Production and assessment of reagents in the development of virus vaccines

by
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ii) I was admitted as a research student in October 2000 and as a candidate for the degree of Doctor of Philosophy in Molecular Virology; the higher study for which this is a record was carried out in the Faculty of Sciences at the University of St Andrews between 2000 and 2003.

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Abstract

The work presented in this thesis consists of three parts: (i) the development of SIV tat as a carrier for enhancing immune responses; (ii) the generation of HIV Gag (P24P17) protein to measure antibody responses in HIV vaccine volunteers; (iii) attempts to deliver the V protein of SV5 into cells to make them non-responsive to interferon.

The work in the first part is considered with the development of effective vaccines against virus infection, focusing on human immunodeficiency virus (HIV). In this work, we investigated the potential of using a protein, SIV tat, with membrane-permeable activity to carry immunogens into target cells to induce CTL responses. The SIV tat protein was shown to bind cell membranes of cultured cells, and furthermore IgG2a antibodies (indicator of Th1 responses) were induced in mice injected with protein antigens fused with SIV tat protein. A basic amino acid rich domain of SIV tat was identified responsible for the binding to cell membranes. In order to examine whether immunization with SIV tat could induce CTL responses, a CTL epitope sequence was cloned into a DNA construct encoding tat fused to the CTL epitope and GST protein. However, after immunizing Balb/c mice with the purified fusion proteins, epitope-specific CTL responses were not detected, even though IgG2a was elicited. Normally, DNA vaccines do not induce strong CTL responses and an additional immunization with live viral vectors is required for the induction of strong CTL responses. To test whether immunization with SIV tat fusion proteins could similarly prime CTL responses, a recombinant adenovirus containing the gene of this fusion protein was made and used in an attempt to boost CTL responses induced by this protein antigen. However, CTL responses were not detected following this prime-boost approach. Although SIV tat was not able to induce CTL responses, the immunogenecity of protein antigens was significantly improved without the presence of adjuvants. Furthermore, Th1 responses were activated, an occurrence not normally achieved by immunizing with proteins. Together, these results suggest that SIV tat protein would

potentially be used as an adjuvant in vaccine modality to induced Th1 and antigen specific antibody responses.

Initially, SIV tat was considered as a carrier to deliver HIV antigens for the induction of HIV-specific CTL responses, and thus P24P17 (part of the gag protein of HIV) was experimentally produced in the baculovirus expression system. However, this project was not continued, since SIV tat was shown incapable of delivering antigens into target cells. Moreover, P24P17 was used as a target antigen for ELISA analyses to measure antibody responses in HIV vaccine volunteers. Only one volunteer from the group receiving the DNA/MVA vaccine regime was found producing antibodies against P24P17.

It is possible to make cell lines non-responsive to interferon (IFN) by constitutively expressing the V protein of SV5, which blocks IFN signaling by targeting STAT1 for degradation. Rather than making cell lines, it could be very useful to add a reagent in the culture medium which inhibits the IFN response. To this end, a tat/v fusion protein was produced. However, STAT1 was not degraded when cultured cells were incubated with purified tat/v, suggesting that SIV tat might not be competent in penetrating cell membranes. Therefore, a recombinant adenovirus containing the V gene was produced to infect cultured cells, and encouragingly the degradation of STAT1 was associated with the expression of the V gene. The potential uses of this vector will be discussed.

Abbreviations

aa	amino acid
ADAR	RNA-specific adenosine deaminase
Ad	adenovirus
AIDS	acquired immunodeficiency syndrome
amp	ampicillin
APC	antigen presenting cell
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CD	cluster designation
cDNA	complementary DNA
CIAP	calf intestine alkaline phosphatase
cpm	counts per minute
CTL	cytotoxic T lymphocyte
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DAPI	4,6-diamino-2-phenylindole
DC	dendritic cell
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
dsRNA	double strand RNA
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EBV	Epstein-Barr virus
E. coli	*Escheri coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme linked immunosorbent assay
env	envelope
ER	endoplasmic reticulum
GAF	gamma activated factor
gag	group specific antigen
GFP	green fluorescent protein
GST	glutathione S-transferase
H-2	designation for the murine MHC locus
HCMV	human cytomegalovirus
HHV	human herpesvirus
HIV	human immunodeficiency virus

His	histidine
HLA	human lymphocyte antigen
HPV	human papillomavirus
HRP	horse radish peroxidase
HSV	herpes simplesvirus
IFN	interferon
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISRE	interferon stimulation response element
kb	kilobases
kpb	kilobase pairs
LB	Luria broth
LCMV	lymphocytic choriomeningitis virus
IL	interleukin
LMP	low molecular weight peptide
LTR	long terminal repeat
mAb	monoclonal antibody
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
mRNA	messenger RNA
MVA	modified vaccinia Ankara
MW	molecular weight
NaAc	sodium acetate
NaCl	sodium chloride
NK	natural killer cell
Ni	nickel
NP-40	nonidet P-40
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pH	pondus hydrogen
PIV	parainfluenza virus
Pk	affinity tag originating from SV5 V protein
PKR	ds-RNA dependent protein kinase
RNA	ribonucleic acid
rpm	revolution per minute
RRE	rev-responsive element
RT	room temperature

SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
STAT	signal transducer and activator of transcription
SV5	simian virus type 5
TAP	transporter associated with antigen presentation
Tat	trans-activator of lentiviral transcription
TBE	Tris-boron-EDTA
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
tet	tetracycline
Th	helper T cell
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
Tris	tris-hydroxymethyl amino methane
Triton	t-octyphenyloxpolyethoxyethanol
tRNA	transfer RNA
Tween-20	polyoxyethylene sorbitan monolaurate
UV	ultraviolet light
v/v	volume per volume ratio
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

Abbreviations for amino acids

Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cystein	cys	C
Glutamine	gln	Q
Glutamic acid	glu	E
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threocine	thr	T
Tryptophan	trp	W
Tyrosine	tyr	Y
Valine	val	V

Genetic code

TTT	phe	TCT	ser	TAT	tyr	TGT	cys
TTC	phe	TCC	ser	TAC	tyr	TGC	cys
TTA	leu	TCA	ser	TAA	stop	TGA	stop
TTG	leu	TCG	ser	TAG	stop	TGG	trp
CTT	leu	CCT	pro	CAT	his	CGT	arg
CTC	leu	CCC	pro	CAC	his	CGC	arg
CTA	leu	CCA	pro	CAA	gln	CGA	arg
CTG	leu	CCG	pro	CAG	gln	CGG	arg
ATT	ile	ACT	thr	AAT	asn	AGT	ser
ATC	ile	ACC	thr	AAC	asn	AGC	ser
ATA	ile	ACA	thr	AAA	lys	AGA	arg
ATG	met	ACG	thr	AAG	lys	AGG	arg
GTT	val	GCT	ala	GAT	asp	GGT	gly
GTC	val	GCC	ala	GAC	asp	GGC	gly
GTA	val	GCA	ala	GAA	glu	GGA	gly
GTG	val	GCG	ala	GAG	glu	GGG	gly

Units

°C	degrees Celsius
g	gram
m	meter
mol	mole
M	molar concentration
l	litre
s	second
min	minute
h	hour
Ci	curie
Da	Dalton
V	volt
A	ampere
rpm	revolutions per minute
g	gravitational acceleration
U	unit of enzymatic activity

Order prefixes

d	deci	10^{-1}
c	centi	10^{-2}
m	milli	10^{-3}
µ	micro	10^{-6}
n	nano	10^{-9}
p	pico	10^{-12}
f	femto	10^{-15}
a	atto	10^{-18}
k	kilo	10^{3}
M	mega	10^{6}
G	giga	10^{9}
T	tera	10^{12}

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Introduction

The aim of works presented in this thesis is to develop vaccines against viral infection in human, especially against human immunodeficiency virus (HIV). There are four main sections in the introduction part of this thesis which cover part of the crucial basic knowledge about the interaction between immune responses and viral infection. In the first section **(A)**, the interferon response, which is a critical innate immune action in response to virus infection, is described thoroughly. In addition, the mechanisms that viruses have evolved to cope with the adaptive and innate immune responses are reviewed. In the second section **(B)**, mechanisms of antigen presentation including the class I, class II and cross-presentation pathways are compared. In addition, the strategies viruses have developed to escape the surveillance of adaptive immune responses are described. Since the space is limited, the basic knowledge of well understood adaptive immune responses is not described in detail in this section. The life cycle of HIV and the globule distribution of HIV subtypes are mentioned at the beginning of the third section **(C)**. The roles of some crucial immune cells (such as dendritic cells, helper T cells and cytotoxic T cells) in responses to HIV infection, are depicted. The final section **(D)** focuses on the development of vaccines against HIV, which include traditional and modern vaccine approaches. The rational of vaccine strategies focusing on early viral proteins, such as tat protein, is discussed at the end of this section.

A. Interactions between innate immune responses and virus infection.

The innate immunity provides the first line of defense after the host's exposure to pathogens. Although interferon responses present several antiviral activities, viruses have evolved mechanisms to interrupt interferon regulatory pathways for their survival.

A.1. Interferon.

Interferons (IFNs) are inducible cytokines that are produced and secreted by cells in response to viral infection. They induce an antiviral state in cells and also help to regulated the immune response. IFNs are commonly grouped into

two types (Finter, 1996; Samuel, 1991). Type I IFNs, also know as viral IFNs, are induced by virus infection and include IFN-α, which is predominately produced by leukocytes, and IFN-β, which is produced by most cell types but particularly by fibroblasts. Type II IFN, also known as immune IFN-γ, consists of the product of the IFN-γ gene and is synthesized by certain cells of the immune system including activated T lymphocytes (CD4+ T helper cells and CD8+ cytotoxic T cells) and natural killer cells in responses to mitogenic or antigenic stimuli (Bach, et al. 1997; Young, 1996).

There are14 different IFN-α genes in human, but only one IFN-β gene and one IFN-γ gene. Although some IFNs are modified posttranslationally by N- and O-glycosylation, the major human IFN-α subspecies are not glycosylated. The IFN-α gene products appear to function in the form of monomers, whereas IFN-β and IFN-γ appear to function as homodimers. Interferons mediate their effects through interactions with type-specific receptors which are different and non-redundant for the type I and type II IFNs (Doly, et al. 1998). Neither IFNα/β-receptor nor IFNγ-receptor knockout mice can mount effective antiviral responses after virus infections (Hwang, et al. 1995). The IFN receptors do not posses enzymatic activities, but are involved in a complex signaling pathway that ultimately induces the expression of a large number of IFN-stimulated genes (ISGs). The products of ISGs are the workhorses of IFN responses and contribute to an antiviral, antiproliferative and immunoregulatory state in the host cells. It is now clear that although IFN levels markedly increase in response to virus infection, the sequence of events, types of IFN that are produced and ISGs that are induced have an important effect on the outcome.

A.2. Interferon-stimulated genes and antiviral responses.

(i) dsRNA-dependent protein kinase (PKR).

Perhaps the most intensely studied type I IFN-induced gene product is the dsRNA-activated serine/threonine protein kinase, which is known as PKR and has multiple functions in control of transcription and translation events (Clemens and Elia, 1997). The N-terminal domain of PKR protein contains two conserved dsRNA binding motifs, and the C-terminal catalytic domain contains

all of the conserved motifs for protein kinase activity (Meurs, et al. 1990). PKR is normally inactive, but on binding to dsRNA, the resulting conformational change in the enzyme unmasks its catalytic domain that results in the phosphorylation of its substrates (Meurs, et al. 1990; Katze, et al. 1991; George, et al. 1996). No RNA sequence specificity is required for dsRNA to bind to PKR, while kinase activation is associated with the formation of a stable dsRNA-PKR complex that requires at least 30 to 50 bp of duplex RNA and is optimal with about 80 bp (Bevilacqua, et al. 1996; Manchie, et al. 1992).

The antiviral effect of PKR is predominantly due to its phosphorylation of the alpha subunit of initiation factor eIF2. This phosphorylation results in the formation of inactive complex that involves eIF2-GDP and eIF2B such that the recycling of eIF2 is interrupted (Clemens and Elia, 1997). Since eIF2B is present in limiting amounts, the translation events are rapidly inhibited.

In response to dsRNA, the PKR protein also plays a role in mediating signal transduction to enhance expression of many immunoregulatory genes. NF-κB is well known as a key transcriptional factor in induction of many of these genes, such as cytokines, chemokine, MHC class I and cell adhesion molecules (Baldwin, 1996). However, NF-κB is normally held in the inactive state in the cytoplasm by association with an inhibitor protein called IκB. On being phosphorylated, IκB becomes ubiquitinated by an E3 ubiquitin lagase and then is degraded by proteasomes, leading to release the associated NF-κB which can enter nucleus and activate transcription (Israel, 2000). PKR can directly phosphorylate IκB or indirectly by activating the IKKβ subunit of the multicomponent IκB kinase (Chu, et al. 1999; Offermann, et al. 1995).

In response to dsRNA, the PKR protein also plays a key role in triggering apoptosis in virus infected cells. Although the downstream targets for PKR-mediated apoptosis remain to be identified, it has been shown that overexpression of PKR can induce apoptosis through a Bcl2- and caspase-dependent mechanism (Lee, et al. 1997). PKR, activated by dsRNA, also

enhances apoptosis by inducing the expression of Fas and Fas ligand (Takizawa, et al. 1995; Balachandran, et al. 1998; Fujimoto, et al. 1998).

(ii) The 2'-5' oligoadenylate synthetase system.

There is another crucial pathway involving in the IFN-mediated antiviral response for the degradation of viral and cellular RNAs. This pathway comprises a group of IFN-induced 2'-5' oligoadenylate (2-5A) synthetases and a endoribonuclease L (RNase L) (Ghosh, et al. 1991; Zhou, et al. 1993). In this multienzyme pathway, IFN-induced 2-5A synthetases are activated by dsRNA, often derived from invading viruses, to produce a series of short 2-5A that activate the 2-5A-dependent RNase L. Activation of RNase L leads to extensive cleavage of single-stranded RNA including mRNA, thereby downregulating protein synthesis (Silverman, 1997). The RNase L has also been shown to cleave 28S ribosomal RNA in a site-specific manner, which in turn inactivates ribosomes and thus inhibits translation (Iordanov, et al. 2000).

Although 2-5A synthetases are activated by dsRNA, there is no obvious structural homology between the dsRNA-binding domains of 2-5A synthetases and those of PKR or ADAR (Ghosh, et al. 1991).

RNase L has been suggested to play a role in apoptosis, since RNase L knockout mice show deficient in apoptosis in several tissues (Zhou, et al. 1997). Although the exact role of RNase L in apoptosis requires further examinations, the 2-5A synthetases/RNase L system seems likely to contribute to the IFN-induced antiviral responses by inducing apoptosis in virus infected cells (Zhou, et al. 1997; Castelli, et al. 1998a, b).

(iii) Mx GTPase proteins.

The myxovirus-resistance (Mx) protein family, including proteins MxA (human cytoplasmic protein) and Mx1 (murine nuclear protein), are also characterized IFN-inducible gene products with antiviral activity. From animal studies, it has been well established that Mx protein alone is sufficient to block the replication of virus in the absence of any other IFN-α/β-inducible proteins (Arnheiter, et al. 1996; Haller, et al. 1998). Mx proteins are GTPases that belong to the

superfamily of dynamin-like GTPases and the intrinsic GTPase activity is required for their antiviral activity (Ptossi, et al. 1993). Mx proteins associate with themselves, and importantly with viral proteins which is believed to interfere with the activity or trafficking of viral proteins such as viral polymerases and nucleocapsid (Kochs, et al. 1999a; Ponten, et al. 1997). The central and C-terminal regions of Mx play important roles in these protein-protein interactions. The expression of Mx proteins is inducible by IFN-α and IFN-β but not by IFN-γ (Arnheiter, et al. 1996; Simon, et al. 1991). The spectrum of antiviral activities of Mx proteins, and mechanisms by which they act to inhibit virus replication are dependent on the specific Mx protein, its subcellular location, and the type of challenge virus.

The human genome contains two related Mx proteins, MxA and MxB, both inducible by IFN-α/β. The MxA protein normally accumulates in the cytoplasm of IFN-treated cells and possesses antiviral activity against a range of RNA viruses, while MxB so far has not been shown to display an antiviral activity (Haller, et al. 1998). The mouse genome, like human genome, encodes two Mx proteins, Mx1 and Mx2. The Mx1 protein, induced by IFN-α/β, accumulates in the nucleus (Dreiding, et al. 1985), and selectively inhibits the replication of members of the *Orthomyxoviridae*, including influenza virus and Thogoto virus (Haller, et al. 1998). The Mx2 gene is nonfunctional in all laboratory strains of mice because of a single-nucleotide insertion that alters the reading frame (Staeheli, et al. 1988). However, the Mx2 protein of feral mouse strains NJL and SPR is functional and, like human MxA, accumulates in the cytoplasm. The IFN-induced expression of feral mouse Mx2 provides protection against VSV infection (Jin, et al. 1999).

The animal model of MxA transgenic mouse with mutations in Mx1, Mx2 and IFN-α/β receptor genes eliminates the possibility that other IFN-induced proteins might act in combination with the MxA protein to confer resistance to virus infection (Hefti, et al. 1999).

The mouse Mx1 protein blocks influenza virus transcription events by interacting with viron-associated polymerase (Krug, et al. 1985; Pavlovic, et al. 1992). In studies with Thogoto virus, it has been shown that IFN-induced human MxA GTPase binds to the NP nucleoprotein component, thus leading to impair the nuclear import of viral nucleocapsids, which consequently prevent viral transcription (Kochs, et al. 1999a, b).

(iv) RNA-specific adenosine deaminase ADAR1.

The RNA-specific adenosine deaminase (ADAR) has been found induced by IFN, but its potential antiviral activity requires further examinations. It has been shown that ADAR is involved in RNA editing by virtue of its ability to deaminate adenosine (A) to yield inosine (I), which has the mutagenic effect to alter the functional activity of viral (particularly negative-strand RNA genomes) and cellular RNAs, and hence by which biological processes can be affected. ADAR recognizes dsRNA as a substrate and unwinds it as a consequence of the A-to-I editing (Bass, 1997; Simpson, et al. 1996). Since I is recognized as G rather than A by polymerases and ribosomes, thus RNA modification potentially alter the protein codes of the transcript and the sequence of replicated RNAs. A novel inosine-specific ribonuclease has been identified and shown to cause degradation of RNAs that previously have been modified by the ADAR (Scadden, et al. 1997). Since many virus go through a dsRNA-based replication intermediate stage, ADAR might preferentially cause genomic substitution and destruction of viral RNAs leading to reduce replication of viral progeny. Selective adenosine deamination is also observed with cellular RNAs, but no information is yet available concerning possible relationships between antiviral responses and editing of cellular pre-mRNAs (Simpson, et al. 1996).

Two immunologically related forms of ADAR, collectively termed ADAR1, have been identified in human cells, including an IFN-inducible 150-kDa protein (p150) which is located both in the cytoplasm and nucleus, and a constitutively expressed 110-kDa protein (p110) which is located predominantly in the nucleus (Patterson, et al. 1995). An additional form of ADAR, designated ADAR2 (80-kDa protein), has also been found which is not IFN-inducible and

does not cross-react immunologically with ADAR1 but possesses the activity of RNA-specific adenosine deamination (Melcher, et al. 1996).

(v) Major histocompatibility complex proteins and proteasomes.

In addition to antiviral effects exerted at the single-cell level that reduce macromolecular synthesis and hence virus yield, INFs modulate a number of immunoregulatory functions involving interaction between immune cells and virus-infected cells. One unarguable role of IFNs in promoting immune responses against virus infections is their ability to upregulate the expression of major histocompatibility complex (MHC, designated HLA in human) on the cell surface (Samuel, 2001).

All IFN family members share the ability to enhance the expression of MHC class I proteins and hence promote the development of CD8+ cytotoxic T-cell responses which plays an important role in the host response to viral infections (Boehm, et al. 1997). These cytotoxic T lymphocytes recognize peptides derived from viral proteins when presented in associated with MHC class I proteins on the surface of virus infected cells (**Fig. 1**). Studies of cells from knockout mice suggest that IFN signaling, Stat1 and IRF1 transcription factors are essential for maintenance of basal expression of the MHC class I protein (Lee, et al. 1999).

In contrast, only IFN-γ is an efficient inducer for the expression of MHC class II proteins on the cell surface, thereby promoting the response of CD4+ T lymphocytes which recognize peptides of viral proteins presented in association with MHC class II proteins (**Fig. 1**) (Bach, et al. 1997). The expression of MHC class II proteins is predominantly regulated by the MHC class II transactivator factor (CIITA). Most cell types do not express basal CIITA. Expression of CIITA leading to upregulation of MHC class II proteins is induced by IFN-γ on many cell types including monocytes, macrophages, microglia, astrocytes, fibroblasts, and endothelia cells (Steimle, et al. 1994). However, the molecular basis for the discordant that CIITA inhibits IL4-dependent class II expression on B cells requires further exploration (Gourley, et al. 1999).

IFN-γ also affects the processing and presentation of antigenic peptides by modulating the expression of enzymatic components of proteasomes and increasing the expression of cellular components required to target antigenic peptides for interaction with MHC class I proteins (York & Rock, 1996). The proteasome is a multisubunit enzyme complex that is responsible for the generation of all peptides that are presented by MHC class I proteins (**Fig. 2**). In unstimulated cells, the proteasome contains three enzymatic subunits x, y, and z. However, following the treatment of cells with IFN-γ, the transcription of these three subunits is decreased and the transcription of three additional enzymatic proteasome subunits LMP2, LMP7, and MECL1 is increased. This leads to the formation of proteasomes of different substrate specificity, thereby altering types of peptides produced and presented to the MHC class I-restricted CD8+ T cells. IFN-γ also enhances the expression of TAP1 and TAP2, which are responsible for transferring peptides (generated by the proteasome) from cytoplasm into the endoplasmic reticulum to bind nascent MHC class I proteins (Trowsdale, et al. 1990; Epperson, et al. 1992). Thus, IFNs play an important role in improving immunogenicity by increasing the quantity and repertoire of peptides displayed in association with MHC class I proteins to CD8+ T cells.

(vi) The IFN regulatory factor (IRF) family.

As a protein family, the IFN regulatory factors (IRFs) have received much attention for their roles in regulating the host response to virus infection. These factors of IRF family (up to nine members have been found), like STAT factors, are important transcriptional regulators in the IFN response (Nguyen, et al. 1997). These factors are homologous to each other in the N-terminal region, corresponding to their conserved DNA binding domain. The IRFs and STATs can function in conjunction with each other to establish signal transduction and gene regulation events leading to establish the antiviral state. IRF-1 was first identified as a regulator of the IFN-a/β gene promoter, and also known to bind to the IFN-stimulated response element (ISRE) found in the promoter region of some IFN-α/β-regulated genes, hence playing an important role in antiviral actions of IFNs (Schindler, et al. 1995). The IRF-9 protein (p48, ISGF-3γ) was

initially identified as a component of a trimeric ISGF-3 complex, along with Stat-1 and Stat-2. It is the only component containing a nuclear localization signal motif and a DNA binding domain (Lau, et al. 2000). The Stat-2 protein appears to form a complex in the cytoplasm with IRF-9 and retains in the cytoplasm in the absence of IFN treatment but rapidly translocates to the nucleus following IFN treatment. IRF-9 is found in both the nucleus and cytoplasm of untreated and IFN-treated cells, while Stat proteins are not found in nucleus of untreated cells. Preassociation of IRF-9 with Stat-2 in the cytoplasm has the potential to poise cells in a state ready to response to signaling by IFN-α/β (Samuel, 2001) At least four members of the IRF family— IRF-1, IRF-3, IRF-5 and IRF-7— act as signal transducers in host IFN-mediated defense. In response to infection, these transcription factors are phosphorylated on serine residues and transported to the nucleus, where they can regulate the expression of either IFNs themselves or IFN-regulated genes (Katze et al. 2002). IRF-3 (constantly expressed) plays an important role in inducing the expression of IFN-β and IFN-α4 at the early stage of response to infection. However, IRF-7 is required for the expression of the other IFN-α genes. Unlike IRF-3, IRF-7 is not constitutively expressed and requires transcriptional activation through the JAK-STAT pathway, as do most IFN-induced genes (Katze, et al. 2002).

(vii) Control of apoptosis.

Apoptosis, or programmed cell death, can serve as a defense mechanism for the host to combat viral infection. The apoptosis response involves a cascade of intracellular signals initiated in response to a wide range of stimuli, including viral infection. Morphological changes associated with apoptosis in virus-infected cells include condensation of chromatin and vesicularisation of cytoplasm. Biological changes include activation of cellular proteases and nucleases and degradation of cell DNA to fragments (Samuel, 2001).

Early destruction of infected host cells may greatly reduce virus yield, leading to suppressed virus transmission in the infected host. However, virus have evolved strategies to antagonize the apoptosis response by encoding viral gene

products to target effector components in the signaling cascade of apoptosis. Suppression or delay of apoptosis response may buy the time for viruses to produce new progenies prior to destruction of infected cells. In contrast, viral gene products posing a function as inducers of apoptosis have been identified, possibly to facilitate the release and spread of progeny viruses from the infected host (Teodoro, et al. 1997; Shen, et al. 1995).

IFNs, like other cytokines, have either pro- or anti-apoptotic activities depending on various factors such as the state of cell differentiation. However, when viruses infect cells, a major function of IFNs is to induce a pro-apoptosis state in infected cells throuh the induction of PKR and the 2'5'A system (Tanaka, et al. 1998; Schindler, 1998). IFNs have also been shown to induce caspases (cysteine, aspartate protease; normally present in the cell as inactive proenzymes) including caspase-1, caspase-2 and caspase-3 and thus to enhance the sensitivity of infected cells to apoptosis (Chin, et al. 1997; Balachandran, et al. 2000; Subramaniam, et al. 1998). IFN-γ has also been demonstrated to enhance the sensitivity to apoptosis by inducing expression of both Fas and Fas ligand (Xu, et al. 1998).

(viii) Inhibition of cell growth.

IFNs can inhibit the replication of some invading viruses through the inhibition of cell growth. However, different cells in culture conditions exhibit varying degrees of sensitivity to the antiproliferative activity of IFNs. As discussed above, IFN-induction of PKR and the 2'5'A system play an important role in the inhibition of cell growth through phosphorylation of eIF2α (Jaramillo, et al. 1995) and activation of RNase L to degrade mRNA and 28S ribosomal RNA (Iordanov, et al. 2000), respectively.

INFs can also exhibit antiproliferative activities in a more direct manner. It has been shown that IFNs can up-regulate the expression of the cyclin-dependent kinase inhibitor p21 (Chin, et al 1996), which is known to be crucial in the progression from G1 into S phase of the cell cycle (Gartel, et al. 1996). When p21 levels are elevated, cyclin-dependent kinase activity is inhibited and

consequently the phosphorylation of the retinoblastoma gene product (pRb) and the related pocket proteins is suppressed (Sangfelt, et al. 1999). Since pRb and the related pocket proteins interact strongly with the transcription factors of E2F family, there is a consequent decrease of free E2F, which is required for the transcription of many genes that are needed for transition from G1 to S phase, resulting in a block of the cell cycle (Iwase, et al. 1997; Goodbourn, et al. 2000).

The p202 gene product and related members of its '200' family are another potent repressors of the cell cycle, following IFN induction (Kingsmore, et al. 1989; Lembo, et al. 1995). These proteins can bind both pRb and the E2F family of transcription factors to form a complex and hence E2F can not bind DNA to stimulate the transcription of genes important for the cell cycle transition from G1 to S phase (Choubey, et al. 1996; Choubey & Lengyel, 1995). Recently, IFNs have also been shown to downregulate the expression of c-myc, a gene product crucial in driving the progression of cell cycle (Ramana, et al. 2000).

A.3 Signal transduction in response to IFNs.

IFNs exert their biological activities through the binding of IFN-α/β and IFN-γ to their cognate cell surface receptors, which results in the activation of distinct but related signal transduction pathways (**Fig. 3**). These signalings ultimately activate the transcription of target genes (known as IFN-stimulated genes, ISGs) that are normally quiescent or are expressed at low levels. Activated transcription factors translocate to the nucleus and bind to the upstream regulatory sequence of ISGs to activate gene expression (Goodbourn, et al. 2000).

The JAK-STAT is the best understood signaling pathway in IFN-mediated activation of cellular gene expression. The signal transducer and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors that become tyrosine phosphorylated by the *Janus* family of tyrosine kinase (JAK) enzymes in response to cytokine stimulation (Stark, et al. 1998). There are

seven members (Stat-1, Stat-2, Stat-3, Stat-4, Stat-5a, Stat-5b, Stat-6) in the STAT protein family and four protein kinases (Jak-1, Jak-2, Jak-3, Tyk-2) in the JAK enzyme family (Leonard, et al. 1998). Tyk-2 and Jak-1 interact with the intracellular domain of IFN-α/β receptor subunits, IFNAR-1 and IFNAR-2, respectively. Jak-1 also interacts with the IFNGR-1 receptor subunit, and Jak-2 interacts with the IFNGR-2 subunit of the IFN-γ receptor. Binding of IFNs to their cognate transmembrane receptor triggers receptor dimerization, which facilitates trnasphosphorylation and activation of receptor-associated JAK kinases. The latent cytoplasmic STAT transcription factors are subsequently phosphorylated by the activated JAK kinases (Bach, 1997). In IFN-α/β signaling, Stat-1 and Stat-2 are phosphorylated and, along with protein p48 (also know as IRF-9), translocate to the nucleus where they form a trimeric complex ISGF-3, which binds the DNA element (designated ISRE) of IFN-α/β-responsive genes and stimulates transcription. For IFN-γ, Stat-1 is phosphorylated, homodimerizes, and translocates to the nucleus. Active Stat-1 homodimers, designated gamma-activated factor (GAF), activates the transcription of IFN-γ-inducible genes through the binding to the cis-acting DNA element, known as GAS (Levy, 1999).

A.4. Production of IFNs.

The regulation of IFN-β synthesis is well characterized and requires the participation of several transcription factors, such as nuclear factor-κB (NF-κB), ATF, and the IFN-regulatory factors (IRFs). These factors are often activated by phosphorylation on serine residues. It is generally assumed that the expression of IFN-β is triggered by virus infection, probably through the production of viral double stranded (ds) RNA. Similar to IFN-β, the expression of IFN-α genes is also activated in response to virus infection and requires phosphorylation on serine residues of specific transcription factors. However, the IFN-α and IFN-β genes are not expressed at the same level after virus infection. There seems to be a crucial positive-feedback loop that controls IFN expression through the action of many IRFs. The IFN-β and IFN-α4 genes seems to be activated early through the action of constitutively expressed IRF3 (Juang, et al. 1998). However, the expression of the other IFN-α requires the

synthesis and activation of IRF7. Unlike IRF3, IRF7 is not constitutively expressed and needs transcriptional activation through the JAK-STAT pathway, as do most IFN-induced genes. IFN-β and IFN-α4 initiate the production of IRF7, thereby leading to the expression of the full spectrum of IFNs (Levy, et al. 2002; Yeow, et al. 2000).

production of IFN-β

The induction of IFN-β is primarily focused at the level of transcriptional initiation and the crucial feature is the redistribution of NF-κB from the cytoplasm to the nucleus (Lenardo, et al. 1989; Visvanathan & Goodbourn, 1989). NF-κB plays an important role in the transcriptional induction of genes involved in immunomodulation, such as other cytokines, MHC class I and cell adhesion molecules (Baldwin, 1996). NF-κB is normally held in a quiescent state in the cytoplasm by association with an inhibitor molecule IκB. Upon receiving stress signals from various stimulators (such as lipopolysaccharide, tumour necrosis factor, interleukin and viral dsRNA), IκB is phosphorylated by a specific multicomponent IκB kinase and subsequently ubiquitinated by an E3 ubiquitin ligase. Once the ubiquitinated IκB is degraded by proteasomes, NF-κB is freed from restraint and can enter the nucleus to activate transcription events (Israel, 2000). In response to dsRNA derived from viral infectons, the dsRNA-dependent protein kinase (PKR) is activated and, in turn, activates the IκB kinase through phosphorylation of its IKKβ subunit (Chu, et al. 1999; Zamanian-Daryoush, et al. 2000). Although the biological mechanism still requires further investigation, it has been shown that PKR can phosphorylate IκB directly (Kumar, et al. 1994; Offermann, et al. 1995).

To activate the expression of IFN-β, NF-κB is required to bind to the promoter region as part of a multiprotein transcription-promoting complex called the enhanceasome (Thanos, 1996), which also contains other transcription factors including HMG-1/Y, ATF-2 homodimers or ATF-2/c-Jun heterodimers (Du, et al. 1993) and a factor (a member of IRF family) that binds to positive regulatory domain I (PRD I). Since many IRF proteins bind to both PRD I and ISRE (IFN-stimulated response element), there may be some functional overlap in these

proteins. The property of functional overlap may bring benefit to the host to ensure that virus infections cannot block induction of IFN-β completely by inhibiting any single IRF protein.

production of IFN-α

In response to virus infection, the products of IFN-α multigene family are predominantly synthesized in leucocytes and can also be synthesized in fibroblastoid cells. The expression of IFN-α genes is IFN-β dependent in fibroblasts, since IFN-α genes are not able to be induced in embryonic fibroblasts derived from mice lacking both copies of the IFN-β genes (Erlandsson, et al. 1998). It is believed that IFN-β is induced by virus infection and in turn can induce the synthesis of IRF-7, leading to transcriptional activation of IFN-α genes (Au, et al. 1998; Yeow, et al. 2000). The induction mechanism of IFN-α in leucocytes has not yet been well illustrated, but it is clearly distinct from induction in fibroblasts, since the presence of IFN-β is not required (Erlandsson, et al. 1998).

production of IFN-γ

The product of IFN-γ gene, rather than being induced directly by virus infection, is synthesized by Th1 CD4+ helper T cells, nearly all CD8+ cells, and natural killer cells in response to recognition of infected cells (Young, 1996). There are two distinct regulatory elements (proximal and distal) located in the promoter region of IFN-γ gene (Aune, et al. 1997). The proximal element is activated by complexes containing c-Jun and ATF-2, while the distal element is activated by GATA-3 and ATG-1 (Penix, et al. 1996; Zhang, et al. 1998a). In naïve and memory CD4+ T cells these two elements are inactivated, while only the distal element is activated in CD8+ cells, leading to the weak production of IFN-γ in CD8+ cells. The p38 and JNK2 mitogen-activated protein kinases (MAP kinase) have been known to involve in activating transcription of the IFN-γ gene, but to understand the complete profile of controlling IFN-γ gene expression requires further investigation (Rincon, et al. 1998; Yang, et al. 1998; Lu, et al. 1999). In response to recognition of infected cells, IFN-γ production is enhanced markedly by cytokines (IL-12 and IL-18) produced by activated antigen-

presenting cells (Okamura, et al. 1998). Neither IL-12 nor IL-18 alone can induce IFN-γ production significantly in unstimulated T cells, but these cytokines together can induce IFN-γ production without the requirement of antigen stimulation (Tominaga, et al. 2000). The mechanism of this is poorly characterized, but may involve activation of Stat-4 by IL-12 and NF-κB by IL-18, and an up-regulation of the IL-18 receptor by IL-12 may also involve in the induction of IFN-γ production (Yoshimoto, et al. 1998). IFN-γ production in activated NK cells is also enhanced by IL-12 and IL-18, but unlike T lymphocytes the recognition of antigens presented on infected cells in not required for IFN-γ production (Singh, et al. 2000).

A.5. Relation to vaccine design.

The knowledge of understanding the mechanism of the induction of IFN in response to viral infection would be potentially useful for the development of vaccines against virus infection. For instance, to generate attenuated viral vaccines, knocking out the viral genes encoding functional proteins to antagonize IFN responses in hosts would be a more straightforward approach, compared to the conventional mutation selection. It has been shown difficult to generate a decent amount of some viruses in the use as vaccines, and therefore making a virus producing cell lines with incompetent capability of IFN responses could be helpful on increasing the yield of interferon sensitive viruses. In addition, the immunogenesity of vaccines could be enhanced by the action of IFNs on promoting immune responses through upregulating the expression of MHC molecules or other associated routes. Based on the understanding of IFN responses, these are some possible applications related to the design of vaccine against viral infection.

A.6. Viruses fight back.

Viruses have evolved mechanisms to block nearly all aspects of the IFN regulatory pathway. This includes the inhibition of IFN production, the disruption of IFN receptor/JAK-STAT signaling events, and other mechanisms that target the antiviral actions of IFN-stimulated genes.

(i) Inhibition of IFN production.

It is generally believed that intracellular dsRNA, derived from viral genome itself or formed as a result of replication of viral genomes, is the inducer of IFN gene expression. Many viruses have been reported to produce dsRNA-binding proteins as part of their life cycle to protect themselves from antiviral mechanisms. For example, the multifunctional NS1 protein of influenza (Lu, et al. 1995), the E3L protein of vaccinia virus (Chang, et al. 1992), the core antigen of hepatitis B virus (Twu & Schloemer, 1989), and products of the NSP3 gene of porcine rotaviruses (Langland, et al. 1994) have been shown to bind dsRNA. The sequestration of dsRNA could inhibit IFN-α/β production and might also act to prevent the activation of dsRNA-induced antiviral gene products such as PKR, 2'5'A oligoadenylate synthetase and ADAR, as well as dsRNA-dependent apoptosis responses.

Interfering with NF-κB activation or function

Following virus infection, NF-κB plays an important role in the activation of IFN-α/β and other immune responses to protect the infected host. Viruses also find the way to suppress IFN-induced antiviral responses by interfering with NF-κB activation or function. For instance, African swine fever virus (ASFV) has been shown to inhibit the activity of NF-κB by encoding a homologue of IκB, a cellular inhibitor of NF-κB (Powell, et al. 1996; Revilla, et al. 1998). NF-κB is also a crucial factor in the induction of anti-apoptosis genes, and any virus that is capable of blocking NF-κB activation may leave itself susceptible to apoptosis. However, the risk of apoptosis associated with inhibition of NF-κB may be circumvented by viral gene products that act to block apoptosis. The product of LMW5-HL gene of ASFV is a Bcl-like protein and exerts anti-apoptosis function (Gillet & Brun, 1996).

viral proteins bind to IRF

The products of viral genes can also block INF-α/β production through binding to the IRF transcription factors that bind to the PRD I region of the IFN-β promoter. The E6 protein of human papillomavirus type 16 (HPV-16) has been shown to bind IRF-3 and inhibit its transcriptional activation function (Ronco,

et al. 1998). However, inhibition of IFN-β production is not blocked completely, suggesting that other cellular factors might substitute for IRF-3. Indeed, IRF-1 and ISGF3 have been shown to potentially substitute for IRF-3 (Miyamoto, et al. 1988; Yoneyama, et al. 1996) , but these factors might be targeted by other viral proteins. For example, the K9 ORF gene of human herpesvirus-8 (HHV-8) can interact with IRF-1 (Zimring, et al. 1998), while the E7 protein of HPV-16 (Barnard & McMillan, 1999) can target the p48 (sometimes referred to as IRF-9) subunit of ISFG3 and thereby prevent its binding to ISRE (an element found in genes that are transcriptionally responsive to type I IFN).

downregulating host mRNA production or protein synthesis

In addition to blocking the activities of transcription factors, viruses may inhibit the production of IFN by generally downregulating host mRNA production or protein synthesis. For example, the matrix M protein of vesicular stomatitis virus can cause a general inhibition of host-cell transcription events, and the L proteinase of foot-and-mouth disease virus can shut off host-cell protein synthesis. Mutations in genes encoding these viral proteins can affect virus pathogenicity and leads to an attenuated virus with efficient IFN-β-inducing properties (Ferran & Lucas-Lenard, 1997; Chinsangaram, et al. 1999).

producing homologues of IL

Viruses may also use more subtle and indirect methods to inhibit IFN production. For example, Epstein-Barr virus (EBV) produces a homologue of IL-10 (normally produced by Th2 cells) to inhibit the ability of monocytes and macrophages to activate Th1 cells by downregulating the expression of MHC class II molecules (Hsu, et al. 1990). Activated Th1 cells produce a number of cytokines, including IFN-γ, that are required for the induction of cytotoxic T lymphocyte responses against virus infection. Similarly, human herpesvirus-6 can upregulate IL-10 production and thereby cause a shift from a Th1 to a Th2 cytokine profile, leading to the induction of less-effective immune response against the virus (Arena, et al. 1999).

(ii) Viral antagonists of IFN signaling.

There are several advantages for viruses when they have the ability to impair IFN signaling. Since there are components in common between signaling pathways, viruses might block both type I and type II IFN pathways by simply targeting one component in the JAK-STAT pathway. Impairment of IFN signaling not only inhibits the induction of cellular antiviral enzymes, but might also downregulates MHC class I molecules within infected cells, making them less sensitive for cytotoxic T cells. In addition, virus-infected cells would be resistant to the antiviral effects of IFNs produced either by activated leukocytes or by infected cells (Goodbourn, et al. 2000).

mimic IFN receptor

Viruses can encode proteins to impair the activity of the JAK-STAT signaling pathway, and there is accumulating evidence showing that multiple mechanisms appear to be involved. Several poxviruses have been shown to encode soluble proteins (vIFN-Rc) that mimic cellular IFN-receptors to bind and sequester IFNs. This mimicry can lead to inhibit the IFN signaling process and subsequently impair the development of an antiviral state (Smith, et al. 1998). For example, M-T7, the first poxvirus receptor homologue identified, was found secreted from myxoma virus-infected cells and acts as a decoy to inhibit the biological activity of rabbit IFN-γ. Other poxviruses also encode vIFN-γRc, including vaccinia virus, where the B8R gene encodes the IFN-γ receptor homologue. Vaccinia virus and several additional orthopoxviruses encode IFN-α/β receptor homologues which bind several IFN-α subspecies as well as IFN-β and block IFN-α/β signaling activities (Alcami, et al. 2000; Colamonici, et al. 1995). Interestingly, in terms of virus host range, both the IFN-α/β and IFN-γ receptor homologues encoded by poxviruses often have a broad species specificity, unlike their cellular counterparts.

proteasome-mediated degradation

Several viral proteins have been shown to disrupt IFN signaling by targeting components of JAK-STAT pathway for proteasome-mediated degradation. For example, infection with simian virus 5 (SV5) or mumps virus leads to degradation of Stat-1 (Didcock, et al. 1999), while infection with parainfluenza

virus 2 (hPIV2, a virus very closely related to SV5 and mumps) causes degradation of Stat-2 (Young, et al. 2000). In the cell infected with human cytomegalovirus (HCMV), the levels of Jak1 and p48 are decreased by a mechanism involving proteasome, leading to disruption of IFN signaling.

Association of viral proteins with transactivation factors of IFN-mediated signaling pathways, without causing degradation, can also provide viruses a mechanism to circumvent the antiviral responses. Sendi virus and hPIV3 appear to block IFN signaling by preventing appropriate phosphorylation of Stat-1, but not targeting Stat-1 or Stat-2 for degradation (Young, et al. 2000; Komatsu, et al. 2000). The T antigen of murine polyomavirus (MpyV) binds to Jak1 thereby preventing the establishment of antiviral state by inhibiting the activation of IFN signaling pathways (Weihua, et al. 1998). The multifunctional E7 protein of human papillomavirus 16 (HPV-16) interacts directly with p48, preventing the formation of ISGF3 and thus the activation of IFN-α/β inducible genes (Barnard & McMillan, 1999). The E6 oncoprotein of HPV-16 selectively binds to IRF-3 and inhibits its transcriptional activity, thereby interrupting the activation of antiviral responses at the early stage of infection (Ronco, et al. 1998). Adenovirus E1A protein can bind to and thus sequester p300, which is the transcriptional co-activator for several transcription factors including Stat-1α (Chakravarti, et al. 2000). E1A can also inhibit the DNA binding activity of ISGF-3 by interacting with the p48 subunit of ISGF-3, since overexpression of p48 restores IFN responses and the transcription of ISRE-driven genes in adenovirus-infected cells (Leonard & Sen, 1997).

mimic vIRF

HHV-8, a gammaherpesvirus associated with Kaposi's sarcoma, encodes a homologue (vIRF) of the IRF family that functions as a repressor of transcriptional activation induced by IFN-α/β and IFN-γ. The HHV-8-encoded vIRF does not appear to inhibit the IFN signaling (Zimring, et al. 1998), but rather represses IRF-1-mediated transcriptional activation, thus inhibiting transcriptional responses to stimuli from IFNs.

(iii) Viral antagonists of IFN-induced antiviral enzymes.

The fundamental importance of the IFN system in response to viral infection is further illustrated by the finding that many viruses encode gene products that antagonize the activity of IFN-induced antiviral enzymes such as PKR and 2'5' oligoadenylate synthetase.

dsRNA-dependent protein kinase PKR

PKR is an IFN-inducible protein kinase and is activated by autophosphorylation, a process mediated by dsRNA, which is dominantly derived from viral infection. The importance of PKR in antiviral responses can be deduced from various mechanisms that are utilized by viruses to inhibit its activity (Gale & Katze, 1998). A number of viruses have been known to encode ds-RNA-binding proteins that sequester dsRNA and thus inhibit the antiviral activity of PKR. Interestingly, some dsRNA binding proteins, such as NS1 of influenza virus (Tan & Katze, 1998) and E3L of vaccinia virus (Sharp, et al. 1998), also bind directly to PKR and inhibit its function, and the OV20.0L gene product of orf virus seems to inhibit PKR in the similar way (Haig, et al. 1998). In addition to sequestering dsRNA, NS1 has also been shown to induce the activation of a cellular inhibitor of PKR termed p58IPK (Lee, et al. 1990).

In addition to binding dsRNA, a variety of viral proteins can inhibit PKR in other ways. Some viral proteins such as NS5A of HCV and PK2 of baculovirus can bind to PKR directly, thus blocking its activity (Gale, et al. 1997; Dever, et al. 1998). Furthermore, vaccinia virus encodes K3L protein, which has structural similarity to the N terminus of eIF2α and binds tightly to PKR, to overcome the action of PKR by inhibiting autophosphorylation and hence prevent phosphorylation of eIF2α (Davies, et al. 1992; Carroll, et al. 1993).

Some viruses can produce abundant short RNA molecules to prevent the activation of PKR. For example, the adenovirus small RNA transcripts termed VAI RNA can form highly ordered secondary structures and hence bind to the dsRNA-binding site on PKR as a competitor for dsRNA. Since the molecule is too short (160 nucleotides), two molecules of PKR can not bind to the same RNA molecule and transactivate each other (Mathews, 1995). Two other small

RNA molecules, EBER-1 and EBER-2, have been found produced by EBV and appear to act in the same manner as VAI of adenovirus to interfere with PKR activity (Sharp, et al.1993). The short RNA, HIV-TAR (Tat-responsive region RNA) encoded by human immunodeficiency virus type 1 (HIV-1) also has been shown capable of inhibiting PKR activity (Gunnery, et al. 1990).

