primary immunization followed by one or three booster immunization spaced 2 weeks. One month or three months after the booster immunization, the animals were sacrificed, splenocytes harvested and used in ELISPOT analysis.

Immune responses skewed towards the Th1 type are considered to be immuno-protective in several infections including HIV/AIDS. We sought to evaluate the nature of the immune responses generated, in the context of Th1 v/s Th2, in immunized mice. Harvested splenocytes were directly incubated, without in vitro stimulation, with Tat peptide pool, consisting of 20-mer peptides spanning the full-length of subtype C Tat consensus sequence, in 96-well ELISPOT plates that were previously coated with cytokine-specific antibodies. Number of spots developed representing antigen-specific lymphocytes secreting cytokines in response to antigen stimulation were enumerated as described in materials and methods.

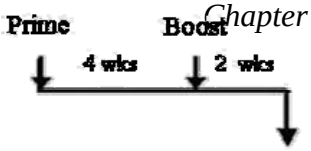
The data summarized in Figures 28 and 29 demonstrate that EF-1α promoter was functionally as efficient as the CMV promoter under all the experimental conditions. The influence of the intron and the T-cell grafting into Tat was positive in that there was significant induction in immune response, however, the magnitude of immune responses varied depending on the conditions as described below. Priming followed by a single booster immunization elicited significantly measurable IFN-γ immune responses from all the Tat expression vectors under both of the viral and cellular promoters and in both of the mouse strains (Figure-28). Importantly, none of the immunizations induced IL-4 response above the back ground level. The induced immune responses represented only IFN-γ secreting cells suggesting strong skewing towards Th1 type immune response. This result was expected, as both the mode of immunization (i.m.) and the nature of the antigen (DNA vaccine) have been reported to predominantly skew the immune responses towards a Th1 type. Codon-optimized Tat expressed higher level IFN-γ immune response than wildtype Tat as demonstrated by us previously (Ramakrishna et al., 2004b). Codon-optimized Tat with an intron engineered between the exons induced higher number of IFN-γ spots as compared to the wildtype or the codon-optimized Tat construct and this

difference was statistically significant (Figure 28). This result was consistent in both of the mouse strains and under both of the promoters.

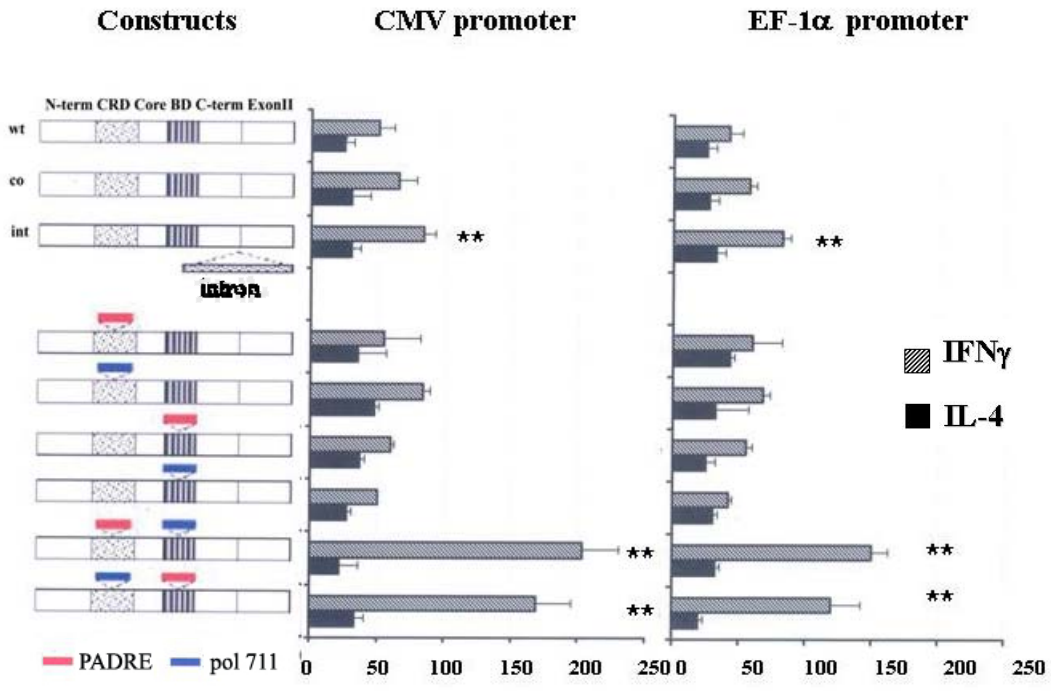
Significantly augmented immune responses were observed with the approach of T-helper epitope grafting. Immune responses in both of the mouse strains were superior when both of the T-helper epitopes were inserted together in either of the combinations as compared to when these epitopes were inserted in isolation, suggesting synergy between the epitopes (Figure-28). Genetic background and the order of the epitopes did not make a significant difference suggesting both of these epitopes could function together thereby enhancing the immune response to the highest level. In contrast, when the two helper epitopes were inserted in isolation, the effect of T-helper grafting appeared to be marginally influenced by the genetic background of the mouse strain. While PADRE epitope appeared to be functional in both of the mouse strains, its contribution was mainly observed in C57BL/6 mice especially when inserted into the cysteine-rich domain. In BALB/c mice, PADRE insertion into either of the domains appeared to have made little difference with respect to Tat_{co}. Furthermore, Pol 711 epitope broadly behaved as the PADRE epitope in BALB/c mice, however, in C57BL/6 mice, this epitope did not demonstrate enhanced immune response from either of the Tat domains. The influence of insertion of single T-helper epitopes, in general, did not make significant influence on immune response with the exception of PADRE insertion into the cysteine-rich domain in C57BL/6 mice (Figure-28B). Importantly, grafting of both of the helper epitopes together into Tat not only elicited the highest IFN-γ immune response but also host genetic differences appeared to be obliterated as comparable frequencies of IFN-γ secreting cells were detected in both BALB/c and C57BL/6 mice, under both the cellular and the viral promoters (Figure-28 A and B).

Absence of discernable immune augmentation with single T-helper insertion was interesting. We delivered two additional booster immunizations to see if Tat constructs with single helper epitopes could be immunogenic under these conditions. Under the one-prime-three-booster regimen, we essentially recaptured the immune responses as per the one-prime-one booster protocol notably at significantly higher magnitude (Figure 29). However, even with additional booster immunizations, we failed to enhance IFN-γ immune response above what is seen with the Tat codon-optimized vector (data not

presented). To examine memory establishment, we rested the mice for three months after the third booster, and enumerated IL-4 and IFN-γ secreting cells in ELISPOT assay. The profile of cytokine secretion essentially remained the same as that of one-prime-one booster regimen (Figure-29). Both of the Tat vectors with two helper epitope inserts induced the highest levels of IFN-γ immune response as before and in the absence of significant IL-4 secreting cells. Additional booster immunizations significantly enhanced immunogenicity of the single helper-epitope Tat constructs especially in BALB/c (Figure-29A), but not in C57BL/6 (Figure-29B) mice, as before (Figure-28). Furthermore, additional booster immunizations also augmented immunogenicity of Tat_{int} in that this construct induced IFN-γ secreting cells at a frequency comparable to the Tat-dual HTL constructs mainly in BALB/c (Figure-29A) and to a lesser extent in C57BL/6 (Figure-29B) mice. The observed results were consistent with both of the promoters.



(A) BALB/c



(B) C57BL/6

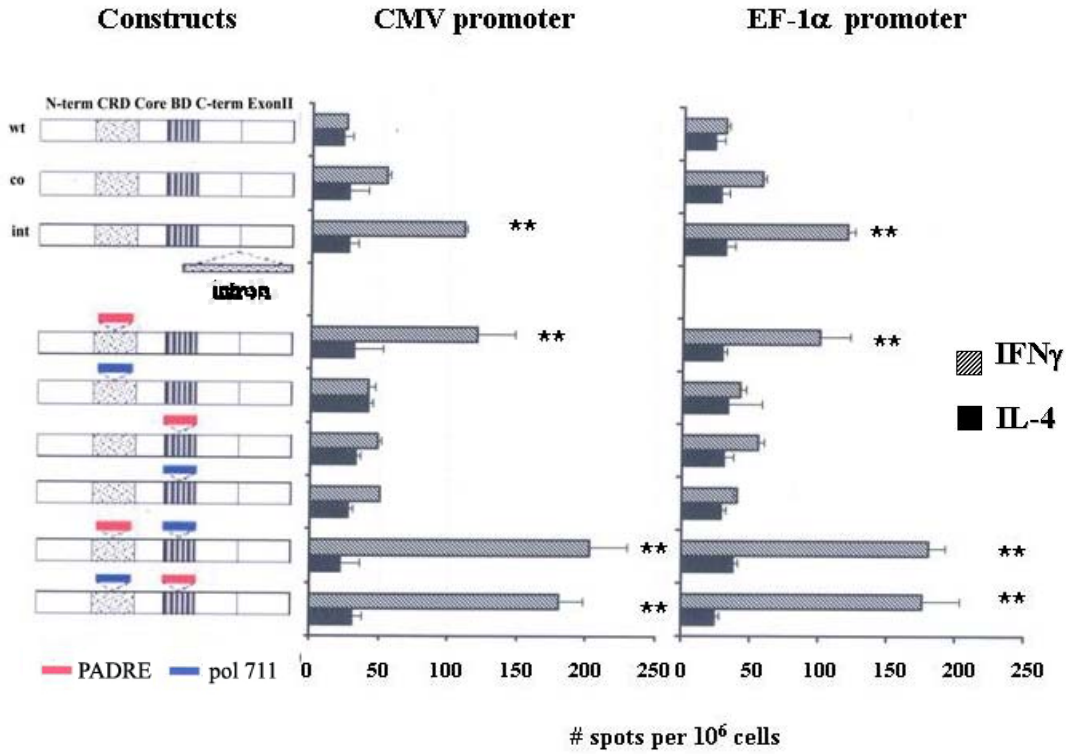


Figure-28: ELISPOT response in **(A)** BALB/c and **(B)** C57BL/6 mice genetically immunized with different Tat constructs. Mice (four or five animals per group) were immunized with 100 μg of plasmid DNA according to the schedule shown in the line diagram. The assay was performed without in vitro stimulation. Each assay consisted of 0.2 x 10^6 splenocytes incubated with Tat peptide pool, and the assay was performed as described in materials and methods. Each bar represents the mean of three individual wells ± standard deviation. Grey bars represent IFN-γ and filled bars IL-4 secreting cells. The panels of Tat expression vectors used in the immunizations have been shown. The number of spots per million cells has been presented on the x-axis. The middle panel corresponds to the immune response elicited by Tat constructs driven by the CMV promoter and the right panel to constructs driven by the EF-1α promoter. The experiment was performed twice and the data presented are representative of these experiments. An asterisks represent statistically significant difference ($P < 0.001$ to 0.01) when various Tat constructs have been compared with the codon-optimized Tat using Student's t-test. wt: wildtype Tat, co: codon-optimized Tat, int: codon-optimized Tat with a synthetic intron.

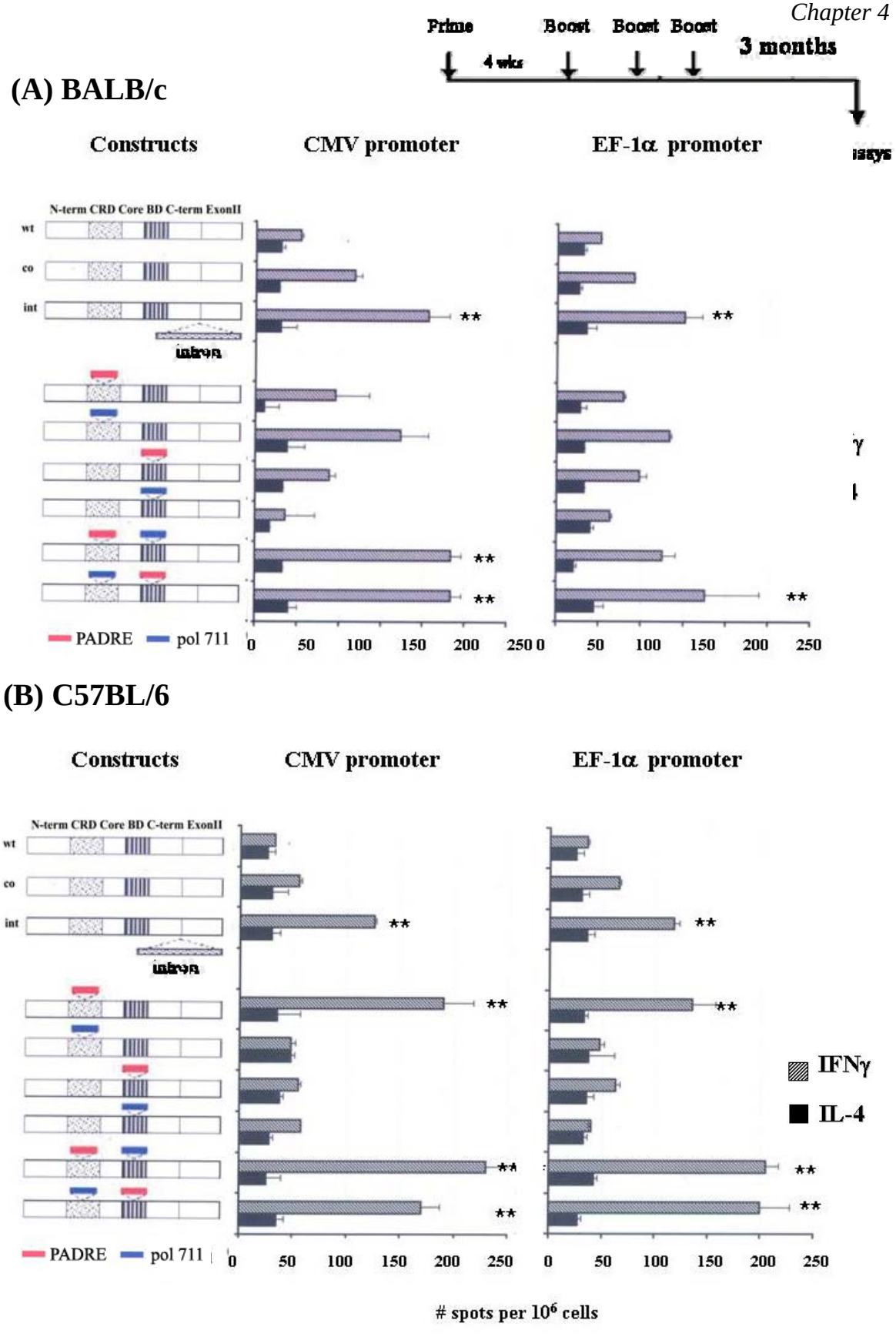

Prime
Boost
Boost
Boost
4 wks
3 months
(A) BALB/c
Constructs
CMV promoter
EF-1α promoter
N-term CRD Core BD C-term ExonII
wt
co
int
intron
**
PADRE
pol 711
0
50
100
150
200
250
(B) C57BL/6
IFNγ
IL-4
spots per 10^6 cells

Figure-29: Highly augmented Th1 biased immune response by the HTL constructs. ELISPOT response in (A) BALB/c and (B) C57BL/6 mice genetically immunized with different Tat constructs. Mice (four or five animals per group) were immunized with 100 μg of plasmid DNA according to the schedule shown in the line diagram. The assay was performed without in vitro stimulation. Each assay consisted of 0.2 x 10^6 splenocytes incubated with Tat peptide pool, and the assay was performed as described in materials and methods. Each bar represents the mean of three individual wells ± standard deviation (error bar). Grey bars represent IFN-γ and filled bars IL-4 secreting cells. The panels of Tat expression vectors used in the immunizations have been shown. The number of spots per million cells has been presented on the x-axis. The middle panel corresponds to the immune response elicited by Tat constructs driven by the CMV promoter and the right panel to constructs driven by the EF-1α promoter. An asterisks represent statistically significant difference ($P < 0.001$ to 0.01) when various Tat constructs have been compared with the codon-optimized Tat using Student's t-test. wt: wildtype Tat, co: codon-optimized Tat, int: codon-optimized Tat with a synthetic intron.