2'5'A synthetase/RNase L system

Since dsRNA is required for the activation of 2'5' oligoadenylate (2-5A) synthetase, viral gene products that sequester dsRNA, such as the E3L protein of vaccinia virus (Rivas, et al. 1998), can inhibit the antiviral activities of both PKR and 2-5A/RNase L system. In addition to sequestering dsRNA, viruses also have evolved strategies to inactivate the antiviral function of the 2-5A/RNase L system. In the HSV type 1 and type 2 infected human Chang cells, 2'5'A derivatives are synthesized and act as competitors of authentic 2'5'A, thereby downregulating the activity of 2'5'A-dependent RNase L (Cayley, et al. 1984). More directly, viruses such as HIV-1 and encephalomyocarditis virus inhibit the antiviral activity of RNase L by inducing the expression of cellular inhibitor of RNase L termed RLI, which inhibit 2'5'A binding to RNase L and thus prevents its activation (Cayley, et al. 1982; Martinand, et al. 1999).

B. Antigen presentation and viral evasion.

Antigen presentation is a crucial process in activating adaptive immune responses against viral infection. However, viruses have evolved to produce various proteins to escape the immune surveillance by blocking antigen presentation.

B.1. Antigen presentations on cell surfaces.

Presentation of peptides from antigenic proteins on MHC molecules is a key event in the generation of immune responses against specific antigens. In general, MHC class I molecules present peptides of proteins, synthesized in the cell, to CD8+ cytototoxic T cells, whereas MHC class II molecules present peptides of extracellular proteins to CD4+ Th cells.

class II pathway

The current model for class II molecules is that in the endoplasmic reticulum, newly synthesized major histocompatibility complex (MHC) class II molecule associate with the invariant (Ii) chain to form a nonameric complex consisting of three class II αβ dimers associated with an Ii chain trimer (Cresswell, 1996). Targeting signals on the cytoplasmic domain of Ii chains direct the class II-Ii complex into an acidic endosomal compartment, where the Ii chain is degraded by proteolytic enzymes resulting in the transient complex composed of a class II αβ dimer and a residual fragment of the Ii chain, called CLIP (class II-associated invariant chain peptides), in the peptide-binding cleft. The transient occupation of CLIP has been proven responsible for blocking the binding of peptides on the class II binding site (Riberdy, et al. 1992). In the acidic endosomal compartment or MHC class II compartment (MIIC) where antigens are proteolytically degraded into peptides, the interaction between HLA-DM (a second class II-like molecule in human cells, H2-M in mice) and MHC class II-CLIP complex induces CLIP dissociation from MHC class II αβ dimmers and facilitates the binding of antigenic peptides (Denzin, et al. 1995; Sloan, et al. 1995; Weber, et al. 1996). The empty class II αβ dimers are stably associated with HLA-DM, thereby preventing their aggregation and prolonging their ability to bind high-affinity peptides derived from internalized proteins. Once loaded with the cognate peptide, the HLA-DM-associated class II molecules can no longer associate with HLA-DM (Denzin, 1996; Kropshofer, et al. 1997). Another class II-like molecule, HLA-DO can negatively regulate HLA-DM to catalyze CLIP dissociation and peptide loading by physically associating with HLA-DM (Denzin, et al. 1997). The mature class II αβ-peptide complexes are then transported to the cell surface and surveyed by CD4+ T lymphocytes.

class I pathway

Endogenous antigens in the cytoplasm which were subjected to be degraded are often covalently linked to a small protein, ubiquitin, on lysine-amino groups near the amino terminus and then degraded in the central channel of proteasomes without affecting other proteins within the cytoplasm (Kierszenbaum, et al. 2000). The 20S proteasome core is composed of four stacked rings (each contains seven protein subunits); two outer α rings and two

inner β rings forming a barrel-shaped structure (Lowe, et al. 1995). The entrance to the lumen is controlled by the 19S cap which is composed of 15 different subunits and associated with the core proteasome to form the complete 26S proteasome. Functions of the cap are presumably responsible for the ubiquitin recognition, and the unfolding and translocation of protein substrates (Van den Eynde, et al. 2001). A set of the β-ring subunits, including β1, β2, β5 is responsible for the catalytic activity of the peptide-bond cleavage. By the induction of IFN-γ , these β subunits are replaced by their homologous subunits, LMP2, MECL1, and LMP7 leading to the formation of immunoproteasome. The cytokine-induced exchanges affect immunoproteasomes to preferentially generate peptides that are recognized more efficiently by class I molecules (Pamer, et al. 1998; Groettrup, et al. 2001). Antigenic peptides are generated by the cytosolic proteolytic system, and subsequently are transported from the cytoplasm into the lumen of rough endoplasmic reticulums (RERs) by an ATP-binding transporter called TAP (for transporters associated with antigen processing). Shortly after synthesis, MHC class I heavy chain associates with the chaperone calnexin and then binds β2-microglobulin leading to the exchange of calnexin for the chaperone calreticulin. Following the chaperone exchange, the incorporation of the thiol oxidoreductase Erp57, two subunits of TAP and tapasin results in the so-called class I peptide-loading complex. The binding of high-affinity peptides derived from proteolytic degradation induces a conformation change in the class I molecules (composed of a class I α-heavy chain and a β2-microglobulin), while lack of peptide bindings results in proteasome-mediated degradation. Finally, the stably assembled class I/peptide complexes release from the class I peptide-loading complex and then exit RERs trafficking through Golgi complexes to cell surfaces for recognition by CD8+ T lymphocytes (Grandea, et al. 2001).

B.2. Antigen cross-presentation in immune responses against virus infection.

T lymphocytes use their T-cell receptors to recognize peptide antigens presented by major histocompatibility complex (MHC) molecules. In the classical antigen-presentation pathway, MHC class I molecules present peptides derived from

proteins synthesized within the cell, whereas MHC class II molecules present peptides derived from exogenous proteins captured from the environment. However, there are strong evidences to suggest that dendritic cells have a specialized capacity, known as cross-presentation, to process exogenous antigens into the MHC class I pathway. This function of dendritic cells provides an important mechanism for the immune system to generate immunity against virus infection (Bevan, 1987).

T lymphocytes can be separated into two subpopulations based on the expression of cell-surface markers, CD4 and CD8. The CD4+ subset is primarily responsible for providing help to other immune cells though direct cell-cell interactions or the secretion of cytokines. After antigen stimuli and receiving help from helper T cells (CD4+ subset), CD8+ T cells can develop into mature cytotoxic T lymphocytes (CTLs), which are best know for their capacity to kill virus-infected cells. Helper T cells recognize peptides presented by MHC class II molecules, whereas CTLs recognize peptides presented by MHC class I molecules (Cresswell & Lanzavecchia, 2001). That CTLs are restricted to MHC class I molecules is important as it ensures that virus-specific CTLs only kill cells that are directly infected with virus. Bystander cells that simply endocytose viral debris from infected cells will not present viral antigens on the MHC class I molecules and thus will not be targeted by CTLs (Heath & Carbone, 2001).

define cross-presentation

For the initial encounter with antigen, T cells rely on dendritic cells (DCs) which can capture pathogen antigens and transport them to draining lymph nodes. In this way, T cells can detect the presence of pathogens simply by scanning antigens presented on DCs that migrate to the secondary lymphoid compartment. In addition to presenting antigens, DCs express co-stimulatory molecules that allow them to activate naïve T cells, which are then able to enter peripheral tissues to fight the invading pathogens (Banchereau & Steinman, 1998).

In the simplest case, DCs themselves could be infected with pathogens, thereby processing pathogen antigens through the MHC class I pathway. However, not all pathogens can infect DCs, and pathogen-infected DCs are often functionally compromised (Salio, et al. 1999; Servet-Delprat, et al. 2000; Engelmayer, et al. 1999). In fact, a pathway termed cross-presentation has been discovered, in which protein antigens that were synthesized in other type of cells could be captured by DCs, processed into the MHC class I antigen-presentation pathway, and used to prime CTL responses (Bevan, 1987).

mechanisms of cross-presentation

Three general mechanisms have been defined for the cross-presentation pathway. **(i)** The first involves direct injection of pathogen-derived antigenic material into the cytosol of DCs (mediated by some viruses and bacteria), which present pathogen antigens on the MHC class I molecules by the normal cytosolic machinery (Yewdell, et al. 1988; Finelli, et al. 1999). **(ii)** The second mechanism involves endosomal processing rather than cytosomal proteasome processing of pathogen antigens. In this mechanism, antigen peptides are generated in the endosomal compartment and subsequently either loaded on the preformed MHC class I molecules (Schirmbeck, et al. 1995; Bachmann, et al. 1995) or regurgitated onto the cell surface for association with preformed MHC class I molecules (Harding, et al. 1994). **(iii)** In the third mechanism of cross-presentation, exogenous proteins are diverted from either the endosomal compartment or directly from the extracellular fluid into the cytosol for processing in the conventional MHC class I pathway. This situation might consist several different processes including macropinocytosis (Norbury, et al. 1997) and cross-presentation of antibody complexes, apoptotic cells, and necrotic cells (Regnault, et al. 1999; Albert, et al. 1998; Lu, et al. 2000). Although cytotoxic T lymphocyte (CTL) responses play a key role in the immune response against virus infections, it is generally not primed by nonliving vaccines. The pathway presenting exogenous proteins on MHC class I molecules in the same manner as endogenous antigens may be useful in the development of nonliving vaccines to elicit CTL responses against virus infections. Therefore, using transductive proteins, such as HIV tat protein, to

introduce protein antigens into cells might activate antigen specific CTL responses.

role of cross-presentation in immune responses against virus infection

Cross-presentation of pathogen antigens could be important for generating CTL immunity to tissue-specific viruses that do not infect DCs, such as papilloma virus, in which the infection is confined to the epithelial cells of the skin (Tindle & Frazer, 1994). In addition, cross-presentation might be vital for generating immunity to viruses that have evolved mechanisms to specifically inhibit conventional MHC class I-restricted antigen processing and presentation (Tortorella, et al. 2000; Hill, et al. 1995; Ahn, et al. 1996; Fruh, et al. 1995; Gilbert, et al. 1996). As well as the specific targeting of antigen presentation, there are emerging examples of viruses (including herpes simplex virus, measles virus, retrovirus, canarypox virus, vaccinia virus and lymphocytic choriomeningitis virus) which have been shown to have detrimental effects on DCs in various aspects (Heath & Carbone, 2001). In these situations, cross-presentation would result in successful CTL priming, even though the function of direct presentation in DCs was damaged by the viral infections.

B.3. Evasion of MHC class I antigen presentation.

Endogenous proteins can be degraded by the proteasome (a large multifunction protease complex) and the resulting peptides are translocated by transporters associated with antigen processing (TAP) molecules into the endoplasmic reticulum (ER), where they are loaded on the binding groove of MHC class I molecules to form a complex and subsequently exported to the cell surface. The presentation of foreign peptides on the cell surface activates and attracts the class I-restricted CD8+ cytotoxic T lymphocytes (CTLs) (**Fig. 2**). The eradication of virus-infected cells relies on the activity of activated CTLs, and thus pathogens that attenuate class I antigen presentation would allow them to escape the surveillance of the immune system. Every step in the assembly and trafficking of the class I complex might be a suitable target for the strategy of immune evasion used by viruses.

transcriptional down-regulation

A number of viruses, such as HPV and adenovirus type 12, can down-regulate the gene expression of the heavy chain of class I molecules, thus preventing the production of class I molecules. Other components of class I presentation pathway including LMP (a proteasome subunit called low molecular weight protein) and TAP are also targets for transcriptional control (Georgopoulos, et al. 2000; Smirnov, et al. 2000). Even partial down-regulation of these components could frustrate T cell recognition of the virus infected cell.

proteolysis

Recognition of virus-infected cells by CTLs requires that the processing of viral proteins into peptides by cellular proteolysis machineries. However, viral pathogens can disrupt this process and interfere with immune recognition. In human cytomegalovirus-infected cells, the viral phosphoprotein pp65 with associated kinase activity can modify other viral proteins leading to limited access to the proteolysis machinery and inhibit the generation of antigenic peptides (Gilbert, et al. 1996). The Epstein-Barr virus encoded EBNA1 protein contains a Gly-Ala repeat domain that inhibits its ubiquitination and thus interferes with its proteasomal proteolysis (Levitskaya, et al. 1997).

peptide transport

Peptides that are produced by the proteasome in the cytosol are delivered by the peptide transporter (TAP) to the lumen of the ER, where they act as a cargo for MHC class I molecules. Both Herpes simplex virus type 1 (HSV-1) and HSV-2 encode a polypeptide inhibitor of TAP, the ICP47, which competes with peptides for the single peptide binding site on the TAP, thus preventing the transportation of antigenic peptides into the ER (Hill, et al.1995; Tomazin, et al. 1996). Human cytomegalovirus (HCMV) encodes a ER-resident glycoprotein that is found in complexes with TAP and MHC class I molecules inhibiting peptide transport during infection (Hengel, et al. 1997).

retention and destruction of class I molecules

Even if assembly and peptide loading of MHC class I molecules are completed successfully, the final complex must be delivered to the cell surface for the recognition by antigen-specific CTLs. Some viruses encode proteins to detain

properly assembled class I molecules at the site of synthesis. Adenoviruses are able to specifically down-regulate the cell surface expression of MHC class I molecules by synthesizing a type I membrane protein, E3-19K, that binds class I molecules in the ER and forces retention through an ER-retrieval signal in its cytoplasmic tail (Paabo, et al. 1989). Similarly, the US3 gene product of HCMV binds to peptide-loaded class I molecules and prevent their intracellular transport (Ahn, et al. 1996). In a subtly different fashion, murine cytomegalovirus (MCMV) produces a type I membrane glycoprotein, gp40, to retain class I molecules in the cis-Golgi compartment rather than in ER, and this function is independent of the cytoplasmic tail (Ziegler, et al. 1997). The HCMV encoded proteins US2 and US11 can trigger destruction of newly synthesized MHC class I molecules. These gene products are capable of dislocating class I heavy chains from the ER to the cytosol and delivering them to the proteasome (Wiertz, et al. 1996). In HIV-infected cells, the Vpu product interferes with an early step in the biosynthesis of MHC class I heavy chains before their egress from the ER (Kerkau, et al. 1997).

internalization of class I molecules

MHC class I molecules may be retained in or purged from the ER, but even when they reach the cell surface, they are not safe from viral proteins that compromise or modify their function. Internalization of class I molecules from the cell surface of virus infected cells is one of those strategies used by viruses to block killing by class I-restricted anti-virus CTLs. The HIV Nef protein modifies the endocytic machinery so that the coreceptor CD4 and MHC class I molecules are endocytosed, resulting in their degradation in lysosomes (Aiken, et al. 1994; Schwartz, et al. 1996). Nef presumably modifies the AP-2 complex, a set of proteins that links cargo molecules (such as receptors) to the coat proteins of the vesicles that transport them, to facilitate endocytosis of CD4 and class I molecules, thus down-regulating their surface expression (Kirchhausen, et al. 1997; Greenberg, et al. 1997).

variants of epitopes

Unrelated to the generation of peptide epitopes, mutations within immunodominant cytotoxic T-lymphocyte (CTL) epitopes can be exploited by

viruses to evade protective immune responses (McMichael, et al. 1995; 1997). Mutations within crucial CTL epitopes can affect either MHC binding or T-cell antigen receptor (TCR) recognition. However, certain types of CTL epitopes that contain substitutions within TCR recognition sites are unable to deliver a full stimulatory signal thus inducing anergy, although they can still interact with TCR. It has been reported that, in hepatitis B virus infected individuals, this type of mutation transforms these epitopes into antagonists, thus actively silencing virus specific T lymphocytes (Bertoletti, et al. 1994).

B.4. Interference with class II-restricted antigen presentation.

The MHC class II-restricted pathway of antigen presentation focuses on peptides generated in the endocytic pathway. Endosomal and lysosomal proteases digest internalized protein antigens and generate peptide fragments appropriate for presentation by MHC class II molecules. Through the constitutive secretory pathway, the newly synthesized MHC class II proteins are delivered to the endocytic pathway where they are loaded with proper antigenic peptides and then transported to the cell surface for recognition by CD4+ T cells (**Fig. 2**) (Wolf, et al. 1995).

The strategies employed by pathogens to interfere with MHC class II expression fall into two classes, namely effects on transcription and post-translation. During infection, adenovirus, MCMV, and HCMV have been shown to down-regulate the gene expression of MHC class II molecules, although the detail mechanisms require further examination. At the post-translational level, the HCMV US2 protein, which has been known to affect MHC class I, apparently also dislocates the DRα and the DMα chain of MHC class II molecules into the cytosol for degradation by the proteasome (Tortorella, et al. 2000; Spriggs, 1996).

Another target involved in interference with MHC class II function is the trafficking along the enodocytic pathway. Pathogens can rearrange the intracellular trafficking machinery either directly or through control of cytokine production. Human papilloma virus and HIV Nef proteins affect vesicle traffic and the function of endocytic machinery, which could affect the intracellular

distribution of class II molecules and the peptides destined for presentation by them (Alcami & Koszinowski, 2000). Interleukin-10 (IL-10) appears to be capable of preventing the surface display of MHC class II molecules by inhibition of their recruitment from intracellular compartments to the cell surface (Koppelman, et al. 1997). The IL-10 homolog encoded by EBV could thus conceivably impede the display of peptide-loaded class II molecules, thereby delaying the call for CD4+ T helper cells.

B.5. Viral evasion of natural killer cells.

Natural killer (NK) cells are lymphocytes that do not express T cell receptors for the recognition of antigens presented by MHC molecules. They do not undergo genetic recombination events to increase their affinity for particular antigens, and are thus considered part of the innate immune system. NK cells mediate direct lysis of target cells including virus-infected cells by releasing cytotoxic granules containing perforin and granzymes, or by binding to apoptosis-inducing receptors on the target cells (Orange, et al. 2002).

Normally, the effector function of NK cells is effectively inhibited by inhibitory signals provided through interaction of receptors on NK cells with self-MHC molecules on target cells. Various inhibitory receptors are consistently expressed by NK cells, including killer-cell immunoglobulin-like receptors (KIR), immunoglobulin-like inhibitory receptors (ILT) and the lectin-like heterodimer CD94-NKG2A (Biassoni, et al. 2001). These receptors bind to host MHC class I molecules and transmit inhibitory signals to the NK cell through intracellular tyrosine-based inhibitory motifs (ITIMs) contained in their cytoplasmic domains.

Many viruses evade cytotoxic T cells recognition by down-regulating class I molecules on the surface of host cells. In theory this might leave infected cells susceptible to NK lysis owing to the reduced opportunity for class I molecules to engage inhibitory receptors on NK cells (Karre, et al. 1997). If the virus are successful in eliminating class I expression, how then does the infected cell avoid attack by NK cells? Some successful viruses have developed specific

mechanisms to evade detection by and activation of NK cells. These mechanisms fall into distinct categories.

expression of virally encoded MHC class I homologs

Although downregulating the expression of MHC class I molecules prevents CD8+ T-cell recognition, cells that downregulated these molecules are targets for NK cells. It has been reported that both mouse and human CMV encodes their own class I homologs, m144 and UL18, respectively, which serve as NK cell decoys and ligate inhibitory receptors to block NK cell cytotoxicity in the absence of host class I molecules (Beck & Barrell, 1988; Farrell, et al. 1997).

In addition to m144, another ten MHC class I homologues were identified in the MCMV genome recently (Smith, et al. 2002). These genes were not detected earlier because they have structural rather than sequence similarity to cellular MHC class I molecules, suggesting that additional undetected class I homologs might exist in other viral genomes as well.

selective modulation of MHC class I protein expression

Modulation of class I protein expression is a common feature in many viral infections (Tortorella, et al. 2000). Viral proteins have been used by various viruses to down-modulate class I molecules that are efficient at presenting viral peptides to CD8+ cytotoxic T cells (CTLs), such as HLA-A and HLA-B, to evade CTL-mediated destruction of infected cells. In contrast, both HLA-C and HLA-E (the dominant ligands for NK cell-inhibitory receptors) are resistant to the virus-induced clearance from the cell surface or their expression which is specifically enhanced by viral proteins.

At least four HCMV proteins, US2, US3, US6 and US11, are reported to cause class I molecules to deviate from their normal progression from the endoplasmic reticulum to the cell surface. In particular, two dominant inhibitory receptor ligands, HLA-C and HLA-E, remain resistant to degradation mediated either by US2 or US11 (Gewurz, et al. 2001; Machold, et al. 1997). Thus, virus-infected cells might evade the killing activity of NK cells by sparing the class I molecules that are least effective at presenting viral peptides to CTLs but most

effective at inhibiting the lysis activity of NK cells. In contrast, US3 and US6 show nonselectivity in their targeting of class I molecules, but it is not clear whether the extent of down-modulation mediated by them is sufficient to make infected cells susceptible to NK cells (Jun, et al. 2000).

The *nef* protein of primate lentiviruses, such as HIV-1, HIV-2 and SIV, can selectively accelerate the endocytosis of HLA-A and HLA-B, but not HLA-C and HLA-E, from the cell surface, leaving infected cells resistant to lysis by NK cells (Cohen, et al. 1999; Le Gall, et al. 1998).

In addition to the specific down-regulation of class I molecules, viruses also up-regulate certain class I molecules to evade surveillance of NK cells. Expression of cellular HLA-E (the ligand for the inhibitory receptor, CD94-NKG2A) on the cell surface requires binding of a nonamer peptide derived from the signal sequence of most HLA molecules. The UL40 protein of HCMV contains a nonamer peptide homologues and thus can enhance the expression of HLA-E on the cell surface and inhibit the effector function of NK cells (Tomasec, et al. 2000; Ulbrecht, et al. 2000).

inhibition of activating receptor function

In addition to having receptors with potent inhibitory capabilities, NK cells have receptors whose signal transmission can induce cytotoxicity, proliferation and cytokine production (Biassoni, et al. 2001). Several means have been employed by viruses to interfere with the process of activating receptor ligation.

The most common mechanism of interference with activating receptor function used by viruses is down-regulation of activating receptor ligands in infected cells. Certain strains of HCMV increase the resistance of their infected host cells to cytotoxic activity of NK cells by down-regulation of LFA-3, the ligand for NK cell-activating receptor CD2 (Fletcher, et al. 1998).

Infection of certain target cells by HIV, human T cell lymphotrophic virus I (HTLV-I), or HTLV-II can induce sialyation of the ligand on target-cell surfaces, resulting in resistance to NK cell cytotoxicity (Zheng, et al. 1992).

Viruses can avoid activating NK cells by producing proteins to prevent the interaction between the activating receptor and its ligands. Ligation of UL16-binding protein (ULBP) with the NKG2D receptor, an activating receptor expressed on NK cells, results in signal transduction via DAP10, leading to NK cell cytotoxicity. HCMV encodes a soluble UL16 protein, which is capable of binding ULBP, thus blocking the interaction between the NKG2D activating receptor and its cognate activating ligand (Sutherland, et al. 2001; Kubin, et al. 2001).

evasion by modulation of cytokines or chemokines

Viruses may evade the NK cell-mediated defense through virus-produced proteins that counteract or modulate the interactions between cytokines or chemokines molecules and their cognate receptors.

Interference with anti-viral NK cell responses involved inhibition or antagonism of cytokines such as IL-12, IL-18, TNF-α, IL1α, IL1β and IL-15, which are important in the induction of NK cell mediated IFN-γ production and cytotoxicity. Both p13 protein (Born, et al. 2000) of ectromelia poxvirus (EV) and MC54L protein (Xiang, et al. 1999) of MCV are homologous to the mammalian IL-18 binding protein, bind murine IL-18 and inhibit IL-18 receptor binding, thus reducing the production of IFN-γ *in vivo*. The E6 and E7 proteins of human papillomavirus (HPV) have been shown to sequestrate IL-18 from its cognate receptor by specific and competitive binding to IL-18 (Cho, et al. 2001) or IL-18Rα (Lee, et al. 2001). Alternatively, viruses could facilitate overproduction or encode homologs of other cytokines, such as IL-10, that are involves in the inhibition of NK cell activity (D'Andrea, et al. 1993).

Among the chemokines, targets for viral modulation could involve those that directly affect NK cell chemotaxis, including MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3 and RANTES, or other chemokines and chemokine receptors involved in recruitment of leukocyte subsets that influence NK cell function (Orange, et al. 2002).

direct viral effects on NK cells

To block NK cell activity, viruses can destroy NK cells or inhibit their effector functions by direct infection, or cause inhibition through presumably direct contact with NK cells. Both HIV and HSV were found capable of infecting NK cells in vitro. Although infection of NK cells with HIV does not affect the overall lytic activity of NK cell cultures, it does induce cytopathic effects, thus reducing viability of NK cells (York, et al. 1993). In contrast, direct infection of HSV can inhibit NK cell cytotoxicity, although the mechanism is unknown (Chehimi, et al. 1991).

Interactions between virus particles and NK cells that do not result in infection may exert other direct effects on NK cells. The major envelope protein (E2) of hepatitis C virus (HCV) has been shown binding to CD81 on NK cells, and thus inhibits NK cell cytoxicity, IL-2-induced proliferation and IL-induced IFN-γ production (Wack, et al. 2001).

C. HIV infection and immune responses to HIV.

Human immunodeficiency virus is a causative agent of acquired immunodeficiency syndrome. Many immune components such as helper T cells and cytotoxic T cells have been shown important in the control of HIV infection.

C.1. Human immunodeficiency virus.

Like all viruses, human immunodeficiency virus (HIV) replicates within living cells of its host. HIV is an enveloped virus, has nine genes and belongs to the lentivirus genus of retroviruses. Each virion contains 72 copies of membrane glycoprotein projections composed of gp120 and 41. The gp41 molecule is a transmembrane protein that crosses the lipid bilayer of the viral envelope. The gp120 molecule is associated with gp41 on the surface of the virion and serves as the viral receptor for CD4 and a coreceptor (a seven-transmembrane protein of the chemokine receptor family), which are present on susceptible cells such as T lymphocytes and macrophages. The viral membrane derives from host cells and contains host-cell membrane proteins, including class I and class II MHC

molecules. Within the viral envelope is the viral core. a nucleocapsid, which contains an outer layer of proteins, made up of p17, and an inner layer of a protein of p24. The viral genome consists of two copies of positive single-stranded RNA, which are associated with a copy of necleoid protein p10 (a protease) and p32 (an integrase), and two copies of reverse transcriptase, p64 (Peterlin & Luciw. 1988).

viral envelope proteins interact with receptors on host cell surfaces

HIV infects cells mainly of the immune system such as T-helper lymphocytes. The envelope protein determines viral tropism and facilitates the membrane fusion process that allows invasion of the viral genome. Upon interaction of gp120 with its receptor CD4 and coreceptor (CCR5 or CXCR4), the envelope complex undergoes a conformational change permitting membrane fusion and entry into the cell (**Fig. 4**) (Chan & Kim. 1998). Although many coreceptors have been targeted in the culture condition, only CCR5 and CXCR4 are known to be used in vivo by R5-tropic and X4-tropic strains, respectively. Besides CD4+ lymphocytes, macrophages and dendritic cells are also targeted by HIV. Macrophages are an important reservoir of infection, including the microglia in the brain (Kaul, et al. 2001). Dendritic cells use their highly expressed DC-specific C-type lectin (DC-SIGN) to capture HIV in the periphery and carry the virus from mucosal ports of entry to the lymph nodes where they enhance infection of T-cells (Geijtenbeek, et al. 2000).

host cell membrane fusion

The envelope protein can also promote the fusion of infected cells with uninfected neighboring cells, a phenomenon called syncytium formation that is readily observed in cultured cells and may be responsible for some of the cytopathic effects of advanced HIV infection (Geijtenbeek, et al. 2000). Isolates of HIV-1 from different sources were formerly classified as syncytia-inducing (SI) or non-syncytium-inducing (NSI). In most cases, these differences correlated with the ability of virus to infect T cells (infected by SI stains) or macrophages (infected by NSI stains). More recent classifications of HIV-1 are based on which coreceptor the virus uses, but there is no absolute correlation

between the use of CXCR4 (present on T cells) and syncytia-inducing ability (Dorsky, et al. 1999; Smyth, et al. 1997).

virus replication

Following entry of HIV into host cells, viral reverse transcriptase converts the viral RNA genome into dsDNA, and the integration of viral DNA into the host-cell genome creates the provirus (**Fig. 4**). The provirus remains latent until events in the infected cell trigger its activation to make new viral particles by using the cellular genetic machinery (Weiss, 2001). High levels of virus replication and cell destruction and turnover occur at all stages of infection, although it takes years before the CD4+ cell count falls below the threshold for symptomatic immunodeficiency to become manifest (McCune, 2001; Richman, 2001).

C.2. Genetic subtypes of human immunodeficiency virus.

In Western Africa the acquired immunodeficiency syndrome (AIDS) is mainly caused by infection of HIV-2, while throughout the rest of the world HIV that causes AIDS is referred to HIV-1. The genetic sequences of HIV-1 and HIV-2 are so divergent that their envelop glycoproteins are not immunologically cross-reactive to neutralizing antibodies (Letvin, 2002). Moreover, members of HIV-1 group are clustered into disparate subtypes (or clades) based on epidemic in distinct geographic regions. There are three branches in the phylogenetic tree of HIV-1 sequences, including M (main), N (new or non-M), and O (outlier) groups. Group M viruses are by far the most widespread, which cause more than 99 % of infections worldwide. Viruses in this group have been classified into distinct genetic subtypes, which are defined as groups of viruses that are genetically more close to each other than they are in other groups (McCutchan, 2000; Robertson, et al. 2000). Currently, there are nine circulating genetic subtypes (A through K) identified in M group. The viruses originally identified as E (the predominant virus involved in heterosexual transmission in Thailand) and I (a small group of viruses in the Mediterranean region) subtypes are now considered as intersubtype recombinants and termed CRF01_AE and CRF04_cpx, respectively. Several intersubtypes recombinant virus referred to CRFs (circulating recombinant forms) have now spread epidemically to

establish distinct lineages, nine of which have been identified (McCutchan, 2000). Viruses of M group and CRFs derived from the M group can be traced back to a natural transmission from a SIV_{cpz} (simian immunodeficiency virus) infected chimpanzee to a human, which might have occurred in the first half of the 20^{th} century in central Africa (Korber, et al. 2000).

C.3. Geographical restriction of HIV-1 infection.

Globally, viruses of subtypes A and C are responsible for most current infections, followed by subtype B (the dominant virus type in Europe, the Americas, and Australia) and intersubtype recombinants CRF01_AE and CRF02_AG. Subtype C, the most prevalent virus type in south Africa and India, currently infects more people worldwide than any other. In central and eastern Africa, subtypes A and D are dominant virus types, while the other subtypes infect only relatively small numbers of people in these area. An intersubtype recombinant termed CRF02_AG is the most prevalent virus in western Africa. In southeast Asia, CRF01-AE intersubtype recombinant, which carries the envelope sequence of subtype E, is the dominant virus type. Intersubtype recombinants from subtypes C and B are becoming common in China (Moore, et al. 2001). The geographic restrictions are increasingly breaking down, as the global transmission of HIV-1 continues. For example, it has been observed that many European residents are infected with multiple genetic subtypes rather than single subtype B (Op de Coul, et al. 2000). However, it should be noted that the genetic subtypes or recombinant lineages of HIV-1 are not analogous to classic viral serotypes, and the degree of genetic, and hence antigenic diversity is enormous compared with that of viruses (such as smallpox) for which effective vaccines have been developed. The sequences of replicating HIV-1 viruses can differ as much as 10 %, even within a single infected individual (Shankarappa, et al. 1999). Therefore the genetic and antigenic diversity is daunting from the perspective of HIV-1 vaccine development.

C.4. Correlation between HLA haplotypes and HIV disease progression.

The central role of CTLs in controlling virus infection is also affected by the haplotype of MHC molecules (HLA haplotype) expressed on the surface of antigen presenting cells. CTLs recognize virus-derived peptides presented by

MHC class I molecules, and different MHC molecules present different group of peptides, thus influencing the quality of CTL responses and the efficacy in viral containment. In fact, correlations have been observed between HLA haplotypes and the disease progression in HIV-1 infected individuals. Statistically, expression of HLA-B27 and HLA-B57 was shown associated with slow progression towards AIDS (Kaslow, et al. 1996), whereas HLA-B*3501 and HLA-C*04 were correlated with rapid disease progression (Carrington, et al. 1999; Gao, et al. 2001). In addition, homozygosity at the MHC class I loci is also associated with more rapid disease progression compared with heterozygosity (Carrington, et al. 1999), which might be part due to less opportunity for a diverse CTL responses. These observations suggest that less breadth of presenting CTL epitope may lead to less capacity for generating CTL responses that can control rival replication.

C.5. Helper T cell responses to HIV.

help from helper T cells is essential for CTLs to control viremia

The loss of adequate CD4+ T cell help in HIV-1 infected patients may be one of the central reason for the failure of functional CTL responses and the eventual failure of controlling viremia. In mice lacking CD4+ T-cell help, the induced CD8+ T cells specific to lymphocytic choriomeningitis virus (LCMV) were shown loosing of antiviral activities, implying that help from CD4+ T-cells is crucial in maintaining the optimal CTL function (Zajac, et al. 1998). Although virus-specific CTL responses were observed in HIV-1 infected individuals, HIV-specific CD4+ T cells, detected by measuring the release of IFN-γ, were found in early infection but disappeared in late stage of infection or in untreated patients (McMichael, et al. 2001). The clinical long-term non-progressors, who maintain high CD4+ T cell counts in their blood for many years, actually present good proliferation of CD4+ T cells which are specific to HIV gag protein (Pitcher, et al. 1999; Rosenberg, et al. 1997). Moreover, early introducing HAART (highly active antiretroviral therapy) in HIV infected patients leads to rescue virus-specific CD4+ T cells and allows selected individuals to maintain low viral loads even after the withdrawal of antiviral therapy (Rosenberg, et al. 1997; 2000). Together, these all suggest that virus-specific CD4+ T cells are important in controlling viremia.

dendritic cells are used as Trojan horses by HIV

It has been observed that HIV-1 infected individuals mount a vigorous virus-specific CD4+ T helper cells response during primary infection, but this response appears to diminish within a few months (Oxenius, et al. 2000). This reduction could result from the selective infection of HIV-specific CD4+ T help cells by HIV-1, which in turn leads to CTL-mediated clearance or direct viral destruction of these help cells. CD4+ T cells specific to HIV might be preferentially infected by HIV. In the periphery, dendritic cells can capture HIV by binding virus membrane glycoprotein gp120 via cellular membrane glycoprotein DC-SIGN, and retain attached virons in an infectious state (Geijtenbeek, et al. 2000). This enable dendritic cells to bring viruses into draining lym ph nodes during primary infection, where CD4+ T cells are activated by antigens which are processed and presented in MHC class II molecules of dendritic cells. The proximity of HIV-associated dendritic cells and CD4+ T cells (which are replication-permissive cells for HIV) is dangerous for the latter (Pope, et al. 1995). HIV-specific CD4+ T cells are preferentially infected and likely to be deleted early in the course of infection. As cited above, this process can be reversed when acutely infected individuals are given HAART (Rosenberg, et al. 1997; 2000).

helper T cells help CD8+ T cells in various aspects

Although effector CTLs can act alone when killing target cells, their differentiation from naïve CD8+ T cells to functional effector cells is often dependent on help from CD4+ helper T cells. T-cell help is known important for activating, maturing and maintaining CD8+ T cells. **(i)** For priming CTL responses, helper T cell activate antigen presenting cells (APC) such as dendritic cells to produce IL-12, which in turn initiates the CD8+ T cell responses (Ridge, et al. 1998). The activation of APC is mediated by CD40-CD40L interactions. CD40L is expressed on the surface of activated CD4+ T-helper cells and is involved in their activation and in the development of their effector functions. Ligation of CD40 on the surface of APCs such as dendritic cells, macrophages and B cells greatly increases their antigen-presentation and co-stimulatory capacity. Some virus can by-pass this step by stimulating

dendritic cells directly, but it is not known whether HIV can do this (Ridge, et al. 1998). Failure of T-cell help could disrupt CD8+ T cells to make new primary immune responses to mutant viruses that have evaded the originally dominant CTL responses.

(ii) Under conditions of CD4+ T cell deficiency, CMV-specific CD8+ T cells are induced in quantity, measured by tetramer staining technique, but lacking of effector functions. These non-functional CD8+ T cells are found immature rather than end-stage effectors, and thus failure of CD8+ T cells to mature could be a consequence of lacking helper T cells (Zajac, et al. 1998). **(iii)** The maintenance of CD8+ T cells may require help for CD4+ T helper cells as well. It has been shown that the persistence of adoptively transferred CD8+ T cells clones in CMV-infected patients depends on the presence of virus-specific CD4+ T cells (Walter, et al. 1995). Survival of infused HIV-specific CD8+ T cells in poor in HIV-infected patients, where CD4+ T-cell help is known being damaged (Brodie, et al. 1999; Tan, et al. 1999). The persistence of these virus-specific CTLs may particularly rely on IL-2 and other factors provided by helper T cells.

It should also be noticed that some subsets of activated CD4+ T cells possess cytotoxic activities in the same fashion as CD8+ CTLs, and are able to release antiviral cytokines/chemokines (Robbins, et al. 1998; Tazume, et al. 2004).

Loss of HIV-1-specific CD4+ T cells may be essential for the pathogenesis of AIDS. Deficiency in T cell help may undermine the functional integrity and survival of virus-specific CD8+ CTL, leading to eventual failure of immune control of viremia and clinical disease progression.

C.6. Functions of HIV-specific CD8+ T cells.

Perforin and granzyme

Virus-specific CTLs possess a range of antiviral activities including the ability to kill infected cells and to produce cytokines and chemokines. The importance of each antiviral activity varies in different viral infections. Perforin is a protein, made by CD8+ T cells, that is present in granules, and together with the

granzymes plays an important role in triggering target-cell death. In the granule exocytosis pathway, the released perforin undergoes a calcium (Ca^{+2}) dependent polymerization and forms pores on the target cell membrane, which permits the entry of granzymes. Granzymes enter these pores and activate a caspase cascade, a chain of proteolytic reaction mediated by cysteine proteases, that results in the apoptotic death of the target cell (Shankar, et al. 1999). Perforin-knockout mice can not recover from LCMV infection, which implies that lysis of infected cells is crucial for controlling this infection (Kagi, et al. 1994). However, these knockout mice handle other viruses efficiently, such as vaccinna and hepatitis B viruses, implying that cytokine/chemokine production is more important in controlling these pathogens (Guidotti, et al. 1996, 1999; Ramsay, et al. 1993).

affect viral replication

Although the answer for which function of CTL is most important for control of HIV infection remains uncertain, CTLs have been shown to efficiently inhibit virus replication in vitro (Price, et al. 1995; Jassoy, et al. 1993). It has been reported that HIV-specific CTLs can produce cytokines that affect viral replication. These cytokines include interferon-γ (IFN-γ), which inhibit HIV replication, and tumor-necrosis factor-α (TNF-α), which can upregulate viral replication through activation of the HIV promoter in the 5' long terminal repeat (LTR). HIV-specific CTLs also produce the CC chemokines MIP-1α, MIP-1β and RANTES, which suppress virus replication by competition for or downregulation of CCR5 (the cellular co-receptor together with CD4 for the entrance of HIV into target cells). In addition, a partially characterized secreted factor, termed CAF (CD8+ T-cell antiviral factor), has been identified to block LTR-mediated transcription in infected cells and hence shuts down virus production (Copeland, et al. 1995).

kill infected CD4+ T cells

Cultured HIV-specific CTLs have been shown to efficiently lyse HIV-infected CD4+ T cells in vitro (Yang, et al. 1996), despite the ability of viral Nef protein to downregulate MHC class I molecules on the surface of infected cells

(Collins, et al. 1998). The rapid turnover of infected cells is shown to be caused by immune-mediated lysis rather than viral cytopathicity, since susceptibility of infected cells to lysis by CTL clones precedes production of extracellular virus (Klenerman, et al. 1996). This implies that lysis caused by CTLs is potentially efficient to control HIV infection. Although lysis of infected cells is predominantly mediated by perforin and granzymes (Shankar, et al. 1999), a minority of CTLs use the alternative pathway where Fas ligand expressed on antigen-activated CTLs triggers apoptosis in the infected cells (Hadida, et al. 1999).

poor killing ability

Finding from several studies show that HIV-specific CTLs taken *ex vivo* have functional defects, a feature that may not be observed in experiments with cultured CTL clones, because culture can readily modify or select for function (Kalams, et al. 1998; Zajac, et al. 1998). Although HLA-epitope tetramer staining shows high frequencies of HIV-specific CTLs in acute or chronic HIV infection, this technique does not measure the function of CTLs. When tetramer staining was combined with intracellular staining for cytokines and chemokines, it was found that most HIV-specific CTLs from patient with chronic HIV disease secreted IFN-γ, TNF-α and MIP-β in the presence of their cognate antigens ex vivo (Appay, et al. 2000). This pattern of cytokine secretion in HIV-specific cells was similar to that observed in CMV-specific cells from HIV-uninfected individuals. However, it was found that less than 15 % of HIV-specific cells produced perforin, which was reflected in poor killing ex vivo, compared with CMV-specific cells from the same patient, 50 % of which contained perforin and performed efficient killing of appropriate target cells (Appay, et al. 2000). Thus, HIV-specific cytotoxic T cells may be less efficient in controlling HIV infection than expected. Since cytotoxic killing effects appear to be a survival pressure for HIV, generation of mutations on specific CTL epitopes renders HIV able to escape from the surveillance of virus-specific cytotoxic T cells (McMichael and Phillips, 1997).

most of HIV-specific CTLs may be immature

It is uncertain why HIV-specific cells poorly express perforin. These cells lose expression of the glycoprotien CD28 on their surfaces, but retain CD27. In contrast, CMV-specific cells lack expression of both glycoproteins, and this is though a specific phenotype of mature effector cells. Thus, most HIV-specific cells *in vivo* may be immature rather than at the functional effector stage (Hamann, et al. 1997). Failure of CD8+ T cell to maturetion may be a consequence of impaired CD4+ T-cell help, which was suggested by experiment in mice infected with LCMV, where virus specific CD8+ T cells were unable to elaborate any antiviral effector function in the absence of CD4+ T-cell help (Zajac, et al. 1998).

C.7. Definition of Th1/Th2.

Th1 secrets IFN-γ and Th2 secrets IL-4

T helper lymphocytes play a central role in immune responses and are required for the induction of cytotoxic T cells and most B cell responses. Based on the cytokines they produce, T helper lymphocytes have been classified into distinctive subsets. Following the discovery of Th1 and Th2 clones in mice, the existence of these subsets of helper T lymphocytes in human beings has been proven as well. After being activated by the antigenic and environmental stimuli in different ways, naïve T helper lymphocytes, Th0, are capable of differentiating into either Th1 or Th2 subsets (Sad, et al. 1994). Th1 cells secret interleukin-2 (IL-2), interferon-γ, and lymphotoxin-α and stimulate type 1 immunity, which is characterized by intense phagocytic activity and CTL responses. Conversely, Th2 cells secret IL-4, IL-5, IL-9, IL-10 and IL-13, and stimulate type 2 immunity, which is characterized by high antibody titers (Seder, et al. 1994). However, it is apparent that many T cells can not be easily classified into Th1 and Th2 on these criteria, as individual cells may exhibit heterogenous pattern of cytokine production (Abbas, et al. 1996). These cytokine patters may be more diverse in human than in murine. Some human Th1 cells also secret IL-10 and IL-13, which are mainly produced by Th2 cells (Yssel, et al 1992; de Waal Malefyt, et al. 1995). This overlap of cytokine production may led to some confusion, so that the conventional definition of subsets of T helper lymphocytes depends strictly on the secretion of IFN-γ or IL-4. The Th1 subset is defined as T helper lymphocytes which secret IFN-γ but

not IL-4, whereas Th2 subset is in the reverse fashion. T helper cells which secret neither IFN-γ nor IL-4 do not belong to the Th1 or Th2 subset (Spellberg, et at. 2001). Some of these cells are naïve Th0 lymphocytes, secreting only IL-2 upon primary stimulation, that have not yet acquired the ability to secret a mature profile of cytokines (Bendelac, et al. 1991).

Th0, Th3, Tr1

Apart from Th1 and Th2, some other subsets of CD4+ T helper lymphocytes, such as mature Th0, Th3 and Tr1, have also been described. Some mature T lymphocytes that secret mixtures of cytokines including both IFN-γ and IL-4 are also called Th0 cells. These mature Th0 cells did not polarize during maturation and thus maintained the cytokine producing profiles of both Th1 and Th2 lymphocytes (Firestein, et al. 1989). Within mucosal tissues, another lineage of T helper lymphocytes are known as Th3 cells that secret transforming growth factor (TGF)-β and are thought to regulate mucosal immunity by suppressing inflammation in the local microenvironment and stimulate B cell secretions of IgA (Fukaura, et al. 1996). T regulatory cell 1 (Tr1), which appears to be similar to Th3, secrets high levels of IL-10, low levels of IL-2, also lower levels of TGF-β and no IL-4. This cell has been shown to suppress antigen-specific immune responses and actively down-regulate pathological immune responses in vivo by suppressing the proliferation of CD4+ T cells in response to antigens (Groux, et al. 1997).

C.8. Polarization of helper T-cell subsets.

It has been demonstrated that Th1 and Th2 cells, presenting distinctive cytokine-producing capabilities, are not derived from different cell lineages, but more likely from the same T-cell precursor (Sad et al., 1994). This precursor is a mature, naive CD4+ T lymphocyte that produces interleukin-2 (IL-2) when first encounters with antigenic stimuli, and its differentiation is mainly induced by the surrounding cytokines (Abas et al, 1996). The preference of it's differentiation depends on the balance between IL-12, which favors Th1 responses, and IL-4, which favors Th2 responses.

Th1

The development of Th1 cells from naïve precursors is induced by IL-12, which signals through STAT4 (signal transducer and activator of transcription). IL-12, a heterodimeric cytokine produced mostly by activated macrophages and dendritic cells in response to microbes, is the key to polarize helper T-cells into Th1 phenotype (Trinchieri,1995). The IL-12 receptor, a member of the hematopioetin receptor superfamily, is composed of beta1 and beta2 subunits. The cytoplasmic domains of beta1 and beta2 are respectively associated with the Janus family tyrosine kinase JAK2 and Tyk2 (Zou, et al. 1997). The stimulation of IL-12 induces the tyrosine phosphorylation and activation of JAK2 and Tyk2. Subsequently, the tyrosine residue in TXXGY^{800}L motif of beta2 subunits is phosphorylated which creates an interaction site for SH2 (for Src Homology) domain of STAT4 (Bacon, et al. 1995; Yao, et al. 1999). STAT4 is then phosphorylated by tyrosine kinase and translocated to the nucleus through an unknown mechanism to form a nuclear DNA-binding complexe that contains STAT4 and STAT3. While STAT3 participates in signal transduction pathways of several other cytokines, STAT4 appears to be activated only by IL-12. The selective activation of STAT4 mediates unique effects of IL-12-induced gene expression on Th1 differentiation (Jacobson, et al. 1995). Consistent with this signaling pathway are the findings that knockout of either IL-12 (Magram, et al. 1996) or STAT4 (Kaplan, et al. 1996; Thierfelder, et al. 1996) results in a marked abrogation of Th1 responses, which includes induction of interferon gamma (IFN-γ), enhancement of natural killer cytolytic function and Th1 differentiation. IFN-γ also induces the development of Th1, partly by enhancing macrophages to secret IL-12, and partly by maintaining the expression of functional IL-12 receptor on CD4+ T cells, which render these cells more responsive to IL-12 (Abbas, et al. 1996). Furthermore, lymphocytes from STAT4-deficient mice present a propensity to the development of Th2 cells (Kaplan, et al. 1996).