4.12 Discussion:

Our study in these immunization experiments targeted three different but complimentary features of Tat vaccine design. One of these objectives was to compare the cellular EF-1α promoter with the viral CMV promoter. The second objective was to examine if insertion of a synthetic intron between the two Tat exons could enhance immune responses. Thirdly, we wanted to evaluate the effect of grafting two different and heterogeneous T-helper epitopes on the immune response. We tested all the Tat expression vectors in two different mouse strains simultaneously and expressing Tat under two different promoters. EF-1α promoter was found to be as efficient as the most popular CMV promoter in eliciting immune responses to Tat. We identified a perfect correlation between these two promoters under all the experimental conditions regardless of mouse strain differences and vector construct variations. The present study is one of the few to evaluate the potential of a cellular promoter for genetic immunization purpose. Our data are quite encouraging in that EF-1α promoter being of cellular origin and demonstrating comparable functional activity as the viral promoter, if not superior, is a suitable replacement for the CMV promoter. EF-1α promoter is less likely to be silenced by the cellular mechanisms, unlike the CMV promoter, and as a consequence may have consistent and reproducible functional activity.

The most striking observation of the present study is the influence of T-helper grafting into Tat on its immunogenicity. Importanly, when both of the HTL epitopes were grafted together, in either of the combinations, the immunizations induced the strongest and comparable immune response suggesting synergy between these helper epitopes. The observed synergy between the two epitopes was independent of the orientation of the grafting. The results obtained are consistent regardless of the mouse haplotype difference and the promoter driving the expression of Tat. It has been well documented that peptides with higher-affinity binding for the MHC skew T-cell response towards a Th1 profile (Alhers JD et al., 2001). The two Universal T-helper epitopes engineered into our Tat constructs have high affinity binding for MHC hence could effectively polarize the immune response towards Th1.

The strategy of epitope grafting would be beneficial not just to Tat, but to any inherently non-immunodominant antigen. Furthermore, insertion of T-helper epitopes into two domains that regulate the most critical functions of Tat also significantly improved the safety profile of Tat thereby making it suitable for internal administration. This particular modification is important when Tat vaccine will be considered for human vaccination. This single feature alone is the most significant improvement of the Tat vaccine design. Previously our laboratory demonstrated that genetic immunization of Tat_{wt} induced a mixed Th1/Th2 response, which was converted into a more desirable Th1 response on administration of additional boosters (Ramakrishna et al., 2004). In the present study, various molecular strategies employed here including codon-optimization of Tat, intron engineering or T-helper epitope grafting, readily pushed the immune response towards a more optimal Th1 type profile with a single booster immunization. This molecular manipulation could obviate a need for multiple booster immunizations. Additionally, intron and T-helper epitopes independently augmented significantly higher immune responses to profile. We are presently evaluating Tat constructs in which both of these strategies have been integrated. Additional experiments are also underway to evaluate the epitopes recognized in the vaccination using pepscan strategy. We have designed 20-mer peptide series spanning the length of Tat-T-helper-epitope antigens and overlapping by 10 residues with the neighbors for the pepscan analysis. Experiments are

also in progress to evaluate the protective nature of the augmented immune responses using two different challenge models as described in materials and methods.

4.13 References

Alexander J, Fikes J et al, The optimization of helper T lymphocyte (HTL) function in vaccine development. *Immunol Res %1998 ;18 (2):79 -92*,(1998a).

Alexander J, Fikes J et al, The optimization of helper T lymphocyte (HTL) function in vaccine development. *Immunol Res %1998 ;18 (2):79 -92*,(1998b).

Alexander J, Sidney J et al, Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity %1994 Dec ;1(9):751 -61*,(1994).

Belliard G, Hurtrel B et al, Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. *Vaccine*, **23**, 1399-1407 (2005).

Black DL: Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem*, **72**, 291-336 (2003).

Blazevic V, Ranki A et al, Helper T-cell recognition of HIV-1 Tat synthetic peptides. *J Acquir Immune Defic Syndr*, **6**, 881-890 (1993).

Borrow P, Lewicki H et al, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*, **68**, 6103-6110 (1994).

Calarota SA, Leandersson AC et al, Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *J Immunol*, **163**, 2330-2338 (1999).

Campbell GR, Pasquier E et al, The glutamine-rich region of the HIV-1 Tat protein is involved in T-cell apoptosis. *J Biol Chem*, **279**, 48197-48204 (2004).

Caselli E, Betti M et al, DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. *J Immunol*, **162**, 5631-5638 (1999).

Cassell D and Forman J: Linked recognition of helper and cytotoxic antigenic determinants for the generation of cytotoxic T lymphocytes. *Ann N Y Acad Sci*, **532**, 51-60 (1988).

Chapman BS, Thayer RM, Vincent KA and Haigwood NL: Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res*, **19**, 3979-3986 (1991).

Davies JD, Leong LY et al, T cell suppression in transplantation tolerance through linked recognition. *J Immunol*, **156**, 3602-3607 (1996).

Duncker BP, Davies PL and Walker VK: Introns boost transgene expression in Drosophila melanogaster. *Mol Gen Genet*, **254**, 291-296 (1997).

Frankel AD, Bredt DS and Pabo CO: Tat protein from human immunodeficiency virus forms a metal-linked dimer. *Science*, **240**, 70-73 (1988).

Furger A, O'Sullivan JM et al, Promoter proximal splice sites enhance transcription. *Genes Dev*, **16**, 2792-2799 (2002).

Goldstein G, Tribbick G and Manson K: Two B cell epitopes of HIV-1 Tat protein have limited antigenic polymorphism in geographically diverse HIV-1 strains. *Vaccine 2001 Feb 8 ;19 (13-14):1738 -46*, **19**, 1738-1746 (2001).

Goulder PJ, Addo MM et al, Rapid Definition of Five Novel HLA-A*3002-Restricted Human Immunodeficiency Virus-Specific Cytotoxic T-Lymphocyte Epitopes by Elispot and Intracellular Cytokine Staining Assays. *J Virol*, **75**, 1339-1347 (2001).

Haas G, David R et al, Identification of a major human immunodeficiency virus-1 reverse transcriptase epitope recognized by mouse CD4+ T lymphocytes. *Eur J Immunol %1991 Jun ;21(6):1371 -7*,(1991).

Hejdeman B, Bostrom AC et al, DNA Immunization with HIV Early Genes in HIV Type 1-Infected Patients on Highly Active Antiretroviral Therapy. *AIDS Res Hum Retroviruses*, **20**, 860-870 (2004).

Hinkula J, Svanholm C et al, Recognition of prominent viral epitopes induced by immunization with human immunodeficiency virus type 1 regulatory genes. *J Virol*, **71**, 5528-5539 (1997).

Huang HW and Wang KT: Structural characterization of the metal binding site in the cysteine-rich region of HIV-1 Tat protein. *Biochem Biophys Res Commun*, **227**, 615-621 (1996).

Huang MT and Gorman CM: Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. *Nucleic Acids Res %1990 Feb 25 ;18 (4):937 -47*,(1990).

Jeang KT, Xiao H and Rich EA: Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem*, **274**, 28837-28840 (1999).

Kim SY, Lee JH et al, The human elongation factor 1 alpha (EF-1 alpha) first intron highly enhances expression of foreign genes from the murine cytomegalovirus promoter. *J Biotechnol*, **93**, 183-187 (2002).

Krone WJ, Debouck C et al, Natural antibodies to HIV-tat epitopes and expression of HIV-1 genes in vivo. *J Med Virol*, **26**, 261-270 (1988).

Kuppuswamy M, Subramanian T, Srinivasan A and Chinnadurai G: Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. *Nucleic Acids Res*, **17**, 3551-3561 (1989).

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

Le Hir H, Nott A and Moore MJ: How introns influence and enhance eukaryotic gene expression. *Trends Biochem Sci*, **28**, 215-220 (2003).

Lieberman J, Fabry JA, Fong DM and Parkerson GR: Recognition of a small number of diverse epitopes dominates the cytotoxic T lymphocytes response to HIV type 1 in an infected individual. *AIDS Res Hum Retroviruses*, **13**, 383-392 (1997).

Livingston B, Crimi C et al, A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. *J Immunol %2002 Jun 1;168 (11):5499 -506*,(2002).

Masemola A, Mashishi T et al, Hierarchical Targeting of Subtype C Human Immunodeficiency Virus Type 1 Proteins by CD8(+) T Cells: Correlation with Viral Load. *J Virol*, **78**, 3233-3243 (2004).

Matsumoto K, Wassarman KM and Wolffe AP: Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J*, **17**, 2107-2121 (1998).

Mishra M, Vetrivel S et al, Clade-specific differences in neurotoxicity of human immunodeficiency virus-1 B and C Tat of human neurons: significance of dicysteine C30C31 motif. *Ann Neurol*,(2007).

Nasim MT, Chowdhury HM and Eperon IC: A double reporter assay for detecting changes in the ratio of spliced and unspliced mRNA in mammalian cells. *Nucleic Acids Res*, **30**, e109(2002).

Nott A, Meislin SH and Moore MJ: A quantitative analysis of intron effects on mammalian gene expression. *RNA*, **9**, 607-617 (2003).

Pauza CD, Trivedi P et al, Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. *Proc Natl Acad Sci U S A*, **97**, 3515-3519 (2000).

Putkonen P, Quesada-Rolander M et al, Immune responses but no protection against SHIV by gene-gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. *Virology*, **250**, 293-301 (1998).

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

Ramakrishna L, Anand KK, Mohankumar KM and Ranga U: Codon Optimization of the Tat Antigen of Human Immunodeficiency Virus Type 1 Generates Strong Immune Responses in Mice following Genetic Immunization. *J Virol*, **78**, 9174-9189 (2004b).

Ranki A, Suni J et al, T-cell recognition of HIV antigens in HIV-seroreverted persons. *AIDS*, **11**, 132-133 (1997).

Reiss P, Lange JM et al, Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. *J Med Virol*, **30**, 163-168 (1990).

Siddappa NB, Venkatramanan M et al, Transactivation and signaling functions of Tat are not correlated: biological and immunological characterization of HIV-1 subtype-C Tat protein. *Retrovirology*, **3**, 53(2006).

Silvera P, Richardson MW et al, Outcome of simian-human immunodeficiency virus strain 89.6p challenge following vaccination of rhesus macaques with human immunodeficiency virus Tat protein. *J Virol*, **76**, 3800-3809 (2002).

Snow EC, Noelle RJ, Uhr JW and Vitetta ES: Activation of antigen-enriched B cells. II. Role of linked recognition in B cell proliferation to thymus-dependent antigens. *J Immunol*, **130**, 614-618 (1983).

van der Burg SH, Kwappenberg KM et al, Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. *J Immunol %1999 Jan 1;162 (1):152 -60*,(1999).

Wieland U, Kuhn JE et al, Antibodies to recombinant HIV-1 vif, tat, and nef proteins in human sera. *Med Microbiol Immunol (Berl)*, **179**, 1-11 (1990).

Zhao J, Hyman L and Moore C: Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol Mol Biol Rev*, **63**, 405-445 (1999).

5. DISCUSSION

It has been more than twenty five years since Human Immunodeficiency Virus (HIV) has been identified as the causative agent for Acquired Immunodeficiency Syndrome (AIDS). More than 60 million people worldwide have been infected with HIV-1, most in the developing world, and nearly half of these individuals have died (Barouch, 2008). Everyday around 6,800 persons become infected with HIV and over 5,700 persons die from AIDS mainly because of inadequate access to HIV prevention and treatment services (UNAIDS, 2007). Despite a concerted effort for the last two and half decades, a vaccine is not in sight yet. The traditional vaccine strategies successful with various other viral pathogens historically, are either not applicable to HIV or have failed to live up to the expectations.

Traditional approaches of HIV vaccine development have attained limited success: The impressive success with vaccine development against a broad range of viral infections provided an impression that HIV vaccine was only a question of time. Despite initial euphoria, efforts towards developing an HIV vaccine have been slower than expected for several reasons. The extraordinary magnitude of genetic diversity, exceptional levels of viral proliferation, diverse mechanisms of immune evasion, integration of the virus into the host genome, elimination and dysfunction of the $CD4^+$ T-cells, prolonged clinical latency, establishment of viral latency, non-availability of a suitable animal model, continued ignorance of immune-protection correlates and many other practical problems made an HIV vaccine a daunting task. An added problem is the imbalanced and unreasonably excessive emphasis on structural antigens mainly on env and partly on gag. Many of these shortcomings of the HIV vaccine efforts have been realized and several strategies are currently underway to circumvent some of the challenges (Johnston and Fauci, 2007).

The failure of the human clinical trials: An indeed large number of human clinical trials failed to reproduce the successful immune responses and protection obtained in

animal models. The first large clinical trial using a recombinant gp120 did not protect against HIV infection (Flynn et al., 2005; Pitisuttithum et al., 2006). Env vaccines and peptides have been tested in several different clinical trials without significant success thus far (Spearman, 2006; Johnston and Fauci, 2007). Further, although significant levels of virus neutralization, in the laboratory experiments, was achieved by env vaccines when laboratory-adapted viral strains were used, neutralizing primary clinical isolates proved to be difficult (Burton et al., 2004). One reason underlining the failure is the possible inability of the recombinant viral env to reproduce the native configuration of the env as present on the viral surface (Crooks et al., 2007). The prime-boost strategy, however, did not show much promise in 1st and 2nd phase human clinical trials. A recent failure of an adenovirus-based vaccine of Merck, HVTN 520, in a human clinical trial has been one of the serious setbacks for HIV vaccine development (Steinbrook, 2007). Repeated failure of the HIV vaccine at the clinic underlines the importance of 'going back to the basics' (Kaiser, 2008).