Th2

IL-4 can stimulate precursor CD4+ T lymphocytes via transferring signals through tyrosine phosphorylation of STAT6 leading to gene expression toward Th2 cell differentiation. Interleukin-4, a pleiotropic type I cytokine produced by

activated T lymphocytes, basophils, and mast cells, controls both growth and differentiation among haemopoietic and non-haemopoietic cells. Its receptor is a heterodimer composed of one IL-4R alpha chain, which binds IL-4 with high affinity and determines the nature of the induced signals, and one IL-2R gamma chain required for the induction of such signals. Upon IL-4 binding, receptor subunits associate, and then facilitate reciprocal tyrosine phosophorylation and activation of JAK1 and JAK3 which are associated with IL-4R alpha chain and IL-2R gamma chain, respectively (Chen, et al. 1997; Malabarba, et al. 1996). Subsequently, the activated JAK kinases phosphorylate various tyrosine residues on the IL-4R alpha chain, leading to the creation of docking sites for downstream signal transduction molecules. IL-4 mediates its biological effects on differentiation of Th2 cells by activating phosphorylation of tyrosine residues at a more distal region between amino acids 557-657 of IL-4R alpha chain, which results in the creation of binding sites for STAT6s and the activation of STAT6 transcription factors by tyrosine phosphorylation as well. (Ryan, et al. 1996; Zhu, et al. 2001). These activated transcription factors dimerize and translocate to the nucleus, where they bind to a promoter sequence of IL-4 gene and activate transcription (Lederer, et al. 1996). Consistent with this signaling model are the findings that knocking out either IL-4 (Koph, et al. 1993; Kuhn, et al. 1991) or STAT6 (Takeda, et al. 1996) results in the reduction of Th2 responses, such as the production of IgG1 or IgE antibodies and the generation of Th2-derived cytokines, IL-5.

C.9. Th1/Th2 effects.

isotype-switching

The isotype switching of immunoglobulin (Ig) is regulated by cytokines produced by T helper lymphocytes. In the mouse, it is now known that IFN-γ mediates switching from IgM to IgG2a and inhibits the production of IgG1, IgG2b, IgG3 and IgE, whereas IL-4 promotes switching to the expression of IgG1 and IgE but markedly inhibits the production of IgM, IgG3, IgG2a and IgG2b (Snapper, et al. 1987). Thus, activation of Th1 or Th2 cells may be expected to affect Ig class and subclass patterns and different Ig patterns induced by infection or immunization may indicate which T helper cell subset has become more dominant. In fact, isotype-switching from IgG to IgE requires

the presence of IL-4 or IL-13, making the production of IgE a perfect indicator for the presence of Th2 cells in vivo (Aversa, et al. 1993). Conversely, the strong association of IgG2a with IFN-γ suggests that predominant IgG2a levels would be a marker for the expansion of Th1 (Snapper, et al. 1987).

cross-regulation

In addition to their stimulatory effects, Th1 and Th2 cells cross-regulate one another through cytokines they secret. IFN-γ, which is secreted by Th1, directly suppresses IL-4 secretion and in turn blocks the ability of naïve Th0 cells to differentiate into Th2 cells. On the other hand, IL-4 and IL-10, produced by Th2 cells, are able to suppress secretion of IL-12 and IFN-γ, thereby leading to inhibit naïve Th0 cells polarizing into Th1 cells. IL-10 also suppresses phagocytosis and intracellular killing, and inhibits antigen presentation to T cells, resulting in T cell anergy (functionally nonresponsive) (Spellberg, et al. 2001).

Th1 effects

Th1 cells are the chief regulator of type 1 immunity, which is characterized by strong cell-mediated immunity and an IgG2a antibody response in mice. Since murine IgG2a has greater affinity for Fc receptors and complement than other IgG subclasses, it is likely the most effective of these subclasses in protection against viruses and other intracellular organisms (Golding, et al 1995). The principle cytokine responsible for these immune effects is IFN-γ which activates macrophages and enhances phagocytosis and intracellular killing of microbes (Dellacasagrande, et al. 1999). IFN-γ also increases expression of class I and class II major histocompatibility complexes (MHC) molecules on a variety of antigen presenting cells, such as monocytes, macrophages, and endothelial cells, in turn, improving antigen presentation to T cells (Johnson, et al. 1990; Volk, et al. 1986). Endogenous antigens bind to MHC I molecules in endoplasmic reticulum, and the antigen-MHC I complexes are transported to the cell surface to present to CD8+ cytotoxic T cells. This results in activation of CD8+ cytotoxic T cells, which then lyse infected cells leading to exposure of intracellular microbes. The complexes of exogenous antigens and MHC II, formed in the phagolysosome, are presented to CD4+ helper T cells, which are

then activated and autocrine-stimulate its own proliferation by secreting IL-2 (Spellberg, et al. 2001). Some Th1 cells in human have been shown to posses cytolytic capacity, and produce cytokines, IL-2 and IFN-γ, to help CD8+ T lymphocytes differentiate into active cytotoxic cells (Parronchi, et al. 1992).

Th2 effects

Th2 responses are usually associated with high titers of antibody responses. In fact, Th2 cells are excellent helpers for B lymphocytes to produce high level of IgM, IgE and non-complement-fixing IgG isotypes such as IgG1 in mice, abd IgG4 in humans (Coffman, et al. 1993). These effects are basically mediated by cytokines produced by Th2 cells. IL-4, IL-10 and IL-13 activate B cell proliferation, antibody productuon, and class-switching. Particularly, IL-4 is the critical cytokine to induce antibody class-switching from IgG to IgE and therefore initiates IgE-dependent, mast-cell-mediated reactions (Galli, et al. 1993).

D. Vaccines against HIV infection.

The development of safe and effective vaccines has been considered as a feasible approach to protect human beings against HIV infection. However, HIV has presents several challenges, and broken the paradigm for classic vaccine development. The modern approaches based on DNA and live vectors were thus investigated.

D.1. Challenges for AIDS vaccine development.

Although the human immunodeficiency virus (HIV) has been identified as the cause of AIDS for two decades, a promising vaccine against this scourge of the twenty-first century is still unavailable. Despite a large effort on the vaccine development, the problem seems to be more difficult than anticipated. HIV has presented several unique challenges for vaccine development.

(i) HIV is a difficult target for neutralizing antibodies. Viral envelope glycoproteins, mediating attachment and entry into host cells, thermostably conceal their conserved receptor- and co-receptor-binding sites which are further masked by loops of highly glycosylated variable sequences (Kwong, et

al. 1998; Myszka, et al. 2000). Antibody-producing B cells recognize the variable loops, but do not frequently recognize the conserved sequences of receptor-binding sites (Parren, et al. 1999), which causes problems in generation of neutralizing antibodies with broad cross-reactivity for different patient isolates (a population of virus that has been recovered from a patient).

(ii) HIV is able to form latent proviral DNA in host cells. In the early step of HIV's life cycle, RNA genome of HIV is reverse-transcripted into DNA and further integrated into the genome of its host cells to form a provirus. The lack of protein expression from provirus renders the virus able to hide from surveillance of the immune system (Finzi, et al. 1998).

(iii) HIV-1 is highly variable (McCutchan, 2000). HIV-1 was introduced from chimpanzees to humans, and in turn evolved in human to form at least 12 genetic subtypes which have diversified further (Gao, et al. 1999). Subtypes of HIV-1 are also called clades, which do not fall into specific categories based on their susceptibility to the recognition of neutralizing antibodies. Antibodies specific to variable loops of isolates from one patient usually do not recognize the variable loops of isolates from other patients, even though these isolates are in the same subtype. The prevalence of intersubtype recombinant strains is increasing and renders HIV-1 more diverse in antigenic (Heyndrickx, et al. 2000). The high error rate of reverse transcription and the rapid turnover of plasma virions provide a broad base of variants that can escape immune recognition (Preston, 1997; Ho, et al. 1995; Evans, et al. 1999). If the vaccine stimulates a response to a few epitopes, the clade difference could restrict the use of the vaccine to clade matched areas. Therefore, development of an HIV vaccine focusing on the induction of broad epitope responses could be extremely important. (McMichael, et al. 2002).

(iv) Immune parameters correlated with protection from HIV infection have not yet been identified. From the natural experiment, it was noticed that milkmaids who had previously contacted cowpox were resistant to smallpox virus infection. This observation leads to the finding that immune responses against cowpox virus are able to cross-react to smallpox virus and therefore used to

develop vaccines against smallpox. A cohort of exposed seronegative sex workers has been identified with resistance to HIV infection in Nairobi, but unfortunately protection is not always long lasting (Rowland-Jones, et al. 1998), and thus definitive markers for guiding vaccine development require further investigation.

(v) Animal models for HIV vaccine research are limited. By far, rhesus macaque models are the most important models in searching for a vaccine to prevent HIV-1 infection. Rhesus monkeys can be experimentally infected with various simian immunodeficiency virus (SIV) of differing virulence, many of which cause acquired immunodeficiency syndrome (AIDS) in monkeys (Nathanson, et al. 1999; Hirsch, et al. 2000). Despite many similarities in the symptoms and pathology between simian and human AIDS, there remain distinctions that could affect vaccine efficacy. With regard to the genomic composition, the Vpx gene product of SIV is not found in HIV-1, the accessory protein Vpu (required for efficient viral assembly and budding) of HIV-1 is not found in SIV, and the functions of protein Vpr are not conserved between these two viruses (Nabel, 2001). Because HIV-1 does not present productive infection in macaques, it can not be used as a challenge virus to assess whether a given vaccine can prevent or ameliorate infection. Although some HIV strains have been shown to cause disease in chimpanzees, the CCR5-tropic isolates (use CCR5 as entrance coreceptor) which is responsible for main transmission between human is not pathogenic in these primates (Novembre, et al. 1997).

D.2. Choice of immunogens.

late viral proteins

HIV encodes more than nine gene products, any of which might be a target for immune recognition and hence serve as candidate antigens for vaccine developments. Three major genes (gag, pol, and env) encode polyprotein precursors that are cleaved to yield nucleocapsid core proteins, enzymes required for replication, and envelope core proteins, respectively. Of the remaining six genes, three (tat, rev, and nef) encode regulatory proteins that are essential for controlling viral protein expression; two (vif and vpu) encode proteins required for viral maturation; one (vpr) encode a weak transcriptional

activator (Cullen, 1998). Proteins (Nef, Rev, and Tat) made early in the course of infection are derived from highly spliced viral messenger RNA, while proteins (Env, Gag, Pol, Vif, Vpr, and Vpu) produced late in the viral life cycle are derived from non- or single-spliced viral messenger RNA. Several of these viral proteins contribute to the structure of virus and are synthesized in high quantity, such as envelope and capsid proteins. Others required for the regulation of viral gene expression are synthesized in lower quantity, such as Tat and Rev proteins. Because CTL responses are essential for controlling viremia and the phenomenon of high frequency of Gag-specific CD8+ T cells was observed in long-term non-progressors, attention has been focused on the use of Gag protein as immunogen to induce CTL responses (Allen, et al. 2002; Hanke and McMichael, 2000). The accessibility of the Env protein on the surface of intact virions would make it an attractive target for neutralizing antibodies. These two gene products of unspliced viral messenger RNA are widely considered as candidate antigens for effective HIV vaccines (Nabel, 2001).

early viral proteins

Among the highly spliced viral RNA products, Nef is not only the largest auxiliary protein, but also is expressed at far higher levels than Tat and Rev. It has been well documented that Nef exhibits a number of distinct activities that undoubtedly contribute to marked increase in viral replication and pathogenicity. In addition to a possible enhancement in virion infectivity due to down-regulation of cell surface CD4 and MHC class I molecule, Nef also enhances virion infectivity by inducing change in the phosphorylation state of matrix proteins (Kestler, et al. 1991). Because Nef is expressed early after the course of infection, immune responses to this protein may limit the burst size of virus from infected cells and hence could contribute to protective immunity. Tat and Rev regulatory proteins have attracted attention because of their potent regulatory activity in viral replication. Rev protein is found primarily in the nucleus and involves in translocation of unspliced viral RNA to cytoplasm for the expression of late viral proteins. Because Rev is expressed at low quantity and not highly immunogenic, it is not considered as an attractive target for vaccine development (Cullen, 1998). In contrast, Tat protein, which is also

found in the nucleus at low quantity, has been widely investigated in several laboratories for the potential utility as a constituent of AIDS vaccines. Tat has been described in extracellular tissues, and several investigators have suggested that its biological activities are associated with the pathogenesis of HIV disease (Emerman, et al. 1998). Although modified forms of Tat proteins can induce immune responses to control viremia in non-human primate models challenged with SHIV (Cafaro, et al. 1999; Pauza, et al. 2000), it is not yet clear whether this protection can be translated into humans with natural infection. The protective effects induced by immunization with modified Tat proteins have not yet been observed in genetic immunization, and this finding has raised questions about the use of Tat as a target antigen. Whether this difference is due to immune responses to Tat by protein versus gene-based delivery, or other undefined variables remains unclear and will require further investigation.

D.3. Benefit from vaccine-elicited cellular immune responses.

success in nonhuman primate model

In recent nonhuman primate model studies, it has been shown that immune responses elicited by a variety of vaccine modalities can control the infection of immunodeficiency virus. In these studies, monkeys have been vaccinated with plasmid DNA, live viral vectors or both, which have been designed to induce cell-mediated immunity. All of the vaccines that have achieved recent success include interleukin-2-adjuvanted DNA vaccines (Barouch, et al. 2001), DNA priming followed by boosters of recombinant modified vaccinia Ankara (rMVA) or recombinant gene-deleted human adenovirous (rAd5), and vaccines that use rMVA or rAd5 for both priming and boosting immunizations (Barouch, et al. 2001; Egan, et al. 2000; Seth, et al. 2000). Immunogens used in these vaccine modalities contain genes of envelope protein (Env), group-specific antigen (Gag), and polymerase (Pol), which are derived from the 89.6 or 89.6P hybrids of simian and human immunodeficiency virus (SHIV) (Karlsson, et al. 1997). The highly pathogenic SHIV-89.6P or SIVsmE660 was used for challenges through intravenous and mucosal routs (Amara, et al. 2001). These vaccinations led to the induction of virus-specific cytotoxic T-lymphocyte (CTL) responses, and the frequency of the elicited CTL was associated with the control of viraemia after the viral challenge. These studies also demonstrated

that the level of detectable viral RNA copies in plasma is correlated with the progression of clinical diseases. Therefore, the vaccine elicited-CTL is potent to contain the viral replication and delay the disease evolution in rhesus monkeys. Furthermore, in the pre-clinical trials of HIV vaccines, a multi-CTL epitope-based vaccine which was delivered in various prime-boost regimes, such as DNA/MVA, SFV/MVA, and DNA/AD5, has been shown effectively eliciting epitope-specifie CTL responses in rhesus monkeys and healthy low-risk volunteers (Hanke, and McMichael, 1999, 2000; Hanke, et al. 2002, 2003; Wee, et al. 2002; Vinner, et al. 2003).

prolong survival and reduce transmission

Although vaccinated animals become persistently infected with SHIV-86.9P, the rapid and complete loss of $CD4^+$ T-cells from the peripheral blood and lymphatic tissues does not occur. Instead, these animals have maintained near-normal $CD4^+$ T-cell counts, diminished viral loads and survived for a prolong period of time compared to unvaccinated controls. Theoretically, the ideal AIDS vaccine should be able to induce sterilizing immunity, preventing or eradicating infection after exposure to the virus. To achieve such sterilizing immunity is still under investigation. However, the findings from these nonhuman primate studies imply that vaccine-elicited CTL responses are likely to provide meaningful protection against the clinical course of HIV-1 infection even if the sterilizing immunity is not achievable at this time (Letvin, 2002)

Some of these vaccine strategies that have been proven effective in nonhuman primate models may also elicit virus specific CTL responses in humans. If these happen, individuals with vaccine elicited-CTL responses may contain viral replication more efficiently and have prolonged survival following infection than do unvaccinated ones. The progression of acquired immunodeficiency syndrome (AIDS) and death have been proven correlated with plasma viral load in HIV-1 infected individuals (Mellors, et al. 1996). With lower viral burdens, infected individuals may have lower risk of transmitting their infections. The HIV-1 infected patients containing viral RNA levels at less than 1000 copies per ml of plasma termed long-term non-progressors tend not to transmit virus to uninfected individuals (Quinn, et al. 2000). Although these vaccines do not

provide protection against infection with HIV-1, they may prolong survival of infected individuals and reduce transmission of epidemic in human population.

D.4. Traditional vaccine approaches.

The studies done to date have provided convincing evidences showing that traditional vaccine approaches such as live attenuated virus, inactivated virus, and subunit vaccines are all likely to be ineffective in controlling HIV infection.

live attenuated virus

By propagation or genetic engineering *in vitro*, viruses can be genetically altered to become attenuated in their *in vivo* pathogenicity. Because these viruses are still capable of replicating *in vivo* and therefore elicit robust immune responses, infection with such pathogenically attenuated viruses has been used successfully to prevent measles, polio, and chicken pox (varicella). Preliminary studies in the rhesus macaque model showed that infection with live but pathogenically attenuated SIV isolate (with deletion in accessory protein gene, *nef*) protects monkeys against challenge by intravenous inoculation of live, pathogenic SIV (Deniel, et al. 1992). A live attenuated SIV with a 12-nucleotide deletion in the *nef* gene was shown to repair this deletion *in vivo* by a sequence duplication event and cause AIDS in infected monkeys (Whatmore, et al. 1995). Moreover, both newborn monkeys and adult monkeys infected for a long period of time with the *nef*-deleted SIV isolate eventually develop AIDS and die (Baba, et al. 1995; Baba, et al. 1999). Similarly, a cohort of Australians infected by a single source of HIV-1-contaminated blood products which were shown harboring virus isolate with a significant mutation in the *nef* gene eventually went on to develop AIDS, even with a delay in onset of disease (Deacon, et al. 1995). These studies show the pathogenic potential of live attenuated HIV-1 vaccine candidates. Therefore, there is no serious consideration at pursuing live attenuated virus for an HIV vaccine.

inactivated virus

The inactivated virus vaccine strategy has been used successfully to prevent infections with influenza and polio in humans, while this strategy has shown disappointing results in SIV/macaque models. The formalin-inactivated SIV

vaccine was shown to protect monkeys from challenge with pathogenic SIV isolate identical to the virus used in this vaccine modality (Murphey-Corb, et al. 1989). However, this vaccine protection was neither broad nor robust. Moreover, further studies suggested that the protection observed in this animal model appears to be an experimental artifact rather than virus-specific immunity (Stott, 1991). Nevertheless, inactivated virus immunogens have been tested in human volunteers but the results were not encouraging (Levine, et al. 1996). Antibody responses capable of neutralizing HIV isolates were not elicited by these immunogens, since most of the viral envelop glycoproteins were depleted during the process of their preparation. It is not surprising that protective immunity is not readily elicited by this strategy, since virus-specific CTL responses are not induced by the immunogen which can not cause generation of viral proteins in target cells. While this strategy appears to be safe, there seems to be no compelling evidence to pursing further studies on inactivated virus vaccine for HIV infection.

subunit viral proteins

Through recombinant DNA technology, highly purified viral proteins have been used as immunogens successfully to prevent hepatitis B virus infection in human. Following this success, similar approach to HIV vaccine design has been evaluated in nonhuman primate AIDS models and in human volunteers, using the monomeric recombinant HIV envelope glycoprotein gp120 as an immunogen. In chimpanzee models, recombinant gp120 vaccine modality has shown protection against challenge with one HIV-1 isolate, but only when the sequences of challenge virus and immunizing gp120 were identical (Berman, et al. 1990). Moreover, since challenge viruses do not replicate to particularly high levels in chimpanzees, it is recognized that these challenges do not represent a rigorous assessment of protection. Nevertheless, the safety and immunogenicity of this type of vaccine have been assessed in early-phase human immunogenicity trials. In this trial, using monomeric gp120 delivered as a protein with adjuvant, antibodies have been elicited but do not neutralize primary patient isolates of HIV-1 (Mascola, et al. 1996). Moreover, further studies reported that vaccine volunteers were not protected from infection with HIV-1, showing inefficacy of this vaccine strategy (Conner, et al. 1998). There

is little optimism in the scientific community, since the immune responses (CTL responses) crucial for HIV containment were not elicited by this approach. Supported by private vaccine manufacturers, phase III efficacy trials were carried out in North America and South Asia, and the results confirmed that an effective vaccine was not on the horizon (Veljkovic, et al. 2003).

Thus, the traditional strategies of vaccine development have been proven not a feasible way to prevent infection with an AIDS virus. Understanding the limitations of these traditional vaccine strategies has directed investigators to explore novel approaches to protect human from infection with HIV.

D.5. DNA vaccines for HIV-1.

The strategy of DNA vaccine refers to administration of naked plasmids encoding specific protein(s) under the control of a eukaryotic enhancer/promoter and polyadenylation signals that confer appropriate expression of protein immunogens. Ideally, when DNA vaccines were inoculated into muscles, cells such as dendritic cells surrounding the inoculation site internalize and transport the plasmids to the nucleus where the immunogen gene is transcripted. Compared to subunit protein vaccines produced in bacteria or yeast, the proteins expressed from DNA vaccines are more likely to assume the native conformation, and their localized expression facilitates uptake by antigen-presenting cells. Thus, the elicited antibodies are more likely to recognize the native proteins of pathogen and provide protection. In addition, since the antigens are synthesized within cells, the class I MHC processing and presentation will be more efficient and thus make the induction of CTL responses more likely.

Various methods for the administration of DNA vaccines have been applied including intramuscular, intranasal and intravaginal routes of adminisration. In addition, by using a gene gun, epidermal inoculation of DNA-coated gold particles have been tested in different animal models (Fosgaard, 1999). These approaches have shown a safe procedure for eliciting immune responses, especially the CTL responses.

advantage of DNA vaccines

The advantages of DNA vaccines over subunit vaccines include the cost of productions and stability of the product. In addition, the incorporation of changes to genes encoding the immunogenic proteins is more flexible. Moreover, a number of studies have suggested that plasmid DNA is quite effective as a priming or initial immunogen in a bimodal vaccine strategy (Amara, et al. 2001).

optimizing gene codons

In rodents, DNA vaccine has been proven effective in eliciting immune responses against a variety of infectious diseases including influenza virus, malaria, tuberculosis, Ebola virus, rabies, lymphocytic choriomeningitis virus and herpes simplex virus (Nabel, 2001). Despite promising results in small laboratory animals, DNA vaccines have been proven less effective in primates, which might be caused by difficulty in achieving sufficient uptake and expression of DNA to induce robust immune responses. Several approaches have been explored to overcome this limitation, for example, using stronger enhancer to increase gene expression. Another approach has been taken to achieve high levels of gene expression by using codon choices preferred in the host species. In some gene-based vectors, the optimization of gene codons has increased the level of protein production significantly, most likely by modifying RNA regulatory structures that prevent export from the nucleus to the cytoplasm thereby preventing effective full-length translation. Most codon-optimized vaccines have focused on the HIV-1 Gag protein, a predominant target antigen of CD8+ T cells in long-term non-progressors (Pontesilli, et al. 1998). By optimizing gene expression, more antigens are available to present to the immune system, which might be helpful in eliciting more effective immune responses to HIV. Further work has shown that the immunity induced by this gag-based DNA vaccine is sufficient to reduce viral replication in monkeys that are challenged with SIV or SHIV (Egan, et al. 2000). On a DNA-based HIV-1 vaccine for pre-clinical trials, two strategies were employed to increase the expression of desired immunogens in human cells. First, the Kozak consensus sequence was deliberately located in front of the open reading frame of the immunogen gene to ensure an efficient initiation of translation. Second, the

substitution with frequently used codons in human genes was introduced to increase nuclear stability and export of heterogenous nuclear RNA which may optimize gene translation (Hanke, and McMichael, 2000).

genetic adjuvants

To improve the efficiency of DNA vaccines, other approaches have been taken which include genetic adjuvants (DNAs that encode a molecule that augments an immune responses), conventional adjuvants, microspheres (a carrier for a vaccine that facilitates immune responses by stabilizing and/or increasing the uptake of the vaccine), and in vivo electroporation. So far, conventional adjuvants, such as alum and copolymers, have not been able to improve the efficacy of DNA vaccines sufficiently to protect infection with immunodeficiency viruses (Shiver, et al. 2002). However, in mouse models, it has been demonstrated that immune responses induced by DNA vaccines can be augmented by modified IL-2 which is fused to the heavy chain of immunoglobulin (Ig) to increase the half-life of IL-2 activity, both when the IL-2-Ig is delivered as a protein (twice a day for 14 days after immunization) or a genetic adjuvant (single inoculation two days after immunization). Interestingly, T-cell immunity was augmented only when the IL-2-Ig genetic adjuvant was administrated after, rather than with, DNA immunization. This restricted requirement is still not well illustrated, but might be due to the ability of IL-2 to increase the number of responding T cells that enter the long-term memory pool (Barouch, et al. 1998).

human trials

In non-human primate models, monkeys that received DNA vaccines augmented with the IL-2-Ig genetic adjuvant were protected against challenge with SHIV (Barouch, et al. 2000). While this approach provides a safe means for eliciting CTL responses, preliminary studies in large animals raised questions in which relatively large amount of plasmid DNA can be inoculated in humans to induce effective immune responses. Nevertheless, a number of plasmid DNA immunogens such as HIV vaccine candidates are planned and ongoing in early-phase human trials (Hanke, et al. 2002; MacGregor, et al. 1998). In addition, since plasmid DNA represents a particularly efficient

priming immunogen, several studies are ongoing to explore the use of bimodal vaccine regimens in which the plasmid DNA is used to prime the immune response and a live vector is sequentially used to boost that response to stimulate a better immunity (Amara, et al. 2001; Hanke, et al. 2000; Vinner, et al. 2003).

D.6. Live vaccine vectors for HIV-1.

Live recombinant vectors are also being explored as vaccine candidates for eliciting immune responses against infection with immunodeficiency viruses. Genes of antigens derived from HIV can be introduced by molecular cloning approaches into live, pathogenically attenuated microorganisms. The resulting recombinant microorganisms can be used as carriers of these antigen genes. Upon infecting with these recombinant live vectors, immune responses are induced to combat both the vector and the antigens derived from genes of HIV carried by these vectors. Such live vectors have been proved particularly useful for eliciting antigen specific CTL responses, since the target antigens are synthesized intracellularly and therefore processed by the antigen presenting cells through the MHC class I pathway (Hanke, et al. 1998; Letvin NL. 2002).

A number of live viral vectors that are being explored as potential vectors for HIV vaccines include several avian and mammalian poxviruses, replication-defective adenoviruses, single-strand RNA alphaviruses, rhabdoviruses, herpesviruses, adeno-associated viruses, semiliki forest virus (Hanke, et al. 2003) and picornaviruses. Each of these viral vectors is unique with respect to the size of the inserted genes they can carry, the range of hosts they can infect, the host pro-inflammatory responses they induce and the immune-evasion strategies they use (Robinson HL. 2002). Given the preclinical success, MVA and Ad5 viral vectors have gained the most attention compared with other viral vectors (Hanke, et al. 1999; Shiver, et al. 2002). The bacterial vector systems that are considered as potential live vectors include the attenuated Mycobacterium Bacille Calmette-Guerin and some of the pathogenically attenuated enteric bacteria such as *Salmonella* (Shata, et al. 2001). All of these approaches will be tested in the near future in human vaccine trials.

(i) Bacterial vector.

Attenuated bacteria expressing various HIV or SIV antigens have also been proposed as potential strategies to induce HIV- or SIV-specific immune responses, especially a more effective mucosal immunity. These attenuated bacteria vectors include bacterial strains *Salmonella typhimurium* and *Streptococcus gordonii*, which have been modified to carry genes encoding HIV gp120 or SIV p27 proteins (Berggren, et al. 1995; Oggioni, et al. 1999; Steger and Pauza, 1997). It has been demonstrated that the to elicit both mucosal and systemic immune responses in nonhuman primate models by using these vectors is achievable. However, whether these elicited immunities are sufficient enough to cope with infections by HIV-1 from mucosal surfaces remains to be investigated.

The use of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) as a vaccine vector and adjuvant to enhance HIV specific immune responses is an attractive approach. This microorganism produces highly immunogenic adjuvant agents such as lipoproteins and heat shock proteins which have been shown to be effective enhancers for the induction of systemic and mucosal immunities (Perraut, et al. 1993). In addition, since mycobacteria can be phagocytosed by macrophages, leading to replication and antigen production in host cells, antigens would be processed through MHC class I pathway, which is critical in the induction of CTL responses. Actually, it has been reported that the induction of delayed-type hypersensitive (DTH) and CTL responses to SIV/HIV Env and SIV Gag proteins can be achieved by using BCG vectors in mouse and macaques monkey models (Honda, et al. 1995; Lim, et al. 1997; Yasutomi, et al. 1993).

More recently the hyperattenuated *Listeria monocytogenes*, engineered to express HIV-1 Gag protein, has been shown capable of eliciting systemic immunity and mucosal immunity against HIV-1 Gag protein in mice through mucosal delivery (Rayevskaya, et al. 2001).

(ii) Herpesvirus vector.

Since immunodeficient virus such as SIV and HIV persist a lifetime in the infected host, vaccine protection against these viruses may require approaches that yield persistant immune responses. Hence a strategy using recombinant herpesvirus as a vaccine vector has been developed and tested. Herpesviruses persist a lifetime in a latent state in infected hosts from which they reactivate periodically. Even for decades after initial infection, humoral and cellular immune responses can be easily detected (Whitley RJ. 1996). Recently, the use of attenuated, replication-deficient and replication-competent recombinant Herpes Simples virus (HSV) strains, which were genetically engineered to express SIV Env and Nef proteins, have been shown to elicit antigen specific antibodies and CTL responses in monkey models. In a preliminary study, animals received these recombinant viral vectors were either solidly protected from infection or showed a sustained reduction in viral load following rectal mucosal challenge with pathogenic SIVmac239 at 22 weeks after the last immunization (Murphy, et al. 2000). This result suggests that HSV might be used as a vaccine vector to induce persistent immunity to provide durable protection against HIV infection. However, as with most viral vectors, pre-existing immunity will be a critical obstacle to overcome.

(iii) Vaccinia virus vector.

The live vectors that have been most extensively studied as potential vaccine vectors are the pox family of viruses. The prototype member of this family is vaccinia virus, which is a live attenuated virus and has been used in the successfully worldwide campaign to eliminate smallpox. Studies in nonhuman primates have shown that immunization with recombinant vaccinia viruses can elicit potent immune responses, especially CTL, to HIV and SIV proteins (Shen, et al. 1991). However, the safety concerns have dampened enthusiasm to move this live vector into human vaccine trials, since vaccinia viruses have been shown to disseminate in immunocompromised individuals, sometimes causing a fatal encephalitis (Redfield, et al. 1987). In addition, a fear has been seriously concerned that a substantial number of those already HIV-infected, immunosuppressed individuals might develop a fatal vaccinia associated

infection when such a vaccination campaign is performed in the region of the world in which HIV infection is endemic.

(iv) Modified vaccinia Ankara.

Since the replication-competent poxviruses can cause disseminated disease in immunocompromised individuals, attention has therefore turned to a number of other poxviruses that are pathogenically attenuated in humans. Perhaps the most interesting of these pox viruses is the modified vaccinia Ankara (MVA), which was generated from a parental vaccinia virus isolate by multiple passages. After over 500 passages in chick-embryo fibroblasts, MVA acquired six large genomic deletions, leaving it infectious and immunogenic but attenuating its replicative potential in primate cells (Antoine, et al. 1998; Meyer, et al. 1991). Recombinant MVA viruses have been proven highly immunogenic and capable of eliciting immunity to protect monkeys from SIV- and SHIV-induced disease. In nonhuman primate models, immunization with a recombinant MVA virus expressing SIV Gag-Pol proteins is able to elicit high frequency of Gag-specific cytotoxic T-lymphocytes which is associated with reduction of viremia after SIV challenge (Barouch, et al. 2001; Seth, et al. 2000). Another similarly generated, multiple gene-deleted vaccinia vector, referred to as NYVAC, has been shown capable of inducing an immune response which is comparable to that elicited by MVA when used as vaccine vector in nonhuman primate models (Letvin NL. 2002).

(v) Canary pox virus.

The most extensively studied poxviruses, which are used as candidate vaccine vectors for HIV, are the avian pox viruses such as canary poxvirus. Canary pox virus can not complete its replication cycle in human cells, but it does initiate sufficient level of protein synthesis for MHC class I processing and therefore elicits immunogen-specific CTL responses in vaccinated individuals. Recombinant canary pox virus encoding HIV-1 Env and Gag proteins has been extensively tested in uninfected human volunteers in recent years. These vaccine modalities have been shown safe and immunogenic, and the eliciting HIV-1 specific antibodies and CTL responses are detectable in peripheral blood in immunized volunteers (Evans, et al. 1999). Moreover, it has been

demonstrated that canary pox vaccines, which encode Env proteins of clade B HIV-1 can elicit broad CTL responses to viruses belonging to various genetically diverse clades of HIV-1 (Ferrari, et al. 1997).

(vi) Replication incompetent Adenovirus.
Perhaps to date replication incompetent adenoviruses are the most promising live recombinant vector used as candidate HIV vaccines. The recombinant adenovirus serotype 5 (Ad5), which was made replication-incompetent by deletion or inactivation of the E1 and E3 genes, originally designed as a gene therapy vector, has been proven cable of eliciting impressive immune responses in both murine and nonhuman primate studies. The replication incompetent adenoviral vaccine vector, expressing SIV gag protein, has been tested in monkey models either as a single modality or as a booster inoculation after priming with a DNA vector. After challenge with a pathogenic hybrid virus SHIV, both vaccine regimens elicits high frequency of gag-specific CD8+ T cells associated with controlling viremia and maintaining CD4+ T cell counts (Shiver, et al. 2002). In fact, this vector has been used in the early-phase HIV immunogenicity trials in humans. Preliminary results in this trial have shown that pre-existing antibody responses to adenovirus type 5 significantly dampen immunogenicity of this vaccine vector. Nevertheless, a number of strategies are currently being explored to circumvent this problem such as using prime-boost vaccination strategies, using unusual serotype adenoviruses, or using adenovirus isolates from nonhuman primate species (Letvin, 2002; Vinner, et al. 2003).

D.7. Advantage and limitation of live-vector (MVA, Ad5).

Since MVA produces its mRNA in the cytoplasm of infected cells, it is not necessary to engineer the recombinant HIV-1 gene to overcome the Rev dependent splicing for efficient gene expression. In contrast to MVA, Ad5 expresses its genes in the nucleus of cells, and therefore recombinant HIV-1 genes have to be modified to avoid Rev dependence (Robinson HL, 2002).

Both MVA and Ad5 have a broad host range for human cells, and both are capable of stimulating the production of pro-inflammatory cytokines that augment immune responses. Infections of MVA mobilize innate immune

responses by stimulating the production of high levels of type I interferons (Blanchard, et al. 1998). Antigens delivered by adenovirus vectors can be efficiently processed for recognition of immune cells, since they can infect dendritic cells and induce maturation of these cells (Zhong, et al. 1999, 2000).

A major limitation of live-vector vaccines is the presence of pre-existing immunity to the vector in human who has been exposed to the virus. People in the older generation, who were vaccinated against smallpox, will have pre-existing immunity to MVA vectors. In addition, if vaccinations for smallpox were to become routine to counter the threat of bioterrorism, the immunity to MVA vectors would become universal in human population. As the result of previous natural infections, about 45 % of US population now has neutralizing antibodies specific to Ad5. The pre-existing immunity to virus vectors, which are used in the vaccine modality, might block the uptake of vector and hence diminish antigen gene expression (Farina, et al. 2001).

The pre-existing immunity to vaccine vectors might be overcome by higher dose of vaccine, and by heterologous prime-boost immunization protocols. Higher doses of vaccine are a brute force approach to immunizing in the presence of pre-existing immunity (Robinson HL, 2002). Since pre-existing immunities specific to DNA vaccine modalities do not exist, the primary immune response, induced by DNA immunization, presumably can focus on the antigen of interest and facilitate the generation of memory T cells specific for the relevant antigen. Once these memory cells are established, viral vectors can augment the primed immune response, while viral vector proteins do not interfere the recall response (Yang, et al. 2003).

D.8. Heterologous prime-boost protocols.

A new and popular strategy for raising high frequencies of antigen specific T cells is the combination of DNA priming and live-vector boosters (Hanke and McMichael, 2000; Schneider, et al. 1999). The remarkable efficiency of this bimodal vaccination protocol is considered to be due to the DNA focusing the immune response on the vaccine antigens rather than stimulating an immune response to the vector. The following live-vector booster enhances this induced

immunity by expressing a larger amount of antigens than that can be achieved by DNA along, and by stimulating the production of pro-inflammatory cytokines that augment immune responses (Robison HL, 2002). A successful study of monkey models has shown that the remarkable strength of antigen specific T-cell and antibody responses can be achieved by this bimodal vaccine protocol. In this study, both the DNA and rMVA (modified vaccinia virus Ankara) encoded Gag, polymerase and Env proteins were derived from SHIV. The frequency of Gag-specific CD8+ T cells was greater for the DNA prime followed by the rMVA booster than for rMVA priming and boosting (Amara, et al. 2001). Similar phenomenon was observed in another study in which monkeys received DNA priming followed by boosting with a replication incompetent adenovirus type 5 (Ad5) vector (Shiver, et al. 2002).

D.9. Focus on Tat protein.

Tat protein encoded by HIV and SIV is a small (14-15 kDa) transcriptional activator that acts in trans by binding nascent viral messenger RNA to facilitate both initiation and elongation of viral transcription. This protein is expressed early in viral life cycle and is know to be capable of inducing both antibody and CTL responses. In addition, Tat possesses some other properties that may be important in immunogenicity and protection. **(i)** Tat is released extracellularly from infected cells and can be rapidly transported into the cytosol of most cells (Ensoli, et al. 1993; Frankel, et al. 1988). **(ii)** Tat has been shown capable of delivering exogenous soluble proteins into MHC class I pathway resulting in the induction of class I restricted CTL responses (Kim, et al. 1997). **(iii)** Extracellular Tat upregulates expression of chemokine receptors and therefore can render bystander cells more susceptible to viral infections (Secchiero, et al. 1999; Huang, et al. 1998). **(iv)** Extracellular Tat may also induce expression of CD95/Fas ligand on monocyte/macrophages consequently causing destruction of T cells (Cohen, et al. 1999). Taken together, these properties of Tat protein suggest that immune responses to Tat may play an important role in controlling viral loads. Firstly, antibodies that are capable of neutralizing extracellular Tat may reduce virus spread by interrupting Tat protein to make bystander cells susceptible to HIV or SIV infection. Secondly, CTL responses against early

expressed Tat may be possible to eliminate virus infected cells prior to production of infectious progeny.

Structure and transcriptional function of Tat protein

The Tat protein of HIV-1 is produced early in the replication cycle and plays a primary role in the transcription of viral genes from a promoter located in the long terminal repeat (LTR). This small early protein composed of 101 amino acid (aa) residues is encoded by two exons. Residues 1-72 encoded by the first exon are necessary for the HIV-1 transactivating activity and contain four domains including the amino-terminal (1-21 aa), the cysteine-rich (22-37 aa), the core (38-48 aa) and the basic domain (49-71 aa). From structural analysis, single residue changes in domain one are well tolerated, while changes in six of the seven highly conserved cysteines in domain two abolish transactivating function.

The best studied region of Tat protein, domain four, contains a basic RKKRRQRRR motif which is important for nuclear localization and uptake of Tat by infected and uninfected target cells. Further, this basic region and RGD sequence (present in the second exon) confer the interaction with heparan-sulphate proteoglycans and with cell surface molecules of the integrin family (Chang, et al. 1995; Caputo, et al. 1999). In addition, this basic peptide motif interacts with the short nascent stem-bulge-loop leader RNA, TAR (trans-activation responsive), located immediately 3' to the LTR transcription start site. Findings indicate that the principle role of HIV-1 Tat is to enhance transcription elongation rather than initiation, and at least two cellular cofactors are involved in this process. The prevailing model is that a cellular protein termed cyclin-T binds to the activation domain of Tat, and in turn increases the affinity and specificity of the resulting complex for the TAR element (Wei et al. 1998). Next, a host cell-encoded protein called CDK9 (cyclin-dependent kinases) is able to interact directly with cyclin-T of TAR-Tat-cyclin-T complex and then induces phosphorylation of the carboxyl-terminal domain of the host cell RNA polymerase II, leading to enhance transcriptionl elongation (Cullen, 1998).

Truncated form of Tat protein

Of interest, it should be noted that a truncated form of Tat containing 86 amino acid residues, which appears in a few laboratory virus strains such as LAI, HXB2, pNL4-3, has been frequently used. Actually, a single nucleotide change at putative residue 87 in these laboratory isolates creates a stop codon resulting in the conversion of natural open reading frame (101 amino acid residues) to the premature termination version. The conserved 101 amino acid form of Tat is found in most *in vivo* isolated of virus, while the 86 amino acid form of Tat is only observed in laboratory isolates, suggesting that this premature termination condon arose artificially during tissue culture passaging (Neuveut & Jeang, 1996). Thus, this might be an interpretation for observations that more than 90 % of independently characterized HIV-1 Tat proteins maintain the 101 rather that 86 amino acid configuration (Jeang, et al. 1999).

Nontranscriptional function of Tat protein

Tat is released by infected T lymphocytes in the extracellular milieu (Ensoli et al. 1990) and internalized via cell surface heparan sulfate proteoglycans (Tyagi, et al. 2001). Uptake of extracellular Tat not only enhances HIV-1 transcription in infected cells, but also affects a range of host cell genes in both infected and uninfected cells. This includes activation of interleukin-6 (IL-6), IL-2, IL-2Rα, IL-10, CD-4, tumor necrosis factor (TNF), transforming growth factor-β1 (TGF-β1), and CD95 ligand. In addition, chemokine receptors CCR5 and CXCR4 (Huang, et al. 1998; Secchiero, et al. 1999) are upregulated which are essential for the infectivity of macrophage- and T-cell-tropic HIV-1 strains, respectively. However, other cellular genes such as p53 and manganese-dependent superoxide dismutase are downregulated. Cells treated with Tat show downregulation of MHC I expression and inhibition of antigen-induced lymphocyte proliferation (Viscide, et al. 1989). Extracellular Tat also promotes T cell destruction by increasing expression of CD95 ligand gene on monocyte/macrophages (Westendorp, et al. 1995) or enhancing Cdk (cell-cycle-dependent protein kinase) activity (Li, et al. 1995), rendering cells susceptible to apoptosis. Interestingly, in a recent study, it has been shown that expression of HIV-1 Tat in immature dendritic cells reprograms gene expression in these cells

and elevates levels of chemoattractant MCP-2 (monocyte chemoattract protein 2) for activated T cells and macrophages. These changes are also observed in SIV (simian immunodeficiency virus) infected monkey models, implying that Tat might facilitate expansion of virus infection via enhancing chemoattractant expression (Izmailova, et al. 2003). These effects of Tat protein on immune cells could represent major contributions to the immunosuppression of HIV-1 infection.

Immune responses specific to Tat contribute the protection

Several studies have shown that immune responses to Tat protein have a protective role, and may control disease progression. Tat protein is well conserved (because of constraints imposed by overlapping reading frames of vpr, rev, env proteins) and immunogenic, and the elicited antibody responses have been shown to correlate with nonprogression to AIDS (Reiss, et al. 1990; Rodman, et al. 1993; Zagury, et al. 1998). A higher antibody titer of both anti-Tat IgM and IgG antibodies has been observed in asymptomatic patients, compared with patients in advanced stages of disease (Fanales-Belasio, et al. 2002). Also, cytotoxic T lymphocytes specific to Tat are frequently detected in asymptomatic infected individuals, and inversely associated with disease progression (Addo, et al. 2001). This correlation has been recently confirmed in vaccine trial of HIV-1 Tat in nonhuman primates challenged with SHIV89.6P (one highly virulent SHIV chimeric virus in which several HIV-1 genes, most commonly tat, rev and env, are substituted for their SIV homologs). Most important, in the early stage of infection in a monkey model, many cytotoxic T lymphocytes recognizing Tat protein are generated and are very efficient at controlling virus replication (Cafaro, et al. 1999, 2000). Mutations within Tat sequence arise while immune responses are developing, which leads to immune escape and long-lasting infection (Allen, et al. 2000). This result implies that induction of Tat-specific T-cell responses by vaccine might cope with HIV infection in human.

Antibodies specific to Tat can block cellular uptake

It has been shown that both monoclonal and polyclonal antibodies specific to Tat protein are readily induced and able to block uptake of Tat in vitro. From

the analysis of monoclonal antibody binding patterns, two major immunogenic region were identified toward amino terminus (aa 5-22) and basic domain (aa 44-62). By measuring the inhibition of Tat-mediated transactivation of HIV-1 LTR reporter genes in tissue cultures, it has been shown that antibodies specific to these two antigenic domains can block cellular uptake of Tat protein. (Brake, et al. 1990). In HIV-1 infected patients, antibodies to amino terminus of HIV-1 Tat were also produced, showing the immunogenic property of Tat in human (Krone, et al. 1988). Most important, monoclonal or polyclonal antibodies to Tat are able to attenuate HIV-1 infection in cell cultures, reaffirming the effect of extracellular Tat for the enhancement of virus infection. A significant reduction and consistent delay of HIV-1 replication in PBMCs (peripheral blood monocytes) from HIV-1 infected subjects was achieved by adding anti-Tat antibodies to tissue culture medium (Re, et al 1995). A correlation of low titer of or absence of antibodies specific to Tat with progression of AIDS disease (Reiss, et al. 1990) further suggests that interdicting extracellular Tat by antibodies might be able to control HIV-1 infection.

Limited antigenic polymorphism of HIV-1 Tat in distinct isolates

The amino acid sequences of Tat proteins from individuals (in Uganda and South Africa) infected with HIV-1 subtype A, C or D are highly conserved in their immunogenic B-cell epitopes, and sera from these individuals are capable of neutralizing the activity of Tat protein derived from subtype B, a distinctly related HIV-1 (Fanales-Belasio, et al. 2002). Furthermore, a sequence data analysis of HIV-1 Tat protein (350 Tat sequences in GenBank) has shown that two immunogenic B-cell epitope sequences have limited or no antigenic diversity within geographically distinct isolates (Goldstein, et al. 2001). Together, these clear evidences of cross-clade recognition in Tat protein suggest that a vaccine approach based on Tat protein may control infection of different viral strains.