Tat remains a potential vaccine candidate: HIV vaccine development traditionally depended on structural proteins like env, gag and regulatory protein Nef all of which are immunodominant. Antibody response to env is certainly critical to prevent or reduce the rate of infection at the entry level. Recent studies have demonstrated the importance of cell-mediated immune responses to gag in restricting viral proliferation in vivo (Kiepiela et al., 2007; Novitsky et al., 2003; Novitsky et al., 2006). In contrast, evidence is also available that gag vaccines failed to induce protective immune response (Saini et al., 2007b; Putkonen et al., 1998). In a head-to-head comparison of Tat vs gag immune responses in a primate model, immune responses to Tat, but not gag, provided protection against viral challenge (Stittelaar et al., 2002). Inclusion of an antigen like Nef in vaccine design could be risky given that Nef is an accessory protein (not an essential protein unlike env, gag or Tat), the presence of which is not critical for the survival of the virus and the virus could efficiently develop resistance against Nef. Although env vaccines conferred protection against autologous viral strains, antigenic variation is a challenge for vaccine design (Osmanov et al., 1996). Further, most of the clinical trials using env and other structural antigens did not provide protective efficacy (Veljkovic et al., 2003;

Kaiser, 2008; Bubnoff, 2007; Steinbrook, 2007). In this backdrop, inclusion multiple antigens in HIV vaccine design and optimizing each individual antigen for efficient immune response is essential. A need for developing multi-component vaccines is being increasingly realized, to induce broader immune responses against the viral infection, by incorporating multiple viral antigens (Ho and Huang, 2002). Extensive work from various laboratories has identified the viral structural proteins, gag and pol, and viral regulatory proteins Nef, Tat and Rev, as potential candidates for vaccine development (Calarota et al., 1999; Evans et al., 1999; Putkonen et al., 1998). Of these non-env candidates, Tat occupies a special place for several reasons.

Why Tat vaccine did not live up to expectations thus far? Despite all its merits, initial attempts of Tat vaccine met with limited success to the extent that there were doubts as per the rationale of Tat use as a candidate vaccine. The primary reason why Tat vaccine did not yield expected results was because all the previous strategies ignored the basics while designing the vaccines. Several technical challenges should have been before expecting Tat to function as a preventive or therapeutic vaccine. Some of the important limitations of the Tat vaccines can be broadly classified into three categories which have been described briefly below (a) poor immunogenicity of Tat, (b) safety concerns since Tat is a toxin and an immunomodulator and (c) restricted antigen presentation as a protein.

1. **Tat is non-immunodominant**: Tat is a small nuclear protein that lacks potential T-helper epitopes. Although T-helper epitopes have been mapped in Tat (Blazevic et al., 1993; Ramakrishna et al., 2004; Ranki et al., 1997; Silvera et al., 2002), in natural infection, several lines of evidence suggest that these T-helper epitopes may not be strong enough. Only a fraction, 10-15% (data from our laboratory), of the seropositive subjects make anti-Tat humoral immune response (Krone et al., 1988; Reiss et al., 1990; Wieland et al., 1990). Of these subjects, only a minority show isotope switching to IgG indicating lack of efficient T-help (Venkatesh PK et al, manuscript in preparation). Likewise, cell-mediated immune responses to Tat were also shown to be scarce in natural infection (Borrow et al., 1994; Goulder et al., 2001;

Lieberman et al., 1997; Masemola et al., 2004; Lamhamedi-Cherradi et al., 1992). The non-immunodominant nature of Tat must be an intrinsic property of Tat given that in experimental immunization also strong immune responses have not been seen in primate (Putkonen et al., 1998; Belliard et al., 2005; Pauza et al., 2000) or human studies (Calarota et al., 1999; Hejdeman et al., 2004). Non-availability of sufficient quantity of Tat in extra-cellular milieu could also be a contributory factor for non-immunodominant nature of Tat in natural infection. Although Tat is believed to be secreted extracellularly (eTat), the data in support of this hypothesis are scanty and wanting. The foregoing suggests that molecular strategies are required to enhance immune responses induced by Tat for this antigen ever to become a candidate vaccine. Nearly all the previous attempts ignored this critical issue and used Tat as a protein, toxoid or DNA without means to enhance immune response.

2. **Tat being a toxin raises safety concerns**: As an extracellular viral factor, eTat is believed to possess pleiotropic effects on host cells and host immune system to enhance viral pathogenesis and infectivity. Some of these properties of eTat could have serious consequences especially in immune-compromised subjects (Huigen et al., 2004).
    i. **Latent virus activation**: eTat could activate latent viruses thus contributing to spreading of the viral infection.
    ii. **Apoptosis of the lymphocytes**: eTat could program uninfected T-lymphocytes (Li et al., 1995), B- cells (Huang et al., 1997) and monocytes, to commit to apoptosis thus increasing the chances immune-suppression in HIV infected subjects. Tat can also inhibit NK cell function contributing to NK cell dysfunction (Zocchi et al., 1998)
    iii. **Coreceptor upregulation**: Tat can upregulate expression of coreceptors CCR5 and CXCR4 on target T-cells thus increasing the chances of viral infection (Huang et al., 1998). Likewise, Tat can modulate expression of a broad range of host genes with serious consequences for the host (Giacca, 2004).

iv. **Neuropathogenesis**: Direct exposure of neurons and astrocytes to Tat is known to enhance cell death leading to neurologic consequences including enhanced dementia (Nath et al., 1998; Mishra et al., 2007).

v. **Perturbing cytokine homeostasis**: Tat can induce cells of diverse phenotype to secrete cytokines and/or chemokines thereby actively perturbing the cytokine homeostasis in the body and consequently contributing to overall immune-suppression (Lafrenie et al., 1997; Nath et al., 1999).

vi. **Immunosuppression**: Tat activates TNF-α secretion from macrophages leading to immune-suppression (Zagury et al., 1998) or through TGF-β (Reinhold et al., 1999). Tat could directly inhibit T-cell proliferation (Zagury et al., 1998; Viscidi et al., 1989). Coexpression of Tat inhibited immune responses to env through the mediation of IL-10 activation (Gupta et al., 2008). In contrast, coexpression of Tat was shown to broaden immune recognition of HIV-1 gag and env demonstrating adjuvant properties (Gavioli et al., 2008). Although Tat is also known to be an immunoactivator (Fanales-Belasio et al., 2002; Gavioli et al., 2004), the conditions that regulate the fine balance between these contradictory functions of Tat have not been well understood.

The Tat vaccine controversy: The recent controversy on the Tat vaccine developed by Dr. Barbara Ensoli's group in Italy revolves around the safety concerns of Tat (Cohen, 2007) and (http://www.i-sis.org.uk/ControversyAIDSvaccines.php). The vaccine developed by this group consists of the functional Tat protein that could have potential hazards associated with human use (Ensoli et al., 2006). No strategies have been employed to answer the question of safety of this Tat vaccine candidate.

Tat toxoid and other inactive forms of Tat: Attempts have been made to formulate Tat protein as a toxoid by chemical treatment (Gringeri et al., 1998; Le Buanec and Bizzini, 2000). Tat toxoid was shown to be safe which also generated moderate immune responses in humans (Gringeri et al., 1998; Gringeri et al., 1999; Noonan et

al., 2003; Moreau et al., 2004) and in primates (Pauza et al., 2000; Richardson et al., 2002; Silvera et al., 2002). Although several studies demonstrated immunogenicity of Tat toxoid, often comparable to the 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; Yang 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 form (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 was reported beyond this animal model (Caselli et al., 1999; Mayol et al., 2007). Oxidized Tat was proposed to be a safe format for vaccination (Cohen et al., 1999).

3. **Tat as a protein or toxoid may not have access to the MHC class-I compartment:** Tat predominantly is an intra-cellular protein although experimental evidence is available to show its secretion into the body fluids. Further, Tat is not exposed on the surface of the virus. Cell-mediate immune responses to Tat, therefore, should be the predominant component to restrict viral expansion in vivo although antibodies are expected to play a significant role. Majority of the previous strategies used Tat as a recombinant protein or toxoid in primate immunization studies (Cafaro et al., 1999; Ensoli and Cafaro, 2000; Pauza et al., 2000; Richardson et al., 2002; Silvera et al., 2002; Tikhonov et al., 2003) or human clinical trial (Ensoli et al., 2006). 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 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. Immune intervention, however, could interfere with immune responses (de et al., 2008; Willis et al., 2006). Further,

preexisting immune response to the viral vector is a significant problem that limits recombinant vector-mediated antigen delivery (Bangari and Mittal, 2006). DNA vaccine, therefore, is 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 these vaccines could be used as a reliable medium of immunization (Dean et al., 2005).