Cytotoxic T-lymphocyte responses against Tat protein

It has been shown that virus specific CTLs (cytotoxic T-lymphocytes) are instrumental in reducing viral loads during primary HIV and SIV (simian immunodeficiency virus) viremia. The appearance of these CTLs in the

peripheral blood is associated with the decline of viremia (Koup, et al. 1994; Borrow, et al. 1994), and the number of CTLs is inversely correlated with viral load (Ogg, et al. 1998). More direct evidences, from macaque models for AIDS, have demonstrated the importance of virus specific CTLs in controlling viremia. In these models, depletion of CTLs by administration of antibodies specific to CD8 (major surface marker of CTL) before or after infection, results in increased viremia, prolonged depletion of CD4+ T-cells and accelerated disease progression (Matano, et al. 1998; Schmitz, et al. 1999; Jin, et al. 1999). However, in HIV-infected individuals, virus specific CTLs can be detected but generally are not capable of eliminating HIV from infected persons or even suppressing the virus to latency (Gea-Banacloche, et al. 2000; Rowland-Jones, et al. 2001).

Broad CTL responses specific to various epitopes on HIV proteins were observed in persons with prolonged infection and low viral load. This suggests that CTL responses to broad range of epitopes might be a crucial component in controlling viremia. Of particular interest, CTLs specific to early expressed regulating proteins Tat, and Rev were only found in long-term asymptomatics. People with progression to AIDS do have comparable CTL responses against other HIV proteins, but not against Tat and Rev (van Baalen, et al. 1997). Also, derived from HIV-1 infected individuals with low virus load, CTLs specific to Tat protein were found in higher numbers and targeting more epitopes, compared with that in patients with high virus load (Addo, et al. 2001). Furthermore, in the cynomolgus macaques models, after infection of SIV, the appearance of CTLs against Tat and Rev correlates with the rapid elimination of virus from peripheral blood (Geretti, et al. 1999).

Tat and Rev are tested as candidate vaccines

It has been reported in many studies that reduction of viremia and attenuation of disease after challenge were achieved by vaccination with Tat and Rev vaccines formula in animal models. The efficient control of the challenge virus in cynomolgus macaques has been achieved by using recombinant virus vectors expressing Tat and Rev in prime-boost vaccine approach (Osterhaus, et al. 1999). The Tat DNA along or with a tat protein boost was found partially

protective in some of the rhesus macaques (Cafaro, et al. 1999). Based on the possibility that extracellular Tat protein may be taken up by cells and processed in the MHC I pathway, vaccination with inactivated Tat protein has shown the capability of controlling viremia in macaque models (Pauza, et al. 2000). Together, these studies indicate that vaccinations with Tat and Rev may be instrumental for combating primary viremia efficiently.

D.10. Early recognition is a critical factor for CTL responses to control HIV infection.

Variable degrees of success in controlling primary viremia have been observed in studies of HIV vaccines which were based on antigens derived from SIV (Mascola, et al. 2001). The relative success of vaccinations in various studies is difficult to compare because different vaccination strategies, challenge viruses and animals are used. Therefore, a direct comparison of vaccination with Tat/Rev and vaccination with Gag/Pol was performed in cynomolgus macaques (Stittelaar, et al. 2002). In this study, the prime-boost vaccination approaches lead to detectable CTL responses against the specific antigen in all animals. Only animals in the Tat/Rev vaccine group have no detectable or lower viremia after challenge, while the Gag/Pol vaccinated or control animals did not control viremia. This vaccine based on the Tat and Rev seems to be more potent. Their open reading frames overlap in part with each other and with that of the envelope. This may refrain the number the mutations that are tolerated and consequently possible to escape detection by specific CTLs. It has been found that the SIV replication was best controlled in macaques that mounted CTL responses against Tat. Nevertheless, rapid accumulation of mutations in Tat epitopes (Rev was not analyzed) leading to escape from CTL recognition was observed, during the resolution of primary viremia. At the same time CTL responses specific to Gag were also found, but their presence did not lead to mutations in their epitopes (Allen, et al. 2000). This implies that CTL responses specific to Tat may be significantly involved in controlling virus replication, and suggests that eliciting CTL responses against early expressed viral proteins might be an attractive approach in HIV vaccine development.

D.11. Rationale of vaccine strategies focusing on early proteins.

Recognition of early viral proteins has been proposed as critical factor in CTL effectiveness against HIV, because more infected cells can be eliminated before releasing progeny infectious virus particles. Thus, less new infections can occur and spreading of virus can be controlled more effectively. It has been concluded that CTL responses specific to early expressed viral proteins put more pressure on virus than did responses to lately expressed viral proteins (Allen, et al. 2000). Mutations which lead to escape from detection by CTLs are readily observed in studies of HIV infection and experimental SIV infection. In all these cases, CTL escape was observed in early expressed protein (Tat) and to a lesser extent in intermediate expressed protein (Env), while escape was rarely found in lately expressed protein (Gag) (McMichael and Phillips, 1999; Gruters, et al. 2002). Therefore, pre-existing and broad CTL responses against early (and to a lesser extent, intermediate) proteins may give better immune control of virus infection resulting in lower viral load consequently less transmission of virus. From the results of our colleague's works, purified SIV tat protein was shown highly immunogenic for the induction of antigen specific antibodies and able to induce Th1 responses which were know in favor for the development of CTL responses. Thus, purified tat protein might be potentially useful in development of HIV vaccines focusing on the induction of antibody and CTL responses.

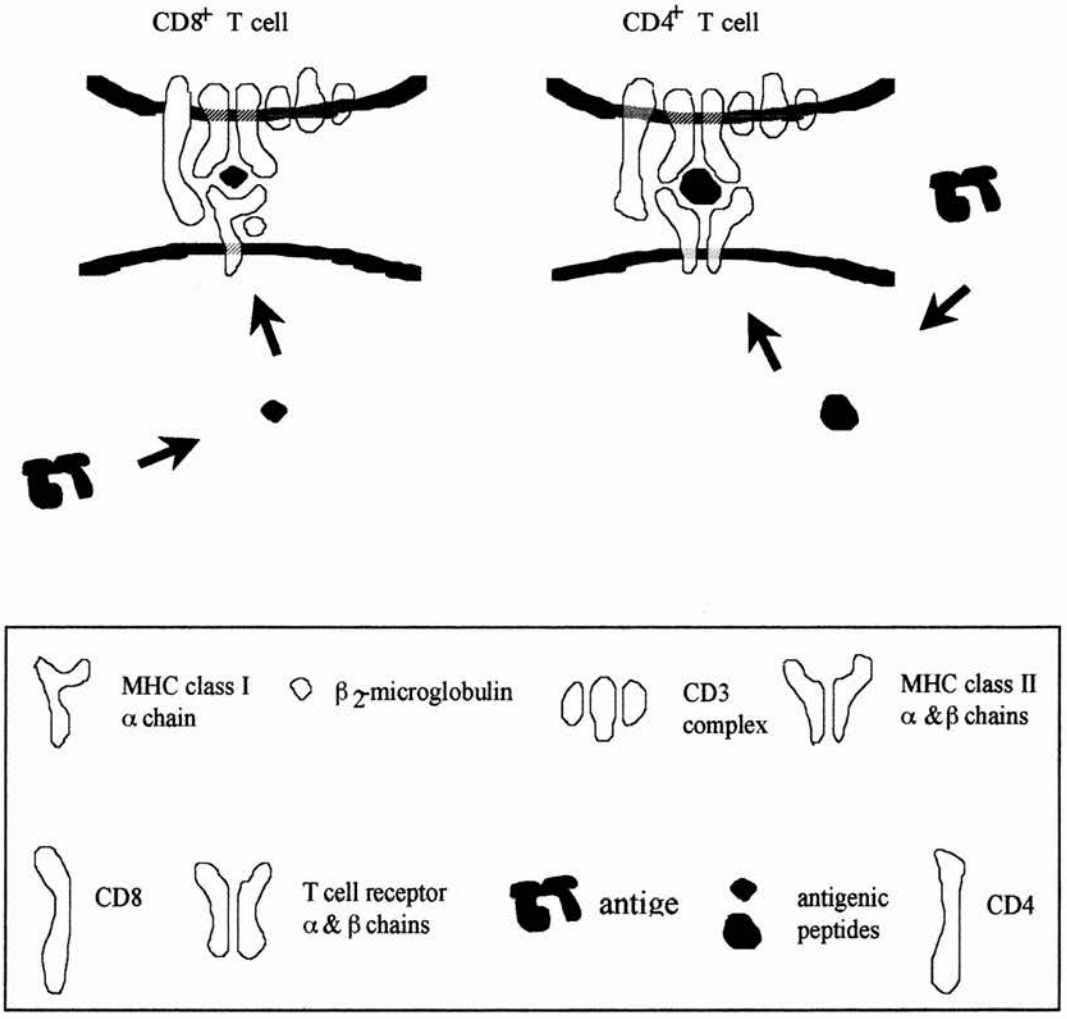

Fig. 1. Schematic diagram of the interactions between the T-cell receptor and the peptide-MHC complex.
Peptides derived from endogenous proteins are presented on class I MHC molecules and recognized by CD8+ T cells, while peptides derived from exogenous proteins are presented on class II MHC molecules and recognized by CD4+ T cells. Bindings of coreceptors (CD4 or CD8) and other accessory molecules (such as DC3 complex) to their cognate ligands enhance signal transduction across cell membranes, leading to the activation of T cells. (adapted from Randall and Hanke, 1994).

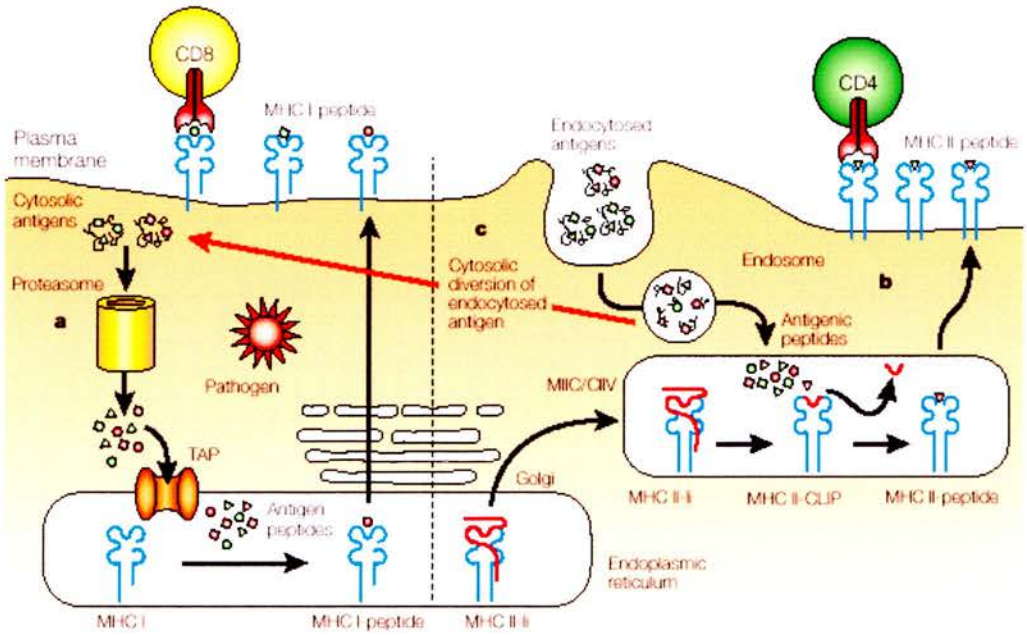

Fig. 2. Different pathways of processing antigens to the class I and class II MHC molecules.

a). Class I pathway. Endogenous proteins were degraded through proteasome machinery and then transported to the endoplasmic reticulum for loading on class I MHC molecules which were them traffic to cell surfaces for the recognition of CD8+ T lymphocytes. b). Class II pathway. Internalized protein antigens move through several acid compartments, in which they are degraded into peptides. Newly synthesized class II MHC molecules are preserved from binding to endogenous antigens by association with the invariant chain (Ii). This complex moves through the Golgi to the MIIC/CIIV compartment where the Ii is degraded to CLIP (class II-associated invariant-chain peptide) which is them exchanged for antigenic peptides from the endocytic route. The resulting peptide loaded class II MHC ultimately traffics to the plasma membrane for the surveillance of CD4+ T lymphocytes. c). Cross-presentation pathway. Dendritic cells can process internalized antigens through the class I pathway and present peptides on the class I MHC molecule for CD8+ T lymphocytes. (MIIC: MHC II loading compartment; CIIV: MHC II vesicles). (adapted from Heath and Carbone, 2001).

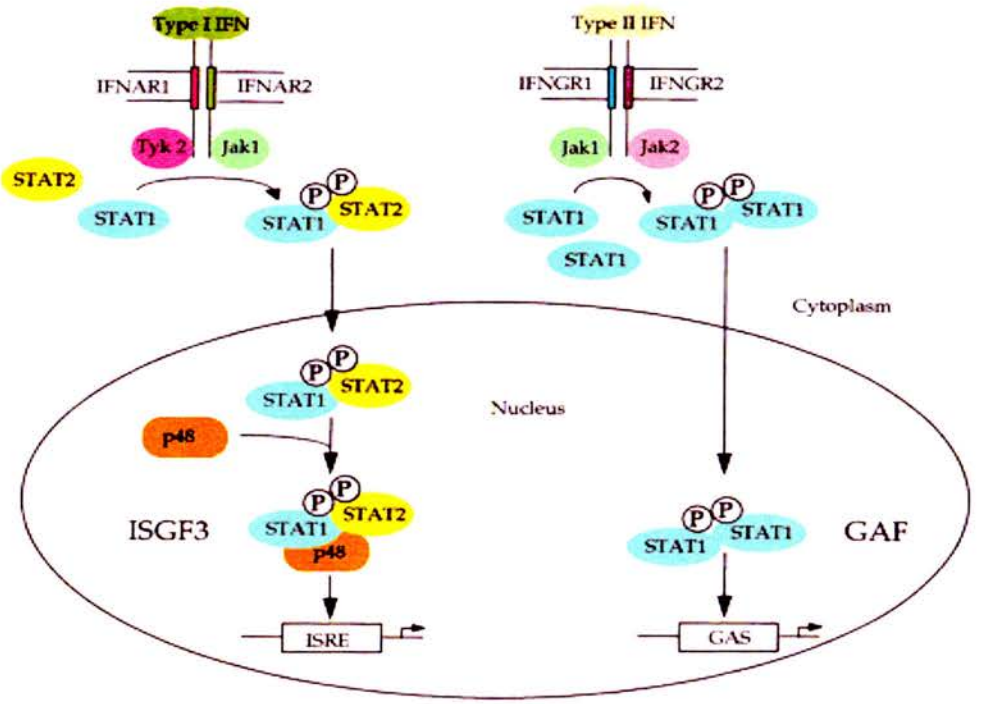

Fig. 3. Signal transduction pathways of type I and II IFNs.
The biological activities of IFNs are initiated by binding to their cognate receptors, which induces the dimerization of receptor subunits and activates receptor-associated tyrosine kinases. Subsequently, activated kinases phosphorylate STAT transcription factors, which then dimerize and translocate to the nucleus, where they can activate transcription of interferon responsive genes. (adapted from Goodbourn, et al. 2000).

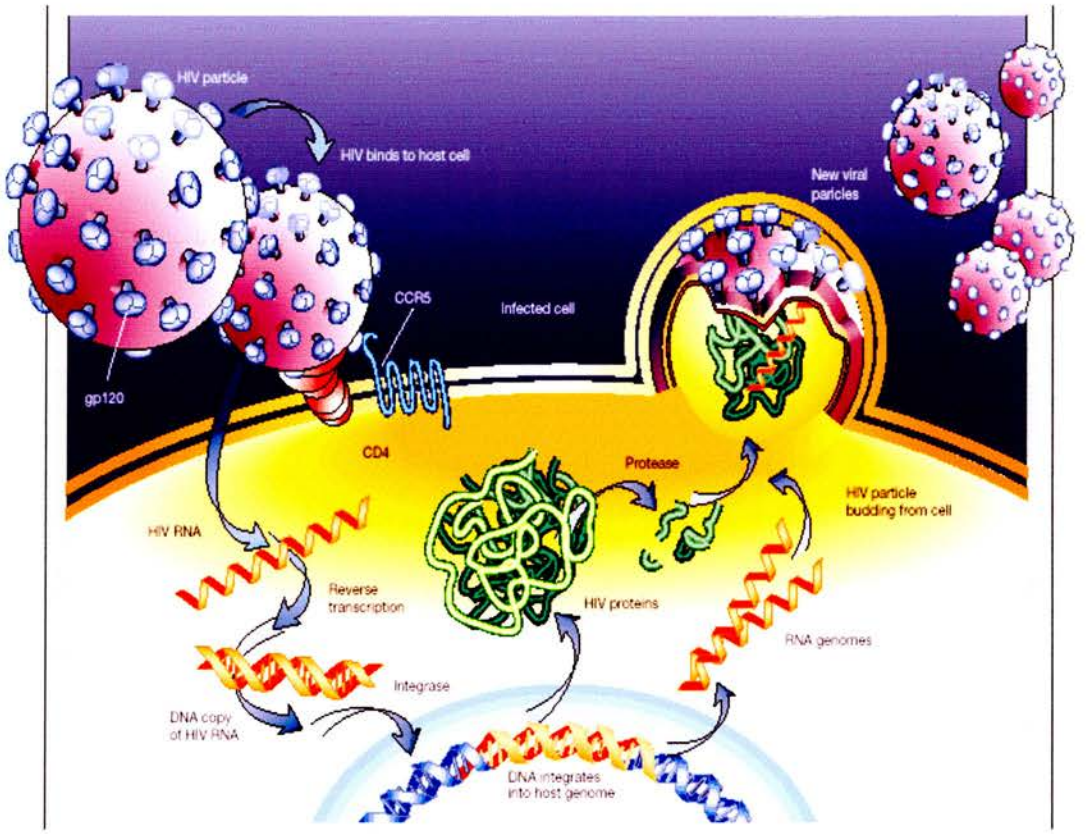

Fig. 4. Replication cycle of HIV.
HIV carries two RNA copies of its genome within its virus particles. Following entry of HIV into target cells, viral reverse transcriptase converts viral RNA genome into dsDNA, which enables HIV genome to integrate into the host DNA, creating the provirus. Provirus remains latent until events in the infected cell triggers its activation, leading to make new virus by using cell's gentic machinery. (adapted from Weiss, 2001).

Materials and methods

1. Plasmid DNA purification.

Procedures of plasmid DNA purification follow the recommended protocol of QIAprep Miniprep Kit. *E. coli*, harboring desired plasmid, was cultured in LB (Luria-Bertani containing 10 g NaCl, 10 g tryptone, and 5 g yeast extract per litre) medium at 37 °C, 200 rpm overnight, and then 5 ml of the overnight culture was harvested by centrifuging at 4500 rpm, 4 °C for 10 min. After centrifuge, pelleted bacterial cells were resuspended in 250 μl of Buffer P1, containing RNase A, and transferred to a 1.5 ml centrifuge tube. Under the alkaline condition, cell resuspension was mixed by gently inverting the tube 6 times with 250 μl of Buffer P2 composed of (NaOH/SDS). Leaving the lysis for more than 5 min was not recommended because long exposure to the alkaline condition can cause irreversibly denatured form of plasmid which is resistant to restriction enzyme digestion. During lysis reaction, vigorous treatment will shear the bacterial chromosome, leading to the contamination of plasmid with chromosomal DNA, so gently inverting the tube is enough. Following the lysis reaction, cell lysate was mixed with 350 μl of Buffer N3 (containing sodium acetate) immediately but gently. The high-salt and low pH value properties of this buffer lead to coprecipitation of chromosomeal DNA, cellular debris and denatured proteins, and leave the correct renaturing plasmid DNA in the solution phase. Then the mixture was centrifuged at 10,000 g, room temperature for 10 min, and supernatants from this step were applied to the QIAprep column by pipetting. The following centrifugation of columns were perform at 10,000 g, room temperature for 1 min. After first centrifuging, columns were washed by applying 0.75 ml of Buffer PE and centrifuged, and then centrifuged for another 3 min to remove residual wash buffer. After columns were transferred to clean microfuge tubes, column matrixes were rinsed with 50 μl of H_2O (containing 1 mM Tris-HCl, pH 8.5) for over one min and columns were centrifuged to elute plasmid DNA.

2. Preparation of competent *E. coli*.

The following simple procedure usually produces competent bacteria with transformation efficiency of about 10^6-10^7 transformed colonies per 1 μg of supercoiled plasmid DNA. The efficiency is high enough for routine plasmid cloning in *E. coli*, even though it will be affected by bacterium strains. In preparation of competent cell from bacterium strain XL1-blue, single colony from overnight LB plate (containing Tetracycline) was picked up and inoculated into 100 ml of LB broth in 1-liter flask. The incubation condition was carried out at 37 °C (220 rpm) until the value of culture $O.D._{600}$ reaches 0.5-0.6 (the viable cell number should be about 10^8 cells/ml). Bacteria were harvested by dispensing culture into two 50-ml polypropylene tubes, then keeping in ice bath for 10 min and centrifuging at 4,000 rpm for 10 min at 4 °C. Each drained pellet was resuspended in 10 ml of ice-cold $CaCl_2$ (0.1 M) and stored on ice for 30 min. After ice bath, bacteria were recovered by centrifugation at the same condition as previously described. Each drained pellet was resuspend in 2 ml of ice-cold $CaCl_2$ (0.1 M) containing 10 % (v/v) glycerol. Suspension of competent cells can be dispensed into aliquots with volume of 100 μl in 0.5-ml chilled eppendorf and then immediately snap-freeze in liquid nitrogen before storing at –70 °C. For transformation of cloning plasmid, 5 μl of DNA ligation reaction solution was transferred into 100 μl of freshly prepared or stored competent cells and mixed well by swirling gently. Then this mixture was treated with the standard heat shock procedure in which the mixture was stored in the ice bath for 30 min, preheated in the water bath (42 °C) for 2 min, and ice bath for more than 2 min. Transfer treated bacteria into 900 ul LB broth in a 30-ml universal tube and incubate at 37 °C (220 rpm) for 45 min to allow bacteria to express antibiotic resistance gene encoded by the plasmid. Up to 100 μl of recovered culture was gently spread onto LB plate (90-mm) with appropriate antibiotics, and then leave it at room temperature until the liquid has been absorbed. The plates were incubated, at invert position, at 37 °C overnight. Generally, obvious colonies should be observed after incubation for 16 hours.

3. T4 DNA ligase ligation reaction.

For constructing proper plasmid constructions, the desired DNA fragments and plasmid vectors were ligated by T4 DNA ligase (200 units/reaction) in the presence of 1x ligation buffer (50 mM Tris-HCl, pH 7.5; 1 mM ATP, 25 μg/ml bovine serum) in the reaction volume of 20 μl at 16 °C water bath overnight. Generally, after proper restriction enzyme digestions, the plasmid vectors were treated with alkaline phosphatase (directly add 10 units/reaction) to remove the phosphate group on the 5' terminal to decrease vector's self-ligation. In the ligation reaction, the number of inserts is roughly five times more than that of plasmid vectors, determined by comparing the UV-emitted intensity of DNA bands on agarose gels.

4. Generating various bacterial vectors encoding different truncation forms of SIV tat protein.

The commercial plasmid pGEM-2T (encoding GST), has been modified to pGHP which encodes a 6x-His and a Pk tags on the C-terminal of GST. The gene of SIV tat protein [containing 131 amino acid (aa) residues] was cloned into pGHP on BamHI and EcoRI sites, resulting in pGTHP. The DNA sequence of serial-three arginine residues of SIV tat was changed to bacterial preference codons, CGTCGCCGT, for improving protein expression in bacteria. These materials described above were produced by our colleagues. Gene fragments of various truncation forms of SIV tat were generated by polymerase chain reactions (PCR) and then cloned into pGTHP on BamHI and EcoRI sites, replacing SIV tat. Each PCR was set up at a volume of 50 μl of water solution which contained dNTP (0.2 mM), forward (F) primers (0.2 μM), backward (B) primers (0.2 μM), mini-prep plamid template (pGTHP, 0.1 μg), reaction buffer (1X), and DyNAzyme II DNA polymerase (1 unit). The program of thermal cycles was set as: 94 °C for 5 min, 25 cycles of three steps (94 °C 30 s, 55 °C 30 s, 72 °C 30 s) and 72 °C for 7 min. Each forward primer contains a BamHI restriction site, and each reverse primer contains a EcoRI restriction site. The N1 (encoding 1-64 aa), N2 (encoding 1-94 aa), 30AA (encoding 65-94 aa), C1 (encoding 95-131 aa) fragments of SIV tat gene, were amplified by four sets of primers: 1 [5'-CGCCG-CggatccATGGA-GACAC-CC-3' (F), and 5'-CCGCC-

GgaattcCCTGG-CAATG-GTAGC-AACA-3' (B)], 2 [5'-CGCCG-CggatccATGGA-GACAC-CC-3' (F) and 5'-CCGCC-GgaattcCAGAT-GTATT-AGCCT-TAGC-3' (B)], 3 [5'-CGCCG-CggatccTTTTG-TTTTC-TTAAA-AAG-3' (F) and 5'-CCGCC-GgaattcCAGAT-GTATT-AGCCT-TAGC-3'(B)], 4 [5'-CGCCG-CggatccTCTGC-ATCAA-ACAACAGA-3' (F) and 5'-CCGCC-GgaattcCTCTG-CCAAG-GC-3' (B)], respectively, and cloned into pGTHP resulting in pGN1HP, pGN2HP, pG30AAHP, pGC1HP.

5. Preparation of plasmid DNA for sequencing.

Plasmids from 5 ml of overnight culture were purified by using the QIAprep Spin Miniprep Kit, and eluted in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The purified plasmid (500 ng) and the proper internal primer (5 pmole) were mixed in sterilized-deionized water up to the volume of 12 μl for the dideoxynucleotide sequencing reation, which was then analyzed on the DNA auto-sequencer ABI 377.

6. Protein purification from *E. coli* bacteria.

An overnight *E. coli* culture was inoculated into 1 litre of LB medium (containing 50 μg/ml of ampicillin) in a 2 litre flask, and grown at 37 °C (220 rpm) until the value of O.D.600 reaches 1.0. The expression of desired proteins can be induced at 37 °C or room temperature. Usually, if the expressed proteins were not soluble at the 37 °C induction condition, cultures will be induced at the room temperature condition. After cultures reach the desired density, cultures were induced with IPTG (prepared in water) at 37 °C for 5 hours, or induced at room temperature overnight. At the room temperature induction condition, cultures were incubated in the water bath (room temperature) for 15 minutes before inducting with IPTG. Cultures were harvested by centrifuging at 4 °C (8,000 rpm) for 10 min. If convenient, bacterium pellets can be leaved at –20 °C for a couple of days. To break bacteria, pellets were resuspend in 5 ml of PBS (containing 0.5 M of NaCl) and passed through a French Press bursting machine. Cell lysates were collected and mixed well with 10 % (v/v) Triton to a final concentration of 1 % (v/v), and then centrifuged at 4 °C (15,000 rpm) for

30 min. Supernatants were collected, filtered with 0.2 μl filters and passed through suitable columns to purify the desired fusion proteins.

To purified GST fusion proteins, the filtered cell lysates were applied into GST affinity columns and washed twice with washing buffer (PBS containing 0.5 M of NaCl). Usually, 88 mg of GST affinity beads were used for purifying proteins from 100 ml of cultures. Beads were swelled in water and washed in the washing buffer (PBS containing 0.5 M of NaCl) twice and collected by centrifuging. Proteins were eluted with the elution buffer which was made by mixing 5 ml of freshly prepared glutathione solution (0.154 g of glutathione in 5 ml 0.1 of M NaOH) with 45 ml of the basic buffer [composed of 2.5 ml 1 M Tris-HCl (pH 8.0), 5 ml 5 M NaCl, 37.5 ml H_2O]. During elution, elution buffer was applied into column with interval of 5 min, each time 1 ml to improve elution efficiency, and flow-outs were collected every 1 ml in different eppendorfs, usually 10 to 20 fractions. After collecting samples, 2.5 μl of flow-out from each fraction was spot onto nitrocellulose paper and stain in Naphthalene solution to check the elution result. According to the intensity of spots, the more concentrated fractions were combined together in dialysis bag (molecular weight cut off 12-14,000 Daltons) and dialyzed in PBS at 4 °C for 3 hours, changing three times. Dialyzed samples were sterilized by passing through 0.2 μm filters and aliquoted in 2 ml tube for storing in –20 °C.

7. SDS-Polyacrylamide Gel Electrophoresis of Protein.

After setting up vertical gel molds on gel-casting stand, prepare 10 ml of 12 % (w/v) resolving gel solution [which is composed of H_2O 3.3 ml, 30 % (w/v) acrylamide mix (29:1) 4.0 ml, 1.5 M Tris-HCl (pH 8.8) 2.5 ml, 10 % SDS 0.1 ml, 10 % (w/v) ammonium persulfate (in water) 0.1 ml, and TEMED 4 μl]. Pipette 3.5 ml of this gel solution into each gel mold, and then layer 0.5 ml of H_2O on top of gel margin. When gels become solid, about 30 min, pour off excess water and then pipette 5 % (w/v) stacking gel solution [which was prepared in 5 ml with H_2O 3.4 ml, 30 % (w/v) acrylamide mix (29:1) 0.83 ml, 1.0 M Tris-HCl (pH 6.8) 0.63 ml, 10 % (w/v) SDS 0.05 ml, 10 % (w/v) ammonium persulfate 0.05 ml, TEMED 5 μl]. Finally insert suitable comb into

the stacking gel and leave it polymerize for 30 min. For being boiled in water for 5 min, each sample should be mixed, at 1:1 ratio, with 2X Loading Buffer [containing 10 % (w/v) SDS 4 ml, 1 M Tris-HCl (pH 8.0) 1 ml, β-mercaptoethanol 1 ml, 50 % (v/v) glycerol (in water) 1 ml, H_2O 3 ml, and a touch of Bromophenol blue powder]. As boiled samples cool down, load 10 μl of sample mixture and perform electrophoresis at 200 constant voltage for 40 min in 1X SDS running buffer, diluted from 10X buffer (prepared with Tris-HCl 30 g, Glycine 144 g, and SDS 10 g in H_2O up to final volume of 1 litre). Migration of protein samples is in proportion to the size of protein, because binding of SDS is of correlation with the size, but not sequence, of protein. However, glycosylation of polypeptide can affect migration of protein, and thus the estimated molecular weight on the gel can not reflect the true mass of glycosylated protein. After electrophoresis, stain SDS-polyacrylamide gels in Coomassine brilliant blue solution (Acetic acid 100 ml, Methanol 400 ml, Coomassie dye 1 g, H_2O 500 ml) for 30 min, and then destain in Destain buffer (Acetic acid 100 ml, Methanol 200 ml, 700 ml H_2O) until protein bands become obvious. For Western blotting, protein can be transferred from resolving gels to nitrocellulose paper in 1X Transfer buffer (10X buffer is composed of Tris-HCl 30 g, Glycine 144 g in water up to the final volume of 1 litre) at 200 mA for 2 hours or 40 mA overnight if convenient. Alternatively, the procedure of protein transfer can be performed with the semi-dry transfer equipment at constant current of 50 mA for each mini SDS gel.

8. Immunofluorescent staining.

Cells were cultured in DMEM medium containing 10 % (v/v) FCS at 37 °C with 5 % CO_2. For immunofluorescent staining, BF or VERO cell were seed, at concentration of $2x10^3$ cells/ml, on multispot microscope slides in square dishes or 13-mm diameter coverslips in 24 well plates overnight. Before staining, cells were treated with tested protein by mixing protein with fresh culture medium at 1 ug/ml and then applying the mixture on cell monolayers, for multispot applying 35 μl, for coverslips 1 ml. Then treated cells were collected at different time points for staining. Briefly, cells were washed with washing buffer [PBS containing 1 % (v/v) FCS twice] and then fixed with the fixing solution

[containing 5 % (v/v) formaldehyde, 2 % (w/v) sucrose in PBS] for 10 min at room temperature. After fixing, cells were washed twice again and permeabilized with the permeabilizing solution [containing 0.5 % (v/v) NP-40, 10 % (w/v) sucrose, 1 % (v/v) FCS in PBS] for 5 min. Because tested proteins contain a SV5-Pk tag on the C terminal end, cells were stained with a mAb (Pk336) for 30 min and washed twice. After primary antibody binding, the secondary antibody (sheep anti-mouse IgG conjugated with TXRD) solution, which contains DNA-binding fluorochrome DAPI 0.5 μg/μl and antibody diluted at 1:500 in washing buffer, was added onto cells for 30 min which were then washed twice again. Following the staining of antibody, samples have to be re-fixed for 5 min to prevent dissociation of antibodies. Cells were examined under a Niko Microphot-FXA immunofluorescence microscope. When cells were grown on multispots, cells were mounted with AF1 solution and covered with cover glass; when cell were grown on coverslips, coverslips were inversely putted on slide mounted with drops of AF1 solution.

9. Western blot.

After proteins were transferred by electrophoresis, the nitrocellulose blot was transfer into a 50 ml blue cap tube and immersed with blocking solution [PBS with 5 % (w/v) non-fat dried milk, 0.1 % (v/v) Tween 20] for 1 hour at room temperature on an rolling shaker. If it is convenient, blot can be left in the staining box containing blocking solution and stored at 4°C overnight. The following incubation of antibodies and washing steps were performed at room temperature on a rolling shaker. Before incubating with the primary antibody solution, rinse the blocked blot with 20 ml of washing buffers [PBS with 0.1 % (v/v) Tween 20] twice. Incubate the blot in 10 ml of diluted primary antibody solution for 30 minutes in which antibody was diluted in washing buffer [containing 1 % (w/v) non-fat dried milk] and the value of dilution factor was determined empirically. Usually, anti-His monoclonal antibody was used at dilution 1/4k (k: 1000), anti-GST polyclonal antibody (derived form rabbits) was used at dilution 1/8k, and anti-Pk monoclonal antibody was used at dilution 1/50k. After incubating with the primary antibody solution, the blot was briefly rinsed with washing buffers twice and then washed for 15 min in 20 ml of

washing buffers. After washing, the blot was incubated in 10 ml of the diluted secondary antibody [conjugated with horse radish peroxidase (HRP)] solution for 30 min and then the following washing steps were performed as described above. Antibody binding was detected by the enhanced chemiluminescence assay (ECL). Briefly, drain off excess washing buffer by holding the blot gently in forceps and touching the edge against a tissue, then placing the protein side up on a suitable surface like staining box, adding 2 ml of mixed detection solution (ECL1 and 2 solution were mixed in the ratio of 1:1) on the surface of blot and incubating for 5 min. For exposure of autoradiography films, the blot was drained again and then wrapped up with transparent without capturing any air bubble. Exposures can vary from 15 seconds to 1 hour.

10. Immunization of mice.

BALB/c mice at age of about three months were intraperitonealy injected with purified fusion proteins, recombinant adenoviruses, or SV5 (simian virus type 5). Each injection formula contains 2 μg of proteins or various amounts of viruses in 0.5 ml of PBS. Usually, mice were injected three times with interval of two weeks. Blood samples were collected from tail veins one day before the third injection for measuring antibody responses by ELISA analysis. Two weeks after the final injection, mice were sacrificed by neck-breaking procedures, and spleens were collected for isolation of splenocytes for CTL assays.

11. Collection of blood samples.

Before collecting blood samples, mice were put on a heating plate (37 °C) for 20 min to increase the circulation of blood stream. Mice were then put on the top of a cage cover and the tail was cut by a blade. Blood samples (200-500 μl) were collected in a 1.5 ml eppendorf tube and left at 4 °C for one hour. Use a yellow tip to lose the aggregated blood clots and then centrifuge samples at 10,000 rpm for 10 min at 4 °C to separate sera which were then transfer to a new 1.5 ml eppendorf tube. These sera are ready for ELISA tests or can be kept at –20 °C until use.

12. Preparation of alum salt associated protein antigens.

It was planed to inject every mouse with 2 μg of protein antigens, and every group will enroll five animals. There are two distinct procedures to prepare alum salt associated protein antigens. In the co-precipitation procedure, protein antigens (12 μg in solution) were mixed with 500 μl of PBS and 50 μl of 1 M $NaHCO_3$ in a 1.5 ml tube. Subsequently, 100 μl of 10 % (w/v) aluminium potassium sulfate was added and mixed thoroughly. The mixture appears as milky white solution and then was centrifuged at 10,000 rpm for 2 min at room temperature. The pellet was resuspended in 3 ml of PBS and ready for injection (500 μl/mice). In the adsorption procedure, PBS, $NaHCO_3$ and aluminium potassium sulfate were mixed first and then centrifuged at the same setting as described above. The pellet was resuspended in 3 ml of PBS which containing 12 μl of protein antigens, and then incubated on ice bath for one hour. This chilled solution was ready for injection (500 μl/mice).

13. Enzyme linked immunosorbent assay (ELISA).

The wells of flat-bottom 96-well plates were coated with 100 μl of the target protein solution (10 μg/ml in PBS) at 4°C overnight or until use, and then blocked with 400 μl of skimmed milk solution [3 % (w/v) skimmed milk powder in PBS] for 1 hour at room temperature. For human serum, 10 % (w/v) skimmed milk solution was used for blocking plates and diluting antibodies and serum samples. The tested serum were serially diluted with 3 % (w/v) skimmed milk solution in the empty 96-well plate and added to the protein coated wells which were then incubated at room temperature for 1 hour. After the first incubation, wells were washed with Tween-20 buffer [0.1 % (v/v) Tween-20 in PBS] three times, and loaded with 100 μl of horseradish peroxidase (HRP)-conjugated antibody solution (for anti-mouse immunoglobulin, 1:2000 to 1:5000 dilutions in 3 % (w/v) skimmed milk were used; for anti-human immunoglobulin, 1/1000 to 1/2000 dilutions were used), again which were incubated and washed in the same procedure as described above. The freshly prepared substrate solution [mixtures of equal volumes of TMB peroxidase substrate (0.4 g/litre 3,3',5,5'-tetramethylbenzidine in a organic base) and peroxidase solution B (0.02 % w/v H_2O_2 in Citric buffer)] was added (100

μl/well) and the reaction was proceeded until the background color develops which was then stop by adding 1 M HCl (100 μl/well). The optical density of the reaction was read at a wavelength of 450 nm.

In the ELISA test for peptide mapping, plates were firstly treated with 1 % (v/v) glutaraldehyde in PBS (100 μl/well) overnight at 4 °C and washed once with PBS, which were then coated with diluted peptide at concentration of 10 μg/ml in PBS (50 μl/well) and incubated overnight at 4 °C, followed by blocking with 10 % (w/v) skimmed milk in PBS (400 μl/well) at room temperature for 3 hours. The appropriate diluted human serum in 10 % (w/v) skimmed milk were added and incubated overnight at 4°C. The rest steps follow procedures in the normal ELISA test, but the wash solution is changed to 1 % (w/v) skimmed milk solution (prepared in PBS).

14. CTL assay.

Two weeks following the final immunization, spleens from mice were removed by using sterile instruments and kept in sterile tubes until being transferred to tissue culture hood. Spleens were washed with PBS twice, transferred to petri dish containing 5 ml of PBS, and mashed through the mesh to squeeze cells out. The suspension was collected into a sterile centrifuge tube and the remaining cells were washed from the mesh with another 5 ml of PBS. Suspensions were mixed together and kept in tube to allow spleen debris to settle down for 30 seconds. The supernatant was transferred to another 10 ml conical plastic tube and span at 1200 rpm for 5 min. The collected pellet was resuspend by tapping tube and then mixed with 8 ml of 0.83 % (w/v) NH_4Cl and stand for 5 min at room temperature. The mixture was underlaid with 1.5 ml of heat inactivated fetal calf serum, span as above, washed once with PBS and resuspend at 7.5×10^5 cells/ml in the complete T cell medium [RPMI 1640 medium containing 10 % (v/v) heat inactivated fetal calf serum (56 °C for 30 min), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M β-mercaptoethanol]. A sixth of the purified splenocytes were either infected with SV5 (10^8 pfu/ml) or incubated with fusion proteins or peptides (20 μg/ml) at 37 °C for 2 hours at the volume of 1 ml, washed twice with PBS, and then

cocultured with the remaining splenocytes at 37 °C for 5-7 days. The source of stimulator cells can be derived from non-immunized mice. These treated splenocytes were termed stimulators while the untreated cells were called responders, and the coculture process was to stimulate responders to form effectors. The whole culture was span down at 500 g for 5 min and resuspend in 1 ml of complete T cell medium. The viable cells were counted by mixing with Trypan blue [freshly mix 0.2 % (w/v) trypan blue and 4.25 % (w/v) NaCl at ratio of 4:1] at ratio of 1:1. These alive effectors were dispensed into U-well microtiter plates and serially diluted to desired concentration [10^7 cells/ml for a starting ratio of 100:1 (effectors: target cells)] in final volume of 100 μl/well. To load CTL epitopes, the target cells (P815 suspension cells) were either infected with SV5 (10^8 pfu/ml) or incubated with proteins or peptides (20 μg/ml) at 37 °C one day prior to the CTL assay. Target cells without epitope loading were used as negative control. These target cells were then labeled with ^{51}Cr by incubating with 1 ml of labeling solution [700 μl PBS, 100 μl 10× medium, 200 μl ^{51}Cr (1 μCi/ml, $Na_2{}^{51}CrO_4$)] at 37 °C for 1 hour. Cells were washed three times with RPMI medium and the last wash was leaved for 1 hour at 37 °C incubator before pelleting. The collected cells were resuspend at 1×10^5 cells/ml in RPMI/10 % (v/v) FCS and 100 μl aliquots were, in triplicate, dispensed into appropriate wells. Plates were span at 800 rpm for 30 seconds to bring targets and effectors together and then incubated at 37 °C for 5 hours. Plates were then span at 1,000 rpm for 10 min, and 50 μl of supernatants were carefully collected and counted by Gamma counter for 2 min. The percent specific ^{51}Cr release was calculated by the following formula: [(sample release – spontaneous release) × 100]/(total release – spontaneous release). Total release was determined from supernatants of labeled target cells that were lysed by addition of 10 % (w/v) SDS [final in 1 % (w/v)]. Spontaneous release was determined from labeled target cells which were not incubated with effector cells.

15. Generation of replication-deficient adenovirus vectors.

An elegant method for the generation of recombinant replication-deficient adenovirus based on the co-transfection of two plasmids (containing overlapping fragments) into a permissive cell line has been developed, and this

method is now wildly used. The large plasmid (pJM17) contains most of the viral genome of Ad5 dl 309, but lacks the DNA packing signal in the E1 region (as a prokaryotic vector was inserted in this region). The transfer vector (pCA14) contains the left inverted terminal repeat (ITR), packing signal, and sequence overlapping the large plasmid. After the gene of interest has been subcloned into the transfer plasmid, these two plasmids are co-transfected into 293 cells (which express E1 gene function), and viruses produced by recombination in transfected cells are isolated through plaque purification and amplified in the same cell line.

One day before the trnasfection experiment, the 293 cells were plated on 6-well plates. Usually the most suitable density of cell was 50-80 % confluent on the day of the experiment. According to the instruction manual of products (FuGENE-6 transfection reagent), 1 μg of the transfer vector pCA14 (containing desired gene) and 6 μg of large plasmid pJM17 were mixed (the total volume of DNA was between 0.5-50 μl) and then gradually added into a tube containing 350 μl of serum-free medium (DMEM) and 21 μl of FuGENE-6. The tube was gently tap to mix the contents, and incubated at room temperature for 15-30 min. These mixture was added into monolayer cell in 6-well plate (each well contains 2 ml of medium), and then, one day after transfection, culture medium was replaced with fresh medium containing 2 % (v/v) FCS. This transfected cells were incubated at 37 °C for 1-2 weeks. During this incubation period, usually CPE will be obserbed in one week aftere transfection. Until the grass CPE was observed, infected cells were collected y centrifugation, and the cell pellet was mixed with 1 ml of supernatant, which was then stored at –70°C as the primary stock. The rest of the supernatant was serially diluted for plaque purification to isolate the desired clone.

16. Plaque assay for adenovirus.

Monolayer 293 cells were grown in 60 mm petri dishes or 6-well plates and allowed to grow till approx 70-80 % confluent. Usually, cells of one 75t confluent flask were split into 5-6 petri dishes (60 mm) and grown in DMEM medium [containing 10 % (v/v) FCS] overnight. It is important to get the cell

monolayer as even across the plate as possible, thus shaking plates vigorously in the incubator before incubation. Virus stocks were serially diluted (one in ten) in DMEM from 10^{-3} to 10^{-9} dilution. Media in cultured dishes were replaced with 2 ml of DMEM media (1 ml for 6-well plates), and then 100 µl of properly diluted virus stocks were added and mixed thoroughly by shaking dishes (or plates). These dishes or plated were kept in sealed the box which was filled with CO_2, and rocked gently in warm room for 1-2 hour. During this time overlay media were prepared in which each 50 ml aliquot contains 15 ml of molten 3 % (w/v) agarose (low melt point agarose made up in pure water and autoclaved), 35 ml of DMEM [contains 2 % (v/v) FCS], 400 µl of sterile $MgCl_2$ (1 M). Before use, these overlay media must be kept at 36°C incubator to keep the agarose molten. Media were replaces with 4 ml of overlay media (2 ml for 6-well plates) and then theses dishes were left in the hood for 30 min to set the agarose. This low melt point agarose has a gelling temperature of 26-30 °C, so it will not gel in the incubator. After incubation for 6-7 days, plaques can be seen without staining. Plaques can be counted and compared easily by staining, in which cells were fixed with 5 % (v/v) formaldehyde (prepared in PBS) overnight. Following removing agarose overlays, 2 ml of staining solutions (comassie blue, methanol, acetic acid) were added and dishes were gently shaken for 10 min which were then washed with tap water and allowed to dry.

17. β-galactosidase staining.

After DNA transfections or virus infections, monolayer cells were rinsed with PBS twice and fixed with 5 % (v/v) formaldehyde (prepared in PBS) for 5 min. Fix solutions were removed and cells were rinsed with PBS twice again to wash out the fixative. The staining solution {5 mM potassium ferricyanide [$K_3Fe(CN)_6$], 5 mM potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$], 2 mM $MgCl_2$, 0.5 mg/ml X-Gal in dimethylformaldehyde} was freshly made up in PBS and applied to the fixed monolayer cells (2 ml is enough to overlay one 60 mm dish), which were then incubated at room temperature or 37 °C. Blue color should appear in a few hours, but staining can be left overnight. Usually, the components of staining solutions were prepared in high concentration (0.5 M

potassium ferricyanide, 0.5 M potassium ferrocyanide, 1.0 M $MgCl_2$, 50mg/ml X-Gal) and kept at –20 °C until used.

18. Immunostaining for confirming purity of Adenovirus stocks.

Like normal plaque assays, the 293 cells (cultured in 60 mm dishes) were infected with proper dilution of recombinant Adenovirus particles (express the wild type SV5 V protein) for 6-7 days and then fixed with 2 ml of fix solutions [5 % (v/v) formaldehyde in PBS] overnight at room temperature. After removing the overlay agarose, the fixed monolayer cells were incubated with 2 ml of permeable solutions [0.5 % (v/v) NP40, 1 % (w/v) sucrose, 1 % (v/v) FCS in PBS] for 10 min to make cell membranes permeable for antibodies in the following steps. Cells were washed three times with washing solutions [1 % (v/v) FCS in PBS] and then incubated with 2 ml of diluted primary antibody solutions [mouse mAb antibodies (recognizing Pk epitope) diluted at 1/1000 in washing solutions] by gently shaking for 1 hour. Again, cells were washed three times and then incubated with 2 ml of diluted secondary antibody solutions [alkaline phosphotase (AP) conjugated anit-mouse Ig antibodies diluted at 1/2k in washing solutions] by gently shaking for 1 hour. After washing three times, cells were incubated with 3 ml of AP substrate solutions for 30 min to 2 hours at room temperature to allow purple-black color develop. If virus stocks are pure without contaminant, every single plaque should appear as purple-black color spot.

19. Growth and purification of recombinant adenovirus.

Permissive cells (293 cells) were cultured in 300 cm^2 flasks with 50 ml of Dulbecco's Minimal Modified Essential medium (DMEM) supplemented with 10 % (v/v) fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin in 37 °C warm room. After seeding cells, flasks were filled with CO_2 for 30 seconds. Until cell density reached to 80 % confluence, culture media were changed to the same medium containing 2 % FCS and cells were infected with desired adenovirus at m.o.i. of 0.1-1. The infection will spread rapidly through the monolayer, and causes cells to become fragile, round up, and eventually detach from the monolayer. Infected cells can be easily detached

if the flask is given a gentle tap. As soon as all cells exhibit a gross cytopathic effect (CPE), about 2-3 days post-infection, the monolayer and detached cells were harvested, transferred to 50 ml centrifuge tubes and recovered by centrifugation at 2,000 rpm for 5 min at room temperature. The supernatant was decant to waste. Actually, the medium contains significant amount of viruses released by lysed cells, but this is not normally used. The cell pellet was resuspend in PBS (1 ml for each flask culture), and extracted with an equal volume of Arklone P (1, 1, 2-trichloro-1, 2, 2-trifluoroethane) with vigorous shaking (but not vortexing) for 30 min at room temperature. After centrifugation at 4,000 rpm for 10 min at room temperature, the aqueous (top) phase was carefully collected, aliquoted, and stored at –70 °C. In general, avoid putting the virus through freeze-thaw cycles as much as possible. The freshly extracted virus can be further purified by CsCl gradient centrifugation.

20. Virus purification by CsCl gradient.

Before proceeding to virus purification, care should be taken to use sterile equipments and reagents to ensure the virus stock is protected from microbial contamination or proteases. Solutions, dialysis tubes, clips, stir bar and buffer container were autoclaved, and centrifuge tubes can be disinfected by washing with 70 % (v/v) ethanol. The previous Arklone p extracted viruses were centrifuged at 4,000 rpm for 10 min at room temperature to remove any precipitated material. During centrifugation, CsCl gradient was prepared by first pipetting 3 ml of 3 M CsCl solution into the centrifuge tube, and then 2 ml of 2 M CsCl solution which was gently layered on top. The tube was than filled up to 2.5 mm from the top with the extracted virus (about 8.5 ml) without disturbing the surface of CsCl solution too much. Tubes were set on the roter 40 Ti and centrifuged at 30,000 rpm for 2 hours at 20 °C. After centrifugation, two bands should be clearly observed. One band containing the virus is present between the high and low density CsCl solutions, while the other band located higher up. To collect the virus, the lower band was gently pulled into a 2 ml syringe by using a 19-gauge needle. The purified virus was dialysed with 1 litre of dialysis buffer [$MgCl_2$ 1 mM, NaCl 135 mM, Tris-HCl 10 mM pH 7.8, Glycerol 10 % (v/v)] at 4 °C cold room for four hours, and dialysis was repeated

once more to ensure efficient removal of CsCl. Viruses were aliquoted in cryotubes, and stored at –70 °C. Avoid multiple freeze-thaw cycles.

21. Clone P24P17 gene into donor plasmid pFastBacHTc.

P24P17 gene was amplified from plasmid pTH-HIVA by PCR using suitable primer set containing NcoI and NotI restriction site on forward and backward primers, respectively, and cloned into pFastBacHTc, resulting in pFastBacHTc/P24P17. As this gene fragment is rich in GC content, PCR was performed in the presence of 5 % (v/v) DMSO to improve amplification efficiency. This 50 μl PCR reaction solution contains 0.1 μg of pTH-HIVA (from mini-prep), 0.4 μM of forward primer (5'-CATGC-ATGccatggGGATG-CCCAT-CGTGC-AGAAC-3'; the start codon ATG is in the NcoI cutting site) and backward primer (5'-AAGGA-AAAAA-gcggccgcTCAGT-AGTTC-TGGGA-CAC-3'; the stop codon UGA is next to the NotI), 4 μM of dNTP, 1 U of Dynazyme, and 5 % (v/v) DMSO, and the reaction was performed at thermal program: 94 °C for 5 min, 25 cycles of three steps (94 °C 30 sec, 55 °C 30 sec, and 72 °C 30 sec), and final extension at 72 °C for 7 min. The start codon of the insert gene, P24P17, locates in the NcoI restriction site. Encoded by this construct, this recombinant protein is in the fusion form of MSYYHHHHHH-DYDIPTTENLYF-GA-P24P17. After being purified by Ni resins and cleaved by rTEV proteases, the final product of this recombinant is P24P17 with extra amino acid residues, GA, at the N terminal.

22. Construction of recombinant bacmids by transposition.

The construction of recombinant bacmids is based on site-specific transposition of an expression cassette, cloned in pFastBac-HTc donor plasmid, into a baculovirus shuttle vector (bacmid) which is propagated in *E. coli*. Upon the function of Tn7 provided by helper plasmid (pMON7124), a mini-Tn7 element can be transposed from a recombinant pFastBac donor plasmid to the mini-attTn7 attachment site on the bacmid. For transposition into bacmid, recombinant pFastBac donor plasmid was transformed into *E. coli* strain DH10Bac (harboring bacmids and helper plasmids) by a heat shuck procedure. Briefly, an aliquot of 0.5 μl of recombinant donor plasmid from mini-prep was

mixed with 100 μl of DH10BAC competent cell in 1.5 ml eppendorf by gently tapping the side of the tube and then incubated on ice for 30 min. After ice bath, the mixture was transferred into 42 °C water bath for 4 min and immediately chilled on ice for over 2 min. To recover transformed cells, this mixture was mixed with 900 μl of LB broth in a 15-ml polypropylene tube and agitated (225 rpm) at 37 °C for 4 h. After incubation, cultures were serially diluted into 10^{-1}, 10^{-2}, 10^{-3} by LB broth and 100 μl of each dilution was evenly spread on selection LB agar plates containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), X-gal (100 μg/ml), IPTG 40 (μg/ml). Because colonies are very small and blue colonies may not be discernible prior to 24 h, those plated should be incubated at 37 °C for 24 to 48 h.

23. Isolation of recombinant bacmid DNA.

After incubation for almost 24 h at 37 °C, large and white colonies were picked up for isolation of recombinant bacmid DNA. Before isolation, it is better to make sure colonies are truly white by streaking colonies on selecting plates (containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, 40 μg/ml IPTG) and incubation at 37 °C overnight. The confirmed white single colony was inoculated into 10 ml LB medium, containing suitable antibiotics as used in the selection plates, at 37 °C for 22 h with shaking at 220 rpm. The overnight culture (5 ml) was harvested by centrifugation at 4 °C 4,500 rpm for 10 min, and then resuspended in 0.3 ml of Solution 1 [15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml Rnase A]. To lysis cells, this cell suspension was mixed with 0.3 ml of Solution 2 [0.2 N NaOH, 1 % (w/v) SDS] by inverting tube for ten times and then incubating at room temperature for 5 min. The suspension should appear as almost translucent after addition of Solution 2. The lysis mixture was gently mixed with 0.3 ml of 3 M potassium acetate (pH 5.5) and incubated on ice bath for 10 min to help precipitate proteins, bacteria debris and genomic DNA. Following ice bath, samples were centrifuged at 4 °C 12,000 rpm for 10 min, and then supernatants were transferred to 1.5 ml eppendorf containing 0.8 ml absolute isopropanol and mixed by gently inverting tube for ten times. Because the size of bacmid is over 135 kb, it is better avoiding vigorously agitation during isolation. At this stage,

samples were placed on ice for 10 min or stored at –20 °C overnight if convenient. After cold treatment can be pelleted by centrifugation for 15 min at 12,000 rpm at room temperature. The supernatant was removed and pellet was washed by adding 0.5 ml 70 % (v/v) ethanol and inverting the tube ten times. The sample was centrifuged again for 5 min at the same setting, and then supernatant was removed as much as possible. As the pellet will become dislodged from tube bottom, supernatant should be carefully aspirated. Pellet was air dried for 10 min and dissolved in 40 μl 1 mM Tris-HCl solution (pH 8.5). To avoid repeated freeze/thaw cycles, DAN was aliquoted in 5 μl and stored at –20 °C until transfecting Sf9 cells.

24. Transfection of Sf9 cells with recombinant Bacmid DNA.

Sf9 cells from suspension culture in mid-log phase were seeded in 6-well plate at final concentration of $9x10^5$ cells per 35-mm well. Usually, cells were cultured in 1 liter flask containing 200 to 500 ml serum-supplemented medium [TC-100 with 7 % (v/v) FCS] from cell number of $5x10^5$. When cultures reach the mid-log phase, the cell density is about $1\text{-}2x10^6$ cells per 1 ml. After seeding appropriate amount of cells, the plate was left at least for one hour allowing cells to attach on the surface of wells. During incubation, for each transfection, Solution A (100 μl TC-100 medium with 5 μg of mini-prep bacmid DNA) and Solution B (100 μl TC-100 medium with 6 μl of CellFECTIN Reagent) were mixed together and incubated for 15-45 min at room temperature. CellFECTIN reagent is a lipid suspension that will settle with time, so the tube was inverted several times to ensure the reagent is homogenous before using. After washing with 2 ml TC-100 medium, monolayer cells were overlaid with the solution mixture (diluted with 0.8 ml of TC-100 medium) and incubated for 5 h in a 28 °C incubator. Then, solution mixtures were replaced with 3 ml of complete TC-100 medium [containing 7 % (v/v) FCS], and cells were incubated at 28 °C for 96 h.

25. Harvest and amplification of recombinant baculoviruses.

When harvesting viruses from the transfected cultures, the culture supernatant (3 ml) was transferred to a 15 ml sterile, capped tube. After centrifugation at

500 g for 5 min, the virus-containing supernatant was transferred into a sterile tube covered with foil and stored at 4 °C. For the small scale amplification of viral stocks, 1 ml of the viral stock was used to infect 80 % full monolayer insect cells in 75t flask containing 30 ml complete medium for 96 h in a 28 °C incubator. The virus titer was determined by plaque assays. For the large scale amplification of viral stocks, 500 ml of suspension cultures were infected with viruses at MOI of 1 for 72 h. The recombinant protein, P24P17, was expressed at the infection condition of MOI of 10 for 72h.

26. Viral plaque assay for baculoviruses.

The infectious potency of baculovirus stocks was determined by performing the plaque formation assay in immobilized monolayer cultures. The exponential culture of Sf9 insect cells at $5x10^5$ cells/ml was prepared, and $1x10^6$ of cells were dispensed into each well of 6-well plates. Cells were allowed to settle to bottom of plate and incubated at room temperature for one hour. During incubation, virus stocks were serially diluted (ten times) in TC-100 medium (without FBS). After incubation, the supernatant in each well was removed, and immediately replaced with virus dilutions (1 ml). Plates were them placed in a covered box with moisture paper towels and incubated at 28 °C for one hour. While incubation, plates were rocked gently at intervals of 20 min to ensure viruses evenly distributed. During incubation, agarose overlay was prepared. Briefly, autoclaved agarose [3 % (w/v) in water] of low-melting temperature was melted in microwave, mixed with TC-100 medium [with 7 % (v/v) FCS] at ratio of 1:2 [agarose (3 % w/v): medium], and then kept at 37 °C incubator until use. After incubation, the virus inoculum was removed by a Pasteur pipet connected to a vacuum pump, and immediately replaced with agarose overlay (2 ml/well). Plates were kept in the hood for 10-20 min to allow agarose overlay to harden. When agarose overlay became solid, 1 ml of TC-100 medium [with 7 % (v/v) FCS] was applied on the top of agarose overlay, and plates were incubated in a humidified box at 28 °C for 3-4 days. After incubation, viral plaques were stained with the neutral red solution. Briefly, the medium (on the top of agarose overlay) was removed, and replaced with 2 ml of freshly prepared neutral red solution [0.025 % (w/v) in water]. Plates were then incubated in the same

setting as described above for 2-4 hours. The staining solution was then removed and plates (up-side down) were stored in dark for 2 hours to overnight. Viral plaqures will appear as red dots.

27. Protein (P24p17) purification with Ni-NTA resins.

The infected insect cells were centrifuged (in one litre centrifuge bottle) at 4 °C 4000 rpm for 30 min, and then pellets were washed in 50 ml tubes with PBS and centrifuged again at the same setting. If convenient, cells can be stored at –70 °C until needed. To lyse cells, pellets from one litre cultures were resuspended in 7 ml of PBS (pH 7.5) containing 0.5 M NaCl and one tablet of protease inhibitor cocktail (mini EDTA-free). Suspensions were sonicated four times each time for 30 seconds and kept in ice bath for 2 min between each sonication interval. The sonicated lysates were mixed with 10 % (v/v) Triton-100 to final concentration of 1 % (v/v), and centrifuged at 4 °C 30,000 rpm (Type 42.1) for 1 h. To avoid blocking columns, supernatants (from 8 litre of cultures) were filtered through 0.2 μm filter before pumping (1 ml/min) into the Ni column (20 ml) which had been equilibrated with phosphate buffer (PBS containing 0.5 M NaCl). After sample loading, the column was washed with 10 volumes of phosphate buffer and with 5 volumes of 10 mM imidazole (in phosphate buffer), and proteins were eluted with 200 mM imidazole (in phosphate buffer). Eluted proteins were dialysised at 4 °C in one litre of phosphate buffer with changing buffer three times each for 2 h. To remove the histidine tag from P24P17, proteins were digested with rTEV proteases in the reaction condition of 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT, and 40 μg/ml rTEV (home made) at room temperature overnight. This home made rTEV protease contains a histidine tag at its amino terminus for easy removal by Ni-NTA resin. After digestion, all reaction solution (4 ml) was repeatedly pumped into Ni column (1 ml) three times and collected in every fraction of 1.5 ml. These collected samples were dialysised in the condition as described previously, filtered through 0.2 μm filter, aliquoted and stored at –20 °C. Totally, 7.5 mg of P24P17 protein was purified from 8 littlers of infected insect cells. The amount of protein was estimated by means of comparing intensity of BSA standard on SDS-gels which were stained with Coomassie blue.

Results

Section I.

A. Use SIV tat protein as an antigen carrier to induce CTL responses.

Summary:

The HIV tat protein has been well reported to carry exogenous proteins into target cells, and this capability is mainly contributed by the arginine rich domain of tat protein. Protein antigens, delivered into cytosol rather than the acid compartments of target cells, should enter the class-I antigen processing pathway to be degraded and the resulting peptides presented on the cell surfaces, thereby inducing CTL responses. In our colleague's previous works, the SIV tat protein (containing the similar arginine rich domain) was genetically fused with the GST protein and expressed in the bacterial expression system. This fusion protein was shown to bind on surfaces of cultured cells by immunofluorescent staining, and to induce the production of IgG2a antibodies (the indicator for Th1 responses) without the presence of adjuvants. In our present works, those results as described above were confirmed, and the amino acid sequence responsible for membrane binding activity of SIV tat protein was narrowed down. Furthermore, sequences of CTL epitopes restricted to the H-2^d mouse haplotype were genetically fused with the SIV tat fusion protein, which were then expressed in the bacterial expression system. These fusion proteins were also shown to bind to cell surfaces, and to induce the production of IgG2a antibodies in BALB/c mice. However, antigen specific CTL responses were not observed in mice injected with these SIV tat fusion proteins.

1. Preparation of GST-Tat-HP (GTHP) fusion proteins.

To determine if the SIV Tat protein is able to deliver exogenous proteins into cells in a similar fashion to the HIV Tat protein, the DNA sequence of SIV Tat gene encoding 133 amino acids was subcloned into pGEX2T expression vectors and expressed in the *E. coli* expression system. This pGEX2T vector has been

modified by adding gene sequences of 6X-histidine (H) and Pk epitope (P) at the 3' end of GST gene, and insertion of SIV Tat gene (codons for RRR have been changed to highly used codons in bacteria) in BamHI and EcoRI cloning sites between GST and His tag generates the expression vector, which encodes the fusion protein, GTHP (composed of GST, SIV tat, and His-Pk tag) (**Fig. tat1a**) (these works have been performed by our colleague, Angelina Diassiti). After induction with IPTG (1 mM) at 37 °C for 5 hours, bacteria which express GHP (composed of GST, and His-Pk tag) or GTHP were broken by sonication in PBS/NaCl (0.5M) buffer, and proteins were purified with glutathione-bead columns. The size, purity and relative amount of purified proteins were checked by 12 % (w/v) SDS-PAGE analysis, and profiles were illustrated by Coomassie brilliant blue staining (**Fig. tat1b**). The purity of GHP is much higher than that of GTHP, since on the gel only one band was observed in the GHP sample while more than one band appeared on the GTHP sample.

2. Improving purity of purified GST-Tat-HP (GTHP).

To improve the purity of purified protein GTHP, different purification strategies were used and compared. Briefly, bacteria were grown at standard conditions as described before, and proteins were purified in three different procedures: (i) purified by glutathione-bead columns at room temperature, (ii) purified by glutathione-bead columns and then by nickel-resin columns at room temperature, (iii) purified by glutathione-bead columns at 4 °C whilst protease inhibitors were added to the cell lysates. Proteins were eluted from glutathione-bead columns with glutathione, and from nickel-resin columns with immidazole. To remove excess glutathione and immidazole, eluted protein samples were dialyzed in PBS buffer. Purified proteins were resolved through 12 % (w/v) SDS-PAGEs and stained with Coomassie brilliant blue (**Fig. tat2**). Purification performed at the condition of low temperature and presence of protease inhibitors improve the purity dramatically, compared with the condition of room temperature and absence of protease inhibitors. This might suggest that the impurity was mainly caused by protease degradation.

As the SIV Tat fusion protein is in the form containing GST at N-terminal end and 6X-histadine and Pk tags at C-terminal end, the two-step purification procedure was used to purify full length proteins. Following purification on glutathione-bead columns, which bind the GST domain, nickel-resin columns were applied to purify fusion proteins containing 6X-histidine at the C-terminal end. Since in the native condition GST protein is formed in dimer, the purified fusion proteins may contain the C-terminal truncated forms which are dimerized with the full length form. This might explain that, even after the two-step purification procedure, the appearance of protein fragments smaller than the full length proteins was observed on the gel. To confirmed this possibility, the two-step purified protein sample was subjected to the Western blot analysis (**Fig. tat3**), in which rabbit anti-GST antibodies and mouse anti-6X-histidine antibodies were applied to probe terminal ends of purified proteins. The HRP conjugated antibodies against rabbit or mouse IgG were used as the secondary antibody, signals were developed in ECL reactions, and the profiles were visualized by X-film exposing analysis. Fragments smaller than the full length protein were detected in anti-GST probing but not in anti-6X-histidine probing, which confirms the possibility described before and suggests that in this case C-terminal truncated proteins were hardly removed by the two-step purification procedure.

3. Storing GST-Tat-HP (GTHP) fusion proteins.

Given that over 90 % of the two-step purified proteins were precipitated after storing at –20°C (data not shown), it was important to find an appropriate storage condition to prevent or reduce protein precipitation. One batch of GTHP was purified with glutathione-bead columns at room temperature and dialyzed in Tris-HCl/NaCl (pH 8.0) buffer, and the other batch of fusion protein was purified through the two-step purification procedure and dialyzed in PBS buffer. After storing at –20 °C for more than one week, these protein samples were centrifuged at 12,000 rpm 4 °C for 10 min to separate soluble and precipitated parts, which were subsequently analyzed by SDS-PAGEs and stained with Coomassie brilliant blue (**Fig. tat4**). Protein samples dialyzed in Tris-HCl/NaCl buffer still contained over 80 % of proteins in the soluble form. In contrast, the

majority of proteins dialyzed in PBS were in the precipitated form, suggesting that Tris-HCl/NaCl buffer is much more suitable than PBS buffer for keeping this protein soluble.

4. GST-Tat-HP (GTHP) fusion proteins can bind cell surfaces of cultured Vero cells.

The ability of SIV Tat to bind to cell surfaces was demonstrated by immunofluorescence staining. Briefly, monkey cells (Vero) were cultured on glass cover slips and then incubated with GHP or GTHP fusion proteins in serum-free DMEM medium at concentration of 2 μg/ml for one hour. Cells were subsequently washed with PBS and fixed in 5 % (v/v) formaldehyde solution, which were then stained with mouse anti-Pk mAb and anti-mouse Ig antibodies conjugated with Texas red. The stained monolayer cells were examined under a immunofluorescence microscope (**Fig. tat5**). These results clearly demonstrated that GTHP fusion proteins, but not GHP fusion proteins, bind efficiently to the surface of cultured monkey cells. However, it was not possible to conclude whether the Tat fusion proteins entered the cells from these results.

5. Identification of the membrane binding domain of SIV tat protein.

The HIV tat protein has been reported to be able to transport into target cells and its basic amino acid region (RKKRRQRRR) is believed to mediate this activity. It was of interest to identify the region of SIV tat which was responsible for the similar activity. To find this essential region, SIV tat protein was prepared in various truncated forms of proteins fused with GST at the N-terminal and His-Pk tag at the C-terminal. Briefly, the gene of SIV tat was amplified by PCR with four sets of primers separately, resulting in four different length of fragments which covered the full sequence of the tat gene. These fragments (named N1, N2, C1, and 30AA) were cloned into pGSX2T individually, and the resulting constructs were expressed in *E. coli* for protein purification (**Fig. tat6**). These truncated proteins were added to monolayered Vero cells and traced by mAb which recognizes the Pk tag. It was clear that truncated proteins N2 (aa 1-94) and 30AA (aa 65-94) rather than N1 (aa1-64) or C1 (aa95-131) were found binding on cell surfaces (**Fig. tat7**), suggesting that

the fragment of SIV tat protein responsible for binding tat to cell surfaces was located in fragment 30AA (FCFLKKGLGI CYEQSRKRRRTPKKAKANTS).

6. Relations between Th1 responses and IgG2a antibodies.

Soluble extracellular protein antigens are notoriously poor stimulators of CD8+ cytotoxic T-lymphocyte (CTL) responses, largely because these antigens have inefficient access to an endogenous cytosolic pathway of the major histocompatibility complex (MHC) class I-dependent antigen presentation. In order to enter the class I pathway, proteins should be endogenously expressed or transported into the cytosol. Since SIV Tat protein could potentially bring exogenous proteins into cells, it was of interest to examine whether SIV Tat can prime CTL responses *in vivo* or not. It has been reported that by the secretion of IL-2 and IFN-γ, CD4+ Th1 cells are able to activate CD8+ cytotoxic T lymphocytes producing CTL responses and also promote B cells to switch to IgG2a production. On the other hand, CD4+ Th2 responses are usually associated with high titers of antibody responses. In fact, Th2 cells are excellent helpers for B lymphocytes to produce high level of IgM, IgE and non-complement-fixing IgG isotypes, IgG1 in mice. Thus, the production of IgG2a is an indication for the induction of Th1 cells and may indirectly be a marker of CTL responses.

7. Immunization with soluble GST-Tat-HP (GTHP) fusion proteins.

To investigate the immunogenecity of GTHP fusion proteins, mice (BALB/c) were intraperitonealy injected with purified GTHP or GHP, and sera were collected for the ELISA analysis in which GST-P was used as the target antigen to detect induced antibodies. The antibody titer is defined as the largest serum dilution value at which ELISA reading value is over 0.5 units at wavelength of 450 nm (**Fig. tat8**). The titer of induced antibody IgG1 was much higher in mice injected with GTHP (>6.4k) than that of mice injected with GHP (>800), indicating that SIV Tat proteins can improve immunogenecity of protein antigens. Encouragingly, IgG2a were efficiently induced in mice injected with GTHP (>1.6k), but were barely induced in mice injected with GHP (<1/50) injected mice, which suggested that SIV Tat protein was responsible for the induction of IgG2a. These results indicate that SIV Tat protein in the fusion

form not only enhance antibody responses but also bias immune responses to Th1 responses which might further enhance CTL responses.

8. Immunization with soluble and insoluble GST-Tat-HP (GTHP) fusion proteins.

The SIV Tat fusion protein (in the soluble form) has been shown able to induce IgG2a production which is a marker of proliferation of Th1 cells. Th1 cells help activate cytotoxic T lymphocytes to generate CTL responses. Thus, in our future works, it may be possible to fuse SIV Tat protein with other antigens to induce antigen specific Th1 responses and also the induction of CTL responses (below). Given that not every protein is soluble in the native purification condition or after low temperature storage, it was of interest to compare the effect of soluble and insoluble protein antigens on the induction of IgG2a antibodies. Mice were intraperitonealy injected with the soluble or insoluble form of GTHP which were prepared as described before (**Fig. tat4**), and sera were collected for ELISA analysis in which the generation of IgG1 and IgG2a was investigated (**Fig. tat9**). The antibody titer is defined as the largest serum dilution value at which ELISA reading value is over 0.5 unit at the wavelength of 450 nm. Both IgG1 (>25.6k) and IgG2a (>12.8k) antibodies were induced in mice injected with the insoluble proteins, but these titers were much less than that induced in mice injected with the soluble proteins (IgG1: >>51.2k; IgG2a:>51.2k). Even though the insoluble SIV Tat fusion proteins are still capable of inducing the production of IgG1 and IgG2a, the capacity is significantly diminished compared with that of soluble proteins.

9. Immunization with alum salt mixed GST-Tat-HP (GTHP) fusion proteins.

Although many materials have been demonstrated as efficient adjuvants on vaccine injections, aluminium-based mineral salts remain the only licensed adjuvant used with vaccines in human. Alum salts trap protein antigens by electrostatic interactions, and thus slow down the release of antigens which may produce sustained stimulation of antigens, favoring Th2 cells development. It has been reported that alum is an adjuvant for antibody induction but not for the induction of cytotoxic immunity. It was of interest to determine whether alum

salt mixed SIV tat fusion proteins were still able to induce antibody and Th1 responses. Additionally, following different procedures, protein antigens can be either co-precipitated with or adsorbed on alum salts to form alum-antigens. It was also of interest to compare effects of these preparation procedures on immune responses. For the co-precipitation procedure, GTHP protein and 10 % (w/v) alum sulfate were mixed first, and then $NaHCO_3$ solution was added to co-precipitate proteins and alum salts. For the adsorption procedure, 10 % (w/v) alum sulfate and $NaHCO_3$ solution were mixed and centrifuged to form alum salt particles, which were then suspended in protein solutions and kept on ice for one hour. In both procedures, alum mixed proteins were centrifuged at 12K for 10 min and re-suspended in PBS buffer for injection in mice. Before injection, antigen samples from both preparation procedures were tested on SDS-PAGEs to confirm that proteins were completely associated with alum salts (**Fig. tat10**). Following the standard procedure, mice were injected with the alum mixed proteins, and sera were collected for the ELISA analysis (**Fig. tat11**).

Mice injected with alum mixed protein antigens, which were prepared either by the co-precipitation or adsorption procedure, generated both strong IgG1 and IgG2a responses. Since ELISA tests on these two subtypes IgG use different antibodies to probe, it is hard to compare the intensity between IgG1 and IgG2a responses. Between these two injection groups, there was no significant difference on IgG1 titer (injected with co-precipitated proteins: >>51.2k; injected with adsorbed proteins: >>51.2k), but the production of IgG2a in the group injected with co-precipitated antigens (>12.8k) was less than that in the group injected with adsorbed antigens (>51.2k). The titer of IgG1 was significantly improved in mice injected with proteins mixed with alum salts (>>51.2k), compared with that (>51.2k) of mice injected with soluble proteins alone. The titer of IgG2a of mice injected with proteins prepared by the co-precipitation procedure was reduced four times (>12.8k), but the titer of IgG2a of mice injected with proteins prepared by the adsorption procedure was maintained at the similar level (>51.2k), compared with that (>51.2k) of mice injected with soluble proteins alone (**Fig. tat9, 11**). It should be noted that ELISA tests shown in Fig. tat9, 11 were performed at the same time in the same condition, so it is fair to compare Fig. tat11 with Fig. tat9 rather than with Fig.

tat8. In the presence of alum salts, antibody responses were significantly increased and SIV tat fusion proteins remained capable of inducing Th1 responses.

10. Generation of double-CTL-epitope-fused fusion proteins.

It has been demonstrated that SIV Tat proteins were able to induce the production of IgG2a antibody after intraperitoneal injection in BALB/c mice (**Fig. tat8**). The production of IgG2a is an indication for the induction of Th1 cells which are in favor of inductions of CTL responses. To test for the generation of CTL responses, lymphocytes from immunized mice have to be examined by CTL assays. Since CTL epitopes restricted to Bacb/c (H-2^d) have not yet been identified on this fusion protein GTHP, two well-known CTL epitopes were genetically added to this fusion protein for CTL assays. The CTL epitope RGPGRAFVTI (restricted by H-2^d haplotype) derived from HIV gp120 was used for CTL assays on BALB/c mice, and the CTL epitope SIINFEKL (restricted by H-2^b haplotype) derived from ovalbumin was used for CTL assays on C57BC/6 mice.

Double strand oligonucleotides encoding the sequence of these two CTL epitopes, RGPGRAFVTI-SIINFEKL (CTLs), were cloned into BamHI restriction site on pGTHP and pGHP vectors, generating pGCTHP (encoding GST-CTLs-Tat-HP fusion protein termed GCTHP) and pGCHP (encoding GST-CTLs-HP fusion protein termed GCHP) (**Fig. tat12a**). Bacteria *E. coli* XL1-blue hosting these expression vectors were induced with IPTG (1 mM) at 37 °C for five hours and then lysed by sonication in Tris-HCl buffer (Tris-HCl 50 mM pH8.0, NaCl 0.5 M). Cell lysates were centrifuged and examined through 12 % (w/v) SDS gels. However, these two fusion proteins were insoluble under this condition (**Fig. tat12b**). Even when expressed at room temperature with low concentration of IPTG (0.05 mM) the solubility was not improved (data not shown). The bacteria were also lysed in various buffer systems including pH4.0 Acetic buffer (0.1 M), pH6.0 Phosphate buffer (0.1 M), and pH8.0 Tris-HCl buffer (50 mM), but these fusion proteins remained insoluble (data not shown). Given that the production of antigen specific IgG2a antibodies was significantly reduced in mice immunized with the insoluble

GTHP fusion protein (**Fig. tat9**), it was considered important to prepare protein antigens in the soluble form. Therefore, proteins were purified with nickel affinity columns in the 8M-urea denature condition, and re-folded by dialyzing in Tris-HCl buffers. The concentration of urea was reduced either gradually or straightaway in the dialysis procedure, but the yield of re-folded soluble proteins was insufficient for these purposes (**Fig. tat13**).

11. Generation of single-CTL-epitope-fused fusion proteins.

The double CTL epitope sequence contains many hydrophobic amino acid residues, which may reduce the solubility of proteins. In addition, the immunization tests focused on BALB/c mice, so the C57BL/6 restricted epitope was removed to reduce the content of hydrophobic amino acid residues. The position of CTL epitope in the fusion protein was also suspected of affecting protein solubility, and therefore the single CTL epitope was cloned at either the N- or C-terminal of Tat protein.

The double stranded oligonucleotide, which encodes the CTL epitope RGPGRAFVTI (I) derived from HIV gp120, was cloned into the pGTHP vector at either BamHI or EcoRI cutting site, resulting in the pGIHP (encoding GST-I-Tat-HP fusion protein termed GITHP) or pGTIHP (encoding GST-Tat-I-HP fusion protein termed GTIHP) construct. In the same fashion, another H-2^d restricted CTL epitope IPKSAKLFF (S) derived from M protein of SV5 (simian virus 5) was cloned into pGTHP, generating pGSTHP (encoding GST-S-Tat-HP fusion protein termed GSTHP) and pGTSHP (encoding GST-Tat-S-HP fusion protein termed GTSHP) (**Fig. tat14**). Bacteria containing expression vectors encoding single CTL epitope fusion proteins were cultured at 37 °C until reaching the density of 0.7 units at O.D. 600 and then induced with IPTG (1 mM) either at 37 °C for five hours or at room temperature overnight. Before induction at room temperature, cultures were incubated in water bath (room temperature) for 15 min. This peculiar procedure has been used by our colleagues to successfully generate soluble recombinant protease rTEV (not shown). Bacteria were lysed by sonication, and after centrifugation lysates were resolved through 12 % (w/v) SDS gels to examine the solubility of expressed

proteins. All of these four desired fusion proteins were shown to be soluble when expressed at room temperature [only GTSHP fusion protein was shown as an example] (**Fig. tat15**), and the position of the CTL epitope sequence did not affect the solubility of expressed proteins.

To examine whether these single-CTL fusion proteins were able to bind on cell surfaces in the same fashion as SIV Tat fusion proteins, immunofluorescent staining was performed. Vero cells were cultured on glass cover slips overnight and incubated with single-CTL fusion proteins (GTIHP or GTSHP) at concentration of 2μg/ml in serum-free medium. After incubation for one hour, cells were fixed and stained with the anti-Pk mAb, and bound antibody was then visualized by a fluorescence microscope (**Fig. tat16**). Clearly, these single-CTL fusion proteins were shown to bind on Vero cells as efficiently as GTHP fusion proteins.

12. Immunization with single-CTL epitope fusion proteins and CTL assays.
To examine whether SIV Tat fusion proteins were able to prime CTL responses, BALB/c mice were intraperitoneally immunized with single-CTL epitope fusion proteins (GTIHP or GTSHP) three times with intervals of two weeks. As a positive control for CTL assays, a group of mice were injected with SV5 (10^7 pfu) once. Two weeks following the last injection, blood samples from mice injected with fusion proteins (6 mice/group) were collected from tail veins. Sera were isolated by centrifugation and pooled together for examining antibody responses by ELISA tests (**Fig. tat17**). These two fusion proteins were shown to induce strong IgG1 (>>51.2k) and IgG2a (>51.2k) responses, and the addition of CTL epitopes appeared not to reduce the immunogenecity of SIV tat fusion proteins.

In CTL assays, suitable target cells are labeled with chromium-51 by incubating the target cells with $Na_2{}^{51}CrO_4$. After the ^{51}Cr diffuses into cells, it binds to cytoplasmic proteins, reducing passive diffusion of the label substance out of cells. When specifically activated CTLs are incubated with such labeled target cells, ^{51}Cr is released from lysed cells. The amount of released ^{51}Cr is directly

related to the number of target cells lysed by the specific CTLs, and represents the lytic capability of CTLs.

In each group, splenocytoes were harvested, pooled together, and cocultured with stimulator cells for one week to enrich antigen specific CTL cells which were termed effector cells. Stimulator cells are splenocytoes from naïve BALB/c mice, which have were incubated with GTSHP or GTIHP or infected with SV5 for two hours. Chromium (^{51}Cr) labeled target cells were syngenic P815 cells, which were incubated with GTSHP or GTIHP or infected with SV5 (10^8 pfu/ml) overnight to present the CTL epitope on cell surfaces. Labeled target cells were incubated with effector cells at various effector-to-target ratios for five hours. After the brief centrifugation, supernatants were harvested, and specific chromium release was calculated by the following formula: [(sample release - spontaneous release) × 100]/(total release - spontaneous release) (**Fig. tat18-1,-2,-3**). Specific CTL responses were not detected in mice either injected with GTSHP or GTIHP, even though mice in both groups generated strong IgG2a responses (>1/51.2k) (**Fig. tat17**). However, a strong CTL response could be detected in mice immunized with SV5, using either SV5 infected P815 cells or P815 loaded with the peptide (IPKSAKLFF) of SV5 M protein (**Fig. tat18-4**).

Interestingly, the group of stimulator cells either incubated with peptides or infected with SV5, when target cells were incubated with peptides, the lytic capability of CTLs was as high as that when target cells were infected with SV5, suggesting that peptide IPKSAKLFF was the dominant CTL epitope in CTL responses against SV5 infection. When stimulator cells were infected with SV5, the lytic capability of CTLs was significantly higher than that when stimulator cells were incubated with peptides, which imply that infection with viruses is more efficient than incubation with peptides for stimulating effector cells.

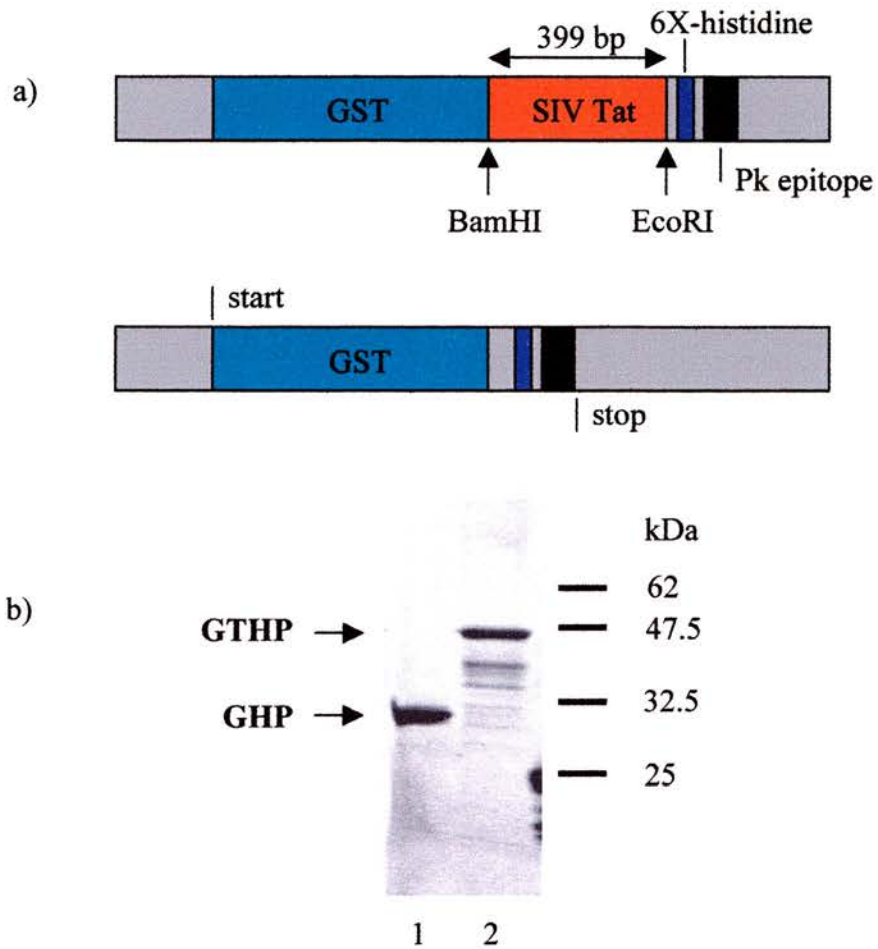

Fig. tat1. (a) Schematic diagram showing the construction of SIV Tat fusion gene. The SIV Tat gene was subcloned into the pGEX2T bacteria expression vector, which has been modified as a vector containing genes of 6X-histidine (H) and Pk epitope (P) at the 3' end of GST gene. By restriction enzyme digestion of BamHI and EcoRI, SIV Tat (codons of RRR have been changed for highly used codons) was inserted in the position between GST and 6X-histidine, generating the GTHP expression plasmid (these works have been performed by our colleague, Angelina Diassiti). (b) SDS-PAGE analysis demonstrating migration positions of purified fusion proteins, GHP (lane 1, purified by Angelina Diassiti), GTHP (lane 2). The fusion proteins were expressed in *E. coli* XL1-blue, and then purified with glutathione-bead columns. Purified proteins were separated through 12% SDS-PAGE and stained with Coomassie blue. Protein migration positions and molecular weights of standard marker were indicated along the gel. GHP: GST-His-Pk; GTHP:GST-Tat-His-Pk.

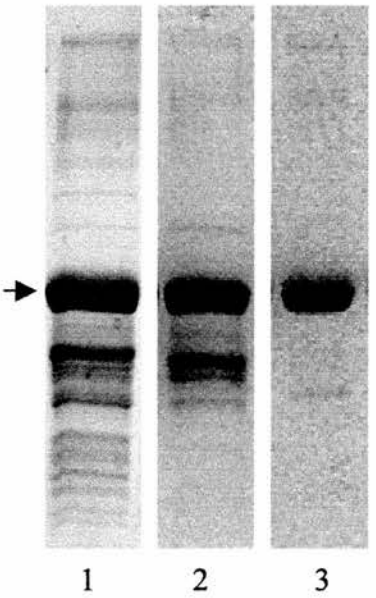

Fig. tat2. SDS-PAGE analysis demonstrating relative purity of GTHP fusion proteins purified in different conditions.
The SIV Tat fusion protein, containing GST protein at N-terminal end and 6X-histidine (H) and Pk tags (P) at C-terminal end, was expressed in bacteria which were induced with 1 mM IPTG at room temperature overnight. Bacteria were harvested and lysed by sonication in PBS/NaCl buffer. The overexpressed proteins were purified in one-step with glutathione columns (lane1) or two-step with glutathione and nickel columns (lane 2) at room temperature. Lysates containing protease inhibitors were purified with glutathione columns in 4 °C cold room (lane 3). Purified proteins were separated through 12% SDS-PAGEs and stained with Coomassie blue. The migration position of desired proteins was indicated by arrow. GTHP:GST-Tat-His-Pk.

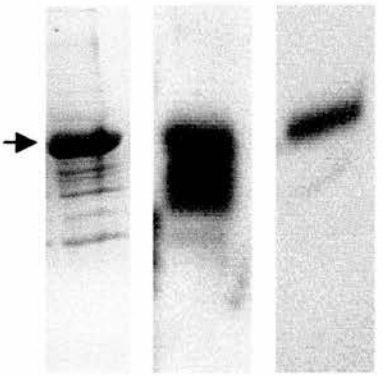

Fig. tat3. Western blot analysis demonstrates that purified GTHP fusion proteins contain C-terminal truncated forms.

The bacterium expressed SIV Tat fusion protein, which contains GST at N-terminal end and 6X-histidine and Pk tags at C-terminal end, was purified in two-step procedures with glutathione and nickel columns. Purified proteins were resolved on 12% SDS-PAGEs which were either stained with Coomassie blue (lane 1) or transferred onto PVDF membranes for Western blot analysis. The N-terminal end of fusion proteins was probed by rabbit anti-GST polyclonal antibodies (lane 2), and the C-terminal end was probed by mouse anti-6X-histidine mAbs (lane 3). The migration position of full-length fusion protein was indicated. GTHP: GST-Tat-His-Pk.

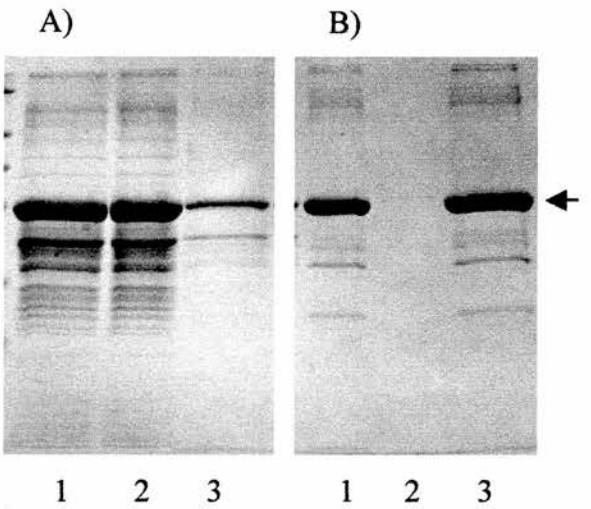

Fig. tat4. SDS-PAGE analysis demonstrating the ratio of soluble and insoluble parts of purified GTHP fusion protein after storing at –20 °C.
The SIV Tat fusion protein, which was designed containing GST at its N-terminal end and two tags of 6x-histidine and Pk at C-terminal end, was expressed in bacteria. Subsequently, the overexpressed protein was purified with glutathione columns at room temperature and dialyzed in **Tris/NaCl buffer** (panel A). The two-step purification procedure was also used to purify the fusion protein at cold room, and **PBS buffer** was used for dialysis in this procedure (panel B). After storing at –20 °C for over one week, the stored samples were thaw (lane 1) and centrifuged to separate soluble (lane 2) and insoluble portions (lane 3), which were then resolved through 12% SDS-PAGEs and stained with Coomasine blue. The arrow indicates the migration position of full-length fusion proteins. GTHP: GST-Tat-His-Pk.

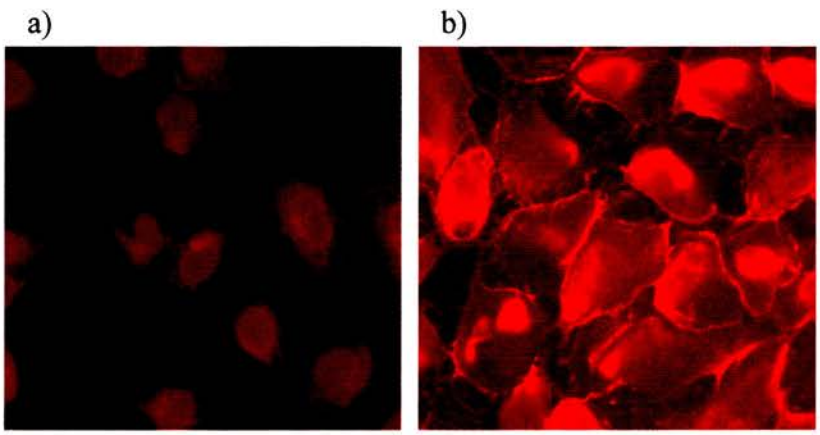

Fig. tat5. Photomicrographs showing GTHP fusion proteins binding on cultured Vero cells.