Innovative features of the present work: This project work enlists several potentially important molecular and immunologic features to address several critical challenges of the Tat vaccine. Many of these novel features are the first-time strategies in the field.

1. **The question of Tat safety**: Given the multitude of harmful effects Tat can exert on the cellular gene expression and host immune system, we have given the highest importance to attenuating Tat using a novel molecular strategy. We disrupted the structural integrity of two of the most important domains of Tat, CRD and BD, that govern several critical biological functions of Tat. We inserted universal T-helper epitopes, PADRE or pol 711, into the CRD and /or BD of Tat causing minimum disruption to the known B-cell or CTL epitopes (Figure-23, p 119). While CRD regulates transactivation, chemokine induction and apoptosis properties of Tat, BD is responsible for nuclear localization, RNA binding and membrane translocation and many other functions. Disruption of just one of the two domains was sufficient to perturb the transactivation property of Tat (Figure25, p 124). Tat without the transactivation capability will be incapable of modulating host gene expression and thereby would fail to transactivate the latent viruses in infected individuals, if administered as a vaccine either in a DNA format or as a protein. Furthermore, Tat with either CRD or BD disrupted caused significantly low level apoptosis in THP-1 cells as compared to Tat with intact domains (Figure 26, p 126) confirming the non-toxin nature of the engineered Tat proteins. We are presently evaluating the effect of Tat domain disruption on several other biological functions of Tat including cytokine induction, cell

differentiation, neuron apoptosis, cellular uptake, upregulation of cellular receptors including FasL and calcium influx. Tat domain disruption has now significantly improved the safety profile of Tat thereby making it suitable for internal administration. This particular modification is important when Tat vaccine will be considered for human vaccination. This single feature of the present study alone is the most significant improvement of the Tat vaccine design.

2. **The question of Tat low-immunogenicity**: We devised and evaluated three different but complementary strategies to augment immunogenicity of Tat. These strategies comprise of T-helper epitope grafting, intron engineering and evaluating a cellular promoter to drive the expression of Tat. Two of these strategies, the intron and the T-helper epitopes independently improved the immunogenicity of Tat either in the context of the CMV or EF-1α promoter. We are presently integrating all the three strategies by constructing Tat expression vectors containing the intron as well as the helper epitopes.

    1) T-helper epitope grafting: In addition to attenuating Tat, T-helper epitope grafting into CRD and BD is also expected to augment Tat-specific immune responses thus circumventing the problem of non-immunogenicity of Tat. We engineered the T-helper epitopes such a way that the known B- and CTL epitopes in Tat are not disrupted. T-helper epitope grafting into Tat had significant impact on Tat immunogenicity. Importantly, when both of the HTL epitopes were grafted together, in either of the combinations, the immunizations induced the strongest and comparable immune response suggesting synergy between these helper epitopes (Figures 28, p 132). The observed synergy between the two epitopes was independent of the orientation of the grafting. The results obtained are consistent regardless of the mouse haplotype difference and the promoter driving the expression of Tat. It has been well documented that peptides with higher-affinity binding for the MHC skew T-cell response towards a Th1 profile (Alhers JD et al., 2001). The two Universal T-helper epitopes engineered into the Tat constructs have high

affinity binding for MHC hence could effectively polarize the immune response towards Th1. The strategy of epitope grafting would be beneficial not just for Tat, but for any other inherently non-immunodominant antigen.

2) **Intron engineering:** Presence of an intron in a transcript can enhance gene expression through several mechanisms. We engineered a small synthetic intron between the two exons of Tat and evaluated the magnitude of immune responses. As expected, the magnitude of immune responses to Tat was significantly superior in the presence of an intron and often nearly comparable to that induced by the HTL Tat constructs. We have constructed Tat expression vectors that integrated both the T-helper epitopes and the intron. Mouse immunizations with the novel Tat-vectors are presently in progress.

3) **Cellular promoter optimization**: In this study, we have compared the performance of a cellular promoter, Elongation factor -1α (EF-1α) promoter, with that of the most commonly used mammalian promoter, the human cytomegalovirus major immediate early (CMV) promoter/enhancer. The comparison comprised of a multilayer and hierarchical strategy using in vitro reporter gene expression, in vivo biochemical evaluation and genetic immunization using Tat. The objective of this undertaking was to evaluate if the EF-1α promoter could serve as a suitable alternative for the most popular CMV promoter for genetic immunization studies. Gene expression from the EF-1α promoter is strong and ubiquitous and this promoter has been successfully used in gene therapeutic applications. The potential of the EF-1α promoter for genetic immunization, however, has not been explored adequately, with the present study being one of the few such studies, to the best of our knowledge. A direct comparison of the EF-1α promoter with the CMV promoter, to drive reporter genes or in genetic immunization, revealed that these two promoters performed with comparable efficiencies in all the assays. These data are in support of the premise that EF-1α promoter is a suitable replacement for the viral promoter for genetic immunization. The

CMV promoter is downregulated in several physiological contexts, as is expected of a viral regulatory element. The cellular promoter EF-1α is less likely to be silenced through these pathways and this prolonged expression of the antigen could be an advantage of the cellular promoter over the viral promoter.

In addition to comparing EF-1α promoter with the CMV promoter in biochemical assays and genetic immunization, we have also attempted to delineate the regulatory elements of the cellular promoter. A previous study carried out a deletion analysis of the EF-1α intron I and alluded to the presence of a putative negative regulatory element since removal of this element significantly improved expression of a reporter gene (Wakabayashi-Ito and Nagata, 1994). This study, however, used a single cell line HeLa that possesses no significance with respect to antigen processing or presentation. Keeping these limitations in view, we generated a different series of deletion mutations in intron I and evaluated these promoters in reporter assays and genetic immunization. An additional objective of the deletion analyses is to identify a smaller EF-1α promoter deletion mutant with undiminished functional activity comparable to the parental promoter. In addition to the HeLa cells, we used two additional cell lines in the analysis. The reporter gene expression analyses using the EF-1α deletion mutants did not substantiate the suppressive role of the putative NRE in the intron, for presence or absence of NRE did not make significant difference among the mutant promoters in all the three cell lines used. Experiments are currently in progress to evaluate these constructs in immunologically relevant cells lines including primary myocytes, monocytes and T-cell lines. Furthermore, data obtained through the reporter analyses using promoter deletion mutants also alluded to the presence of two important transcription factor binding sites, Sp1 and CdxA, not identified previously in the intron. The functional significance of these findings remains to be confirmed.

3. **Tat antigen presentation to the MHC I pathway**: Like viruses, genetic vaccines can introduce encoded antigens efficiently into the MHC class I pathway thereby eliciting high magnitude cell-mediated immune responses. It is for this reason we opted to deliver Tat as a genetic vaccine in our studies. However, DNA vaccines suffer from a serious limitation in their inability to induce strong immune responses in larger animals including the human beings. The choice of unifying a non-immuno-dominant antigen like Tat and inefficient immunization medium like the DNA vaccine in the present study is intentional, not coincidental, as this combination offers the advantage of succinct evaluation of the immune-modulatory molecular strategies. In the present study, data from the DNA immunizations induced strong and Th-1 type with little or no Th-2 type immune responses in two different mouse strains and under different vaccination regimens. Additional experiments are presently in progress to further characterize the antigen-induced immune responses. These include the following: establishment of the memory T-cell, central vs effector memory T-cell using flow cytometry; characterization of the activation and proliferation markers, effector-T cell phenotyping (both CD4 and CD8) for multiple cytokine secretion including IFN-gamma, TNF-alpha and IL-2 using intracellular cytokine assay.