The Vero cell line was cultured on glass cover slips overnight and then incubated in serum-free medium containing 2 μg/ml of GHP (a) or GTHP (b) fusion proteins for one hour. Monolayers were then fixed and immunofluorescence stained by mouse anti-Pk mAbs and anti-mouse Ig antibodies conjugated with TXRD. GHP: GST-His-Pk; GTHP: GST-Tat-His-Pk.

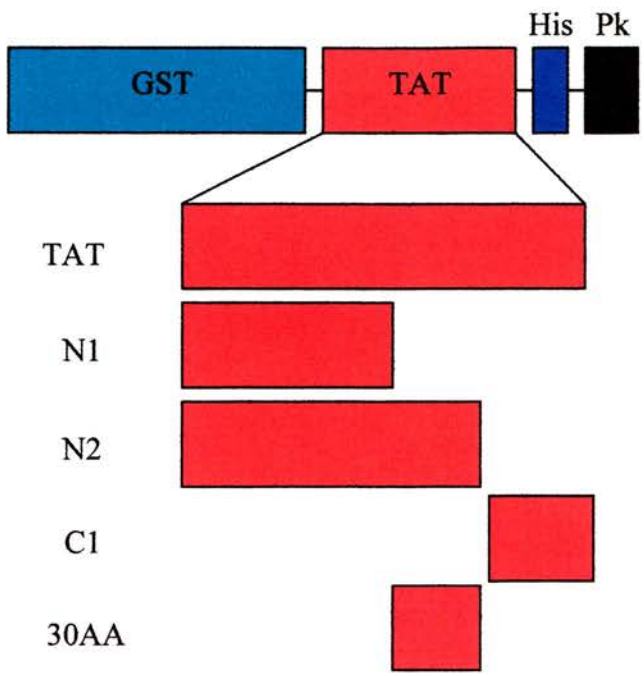

Fig. tat6. Diagram showing various truncated fragment of SIV tat protein.
By genetic engineering, each truncated protein was fused with GST at the N-terminal and fused with His-Pk tag at the C-terminal. These fusion proteins were expressed in *E. coli* and purified by GST columns. TAT: the full length form of SIV tat protein containing 131 amino acid (aa) residuals; N1: aa residuals 1-64; N2: aa residuals 1-94; C1: aa residuals 95-131; 30AA: aa residuals 65-94.

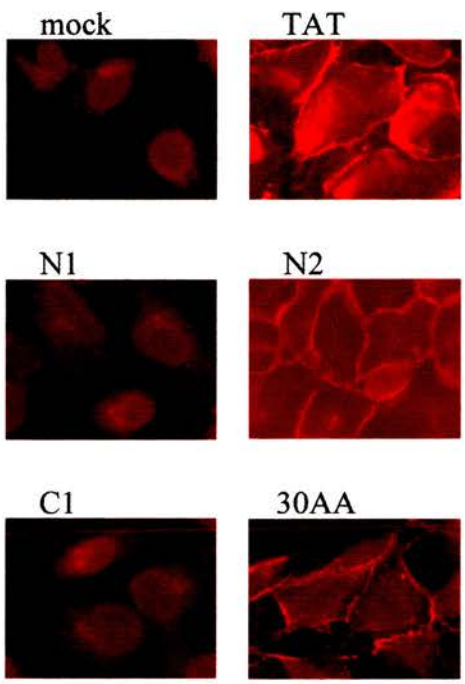

Fig. tat7. The membrane-binding activity of various truncated SIV tat fusion proteins was examined by immunofluorescent staining analyses.
Each truncated SIV tat protein contains GST on the N-terminal and His-Pk tag on the C-terminal, and was purified by the GST column followed the expression in *E. coli*. The purified proteins were added to the Vero cell cultures, which were grown on the coverslips, and probed by mAbs which recognize the Pk tag on the C-terminal of fusion proteins. TAT: full length form of SIV tat protein containing 131 amino acid (aa) residuals; N1: aa residuals 1-64; N2: aa residuals 1-94; C1: aa residuals 95-131; 30AA: aa residuals 65-94.

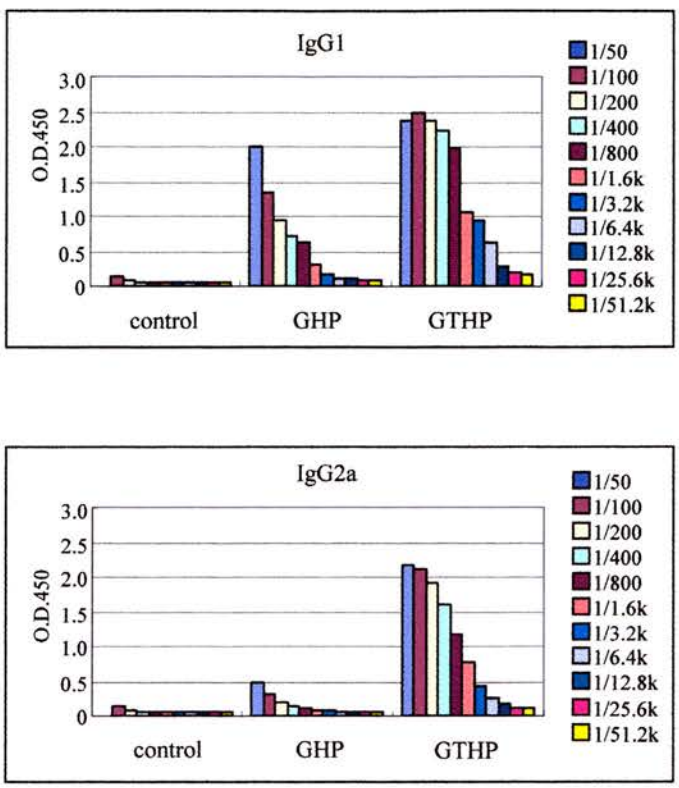

Fig. tat8. Antibody responses after immunization of GHP or GTHP fusion proteins in BALB/c mice.

BALB/c mice at age of about three months were intraperitonealy injected with GHP or GTHP fusion proteins three times with interval of two weeks. Each injection contains 2 μg of fusion proteins in PBS buffer (0.5 ml), and each group contains 5 animals. Blood samples (0.1-0.3 ml) were collected from tail veins two weeks after the final injection. Sera were equivalently pooled in each injection group and the induced IgG subtype antibodies, IgG1 and IgG2a, which were specific to GHP fusion proteins, were examined by ELISA analysis. (control: sera from mice without immunization; GHP: injected with GST-HP fusion proteins; GTHP: injected with GST-Tat-HP).

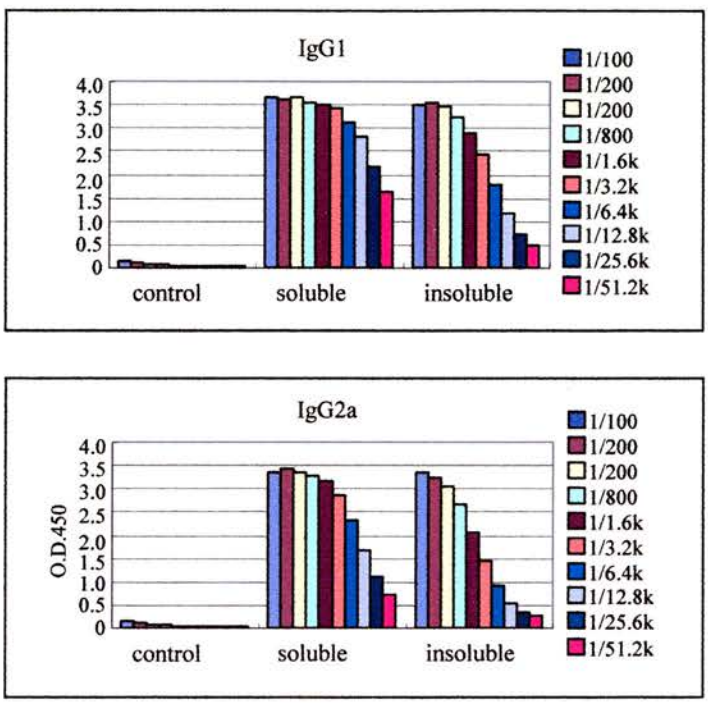

Fig. tat9. Antibody responses after immunization of soluble or insoluble form of GTHP fusion proteins in BALB/c mice.

BALB/c mice at age of about three months were intraperitonealy injected with 2 μg of soluble or insoluble form of GTHP fusion proteins in 0.5 ml of PBS buffer three times with interval of two weeks. Blood samples (0.1-0.3 ml) were collected from tail veins two weeks after the final injection. Sera were equivalently mixed in each injection group which contains five animals. The level of IgG subclasses containing IgG1 and IgG2a, which specifically recognize GTHP fusion proteins, was examined by ELISA analysis. (control: serum samples from mice without immunization; soluble: injected with soluble proteins; insoluble: injected with insoluble proteins). GTHP: GST-Tat-His-Pk.

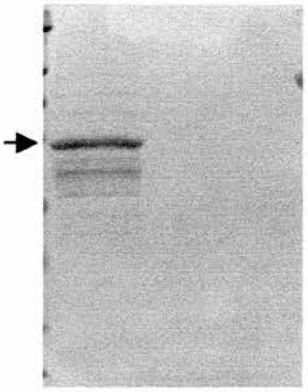

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Fig. tat10. Alum salts can efficiently trap GTHP fusion proteins.
The fusion protein, GTHP (1), was mixed with 10% alum sulfate and then with $NaHCO_3$ solution to co-precipitate with alum salts. After centrifugation, aliquots of the supernatant were examined on 12% SDS gels (2), and pellets were suspended in PBS buffer for injection. Alum mixed antigens were also prepared in the adsorption procedure, in which 10% alum sulfate and $NaHCO_3$ were mixed to form precipitated particles which were then collected by centrifugation and suspended in protein solution. The mixture was kept on ice for one hour to let proteins adsorbed on alum salts which were then span down and resuspended in PBS buffer for injection. The supernatant was examined on 12% SDS gels and stained with Coomassie blue (3). GTHP: GST-Tat-His-Pk.

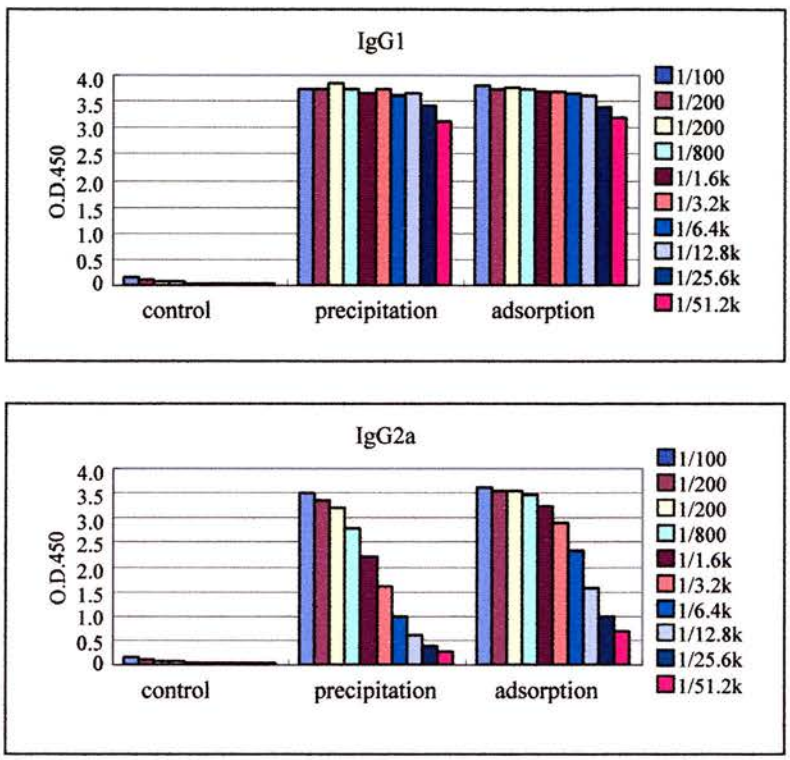


Fig. tat11. Antibody responses after injection of GTHP fusion proteins which were mixed with alum salts either by co-precipitation or adsorption.
BALB/c mice at age of about three months were intraperitonealy injected with 2 μg of alum mixed GTHP fusion proteins three times, with interval of two weeks. Alum mixed proteins were prepared by two different procedures. Proteins were either co-precipitated with alum sulfate and $NaHCO_3$ or mixed with the preformed alum salts. Blood samples (0.1-0.3 ml) were collected from tail veins two weeks following the final injection. Sera were equivalently mixed in each injection group and the induced IgG subtype antibodies, IgG1 and IgG2a, which were specific to GTHP fusion proteins, were examined by ELISA analysis. (control: sera from mice without immunization; precipitation: mice injected with proteins prepared by the co-precipitated procedure; adsorption: mice injected with proteins prepared by the adsorption procedure). GTHP: GST-Tat-His-Pk.

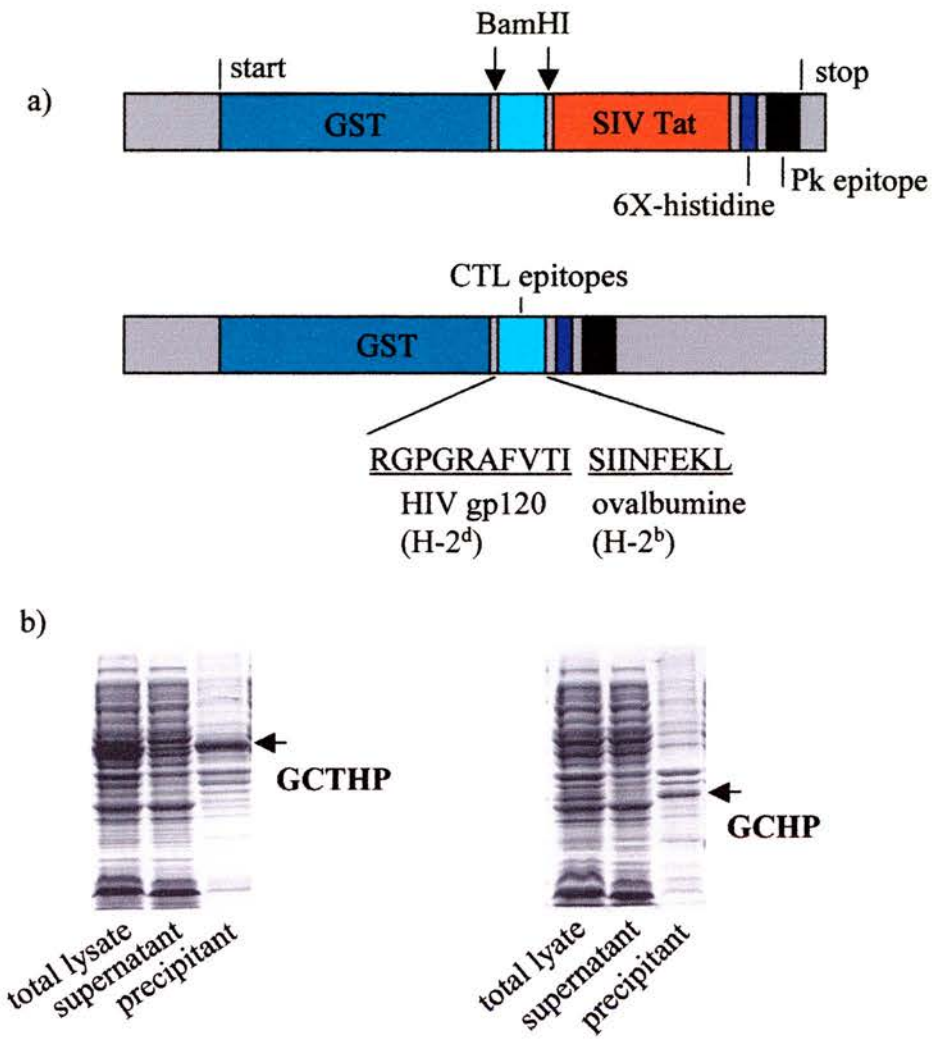


Fig. tat12. (a) Clone double CTL eptiopes into pGTHP and pGHP.
The double-stranded oligonucleotide encoding CTL epitopes was directly cloned into the BamHI cutting site of pGTHP or pGHP, in frame with the GST open reading frame, to generate the expression plasmid pGCTHP or pGCHP. This CTL epitope sequence consists of RGPGRAFVTI (restricted to H-2^d haplotype) from HIV gp120 and SIINFEKL (restricted to H-2^b haplotype) from ovalbumine. (b) Fusion proteins containing the double epitope sequence were insoluble. Bacteria *E. coli* XL1-blue harbouring expression vectors, pGCTHP or pGCHP, were induced with IPTG (1 mM) to express fusion proteins, GST-CTLs-Tat-His-Pk (GCTHP) or GST-CTLs-His-Pk (GCHP), at 37 °C for five hours. Bacteria were harvested and broken by sonication followed by centrifugation. The total lysate, supernatant and precipitant were examined through 12% SDS gels. The migration position of induced proteins was indicated by arrow.

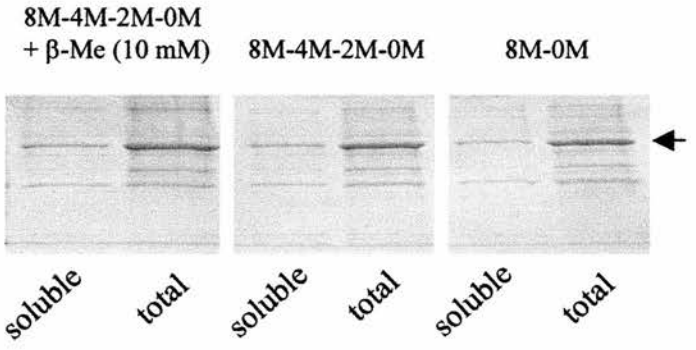


Fig. tat13. Refolding of GCTHP fusion proteins.
The GCTHP fusion protein, was expressed in *E. coli* XL1-blue at 37 °C for five hours and bacteria were broken by sonication in Tris-HCl buffer (pH 7.8) containing 8 M urea. Proteins were purified with affinity nickel columns and eluted in the same buffer containing immidazole (100 mM). The purified proteins were dialyzed in Tris buffer or in the same buffer containing urea which was gradually reduced from 8 M to 0 M. These buffers were prepared either with or without β-mercaptoethanol (10 mM). After dialysis, the total protein solution were centrifuged to separate soluble and insoluble portions which were then resolved through 12% SDS gels and illustrated by Coomassie blue staining. The migration position of desired proteins was indicated by arrow. GCTHP: GST-CTLs-Tat-His-Pk.

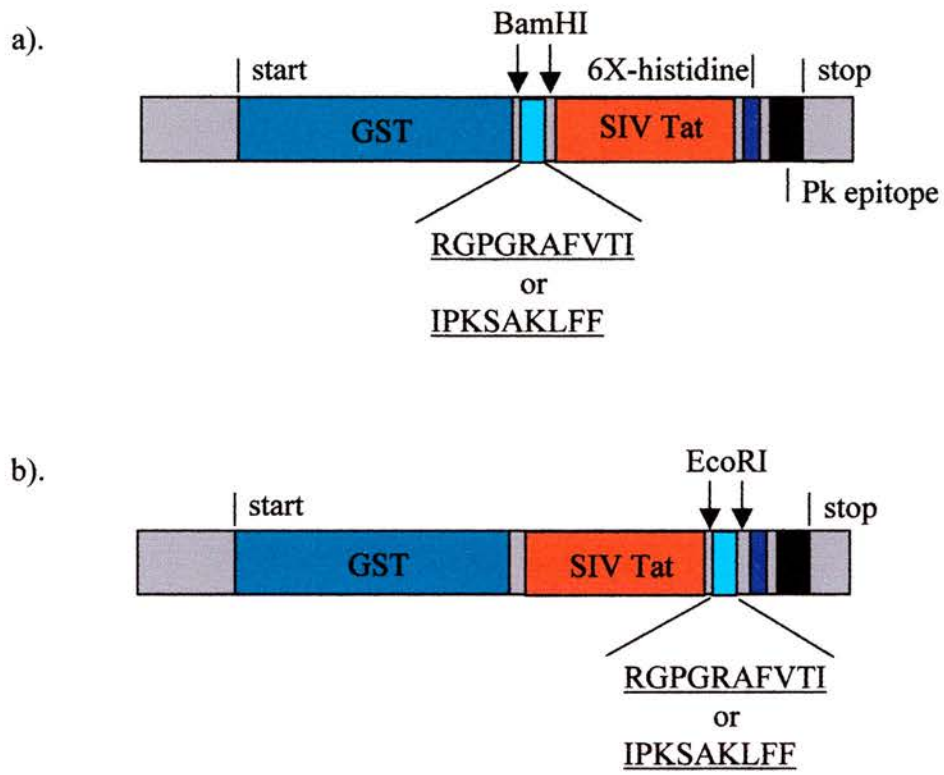


Fig. tat14. Clone single CTL epitope sequence into pGTHP.
(a) The double strand oligonucleotide encoding CTL epitope RGPGRAFVTI (termed I; restricted to H-2^d haplotype) derived from HIV gp120, was cloned into pGTHP vector on either BamHI or EcoRI cutting site to generate expression vector pGITHP or pGTIHP. (b) Following the same procedure, the CTL epitope IPKSAKLFF (termed S; restricted to H-2^d haplotype) from M protein of SV5 (simian virus 5) was cloned into pGTHP, resulting in pGSTHP and pGTSHP constructs.

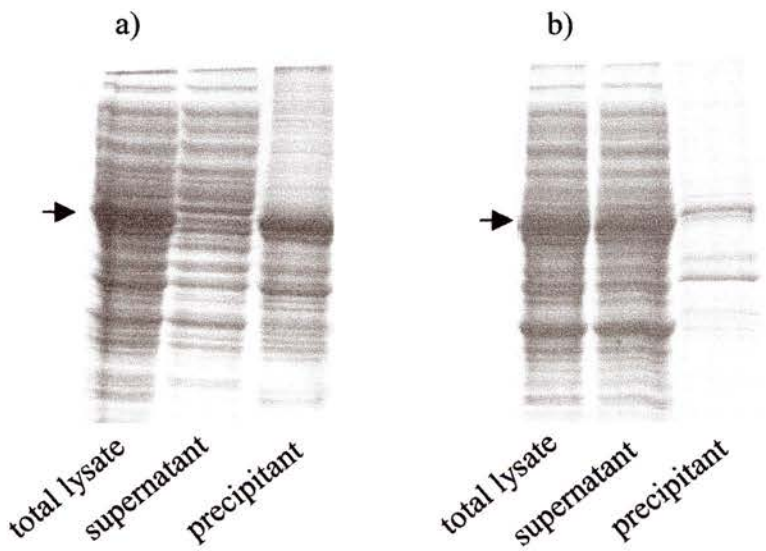


Fig. tat15. The GTSHP fusion protein was soluble when expressed at room temperature.

Bacteria *E. coli* XL1-blue harboring pGTSHP were induced with IPTG (1 mM) at 37 °C (a) or room temperature (b) to express GTSHP fusion proteins [S: the CTL epitope derived from M protein of SV5 (simian virus 5)]. Bacteria were harvested and then broken by sonication followed by centrifugation. The total lysate, supernatant and precipitant were examined through 12% SDS gels. GTSHP: GST-Tat-S-His-Pk.

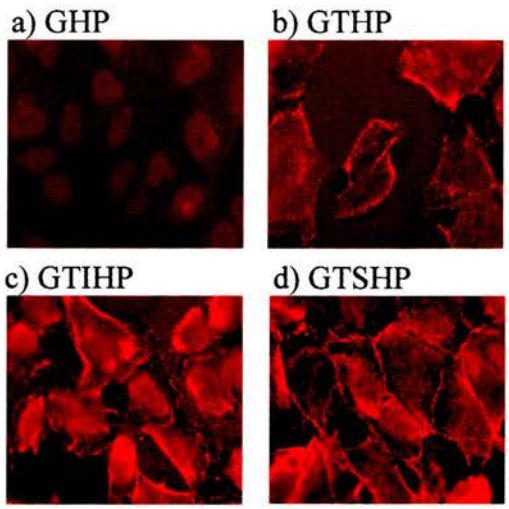


Fig. tat16. Single CTL epitope fusion proteins were shown to bind to cell surfaces of Vero cells.

Vero cells were cultured on glass cover slips overnight and then incubated with the GHP (a), GTHP (b), GTIHP (c), or GTSHP (d) fusion protein in serum-free medium for one hour (I: the CTL epitope derived from HIV gp120; S: the CTL epitope derived from M protein of SV5). Anti-Pk mAbs were used to probe these fusion proteins which contain the Pk tag (P) on their C-termini, and the TXRD conjugated anti-mouse Ig antibody was used as the secondary antibody. Cells were fixed and visualised by a fluorescent microscope. GHP: GST-His-Pk; GTIHP: GST-Tat-I-His-pk; GTSHP: GST-Tat-S-His-Pk.

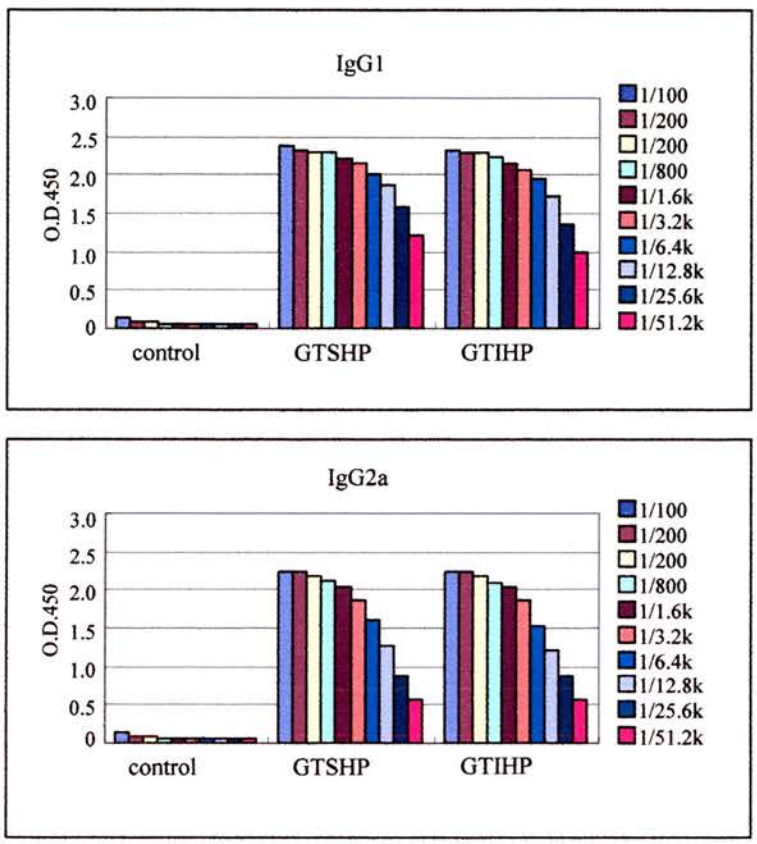


Fig. tat17. Antibody responses after immunization of single-CTL epitope fusion proteinns.

BALB/c mice at age of about three months were intraperitonealy injected with 2 μg of soluble fusion proteins (GTIHP or GTSHP) in PBS buffer (0.5ml) three times with interval of two weeks. Blood samples were collected from tail veins two weeks after the final injection. Sera were isolated by centrifugation and equivalently pooled together in each injection group (6 mice/group). The level of induced IgG1 and IgG2a antibodies specific to GTHP fusion proteins was examined by ELISA analysis. (control: serum samples from mice without immunization; GTIHP: injected with GST-Tat-I-HP; GTSHP: injected with GST-Tat-S-HP).

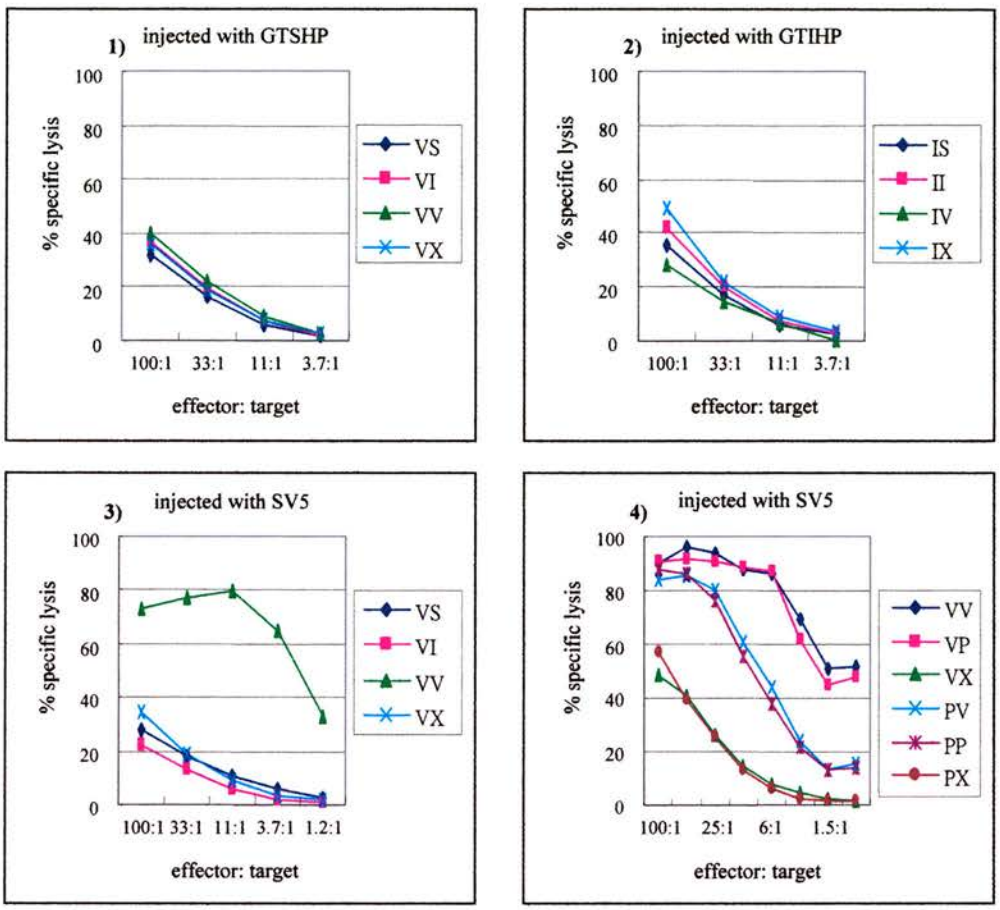


Fig. tat18. Measure CTL responses specific to epitopes derived from SV5 and HIV.

BALB/c mice at age of about three months were intraperitonealy injected with soluble GTHP (**1**) or GTIHP (**2**) in PBS buffer (0.5 ml) three times with interval of two weeks. As positive control, a group of mice were injected with SV5 (10^7 pfu) once (**3**). In each group, splenocytes were harvested, pooled together and cocultured with syngeneic stimulator cells for a week. Stimulator cells were splenocytes from naïve BALB/c mice, which were either incubated with GTSHP or GTIHP or infected with SV5 for two hours. Chromium labeled target cells were syngeneic P815 cells, which have been either incubated with GTSHP or GTIHP or infected with SV5 overnight. (**4**) To confirm that IPKSAKLEF is a dominant CTL epitope, a group of mice were injected with SV5 (10^7 pfu) for two weeks. Syngenic stimulator splenocytes and target cells were either incubated with peptide IPKSAKLFF (20 μg/ml) or infected with SV5. (the first letter: treatment on stimulator cells; the second letter: treatment on target cells; V: infected with SV5; P: incubated with peptide; X: without any treatment; S: incubated with GTSHP; I: incubated with GTIHP).

Section I.

B. Use of recombinant adenovirus to boost Th1 responses primed by SIV tat fused protein antigens.

Summary:

Although in our preliminary results, CTL responses were barely induced, if at all, by this approach, the induction of IgG2a antibody indicated that Th1 type responses were induced. Here, we attempt to use the live viral vector (recombinant adenovirus) vaccine to boost any CTL response which was primed by the SIV tat fused protein antigens. In this study, the recombinant adenovirus vector (human Ad type 5), which carries the gene of fusion protein GTSHP (composed of GST, SIV Tat, CTL epitope from M protein of SV5 virus, 6x-His tag, Pk tag) used in the previous study, was constructed. The fidelity of vector construct was confirmed by DNA sequencing and transient expression analysis. The recombinant adenovirus was generated by homologous recombination in 293 cells, and the single virus clone was isolated by plaque purification. Purified virus was prepared by CsCl density gradient centrifugation. The infectivity of purified viral particles and the expression of GTSHP fusion protein were demonstrated by immunofluorescent staining. After injecting mice with this recombinant adenovirus, a CTL response specific to the SV5 CTL epitope was not detected. Although the preliminary results were not encouraging, there are still some parameters which needed to be tested, such as the dose of virus, so this study will be followed up in the near future.

1. Generation of recombinant adenovirus (Ad/GTSHP).

An elegant method for the generation of recombinant replication-deficient adenovirus which is based on homologous recombination in permissive cell line (293 cells) has been developed and widely used. This method uses two plasmids containing overlapping fragments that recombine. The large plasmid (pJM17) contains most of the viral genome (from Ad5 dl 309) in the circular form, but lacks the DNA packing signals and a functional E1 region. The transfer vector (pCA14) contains the left inverted terminal repeat (ITR), packaging signal, and sequence overlapping the large plasmid. After the gene of interest has been

introduced into the transfer plasmid, these two plasmids are co-transfected into 293 cells (which express E1 gene function) and viruses produced by recombination in transfected cells are isolated through the plaque purification.

To generate recombinant adenovirus, which carries the gene of GTSHP fusion protein, the desired gene was amplified from pGEX2T/GTSHP by polymerase chain reaction (PCR) and subcloned into pCA14 transfer vector using the restriction enzyme sites XhoI and HindIII, resulting in the construct pCA14/GTSHP (**Fig. Ad1**). The fidelity of this construct was confirmed by DNA sequencing (using ABI sequencer), and the expression of GTSHP fusion protein was examined in 293 cells by transient transfection (using Fugene transfection reagent) and expression analysis. The transfected cells were lysed in disruption buffer two days after transfection, and lysates were resolved on 12 % (w/v) SDS PAGE. As the C-terminal of this fusion protein contains a Pk epitope, anti-Pk mouse monoclonal antibody was used to probe the expressed protein in the Western blot analysis. Only one band with molecular weight of about 47 kDa, the expected size of GTSHP fusion protein, was detected, confirming the fidelity of this construct and indicating that this fusion protein was properly translated (**Fig. Ad2**).

The approach for generation of replication-deficient adenovirus is based on the homologous recombination in 293 cells (**Fig. Ad3**), which possess the gene to compensate the function of deficient viral E1 gene. The constructed transfer vector pCA14/GTSHP and the large plasmid pJM17 were mixed in the ratio of one to six and then co-transfected into monolayer 293 cells using Fugene transfection reagents. One week after transfection, cytopathic effects (CPE) appeared on the infected cells, and one to two days later a gross CPE was observed. Infected cells were detached by scraping and pelleted by centrifugation, and stored with part of culture supernatant at -70°C (as primary viral stock). The supernatant was also collected and serially diluted in serum free DME medium for plaque isolation. To perform the plaque assay for isolating virus clones, 293 cells were cultured in six-well plates one day before infection with the appropriate dilution of virus. After the formation of plaque,

virus clones were picked from individual plaque and amplified in 293 cells in 24-well plates until a gross CPE appears (usually two to four days).

2. Detect the correct clone of recombinant adenovirus.

To determine the percentage of correct recombinant viruses which were generated by this method, the original plates of plaque assay were immune stained for expression of GTSHP, using the anti-Pk mAb. Alkaline phosphotase conjugated anti-mouse Ig antibody was used as the secondary antibody, so the positive plaques appear as dark blue rings. Since the negative plaques were not easily visualized on the immune-stained plate, Coomassie blue staining was subsequently applied to the same plate, lighting up all the plaque (**Fig. Ad4**). By comparing photos taken from each staining result, the percentage of positive plaque was estimated about 75 %, indicating that viruses generated from the homologous recombinant method are not single genotype population. This result strongly demonstrates that plaque purification is necessary in the isolation of recombinant adenovirus.

3. Isolataion of recombinant adenovirus clones by plaque purification.

The individual virus clones were randomly picked up from single plaques and amplified in 293 cells grown in 24-well plates. After the gross CPE appeared, the cell culture medium of infected cells were collected and stored at -70°C as secondary virus stocks. Infected cells were also examined by Western blot (**Fig. Ad5**) using the anti-Pk mAb to probe the expressed fusion protein GTSHP. Cells inoculated with virus clones number one and two showed the expression of the desired fusion protein, indicating that these two virus clones contain the gene coding for the GTSHP fusion protein, consistent with the result of immune staining on plaque assay plates (**Fig. Ad4**).

A virus clone (number one) encoding for GTSHP was further amplified in monolayer 293 cells grown in one 75t flask. Following infection, virus spread rapidly through the monolayer, and caused the cells to round up and eventually detach from the monolayer. The best time to harvest virus was when infected 293 cells readily detached when the flask was given a gentle tap. Viruses were partially purified from infected cells by extraction with the organic solvent

Arklone P in 1 ml of PBS, prior to titration (~ 10^8 pfu/ml). This crude extraction was used as the starting material for further large scale amplification of virus.

4. Purification of recombinant adenovirus by CsCl gradient centrifugation.

For large scale purification of virus, monolayer 293 cells were grown to 80 % confluence in 300t flasks and infected with virus at low m.o.i., usually from 0.01 to 0.1 pfu/cell. Until the proper timing for harvesting virus, as described above, infected cells were extracted with Arklone P with vigorous mixing but not vortexing. This virus extraction is still a crude preparation, heavily contaminated with cellular proteins, so further purification by CsCl gradient centrifugation is required. The gradient was prepared by adding 3 ml of 3M CsCl solution, 2 ml of 2M CsCl solution and the virus extraction in the order gently to the ultracentrifuge tube (for Beckman SW41 rotor). The virus extraction (usually 9 ml) from ten 300t flasks of infected cells can fill up one tube, and after high speed centrifugation two bands should be visible. An opalescent band between the high and low density CsCl solutions contains the virus, while a second band containing cellular/viral proteins is present higher up the tube (**Fig. Ad6**). Virus was collected by using a 19-gauge needle, and dialyzed in 1 liter of Tris-HCl/glycerol buffer (described in Material and Methods) twice at cold room, each time for 2-3 hours. Usually, after dialysis, the volume of virus sample (about 1 ml/band) will not reduce much. The PBS was not recommended for dialysis, since most of the virus particles will precipitate in this buffer condition and the virus titer will drop dramatically, usually from 10^{10} to 10^3 pfu/ml (data not shown). During purification, virus samples were collected in each main step for further titration.

5. Titration of purified recombinant adenovirus.

To do titration, virus samples were serially diluted for plaque assay on monolayer 293 cells grown in 6-well plates. After the formation of visible plaques, usually appearing at one week postinfection, cells were fixed with formaldehyde solution and stained with Coomassie blue. After staining, plaques appear as transparent spots compared to the dark blue background of uninfected cells (**Fig. Ad7**). The titer of virus sample from the crude Arklone P extraction is $7x10^9$ pfu/ml, so totally ten 300t flasks of cells (about $2x10^8$ cells in 9 ml of

extraction) can generate about $6x10^{10}$ infectious virus particles. After CsCl gradient centrifugation without dialysis, the virus titer increases ten times to $7x10^{10}$ pfu/ml. Since the volume of collected virus sample is about ten times less (from 9 ml to 1 ml), the total yield of infective virus particles does not change significantly, indicating that CsCl gradient centrifugation can concentrate the virus sample without affecting the infectivity of viruses. After being dialyzed in Tris-HCl/glycerol buffer and stored at -70°C even for three weeks, the purified viruses still maintain the infectivity, since the titer did not drop. The titer of virus stored at 4°C reduced to two-thirds of that of virus stored at –70°C, suggesting that -70°C would be a proper storage temperature for purified adenovirus.

6. Recombinant adenovirus infects cell line derived from mice.

It has been reported that adenovirus mediated gene transfer is inefficient in cells lacking the primary receptor, the coxsakievirus and adenovirus receptor (CAR). To determine if the purified adenovirus (Ad/GTSHP) is capable of infecting cell lines derived from mice, the BF cells inoculated with adenovirus were examined by immunofluorescent staining (**Fig. Ad8**). The BF cells were grown on glass coverslips overnight and inoculated with Ad/GTSHP with m.o.i. of 10. At 2 days p.i., cells were fixed with formaldehyde and cells expressing the fusion protein GTSHP were detected by staining the cells with a anti-Pk mAb. In the cells infected with adenovirus, nuclear localization (SIV tat protein is a nuclear protein) of GTSHP was observed, while fluorescence was not observed in uninfected cells, indicating that the adenovirus vector can infect the mouse BF cell and the transgene is expressed. However, even at a m.o.i. of 10, only quarter of cells were positive for fluorescence.

7. Cytotoxic T lymphocyte (CTL) assay.

To assess if any CTL response primed by the protein antigen GTSHP can be boosted by the recombinant adenovirus Ad/GTSHP. Mice (BALB/c) were primed with GTSHP (2 μg/injection) and boosted with either GTSHP (2 μg/injection) or Ad/GTSHP (10^8 pfu/injection) once at 2 weeks post priming injection (**Fig. Ad9-1**). Also, we tested the effect of virus dose on the prime-

boost immunzation regime. Mice were divided into three groups, in which mice were injected with either high titer (10^8 pfu/injection) or low titer (10^5 pfu/injection) of virus or mock infected, and boosted with one dose (10^8 pfu/dose) of virus at 2 weeks post priming injecting (**Fig. Ad9-2,-3,-4**). Two weeks after the final injection, spleen lymphocytes were collected and pooled together (3 mice/group) for CTL assays. Since CTL epitope (restricted to H-2^d haplotype) from the SV5 M protein was present both in the GTSHP fusion protein and the Ad/GTSHP recombinant virus, the CTL assay focused on this epitope. Spleen lymphocytes from immunized mice were cocultured with activator cells (spleen lymphocytes from naïve mice and infected with SV5) for five days. The target cells (P815 cell line) were infected with SV5 or mock infected one day before the CTL assay and labeled with chromium (^{51}Cr) for one hour prior to the assay.

In each immunization group, no clear difference in the killing effects on the naïve and SV5 infected target cells was observed, indicating that CTL responses specific to this epitope were not induced in any of these immunization regimes. Interestingly, the killing effects in groups injected with virus (prime-boosted with high titer of virus, with high/low titer of virus, and injected only once with high titer of virus) were higher than that in the group prime-boosted with proteins.

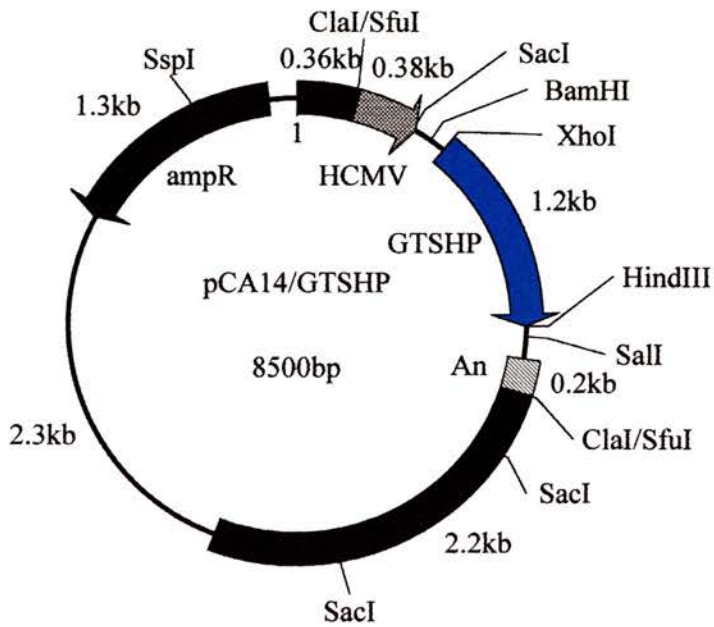
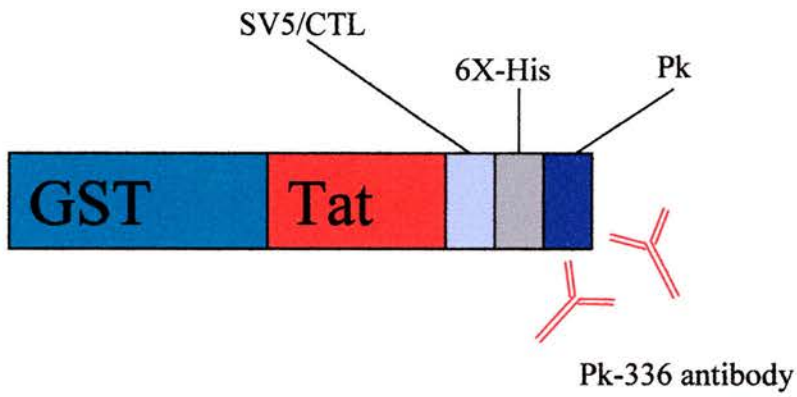


Fig. Ad1. Construction of pCA14/GTSHP for generating recombinant adenovirus.

The fusion protein gene GTSHP (containing GST, SIV Tat, SV5 M protein CTL epitope, 6x-his tag, and Pk tag) was amplified by PCR from pGEX2T/GTSHP and subcloned into pCA14 transfer vector on restriction enzyme sites XhoI and HindIII. The size of each sequence region is indicated in kilo base pair (kb). HCMV: promoter region from human CMV. ampR: ampicilline resistance gene. An: polyadenine sequence.

a).



b).

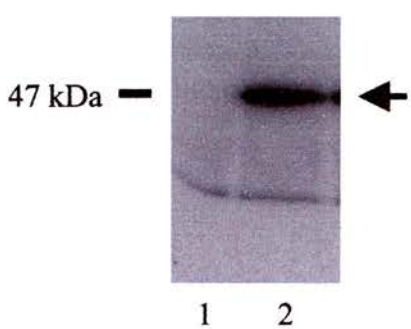


Fig. Ad2. Expression of GTSHP fusion protein from pCA14/GTSHP construct in 293 cells.

a). Diagram of GTSHP fusion protein. This fusion protein, from n-terminal to c-terminal is composed of GST, SIV Tat, CTL epitope of SV5 M protein, 6x-His tag, and Pk tag. b). Western blot showing transient expression of GTSHP fusion protein in 293 cells. Lane 1: naïve 293 cells; lane 2: 293 cells transfected with the pCA14/GTSHP construct. The expressed protein, indicated by arrow, was detected by mouse monoclonal antibody (Pk-336) which specifically recognizes Pk epitope.