4. **Novel challenge models**: Two novel in vivo challenge models, the EcoHIV challenge model and the syngenic tumor model, have been optimized to evaluate the augmented anti-Tat immune responses. Some of the Tat constructs, driven by the EF-1α promoter or its deletion mutants, especially the ApaI-SacI and the SacII constructs, appear to have reduced the pro-viral load significantly. The viral suppression was pronounced when additional boosters were administered. Additional experiments are presently underway to ascertain these observations and to estimate the contribution of anti-Tat responses in the control of the virus proliferation. The syngenic tumor challenge model that we have standardized would enable us to answer that question. Overall this study would open up more

avenues for the exploration of the cellular promoter EF-1α for vaccination studies.(Saini et al., 2007a)

Reference List

Bangari DS and Mittal SK: Current strategies and future directions for eluding adenoviral vector immunity. *Curr Gene Ther*, **6**, 215-226 (2006).

Barouch DH: Challenges in the development of an HIV-1 vaccine. *Nature %2008 Oct 2;455 (7213):613 -9*,(2008).

Belliard G, Hurtrel B et al, Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. *Vaccine*, **23**, 1399-1407 (2005).

Blazevic V, Ranki A et al, Helper T-cell recognition of HIV-1 Tat synthetic peptides. *J Acquir Immune Defic Syndr*, **6**, 881-890 (1993).

Borrow P, Lewicki H et al, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*, **68**, 6103-6110 (1994).

Bubnoff vB: 'Stopping a steam train': Immunizations and enrollment in a second trial with MRKAd5 have now been permanently stopped, and volunteers in the Phambili and STEP trials are being unblinded. *IAVI Report*, **11**, 1-6 (2007).

Burton DR, Desrosiers RC et al, HIV vaccine design and the neutralizing antibody problem. *Nat Immunol*, **5**, 233-236 (2004).

Cafaro A, Caputo A et al, Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. *Nat Med*, **5**, 643-650 (1999).

Calarota SA, Leandersson AC et al, Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *J Immunol*, **163**, 2330-2338 (1999).

Caselli E, Betti M et al, DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. *J Immunol*, **162**, 5631-5638 (1999).

Cohen J: AIDS RESEARCH: Feud Over AIDS Vaccine Trials Leads Prominent Italian Researchers to Court. *Science*, **317**, 738-739 (2007).

Cohen SS, Li C et al, Pronounced acute immunosuppression in vivo mediated by HIV Tat challenge. *Proc Natl Acad Sci U S A*, **96**, 10842-10847 (1999).

Crooks ET, Moore PL et al, A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. *Virology*, **366**, 245-262 (2007).

de MA, Lambeck AJ et al, Viral vector-based prime-boost immunization regimens: a possible involvement of T-cell competition. *Gene Ther*, **15**, 393-403 (2008).

Dean DA, Strong DD and Zimmer WE: Nuclear entry of nonviral vectors. *Gene Ther*, **12**, 881-890 (2005).

Ensoli B and Cafaro A: Control of viral replication and disease onset in cynomolgus monkeys by HIV-1 TAT vaccine. *J Biol Regul Homeost Agents*, **14**, 22-26 (2000).

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

Evans TG, Keefer MC et al, A Canarypox Vaccine Expressing Multiple Human Immunodeficiency Virus Type 1 Genes Given Alone or with Rgp120 Elicits Broad and Durable CD8+ Cytotoxic T Lymphocyte Responses in Seronegative Volunteers. *J Infect Dis*, **180**, 290-298 (1999).

Fanales-Belasio E, Moretti S et al, Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. *J Immunol*, **168**, 197-206 (2002).

Flynn NM, Forthal DN et al, Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis*, **191**, 654-665 (2005).

Gavioli R, Cellini S et al, 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**, 727-737 (2008).

Gavioli R, Gallerani E et al, HIV-1 Tat Protein Modulates the Generation of Cytotoxic T Cell Epitopes by Modifying Proteasome Composition and Enzymatic Activity. *J Immunol*, **173**, 3838-3843 (2004).

Giacca M: The HIV-1 Tat Protein: A Multifaceted Target for Novel Therapeutic Opportunities. *Curr Drug Targets Immune Endocr Metabol Disord*, **4**, 277-285 (2004).

Goulder PJ, Addo MM et al, Rapid Definition of Five Novel HLA-A*3002-Restricted Human Immunodeficiency Virus-Specific Cytotoxic T-Lymphocyte Epitopes by Elispot and Intracellular Cytokine Staining Assays. *J Virol*, **75**, 1339-1347 (2001).

Gringeri A, Santagostino E et al, Tat toxoid as a component of a preventive vaccine in seronegative subjects. *J Acquir Immune Defic Syndr Hum Retrovirol*, **20**, 371-375 (1999).

Gringeri A, Santagostino E et al, Safety and immunogenicity of HIV-1 Tat toxoid in immunocompromised HIV- 1-infected patients. *J Hum Virol*, **1**, 293-298 (1998).

Gupta S, Boppana R et al, HIV-1 Tat suppresses gp120-specific T cell response in IL-10-dependent manner. *J Immunol*, **180**, 79-88 (2008).

Hejdeman B, Bostrom AC et al, DNA Immunization with HIV Early Genes in HIV Type 1-Infected Patients on Highly Active Antiretroviral Therapy. *AIDS Res Hum Retroviruses*, **20**, 860-870 (2004).

Ho DD and Huang Y: The HIV-1 vaccine race. *Cell*, **110**, 135-138 (2002).

Huang L, Bosch I et al, Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. *J Virol*, **72**, 8952-8960 (1998).

Huang L, Li CJ and Pardee AB: Human immunodeficiency virus type 1 TAT protein activates B lymphocytes. *Biochem Biophys Res Commun*, **237**, 461-464 (1997).

Huigen MC, Kamp W and Nottet HS: Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection. *Eur J Clin Invest*, **34**, 57-66 (2004).

Johnston MI and Fauci AS: An HIV vaccine--evolving concepts. *N Engl J Med*, **356**, 2073-2081 (2007).

Kaiser J: AIDS RESEARCH: Review of Vaccine Failure Prompts a Return to Basics. *Science*, **320**, 30-31 (2008).

Kiepiela P, Ngumbela K et al, CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med*, **13**, 46-53 (2007).

Kim DT, Mitchell DJ et al, Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. *J Immunol*, **159**, 1666-1668 (1997).

Krone WJ, Debouck C et al, Natural antibodies to HIV-tat epitopes and expression of HIV-1 genes in vivo. *J Med Virol*, **26**, 261-270 (1988).

Lafrenie RM, Wahl LM et al, Activation of monocytes by HIV-Tat treatment is mediated by cytokine expression. *J Immunol*, **159**, 4077-4083 (1997).

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

Le Buanec H and Bizzini B: Procedures for preparing biologically inactive, but immunogenic HIV-1 Tat protein (Tat toxoid) for human use. *Biomed Pharmacother*, **54**, 41-44 (2000).

Li CJ, Friedman DJ et al, Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. *Science*, **268**, 429-431 (1995).

Lieberman J, Fabry JA, Fong DM and Parkerson GR: Recognition of a small number of diverse epitopes dominates the cytotoxic T lymphocytes response to HIV type 1 in an infected individual. *AIDS Res Hum Retroviruses*, **13**, 383-392 (1997).

Masemola A, Mashishi T et al, Hierarchical Targeting of Subtype C Human Immunodeficiency Virus Type 1 Proteins by CD8(+) T Cells: Correlation with Viral Load. *J Virol*, **78**, 3233-3243 (2004).

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

Mishra M, Vetrivel S et al, Clade-specific differences in neurotoxicity of human immunodeficiency virus-1 B and C Tat of human neurons: significance of dicysteine C30C31 motif. *Ann Neurol*,(2007).

Moreau E, Belliard G et al, Important B-cell epitopes for neutralization of human immunodeficiency virus type 1 Tat in serum samples of humans and different animal species immunized with Tat protein or peptides. *J Gen Virol*, **85**, 2893-2901 (2004).