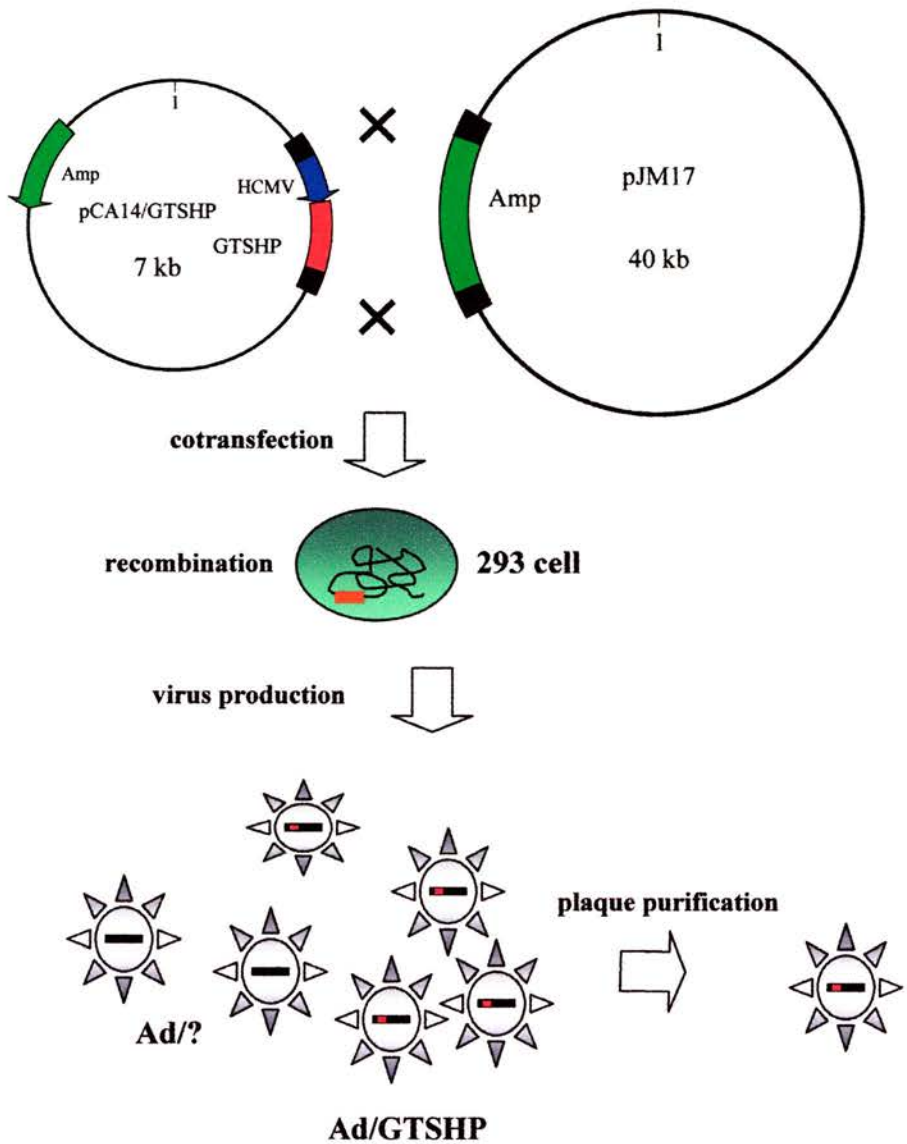
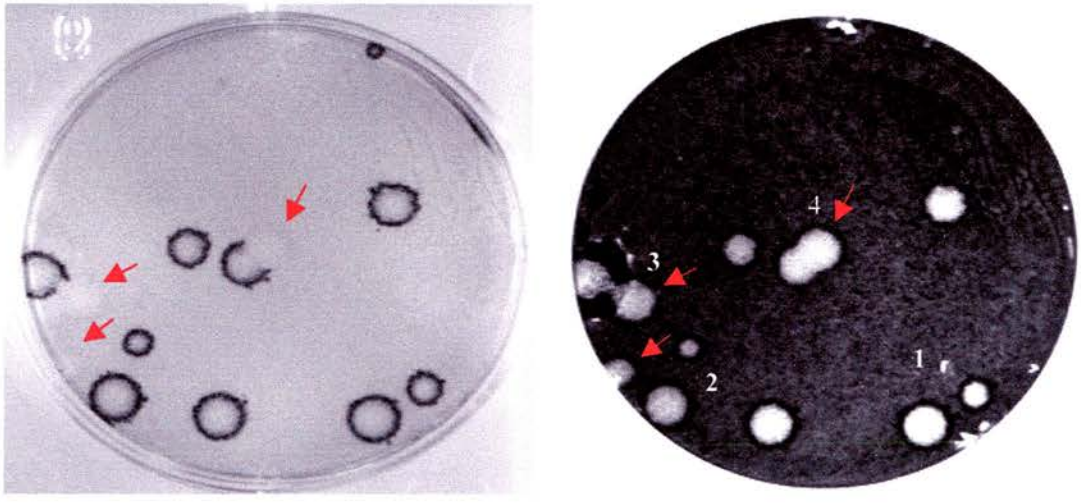


Fig.Ad3. Cartoon showing the main events in the generation of recombinant adenovirus.

The transfer vector pCA14/GTSHP and large plasmid pJM17 were contransfected into permissive cells (293 cells), where gene recombination occurred. A mixed population of recombinant adenovirus containing the desired gene (Ad/GTSHP) or unknown gene (Ad/?) was generated from transfected cells, and further plaque purification procedure was required to isolate the desired virus clone.



Immune staining Coomassie blue staining

Fig. Ad4. Plaque purification of recombinant adenovirus (Ade/GTSHP).
Monolayer 293 cells are infected with serially diluted crude extraction from 293 cells cotransfected with pCA14/GTSHP and pJM17. After the formation of plaques, recombinant virus clones are picked up from individual plaques and amplified in 293 cells. The infected monolayer cells were proceed to immune staining and Coomassie blue staining. In the immune staining, mouse monoclonal antibody (Pk-336) was used to probe the expression of GTSHP fusion protein, and anti-mouse-AP (alkaline phosphotase) was used as secondary antibody. The plaques that appear as negative staining in immune staining were indicated by red arrows. 1-4: plaques that were isolated and then amplified in 293 cells.

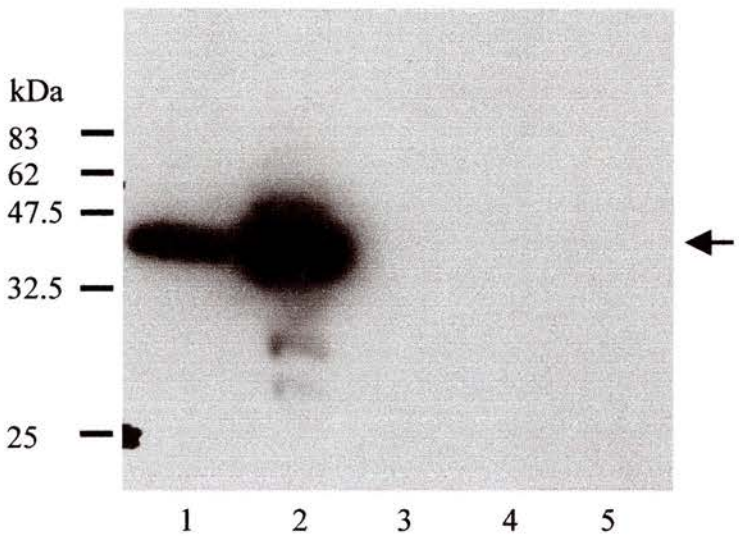


Fig. Ad5. Western blot showing the expression of GTSHP fusion protein in 293 cells infected with individual virus clones isolated form plaque purification.
Lane 1-4: cells infected with isolated virus clones 1-4 (shown in Fig. Ad4), respectively; lane 5: naïve 293 cell. The expressed fusion protein GTSHP (indicated by arrow) was probed by mouse monoclonal antibody (Pk-336) which specifically recognizes the Pk epitope. The migration position and molecular weight (in kDa) of standard protein marker were shown along the left of the figure.

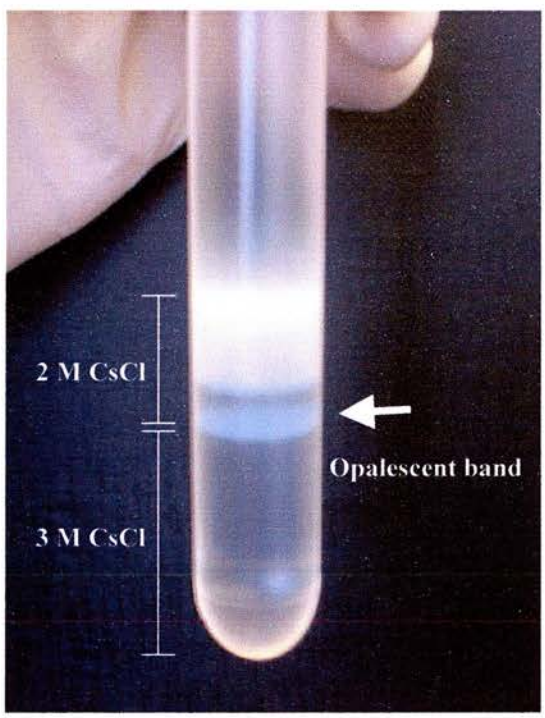


Fig. Ad6. Purification of recombinant adenovirus by CsCl gradient.
The crude extraction of recombinant virus was further purified by CsCl gradient (contains 3 M and 2 M CsCl solution) high speed centrifugation. The opalescent band containing concentrated virus particles was located between the phase of 3 M and 2 M CsCl solutions.

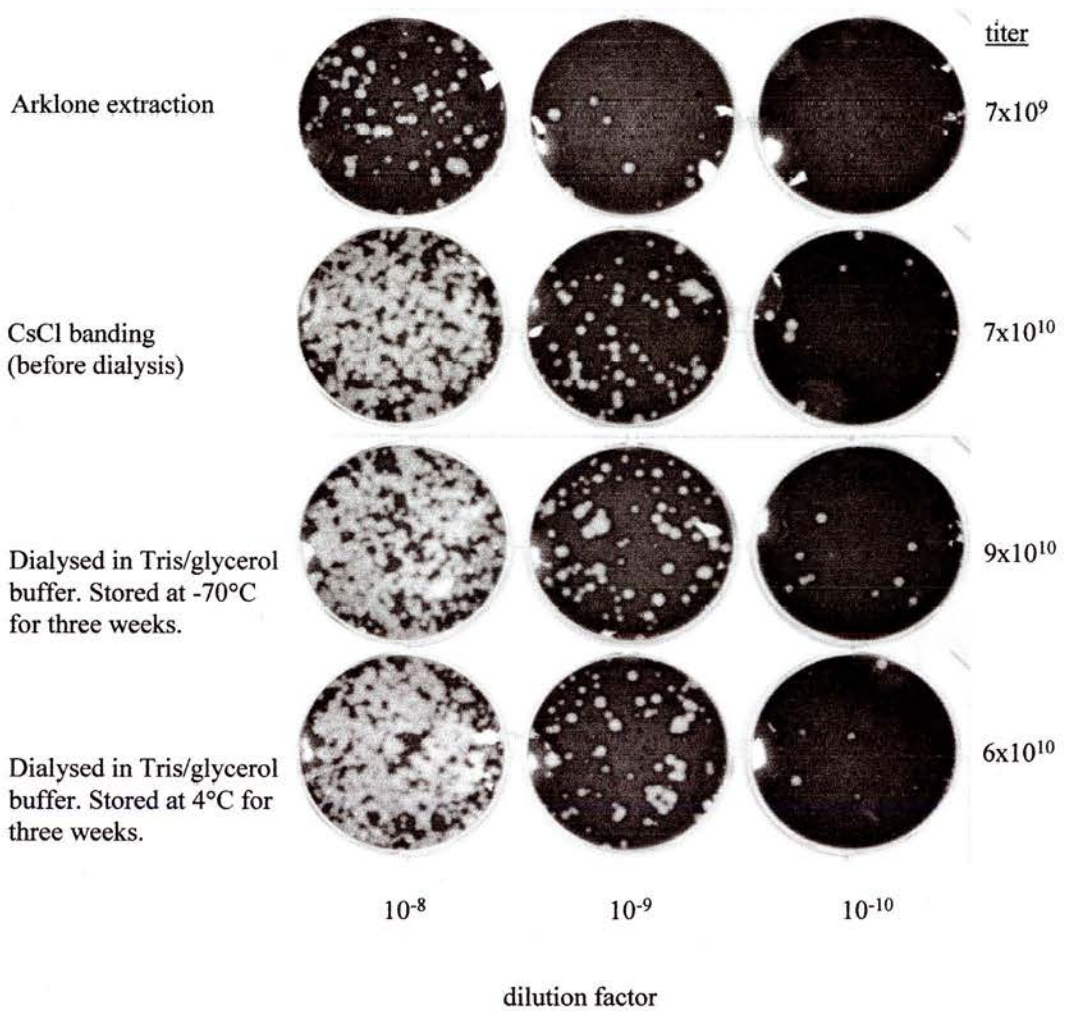
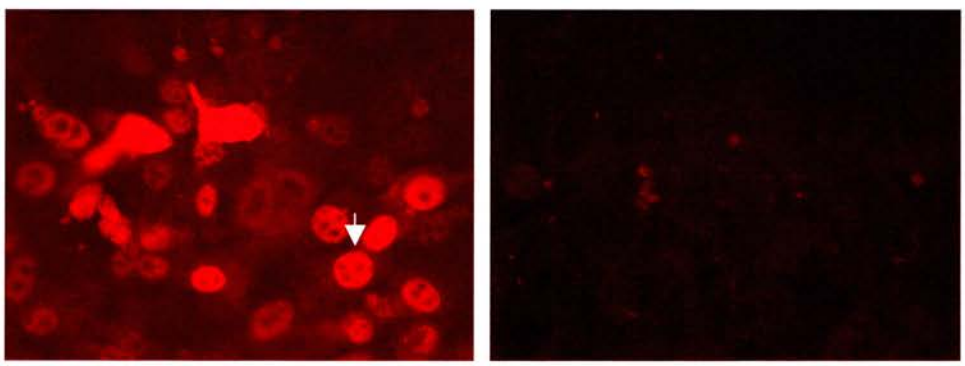


Fig. Ad7. Ad/GTSHP virus plaque titration.
Viruses were purified from infected 293 cells by arklone extraction, processed to CsCl banding and then dialyzed in Tris/glycerol buffer to wash out the CsCl salt. The titer of virus sample from each step of purification was examined by plaque assay on monolayer 293 cells. Through Coomassie blue staining, uninfected cells appear in dark blue while the viral plaques appear in transparent spots.



Ad/GTSHP infected naive

Fig. Ad8. Recombinant adenovirus (Ad/GTSHP) infects mouse cell line (BF cell).

BF cells were cultured on glass coverslips overnight and infected with recombinant adenovirus (Ad/GTSHP) for two days. The expression of GTSHP gene carried by recombinant virus was detected by immunofluorecent staining in which mouse monoclonal antibody (Pk-336) was used as primary antibody to probe the C-terminal Pk tag of the expressed fusion protein GTSHP. The white arrow indicates the nuclear localization of expressed fusion protein GTSHP.

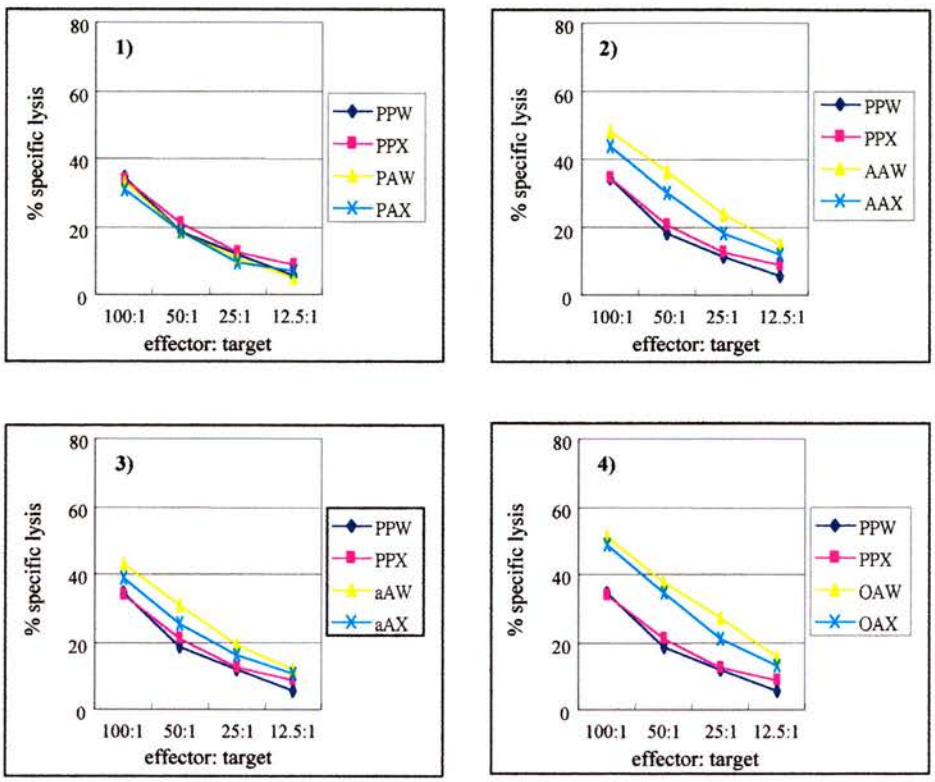


Fig. Ad9. Measure CTL responses in mice injected with Ad/GTSHP.
Following prime-boost immunization regimes, mouse spleen cells are collected and stimulated with SV5 W3 virus for 6 days. The specific killing activity was determined by the release of chromium from P815 target cells. The three letter symbol in the charts indicate the prime-boost immunization procedures (first two letter) and the treatment of target cells (the third letter). P: injection with purified GTSHP fusion proteins (2 μg/injection); A: injection with high titer (10^8 pfu/injection) of recombinant adenovirus (Ad/GTSHP); a: low titer of virus (10^5 pfu/injection); O: without any injection. Target cells were either infected with (W) or without (X) SV5 W3 virus.

Section II.

Detection of antibody responses induced by immunization with HIV vaccines.

Summary:

In order to identify induced antibody responses to recombinant HIV gag proteins (P24P17) in HIV vaccine immunized volunteers, serum samples were subjected to ELISA analyses. These vaccine volunteers from Oxford were immunized either with DNA plasmids, modified vaccinia virus Ankara (MVA) or DNA/MVA prime-boost vaccine regimen which contained the gene of P24P17. As target antigens on ELISA analyses, P24P17 recombinant proteins were produced in baculovirus expression systems and purified by nickel affinity columns. In 18 of DNA, 8 of MVA and 9 of DNA/MVA vaccine volunteers, only one volunteer in the DNA/MVA group was identified producing anti-P24P17 antibodies after immunization. Serum samples from most of the vaccine volunteers presented high background values. Western blot analyses showed that pre-immunized sera contained antibodies cross-reacting to P24P17 recombinant proteins. If the cross-reactive epitopes in the amino acid sequence of P24P17 were identified, knocking out this/these epitopes might reduce background values in ELISA tests. To map out the cross-reactive epitopes, ninety sets of synthesized peptide fragments derived from the amino acid sequence of P24P17 recombinant proteins were used as target antigens for ELISA analyses. One peptide fragment was identified to react with pre-existing antibodies in serum samples of vaccine volunteers and non-immunized individuals.

1. Preparation of recombinant HIV gag proteins, P24P17.

The recombinant HIV gag protein (P24P17) was required in large amounts for detecting anti-P24P17 antibody responses in HIV vaccine immunized volunteers. Given the gene sequence of P24P17 protein is GC rich (>60 %) and contains large amount of rare codon sequences which were not suitable for bacterial expression systems, and the posttranslational modification in insect cells is similar to that in mammalian cells, P24P17 was expressed in baculovirus

expression systems. Briefly, the gene encoding P24P17 protein was amplified by PCR from pTH-HIVA plasmid [This plasmid consists of 73 % of the gag proteins (derived from the sequences of HIV-1 clade A, the predominant HIV clade in Nairobi) fused to a string of 25 partially overlapping CTL epitopes.] and cloned into a pFastBacHTc donor plasmid (Fig. ox1). The recombinant plasmid was transformed into Escherichia coli DH10Bac competent cells which contain the baculovirus shuttle vector (bacmid) and the helper plasmid (pMON7124). The bacmid (bMON14272) contains a kanamycin resistance marker, and a segment of DNA encoding the lacZα peptide from a pUC-based cloning vector. Inserted into the N-terminus of the lacZα, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-att Tn7) that does not interrupt the reading frame. The bacmid propagates as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that are blue in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG. The mini-Tn7 element on the pFastBacHTc donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid, which results in the gene of interest being cloned into the bacmid. Colonies containing recombinant bacmids were identified by disruption of the lacZα gene. High molecular weight mini-prep DNA (>135 kb) was prepared from selected E. coli clones containing the desired recombinant bacmid, and this DNA was then used to transfect monolayer insect cells (Sf9) to produce recombinant baculoviruses. Suspension insect cells were infected with virus at a multiplicity of infection (MOI) of 10 to express recombinant P24P17 proteins, which also contain 6x histidine affinity tag at its amino terminus. The fidelity of expressed recombinant proteins was confirmed by mass spectrometer. The 6x histidine affinity tag has a strong affinity for Ni-NTA resin, allowing the desired protein to be purified easily with nickel columns. The spacer region between histidine tag and P24P17 contains rTEV protease recognition site. The purified recombinant proteins were digested with rTEV proteases to remove the histidine tag. The protease also contains a 6x histidine tag at its amino terminus for easy removal by affinity chromatography. As shown in Fig. ox2, after protease treatment the migration position (about 47

kDa) of purified proteins slightly faster, indicating that 6x histidine tag was efficiently removed by protease digestion. The rTEV proteases and nonspecific proteins were removed by the second purification with nickel affinity columns, as the intensity of corresponding bands on the Coomassie blue stained gel was reduced enormously. These purified proteins were then coated on micro-well plates for ELISA analyses.

2. Detecting anti-P24P17 antibody responses in vaccine volunteers by ELISA analyses.

In order to test if antibody responses (specific to recombinant HIV gag protein domain P24P17) can be raised in HIV vaccine volunteers, ELISA analyses were performed to measure antibody reponses. These vaccine volunteers were classified into three groups which were injected either with plasmid DNA, recombinant MVA, or DNA/MVA in a prime-boost vaccine regime. Serum samples from vaccine volunteers were collected before and after immunization at different time points. The ELISA micro-well plates were coated with recombinant P24P17 proteins which were expressed in baculovirus expression systems and purified with nickel affinity chromatography. As shown in Fig. ox3C1, by comparing the optical density values, only one vaccine volunteer (number 3) in the DNA/MVA prime-boost group was identified producing anti-P24P17 antibody responses after vaccine immunization, as serum samples collected after immunization presented significantly higher optical density values in ELISA tests than that of sera collected before immunization. In this case, antibody responses were not detected at 7 days after immunization, but appeared at 21 days after immunization, indicating that antibody responses were induced at some point between one week and three weeks after immunization. After induction, antibody titers gradually increased until 77 days after immunization and then declined but were still detectable at 189 days after immunization, showing that induced antibody responses can extend for more than six months. Even though serum samples were diluted at a ratio of 1/800, the optical density value of the post-immune serum sample d77 (collected 77 days after immunization) was still twice more than that of pre-immune sera, thus the titer of induced antibodies at that time point was defined as >800 (Fig. ox3C2). In ELISA analyses, serum samples from the other vaccine volunteers

did not show any significant increase of optical density in post-immune sera, compared with that of pre-immune sera. This indicatee that strong antibody responses were not generally induced by these DNA-based vaccine regimens.

3. High background values appear in ELISA analyses.

Serum samples from plasmid DNA and MVA vaccine volunteers did not show the induction of antibody responses, but presented high background values at serum dilution ratio of 1/10 in ELISA analyses (Fig. ox3A, 3B). These high background values might mask the detection of weak antibody responses which were induced by vaccine injections. When serum samples were further diluted to ratio of 1/80, background values were reduced proportionally, but significant differences of optical density values between pre-immune and post-immune sera were still not detected. Also, some volunteers' serum samples, even when diluted to 1/80, still presented high background values. The background values (<0.2 unit) of the DNA/MVA prim-boost vaccine volunteer (number 3), who was the only one showing induction of antibody responses after immunization, was far less than that of the other volunteers in the same vaccine group. However, in this vaccine group, some volunteers' serum samples also presented high background values (>0.5 unit), even though sera were diluted at ratio of 1/100.

4. Skimmed milk solutions do not cause the problem of high background values.

We have successfully performed ELISA analyses with mouse sera in which 3 % (w/v) skimmed milk solutions were used for diluting serum samples and blocking micro-well plates, without showing any problem of high background values. However, in the same skimmed milk solution condition, high background values appeared in analyses of human serum samples from vaccine volunteers. There are some possibilities which might be related to this problem: (i) the working concentration (3 % w/v) of skimmed milk solutions might not be high enough to block nonspecific antibody binding in these analyses, so higher concentration of skimmed milk solution needed to be tested; (ii) serum proteins or cell debris presented in human sera might enhance nonspecific antibody binding, so extra manipulations such as centrifuging serum samples or purifying

antibodies to clean serum samples might be necessary; (iii) human sera might contain antibodies that can recognize protein components of skimmed milk, so solutions such as BSA or ovalbumin might be another choice; (iv) these tested human sera might posses cross-reactive antibodies against P24P17 protein, so identification of the cross-reactive epitopes on P24P17 protein will be important for the strategies to solve the problem of high background values in future works. To verify the possibilities as described above (i, ii, iii), control pooled sera (donated by three people, who were not immunized or infected with HIV, from St Andrews University) were tested against skimmed milk, BSA, ovalbumin in ELISA analyses (Fig. ox4). The pooled sera were centrifuged to remove debris and precipitated proteins, and ammonium sulfate was added to the supernatant to precipitate antibodies. Antibodies were resuspended with phosphate buffer saline in the same volume of serum used for purification. These manipulated and un-manipulated sera were diluted at ratio of 1/25 (A) and 1/100 (B) in low (1 % w/v) and high (5 % w/v) concentration of skimmed milk solutions and analyzed by ELISA tests on blank micro-well plates, which were coated with 10 % (w/v) of skimmed milk solutions. These sera were also tested against BSA and ovalbumin in the same fashion as described for skimmed milk. Antibody bindings were detected with HRP-conjugated anti-human IgG antibodies in the presence of HRP substrates (TMB) and read at a wavelength of 450 nm. When whole sera and manipulated sera were diluted in skimmed milk solutions, the reading values were much lower than that of sera diluted in BSA or ovalbumin solutions. These lower reading values (<0.1 unit in 5 % solution; <0.2 unit in 1 % solution) were close to the base line value (around 0.06 unit) in which wells were not coated with any solution or incubated with any human serum, but only with HRP-conjugated antibodies which were either diluted in 1 % (w/v) or 5 % (w/v) of skimmed solutions. The base line values were all in the range of 0.05 to 0.06 unit, which were not affected by the dilution solution (skimmed milk, BSA, or ovalbumin solution) or its percentage (1 % w/v or 5 % w/v). These indicated that skimmed milk solution can not cause a significant amount of background value in ELISA analyses of human sera, especially, when sera were diluted in high concentration (5 % w/v) of skimmed milk solution. Compared with the whole sera, manipulated sera presented lower reading values in these three solution

conditions, but the difference was not obvious when sera were diluted at ratio of 1/100. This implied that when serum samples were diluted over 1/100, extra manipulation on serum samples to reduce background values in ELISA analyses might not be necessary. However, when the weak antibody response was considered, the purification of antibodies by ammonium sulfate could be very useful in reducing background noises. Given previous analyses of serum samples from plasmid DNA and MVA vaccine volunteers were performed in 3 % (w/v) of skimmed milk solution, we suspected that this concentration of skimmed milk solutions might account for the high background values. So, the analyses of serum samples from one MVA vaccine volunteer (number 3), which presented the greatest background values in this vaccine group, was re-performed in 10 % (w/v) skimmed milk solution which was used to block plates and dilute serum samples. The result of this test was similar to that performed in 3 (w/v) % skimmed milk solution, presenting high background values and no induction of antibody responses (data no shown). Together, these results might indicate that the problem of high background values was not caused by skimmed milk solutions.

5. Antibody cross-reaction in human serum samples.

Although antibody-antigen recognition is highly specific, the elicited antibodies, in some cases, can cross-react with unrelated antigens. If different antigens share the same epitope or unrelated epitopes possess similar structural/chemical properties, such cross-reaction may occur. It is possible that the high background values were caused by these cross-reacting antibodies. To test for this possibility, sequential serum samples from one MVA vaccine volunteer (number 3), whose sera presented the greatest background value in this vaccine group and the reading values of post-immune sera were higher than that of pre-immune sera, were subjected to Western blot analyses. Briefly, the purified recombinant HIV gag proteins (P24P17) were separated on SDS-PAGEs and transferred onto nitrocellulose membranes, which were then cut into strips and probed with serum samples separately. As shown in Fig. ox5, antibodies that recognize P24P17 were detected in serum samples collected both before (ds, d0) and after (d14, d21, d35, d49, d77, d189) immunization, and in the control serum from a donor (signified as A) whom had not been immunized with the

HIV vaccine. There was no binding signal presenting in the control strip which was not incubated with serum samples but with secondary antibodies, implying that those positive binding signals were due to antibodies existing in serum samples. The presence of positive binding signals in serum samples, collected before immunization, might explain why high background values appeared in ELISA analyses of vaccine volunteers.

6. Identifying cross-reactive epitopes on recombinant P24P17 proteins.

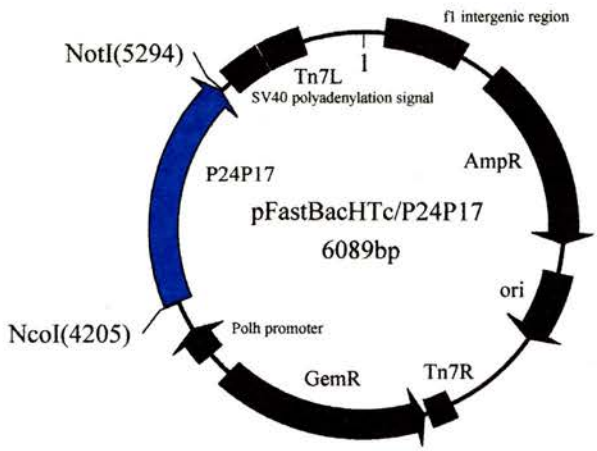
By Western blot analyses, it was shown that the presence of high background values in ELISA analyses of vaccine volunteers might be caused by antibody cross-reaction with P24P17. Therefore, it may be possible to reduce the intensity of background values by knocking out the cross-reactive epitopes in P24P17. In order to map out these epitopes, a set (1 to 90) of 15-amino acid peptide fragments covering the majority of amino acid sequence of recombinant P24P17 proteins with 11-amino acid overlapping were received from Oxford, and applied for ELISA analyses. Serum samples from MVA vaccine volunteer (number 3), which were collected before (d0) and after (d14, d21, d49, d77, d189) immunization at different time points, and control serum samples donated by individuals (signified as A, G, R) from St Andrews University were diluted in skimmed milk solutions and incubated with micro-well plates that have been pre-coated with peptide fragments. Peptide fragments were identified serially by numbers from 1 to 90. Since peptide fragments numbered 3, 4, 37, 88 were missed, the corresponding wells on the micro-well plate were not coated with any peptide fragment. Therefore, the highest reading value from these four wells was defined as the base line value, while reading values over base line value were considered as positive antibody bindings in each individual. As shown in Fig. ox6, the control serum sample, A, shown reacting with peptide fragments 1, 65, 85, sample G with fragment 65, and sample R with fragments 41, 65, 85. Serum samples from the MVA vaccine volunteer all presented positive binding with peptide fragment 65, and one of the post-immune serum samples (d21) showed one more positive binding with peptide fragment 85. Apparently, peptide fragment 65 was commonly recognized by antibodies from both serum sample groups (controls and vaccine volunteers), which might indicate that one cross-reactive epitope was located in this fragment.

If this is the case, peptide 65 may compete with P24P17 proteins in antibody bindings, hence leading to reduction of reading values in ELISA analyses. To test for this, 1 ml of serum samples (diluted at ratio of 1/100 in skim milk solutions) were incubated with peptide fragment 65 (10 μg) at room temperature for one hour, prior to ELISA analyses on micro-well plates coated with P24P17 proteins. It was estimated that the molar ratio of this peptide fragment was thirty times greater than that of total antibodies in this mixture. However, the reduction of reading values was not observed in these tested serum samples [including control serum A, and serum samples d14, d77 from MVA vaccine volunteer (number 3)], showing that this commonly recognized peptide fragment did not compete with P24P17 in this test (data not shown).

7. Antibody responses to peptide 65 were not boosted in MVA vaccine volunteer number 3.

In the MVA vaccine group (Fig. ox3B), serum samples of volunteer number 3 showed the greatest background value, even diluted to ratio of 1/80. Nevertheless, the ELISA reading value of the first collected post-immune serum sample (d14) of this volunteer was about 20 % higher than that of pre-immune serum samples. However, the other volunteers did not show any significant difference between pre- and post-immune sera, even though, at dilutions of 1/80, their background values (<0.1-0.3) were far less than that of volunteer 3 (0.5 unit). Together, these data might suggest that, in this volunteer (number 3), antibody responses were weakly induced. Furthermore, in the peptide ELISA analyses (Fig. ox6B), it was shown that pre- and post-immune serum samples of this volunteer reacted with the peptide fragment 65. Consequently, if weak antibody responses were induced following immunization, they may have been directed to the epitope of peptide fragment 65. Therefore, the intensity of antibody responses to peptide fragment 65 in pre- and post-immunization serum samples were tested. Micro-well plates were coated with peptide fragments 30, 41 (these two peptides were used as negative controls) and 65 in separate wells. As shown in Fig. ox7, there was no increase in the antibody levels to peptide fragment 30 and 41. However, between pre- and post-immune serum samples, there was also no significant differences of antibody responses to peptide

fragment 65, indicating that specific antibody responses were not induced after MVA vaccine immunizations.



5'-CTCGGTCCGAAAACC ATG TCG TAC TAC CAT CAC CAT CAC CAT
M S Y Y H H H H H
6x histidine affinity tag

CAC GAT TAC GAT ATC CCA ACG ACC GAA AAC CTG TAT TTT CAG
H D Y D I P T T E N L Y F Q**
spacer region rTEV protease cleavage site

EheI NcoI BamHI EcoRI StuI SalI SstI
GGC GCC ATG GGG ATC CGG AAT TCA AAG GCC TAC GTC GAC GAG
G A M G I R N S K A Y V D E

SpeI NotI NspV XhoI PstI XhoI
CTC ACT AGT CGC GGC CGC TTT CGA ATC TAG AGC CTG CAG TCT
L T S R G R F R I Stop

SphI KpnI HindIII
CGA GGC ATG CGG TAC CAA GCT TGT CGA GAA GTA CTA-3'

Fig. ox1. Map and construction of pFastBacHTc/P24P17 expression vector which expresses recombinant P24P17 protein in the baculovirus expression system.

The amino acid sequence of the 6x histidine affinity tag, spacer region and rTEV protease cleavage site are underlined. The rTEV cleavage, signified as "**", appears between glutamine (Q) and glycine (G) amino residues. The gene of P24P17 containing 1089 bp was subcloned into NcoI and NotI restriction sites, and the start codon was located in the NcoI site.

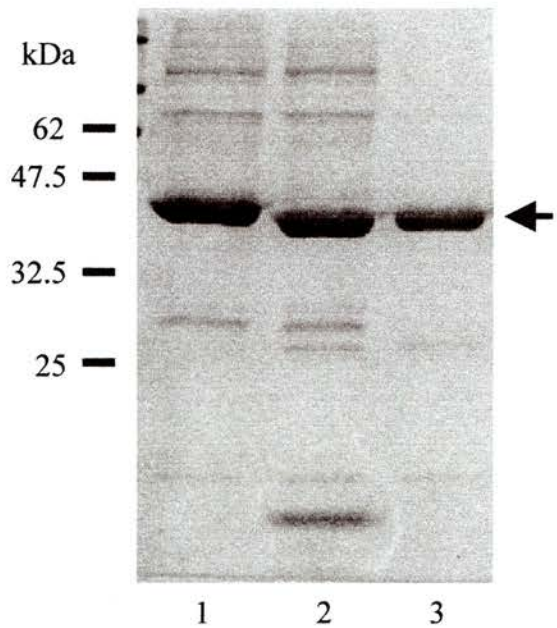
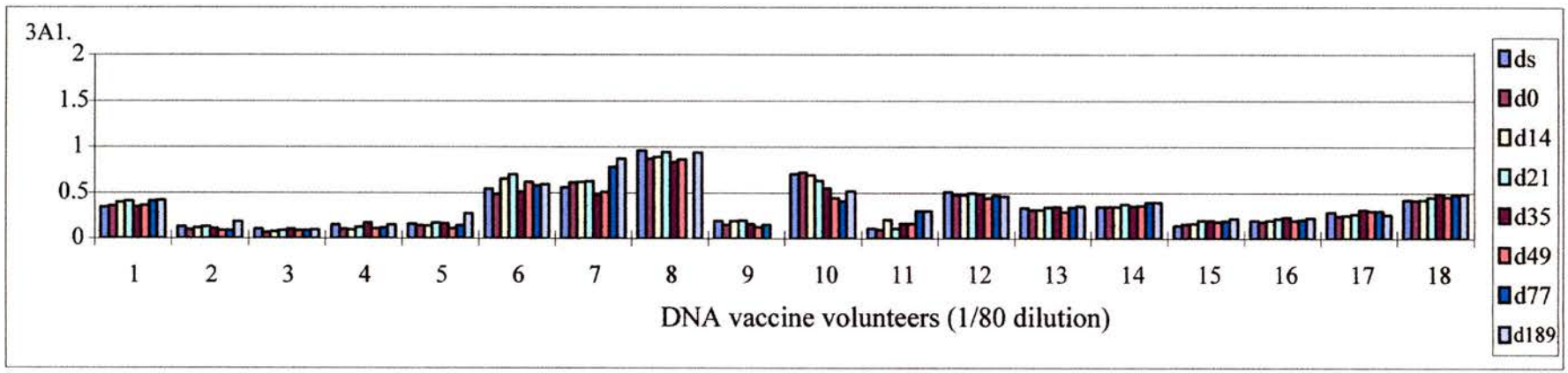


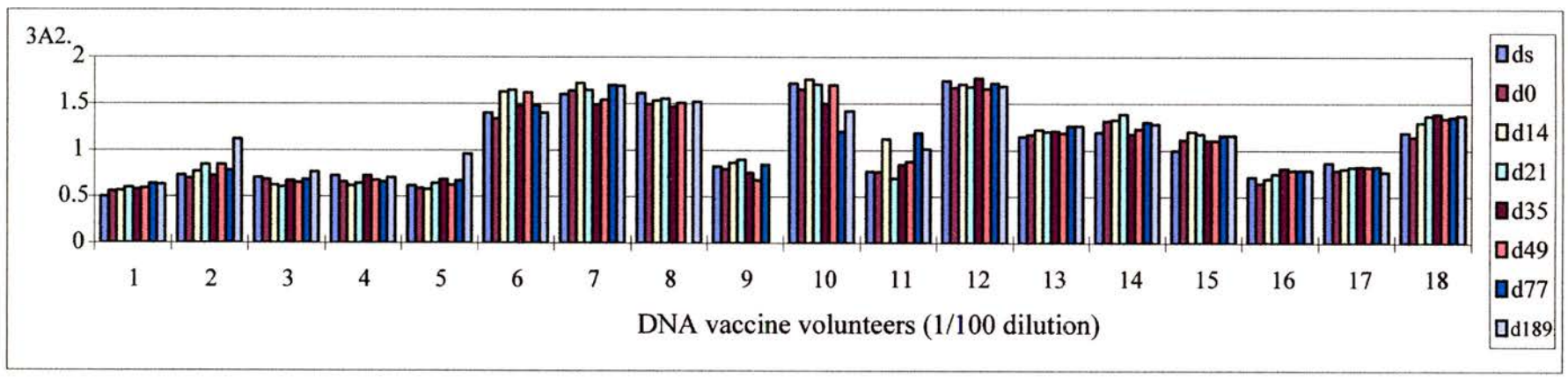
Fig. ox2. Purification of recombinant HIV gag protein, P24P17, expressed by the baculovirus expression system.

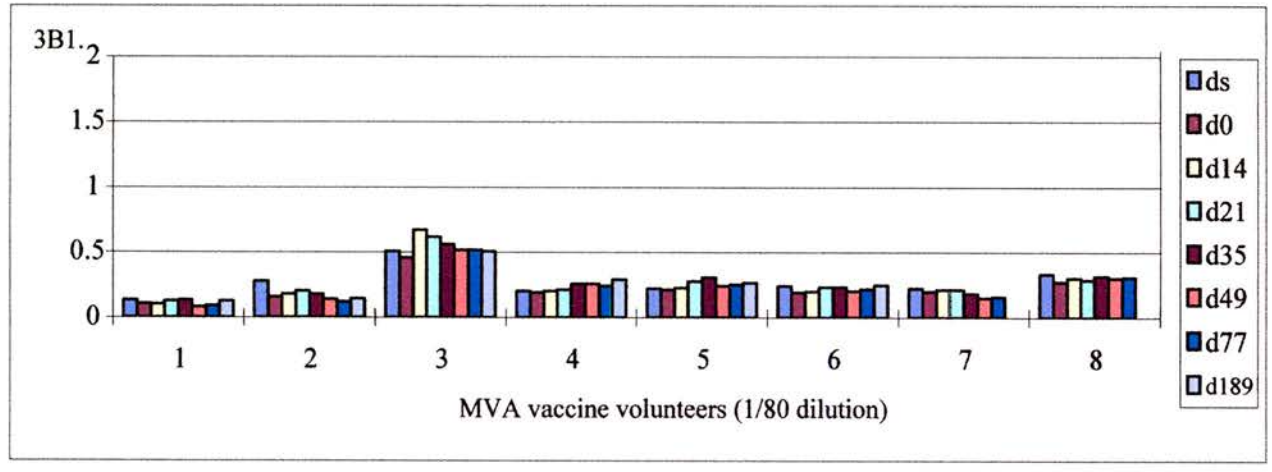
Insect cells (Sf9) were infected with recombinant baculovirus carrying P24P17 gene at MOI of 10, and incubated for three days to express the recombinant protein. The desired proteins were purified by nickel affinity columns (1) and then digested with rTEV proteases to cleave the histidine tag (2). The protease treated mixtures were loaded into nickel affinity columns to remove of histidine tags and rTEV proteases (3). Protein samples were resolved in 12% SDS-PAGEs. The migration positions and molecular masses of protein standard markers were shown along the left of the SDS gel which was stained with Coomassie blue, and the migration position of purified P24P17 protein (about 47 kDa) is indicated by the arrow. The yield of purified P24P17 was about 1mg per litre of insect cells.

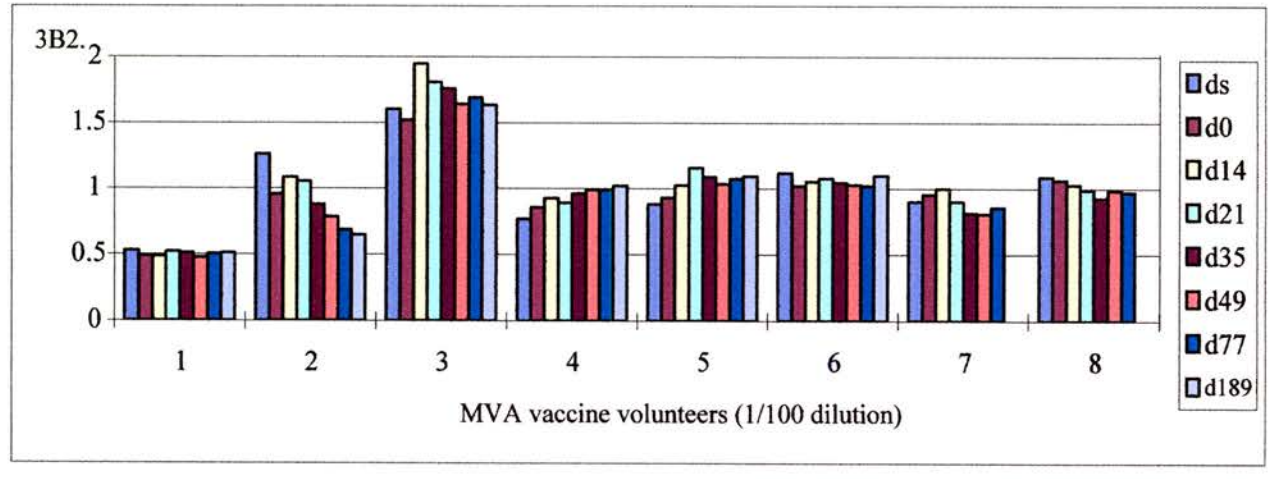
Fig. ox3. Antibody responses against P24P17 protein were not induced in most of vaccine volunteers.

The vaccine volunteers were immunized with either DNA (A), MVA (B) or DNA/MVA prime-boost (C) vaccine regimens, and the serum samples were collected before (at ds, d0) and after immunization (at d14, d21, d35, d49, d77, d189). Serum samples were diluted in skimmed milk solutions and analysed by ELISA tests in duplicate on micro-well plates which were coated with purified recombinant P24P17 protein. Bound antibodies against P24P17 protein were detected with HRP-conjugated anti-human IgG antibodies in the presence of HRP substrates [Sigma were used in (A, B), TMB were used in (C)]. The intensity of products from peroxidase reactions was read at a wavelength of 490 nm (A, B) or 450 nm (C) by the ELISA reader.