Nath A, Conant K et al, Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J Biol Chem*, **274**, 17098-17102 (1999).

Nath A, Geiger JD et al, Role of Viral Proteins in HIV-1 Neuropathogenesis with Emphasis on Tat. *NeuroAids*, **1**, (1998).

Noonan DM, Gringeri A et al, Identification of Immunodominant Epitopes in Inactivated Tat-Vaccinated Healthy and HIV-1-Infected Volunteers. *J Acquir Immune Defic Syndr*, **33**, 47-55 (2003).

Novitsky V, Gilbert P et al, Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection. *J Virol*, **77**, 882-890 (2003).

Novitsky VA, Gilbert PB et al, Interactive association of proviral load and IFN-gamma-secreting T cell responses in HIV-1C infection. *Virology*,(2006).

Osmanov S, Heyward WL and Esparza J: HIV-1 genetic variability: implications for the development of HIV vaccines. *Antibiot Chemother*, **48**, 30-38 (1996).

Pauza CD, Trivedi P et al, Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. *Proc Natl Acad Sci U S A*, **97**, 3515-3519 (2000).

Pitisuttithum P, Gilbert P et al, Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis*, **194**, 1661-1671 (2006).

Putkonen P, Quesada-Rolander M et al, Immune responses but no protection against SHIV by gene-gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. *Virology*, **250**, 293-301 (1998).

Ramakrishna L, Anand KK, Mohankumar KM and Ranga U: Codon Optimization of the Tat Antigen of Human Immunodeficiency Virus Type 1 Generates Strong Immune Responses in Mice following Genetic Immunization. *J Virol*, **78**, 9174-9189 (2004).

Ranki A, Suni J et al, T-cell recognition of HIV antigens in HIV-seroreverted persons. *AIDS*, **11**, 132-133 (1997).

Reinhold D, Wrenger S, Kahne T and Ansorge S: HIV-1 Tat: immunosuppression via TGF-beta1 induction. *Immunol Today*, **20**, 384-385 (1999).

Reiss P, Lange JM et al, Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. *J Med Virol*, **30**, 163-168 (1990).

Remoli AL, Marsili G et al, Intracellular HIV-1 Tat protein represses constitutive LMP2 transcription increasing proteasome activity by interfering with the binding of IRF-1 to STAT1. *Biochem J*, **396**, 371-380 (2006).

Richardson MW, Mirchandani J et al, Immunogenicity of HIV-1 IIIB and SHIV 89.6P Tat and Tat toxoids in rhesus macaques: induction of humoral and cellular immune responses. *DNA Cell Biol*, **21**, 637-651 (2002).

Saini M, Hadas E, Volsky DJ and Potash MJ: Vaccine-induced protection from infection of mice by chimeric human immunodeficiency virus type 1, EcoHIV/NL4-3. *Vaccine 2007 Dec 17;25 (52):8660 -3 Epub 2007 Oct 31*, **25**, 8660-8663 (2007).

Saini M, Hadas E, Volsky DJ and Potash MJ: Vaccine-induced protection from infection of mice by chimeric human immunodeficiency virus type 1, EcoHIV/NL4-3. *Vaccine*,(2007b).

Silvera P, Richardson MW et al, Outcome of simian-human immunodeficiency virus strain 89.6p challenge following vaccination of rhesus macaques with human immunodeficiency virus Tat protein. *J Virol*, **76**, 3800-3809 (2002).

Spearman P: Current progress in the development of HIV vaccines. *Curr Pharm Des*, **12**, 1147-1167 (2006).

Steinbrook R: One step forward, two steps back--will there ever be an AIDS vaccine? *N Engl J Med*, **357**, 2653-2655 (2007).

Stittelaar KJ, Gruters RA et al, Comparison of the efficacy of early versus late viral proteins in vaccination against SIV. *Vaccine*, **20**, 2921-2927 (2002).

Tikhonov I, Ruckwardt TJ, Hatfield GS and Pauza CD: Tat-neutralizing antibodies in vaccinated macaques. *J Virol*, **77**, 3157-3166 (2003).

Veljkovic V, Muller S and Kohler H: AIDSVAX results: an important open question. *Vaccine*, **21**, 3528-3529 (2003).

Viscidi RP, Mayur K, Lederman HM and Frankel AD: Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. *Science*, **246**, 1606-1608 (1989).

Wakabayashi-Ito N and Nagata S: Characterization of the regulatory elements in the promoter of the human elongation factor-1 alpha gene. *J Biol Chem %1994 Nov 25 ;269 (47):29831 -7*,(1994).

Wieland U, Kuhn JE et al, Antibodies to recombinant HIV-1 vif, tat, and nef proteins in human sera. *Med Microbiol Immunol (Berl)*, **179**, 1-11 (1990).

Willis RA, Kappler JW and Marrack PC: CD8 T cell competition for dendritic cells in vivo is an early event in activation. *Proc Natl Acad Sci U S A*, **103**, 12063-12068 (2006).

Yang Y, Tikhonov I et al, Monocytes Treated with Human Immunodeficiency Virus Tat Kill Uninfected CD4(+) Cells by a Tumor Necrosis Factor-Related Apoptosis-Induced Ligand-Mediated Mechanism. *J Virol*, **77**, 6700-6708 (2003).

Zagury D, Lachgar A et al, Interferon alpha and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. *Proc Natl Acad Sci U S A*, **95**, 3851-3856 (1998).

Zocchi MR, Rubartelli A, Morgavi P and Poggi A: HIV-1 Tat inhibits human natural killer cell function by blocking L-type calcium channels. *J Immunol*, **161**, 2938-2943 (1998).

Appendix

Solution-1 for TSM: GTE
50 mM Glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)
autoclaved and stored at 4^0C

Solution-2 for TSM:
0.2 N NaOH (diluted from 10 N stock)
4% Triton-X 100
in sterile distilled water, can be stored at 4^0C for up to 1 month

Wash solution for TSM:
60% ethanol
10 mM Tris (pH 8.0)
100 mM NaCl
1 mM EDTA (pH 8.0)
store at room temperature for several months.

2X HBS for $CaCl_2$ mediated transfection
270 mM NaCl
0.001 mM Na_2HPO_4
0.054 mM HEPES
in sterile distilled water, adjust pH to7.0 and store in frozen aliquots. Discard after a single use.

TNN buffer for cell lysis
50 mM Tris (pH 8.0)
150 mM NaCl
10% Glycerol
20% Triton-X 100
25 mM NaF
Stored frozen at -20° C. Add PMSF to 100 μg/ml just before use.

Lysis buffer for β-gal assay
0.1 M Tris (pH 7.8)
0.5% v/v Triton-X 100
stored at room temperature for up to a month.

0.1 M sodium phosphate buffer (pH 7.5)
Mix 41 ml of 0.2 M $Na_2HPO_4.2H_20$ (35.61 g/lt) and
9 ml of 0.2 M NaH_2PO_4. $2H_2O$. Make up volume to 100 ml by sterile distilled water.

1X ONPG for β-gal assay
4 mg/ml in 0.1 M sodium phosphate (pH 7.5).

100X Mg^{2+} solution for β-gal assay
0.1 M $MgCl_2$
4.5 M β-ME
store at –20 ^{0}C for up to a month.

2X SEAP buffer
2 M Diethanolamine
1 mM $MgCl_2$
20 mM L-Homoarginine
in sterile distilled water, store at 4^0C.

TBS
25 mM Tris
14 mM NaCl
0.05% Tween-20
Adjust pH to 7.5. Make a 10X stock, autoclave and store at room temperature.

G418 solution
100 mg/ ml solid dissolved in unreconstituted RPMI-1640 medium. Aliquot and store at $–20^0$C.