3A1.
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
DNA vaccine volunteers (1/80 dilution)
ds
d0
d14
d21
d35
d49
d77
d189


3A2.
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
DNA vaccine volunteers (1/100 dilution)
ds
d0
d14
d21
d35
d49
d77
d189


3B1.
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
ds
d0
d14
d21
d35
d49
d77
d189
MVA vaccine volunteers (1/80 dilution)


3B2.
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
ds
d0
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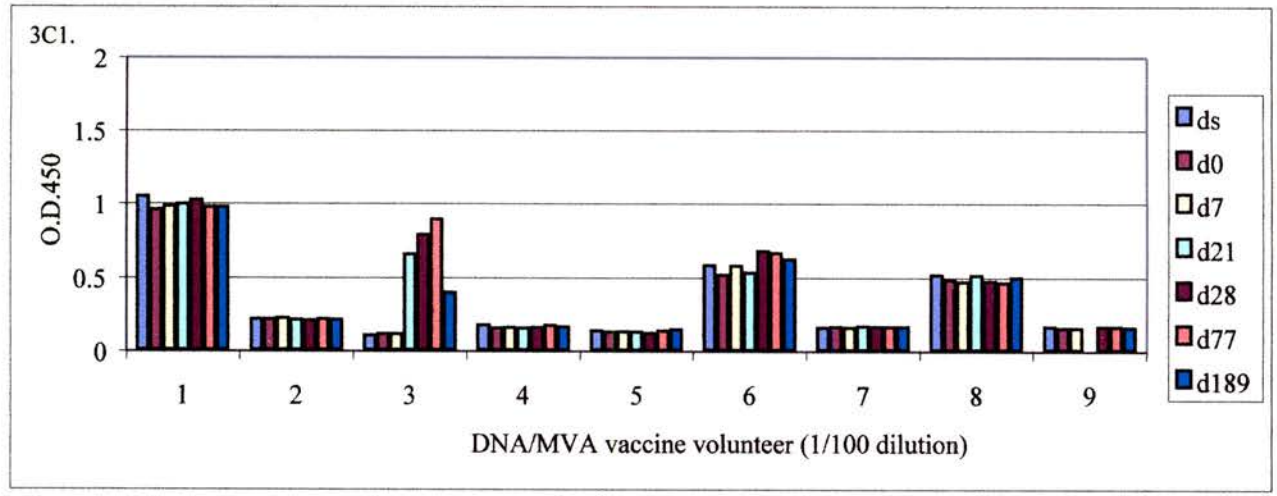
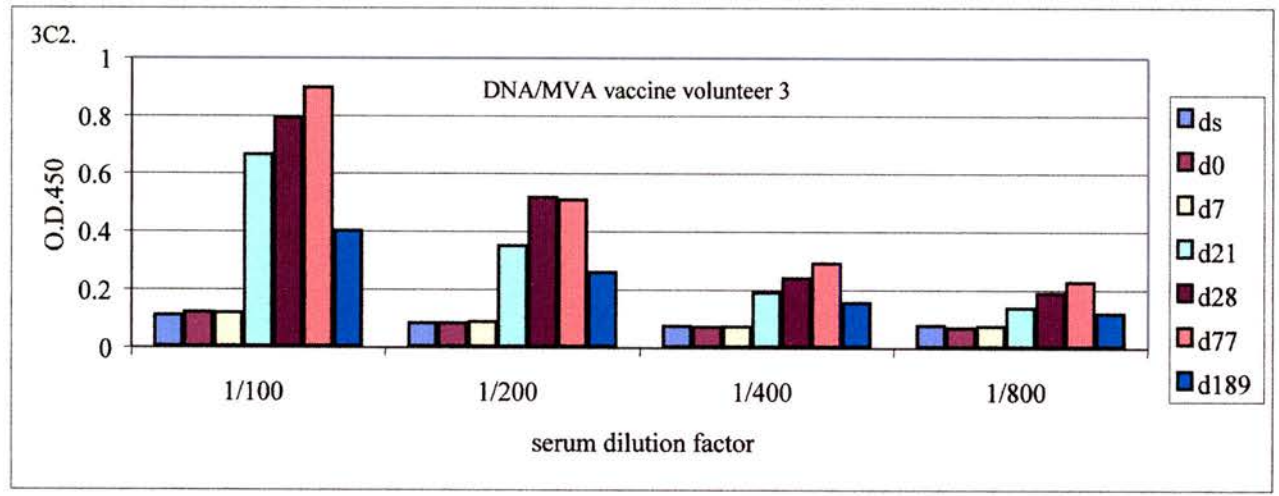
d21
d35
d49
d77
d189
MVA vaccine volunteers (1/100 dilution)


3C1.
2
1.5
1
0.5
0
O.D.450
1
2
3
4
5
6
7
8
9
DNA/MVA vaccine volunteer (1/100 dilution)
ds
d0
d7
d21
d28
d77
d189


3C2.
DNA/MVA vaccine volunteer 3
1
0.8
0.6
0.4
0.2
0
O.D.450
1/100
1/200
1/400
1/800
serum dilution factor
ds
d0
d7
d21
d28
d77
d189

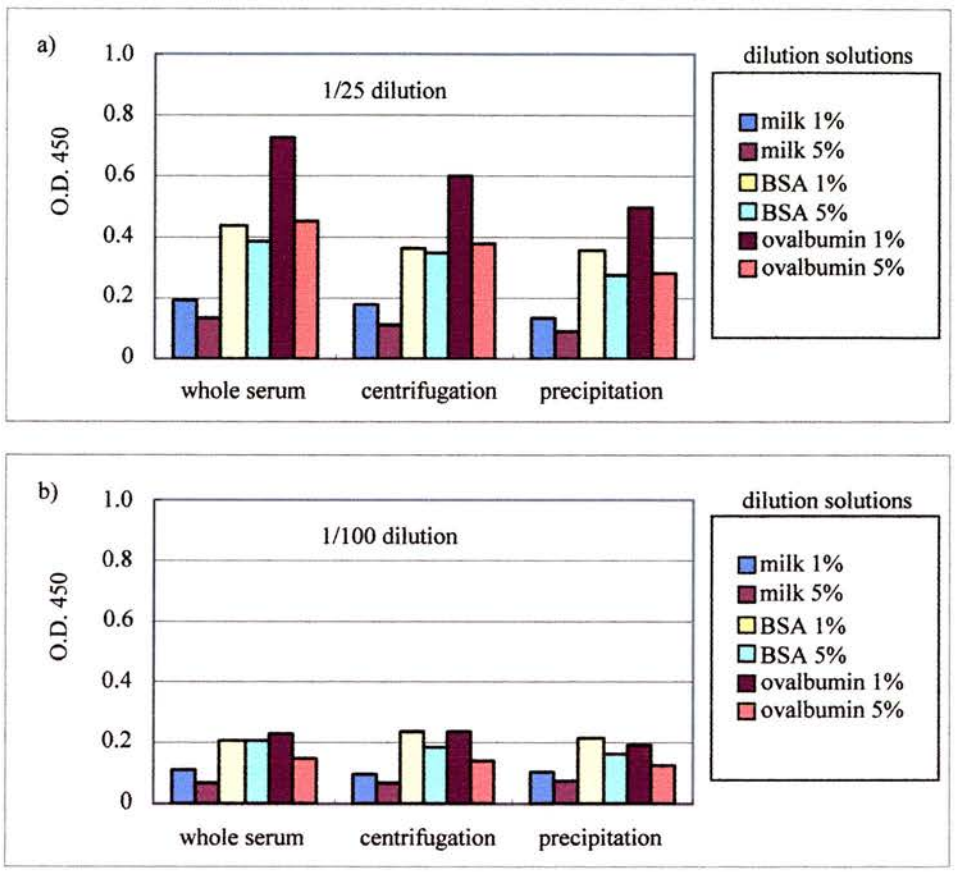


Fig. ox4. Skimmed milk solutions do not cause high background values in ELISA analyses of human serum.

The control human sera (donated by three people, who were not immunized with any HIV vaccine, from St Andrews University) were equally pooled together (whole sera). The pooled sera were centrifuged to remove debris and precipitated proteins (centrifugation), and ammonium sulfate was added to the supernatant to precipitate antibodies. Antibodies were resuspended with phosphate buffer saline in the same volume of serum used for purification (precipitation). These manipulated sera were diluted at ratio of 1/25 (a) and 1/100 (b) in 1% and 5% of skimmed milk solutions and analyzed by ELISA tests on blank micro-well plates, which were coated with 10% of skimmed milk solutions. The sera were also tested against BSA and ovalbumin in the same fashion as described for skimmed milk. Antibody responses were detected with HRP-conjugated anti-human IgG antibodies in the presence of HRP substrates (TMB) and read at a wavelength of 450 nm.

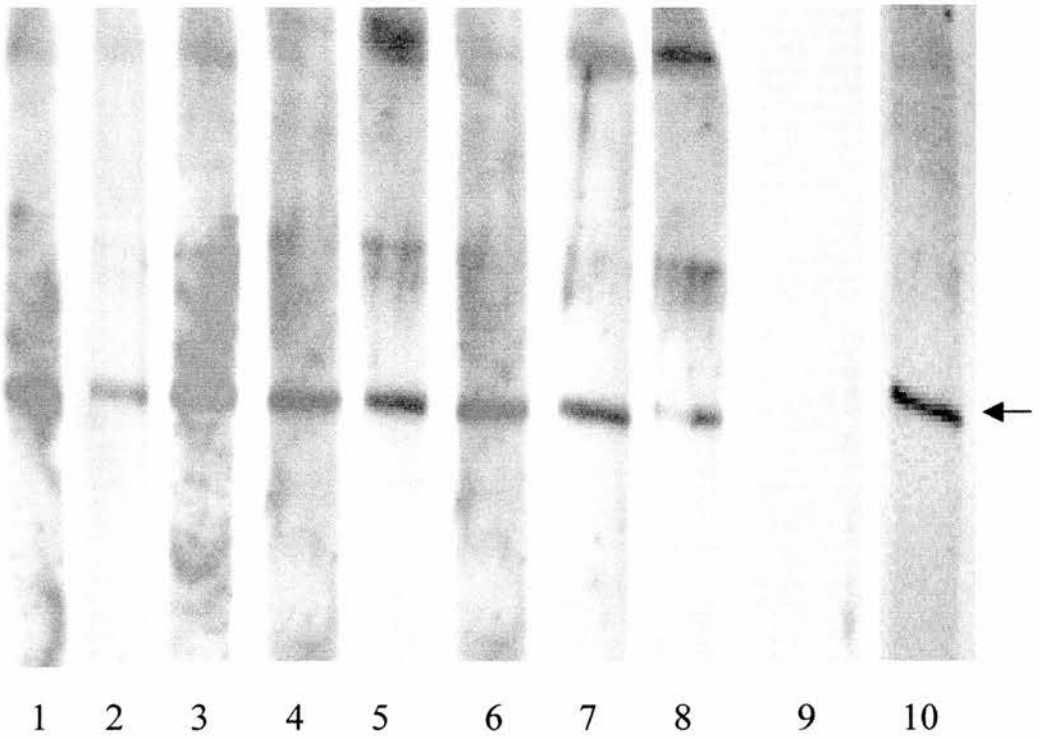
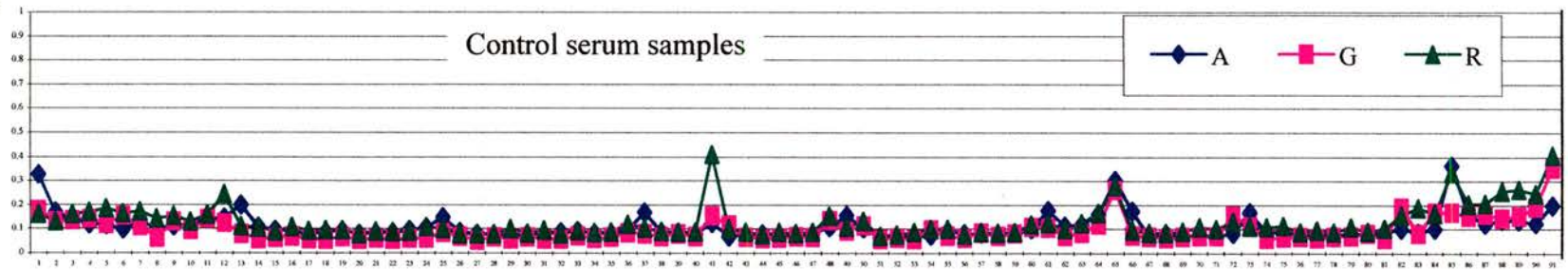


Fig. ox5. Western blot analyses showing human sera (from MVA vaccine volunteer number 3) recognising P24P17 protein.
Purified recombinant HIV gag protein (P24P17) were resolved on the 12% SDS-PAGE and transferred to a nitrocellulose membrane which was then cut into strips and blocked with skim milk solutions for Western blot analyses. Strips 1 to 8 were separately incubated with serum samples (1:100 dilution) which were collected before (at ds, d0: lane 1, 2 respectively) and after (at d14, d21, d35, d49, d77, d189: lane 3 to 8 respectively) immunisation from MVA vaccine volunteer (number 3). Strip 9 was not incubated with any serum sample but with the HRP-conjugated anti-human IgG antibodies. In strip 10, the serum sample was donated by a person (signified as A) from St Andrews University, not been immunised or infected with HIV. The migration position of P24P17 was located by the arrow.

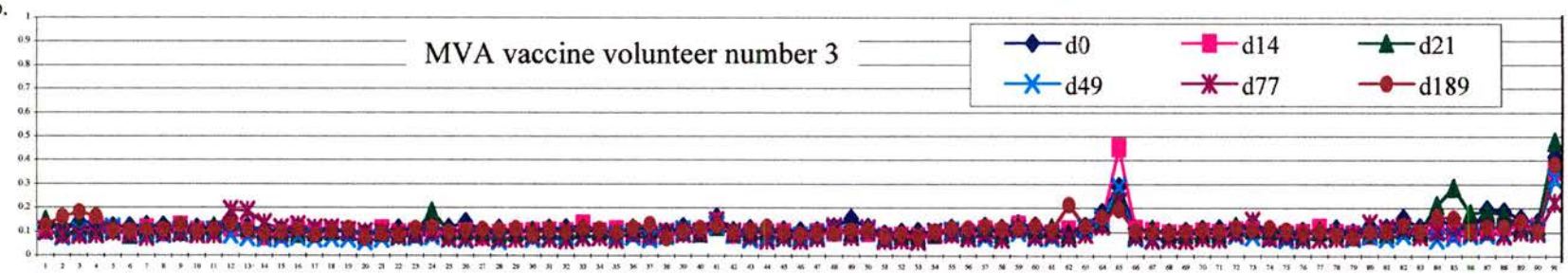
Fig. ox6. Human sera recognize synthetic peptide fragments derived from recombinant P24P17 protein.

15-amino acid peptide fragments, with 11-amino acid overlapping, that cover the majority of P24P17 were numbered serially from 1 to 90 and coated on micro-well plates for ELISA tests. The control serum samples (a) donated by individuals (signified as A, G, R) from St Andrews University and samples (b) from MVA vaccine volunteer (number 3) were diluted at ratio of 1:100 in skimmed milk solution and incubated with the peptide coated plates. Bound antibodies were detected with HRP-conjugated anti-human IgG antibodies in the presence of HRP substrates (TMB) and the intensity of products from peroxidase reactions was read at a wavelength of 450 nm. It should be noted that number 91 indicates that wells were coated with P24P17 proteins and that there were no peptide-coating in wells 3, 4, 37 and 88.

6a.


Control serum samples
A
G
R
peptide numbers

6b.


MVA vaccine volunteer number 3
d0
d14
d21
d49
d77
d189
peptide numbers

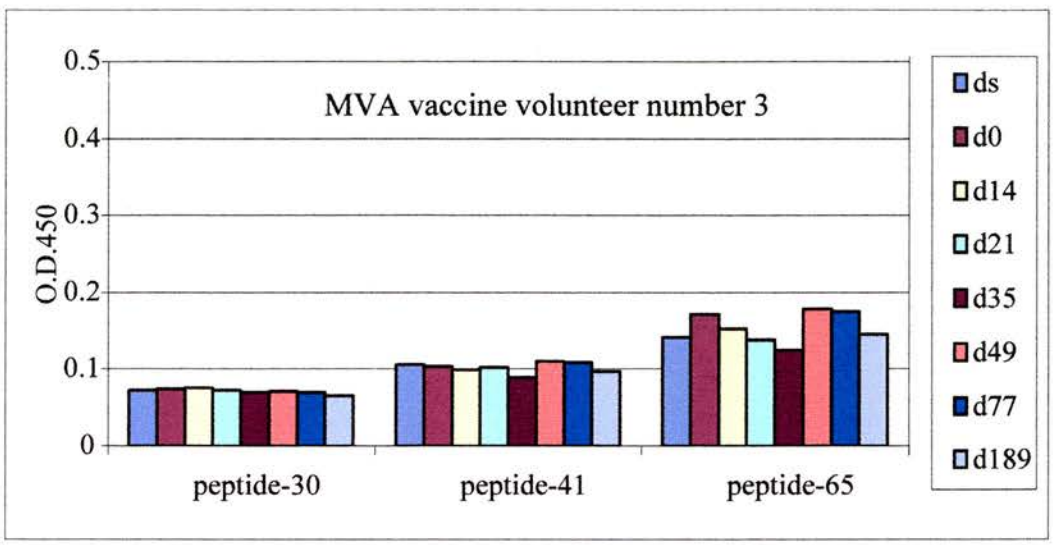


Fig. ox.7. Antibody responses to peptide fragment 65 were not boosted in MVA vaccine volunteer number 3.
Serum samples (1:100 dilution) from MVA vaccine volunteer 3 were collected before (ds, d0) and after (d14, d21, d35, d49, d77, d189) immunization, and subjected to ELISA analyses on micro-well plates which were coated with peptide 30, 41, and 65 in different wells.

Section III.

Transfer SV5 V protein or gene into target cells.

Summary:

In our previous studies, it has been shown that the tat protein of SIV is able to bind the surface of cultured cells, but the ability to transfer proteins into target cells is still not proved. Therefore, in this study we aim to illustrate this ability by making a functional assay, in which the SIV tat protein was fused with the V protein of SV5 virus by gene cloning approaches. The SV5 V protein has been well known to cause proteasome-mediated degradation of STAT1 proteins in host cells. Therefore, the degradation of STAT1 proteins might be observed in cells which were cultured with the purified tat/v fusion protein, if the protein transferring ability of SIV tat protein is functional. Furthermore, the presence of STAT1 proteins is critical in interferon responses against virus infection, and the degradation of STAT1 combined with other unknown functions of V protein can lead to improve the yield of viruses which are interferon sensitive. For the safety concern in the development of virus vaccines, to abolish STAT1 by introducing the SV5 V protein into virus-producing cells rather than making a V expressing cell line would avoid the risk of generating virus offsprings which contain the SV5 V gene. In this study, the purified tat/v fusion protein was shown to bind the surface of host cells by immunofluorescent staining, but the degradation of STAT1 proteins was not observed by Western blot analysis. To understand if the function of V protein was retarded after being fused with tat protein, the tat/v gene was subcloned into a mammalian expression vector. The expression of tat/v was shown to cause degradation of STAT1, indicating that tat protein can hardly affect, if at all, the function of V protein. Interestingly, after transient gene transfection, the distribution of expressed tat/v did not show the trend of spreading to surrounding cells, which might indirectly indicate that SIV tat protein in this fusion construction does not possess the same function as HIV tat protein to translocate through cellular membrane freely. Since the green fluorescent protein (GFP) has been widely and successfully used to demonstrate the translocation activity of HIV tat protein and peptides, here GFP was subcloned with tat/v gene for bacterial expression. Again, the purified GFP

fused tat/v protein was shown to bind cell surfaces but not to cause degradation of STAT1 proteins in host cells. Although at the moment we can not successfully use SIV tat protein to introduce SV5 V protein into host cells, to abolish STAT1 for vaccine development is still an attractive idea. Therefore, we took advantage of recombinant adenovirus to introduce V gene transiently into host cells, and in our preliminary results the degradation of STAT1 was successfully achieved.

1. Preparing and testing tat/v fusion protein.

To generate tat/v fusion protein, the gene of SIV tat protein was amplified by PCR and subcloned into the pGEX-4T/TEV/v vector, resulting in the construct of pGEX-4T/TEV/tat-v (**Fig. tv1a**) which was then expressed in bacteria *E. coli* BL21 under the induction of IPTG at room temperature condition. The expressed fusion protein contains glutathione sulfate transferase (GST) domain, TEV protease recognition sequence, SIV tat protein, and V protein of SV5 virus. The expressed proteins, termed GST-tat/v, were purified by GST bead columns and then separated on the SDS-gel to check the purity (**Fig. tv2a**). The desired protein was located at the migration position of the molecular weight of around 68 kDa, but there were some other unexpected protein bands appearing on the gel. In order to avoid the possible steric hindrance affection on the translocation activity of tat protein, the recombinant TEV protease was used to remove the GST domain from GST-tat/v protein, resulting in the tat/v fusion protein located at the migration position of the molecular weight of around 42 kDa (**Fig. tv2b**). Obviously, after the treatment of TEV, the intensity of GST on the gel was increased much more than expected, and most of those unexpected protein bands were disappearing, which might suggest that these disappearing bands came from non-completely translated GST-tat/v proteins.

To understand if the tat protein domain of tat/v protein can be a carrier to transfer the whole fusion protein into mammalian cells, proteins with (GST-tat/v, tat/v) or without (GST, V) the tat domain were added to the monolayered Vero cell culture and traced by mAb which recognizes the Pk epitope on the V protein domain (**Fig. tv3**). Clearly, through the fluorescent microscope, proteins containing the tat domain were observed on the surface of almost all of the

cultured cells, while proteins without the tat domain were not observed on any single cell. This might indicate that the surface binding was specifically contributed by the tat protein domain, and the binding activity was not hindered by the GST domain. Since the distribution of the tat fused proteins was barely observed, if any, inside cells, it is still uncertain whether the tat protein can bring proteins into cells or not. However, there might be a small portion of tat fused proteins to be transferred into target cells, and this amount of protein is not enough to be detected by the approach used here. Given the V protein of SV5 virus can cause proteasome-mediated degradation of STAT1 proteins, the entrance of tat fused V protein into incubated cells might be proved by the degradation of STAT1 protein. Therefore, monolayered Vero cells were incubated with tat fused V proteins (GST-tat/v, or tat/v), and the integrity of STAT1 protein was checked by Western blot analysis at different time points (5h, 10h, 24h). Compared with the mock incubation, there was no obvious reduction of STAT1 protein in cells incubated with tat/v proteins at any time point (**Fig. tv4**). This might indirectly indicate that tat can not successfully transfer V protein into target cells or the quantity of transferred V protein is not sufficient to cause degradation of STAT1 proteins.

2. Transient expression of tat/v in mammalian cells.

In the previous experiment, STAT1 protein was not degraded when the target cells were incubated with purified tat/v protein which was produced in bacteria. This might suggest that the V protein domain was not functional in this expression/purification system, or fusion with tat protein could disable V protein. Therefore, gene of SIV tat protein was amplified by PCR and cloned into pEF-plink2/v vector (contains the V gene of SV5 virus), forming the pEF-plink2/tat-v construct (**Fig. tv1b**), for examining the tat/v protein in mammalian expression condition. To investigate if the V protein domain of tat/v protein expressed in mammalian cells is functional in causing degradation of STAT1, the monolayered 293 cells were co-transfected with pEF-plink2/tat-v, luciferase reporter plasmid, and lacZ expressing vector for the functional assay. Since STAT1 is a transcription factor of interferon responsive genes, degradation of STAT1 will block interferon signaling and thus inhibit the expression of luciferase gene which was controlled by the interferon-α/β-responsive

promoter. These cells were stimulated either with or without interferon, and the luciferase activity was measured and normalized to lacZ activity. The relative activity value of cells transfected with pEFplink2 and treated with interferon was arbitrarily set as 100 %. The expression of protein V or tat/v was confirmed by Western blot (**Fig. tv5**). The relative luciferase activity of cells stimulated with interferon and transfected with pEF-plink2/v or pEFplink2/tat-v was almost equal to that of cells without interferon stimulation, indicating that interferon signaling was completely blocked by V or tat/v protein (**Fig. tv6**). This result also suggested that the function of V protein in causing STAT1 degradation was not handicapped when the V protein was fused with tat protein and expressed in mammalian cells.

It has been well demonstrated that the HIV tat protein can be released extracellularly from infected cells and traverse cell membranes of most cells efficiently. This membrane-penetration activity is mainly contributed by the basic amino acid domain. Although the SIV tat protein contains a similar basic amino acid domain, in our previous studies we barely observed the evidence showing that SIV tat protein can penetrate cell membranes of cultured cells. If the SIV tat protein can penetrate cell membranes in the same fashion as HIV tat protein, the transient expressed tat/v protein might spread to the surrounding cells. To examine this presumption, monolayered 293 cells were transfected with pEF-plink2/tat-v or pEF-plink2/v mammalian expression vector, and the encoded tat/v or V protein was probed by the mAb which recognizes the Pk epitope of V protein (**Fig. tv7**). After immunofluorescent staining, the transfected cells were located, but the expressed tat/v protein did not spread to the surrounding cells. There was no obvious difference in the distribution pattern between tat/v and V proteins, implying that the SIV tat protein might not be able to traverse cell membranes.

3. Preparing and testing GFP-tat/v fusion protein.

Since the green fluorescent protein (GFP) has been widely and successfully used to demonstrate the membrane-penetration activity of HIV tat protein and peptides, we try to apply the same approach to examine the SIV tat protein. Therefore, the tat/v gene was cloned into the bacterial expression vector

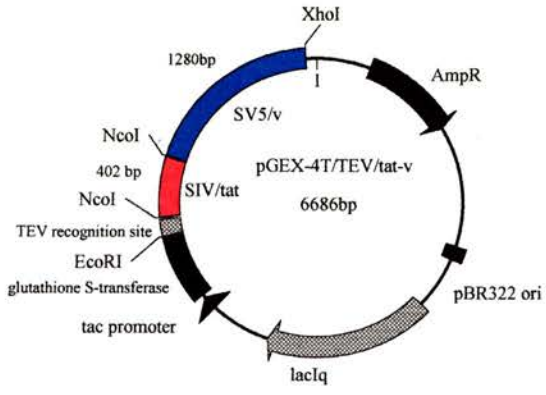
pHISEGFPTEV, forming the pHISEGFPTEV/tat-v construct (**Fig. tv8**). The expressed tat/v protein contained the His tag and GFP at the N-terminal, and was purified by the Ni-resin column. The monolayered Hep2 cells were incubated with the purified GFP-tat/v and then examined under the fluorescent microscope. The GFP-tat/v appeared in green was observed surrounding the cell surface rather than inside the cell (**Fig. tv9**). However, the emission of green was not observed when the cells were incubated with the GFP (not shown), suggesting that tat was responsible for the binding on cell surfaces. In the further examining analysis, when cells were incubated with IFNα (to increase the expression of STAT1) and GFP-tat/v, there was no obvious decrease of STAT1, showing that GFP-tat/v can hardly cause, if any, degradation of STAT1 in cultured cells (**Fig. tv10**). Together, these results might be another evidence suggesting that SIV tat does not possess the membrane-penetration activity as HIV tat does.

4. Generation of recombinant adenovirus to transfer SV5 V gene into target cells.

Combined with other undocumented functions of V protein, the expression of SV5 V protein in the host cells can cause degradation of STAT1 and lead to increase the production of interferon sensitive viruses. Furthermore, the size of virus plaques in the host cells expressing the V gene is bigger than that in the naïve cells. If the tat domain of tat/v fusion protein can traverse cell membranes, the tat/v protein might be used to help isolate and generate interferon sensitive viruses in various cell lines, without making specific V expressing cell line which is a time consuming process. However, in our previous study this approach was not successfully achieved, and thus transferring the V gene rather than the V protein into host cells would be another considerable choice. Since the recombinant adenovirus can transfer foreign genes into target cells efficiently, and the expression of the transferred gene is transient without the process of gene integration, hence the recombinant adenovirus (rAd) carrying the SV5 V gene was made. Briefly, the V gene was amplified by PCR and subcloned into pCA14 vector, forming the pCA14/v construct (**Fig. tv11**), which was then cotransfected with the pJM17 vector into the virus-generation competent cells (293 cell line) to generate rAd. The desired virus was isolated

by the standard plaque purification procedure and then amplified in 293 cells for large scale purification (described before). To examine the purified rAd/v (rAd containing the V gene of SV5), monolayered Vero cells were infected at m.o.i. of thirty for two days and the expression of V protein was detected by Western blots and immunofluorescent analyses (**Fig. tv12**). The expression of V protein was observed in infected cells, and associated with the degradation of STAT1, indicating that recombinant adenovirus can efficiently transfer functional V gene into target cells.

a)



b)

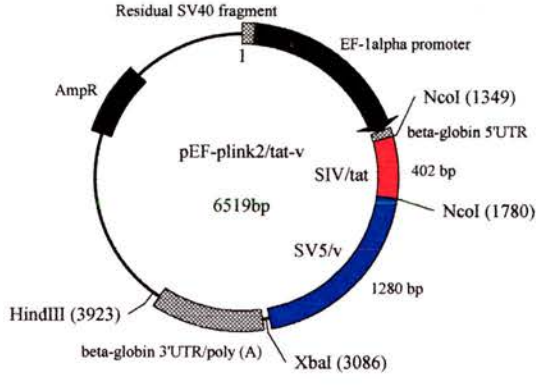


Fig. tv1. Subcloning SIV/tat and SV5/v genes into bacterial and mammalian expression vectors.

a) SIV/tat gene was amplified by PCR and subcloned into pGEX-4T/TEV/V on NcoI site between TEV protease recognition site and SV5/v gene, resulting in the pGEX-4T/TEV/tat-v construct. The fusion protein, containing GST, TEV recognition sequence, SIV/tat, SV5/v, was expressed in *E. coli* BL21 under the induction of IPTG at room temperature. b) By using the same strategy, SIV/tat gene was inserted into pEF-plink2/V, forming pEF-plink2/tat-v construct. The expression of fusion protein tat-v, composed of SIV/tat and SV5/v, was controled by EF1-alpha promoter in mammalian cells after transient transfection.

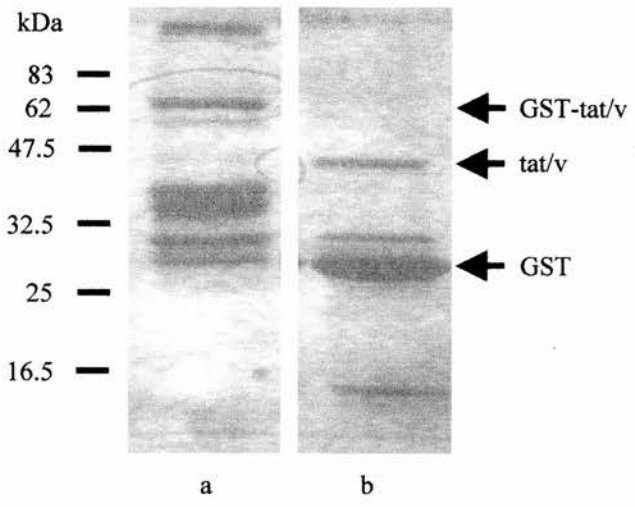


Fig. tv2. Expression and purification of SIV/tat fused SV5/v protein.
Encoded from pGEX-4T/TEV/tat-v, the fusion protein GST-tat/v, composed of GST, TEV protease recognition site, SIV/tat, and SV5/v proteins was expressed in *E. coli* BL21 under the induction of IPTG at room temperature. The expressed fusion proteins were purified by GST bead columns (a), and then digested with rTEV proteases to remove the GST domain (b).

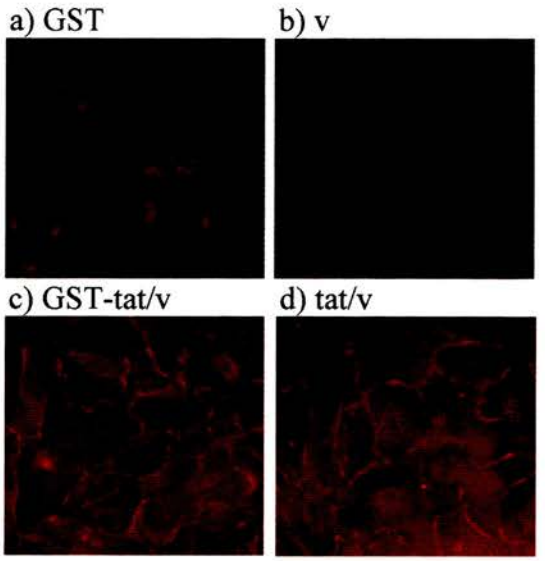


Fig. tv3. Purified tat/v fusion proteins can bind cell surfaces of Vero cells.
On coverslips, monolayered Vero cells were incubated with purified proteins GST(a), v(b), GST-tat/v(c), tat/v(d) for two hours, and then immunostained with mAb which recognizes Pk epitope on each protein. (GST: glutathione sulfate transferase; tat: tat protein of SIV; v: v protein of SV5 virus). Note: the v protein domain contains a natural Pk epitope and the GST possesses a Pk epitope which was artificially introduce on its C-terminal.

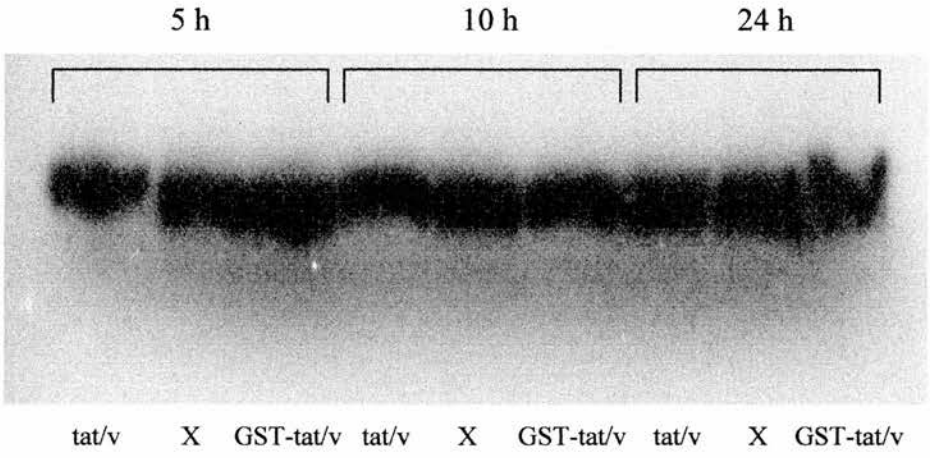


Fig. tv4. Incubation with SIV/tat fused SV5/v protein does not cause degradation of Stat 1 protein in target cells.
Monolayer Vero cells were incubated with purified proteins (tat/v, GST-tat/v), and the integrity of Stat 1 protein was detected by Western blot at various time points (5h, 10h, 24h). tat/v: SIV/tat fused SV5/v protein, GST-tat/v: GST fused tat/v protein; X: mock incubation.

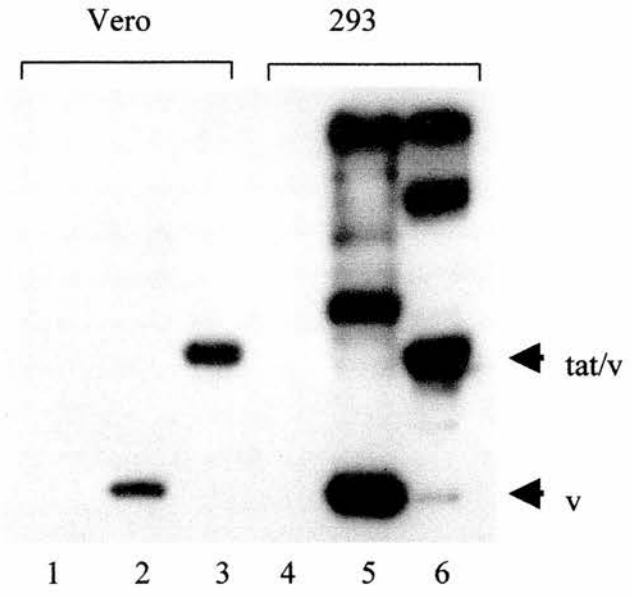


Fig. tv5. Transient expression of tat-v and v proteins in mammalian cells. Monolayered Vero (lane 1, 2, 3) and 293 (lane 4, 5, 6) cells were respectively transfected with pEF-plink2 (lane 1, 4), pEF-plink2/v (lane 2, 5), and pEF-plink2/tat-v (lane 3, 6) constructs for two days. The expression of tat-v and v proteins were detected by Western blot in which mAb recognizing the Pk epitope in the v protein was used.

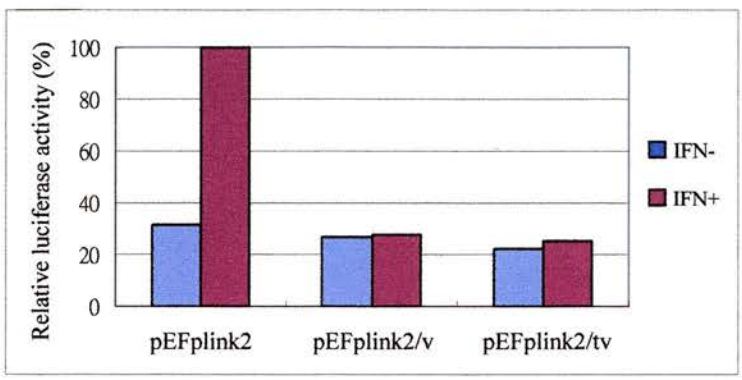


Fig. tv6. Fusion of SIV/tat protein does not affect the activity of SV5/v protein in blocking interferon signaling.
Monolayered 293 cells were transfected with different pEF constructs (pEFplink2, pEFplink2/v, pEFplink2/tv) and together with a reporter plasmid, which contains a luciferase gene under the control of IFN-α/β-responsive promoter, and a lacZ expressing plasmid for two days. At 4 h before harvesting cells, the culture medium was supplemented either with or without interferon (IFN). The luciferase activity was measured and normalized to the β-galactosidase activity. The relative activity value of cells transfected with pEFplink2 and treated with IFN was arbitrarily set as 100%.

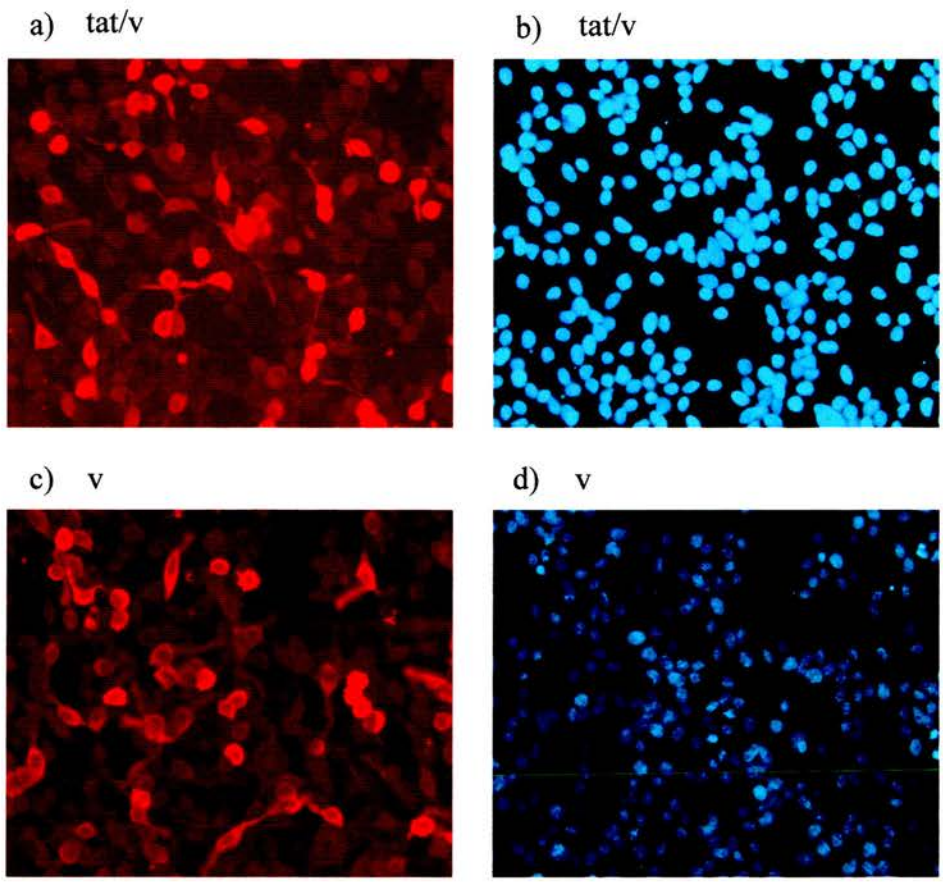


Fig. tv7. Immunofluorescent staining shows transient expression and distribution of SV5/v and tat-v proteins in 293 cells.
Grown on coverslips, 293 cells were either transiently transfected with pEF-plink2/tat-v (a) or pEF-plink2/v (c), and expressed tat-v and v proteins were detected by the mAb recognizing the Pk epitope of v protein. On the same coverslip, cells were stained with DAPI (b, d) at the same time to localize the nucleolus.

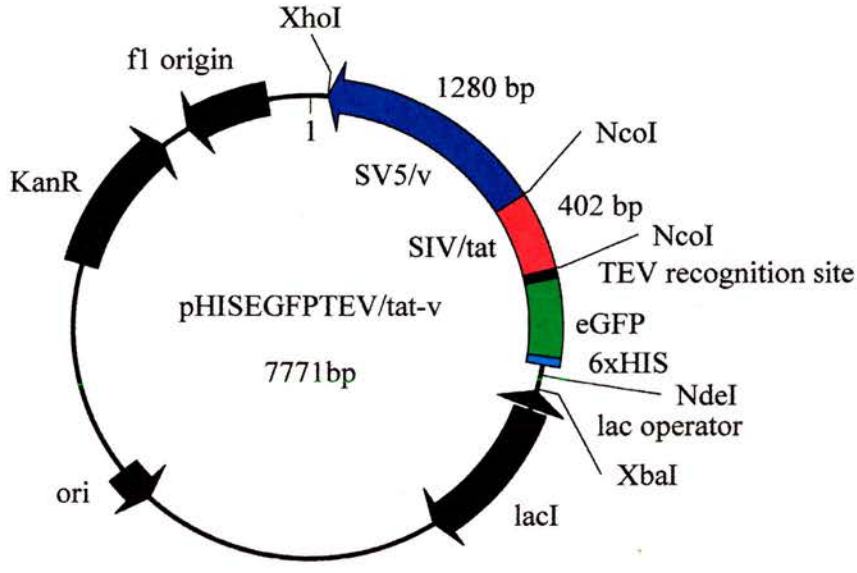


Fig. tv8. Subcloning SIV/tat and SV5/v genes into the bacterial expression vector for generating eGFP fusion protein.

The gene fragment of SIV/tat and SV5/v was introduced into pHISEGFPTEV on restriction enzyme digestion sites XhoI and NcoI, resulting in pHISEGFPTEV/tat-v construct. The fusion protein is mainly composed of three proteins, eGFP, SIV/tat and SV5/v. The TEV protease recognition site is located between eGFP and SIV/tat, and the 6xHIS tag sequence is fused on the N-terminal of eGFP.

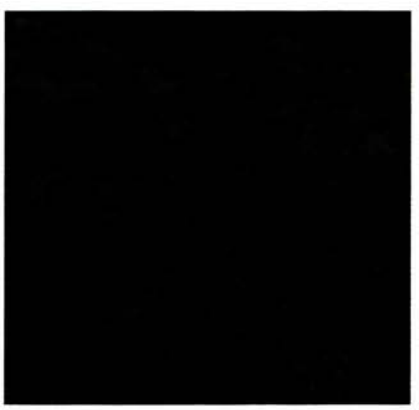


Fig. tv9. Purified GFP-tat/v fusion protein can bind the surface of Hep2 cells. Hep2 cells were grown on coverslips overnight and then incubated with purified GFP-tat/v fusion proteins for two hours. tat/v: fusion protein composed of SIV/tat and SV5/v proteins.

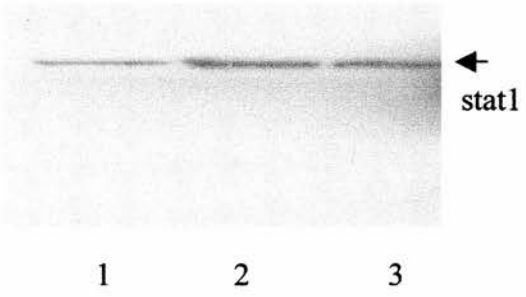


Fig. tv10. Incubation with GFP-tat/v fusion proteins does not cause degradation of Stat 1 in Hep2 cells.
Monolayered Hep2 cells were incubated with IFNα and together with purified GFP-tat/v proteins (3) for six hours. (1): naïve cells; (2): only treated with IFNα. The existence of Stat 1 was detected by Western blot using mouse mAb.

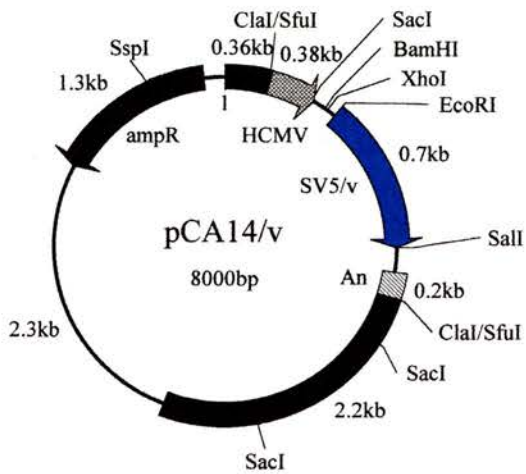


Fig. tv11. Subcloning the gene of SV5/v protein into pCA14 for generating recombinant adenovirus.

The gene of SV5/v protein was amplified by PCR and subcloned into pCA14 on restriction enzyme sites EcoRI and SalI, which were designed in the sequence of primers. The expression of SV5/v protein was driived by promoter derived from human CMV. The size of each gene fragment is shown in kilo base pair (kb). ampR: ampicilline resistance gene. An: polyadenine sequence.

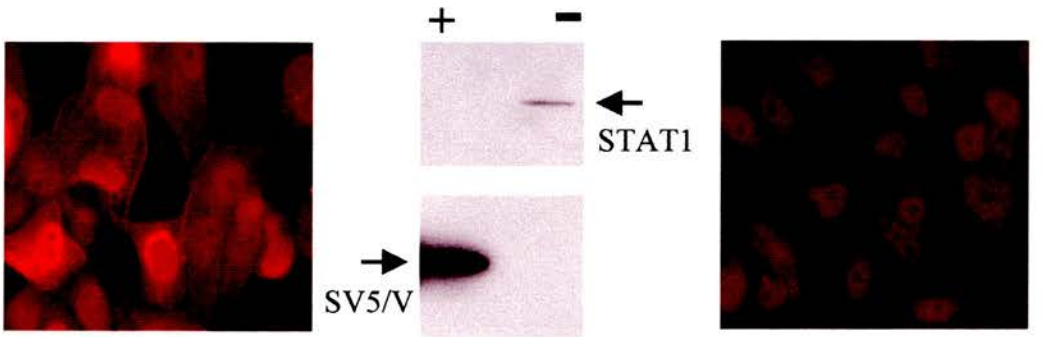


Fig. tv12. Recombinant adenovirus can be used to transfer SV5/v gene into and cause degradation of Stat 1 protein in target cells.
Vero cells were grown on coverlips overnight and then infected with recombinant adenovirus ,which contains the SV5/v gene, for two days. The expression of SV5/v protein was detected by immunofluorescent staining and Western blot. The existence of Stat 1 protein was detected by Western blot. +: infected with rAd/v; -: naïve cells.

Discussion

1. Detection of antibody responses induced by immunization with HIV vaccines.

An excellent multiple-epitope DNA construction of an experimental HIV-1 vaccine for a clinical trial in Kenya and United Kingdom has been made (Hanke, and McMichael, 2000). This DNA construct encodes a immunogen HIVA, which consists of about 73 % of the gag protein fused to a string of 25 partially overlapping CTL epitopes, and was delivered by a DNA vector and modified vaccinia virus Ankara (MVA). These two vaccine vehicles were used in a prime-boost vaccination protocol, focusing on the induction of cellular immune responses mediated by a concerted action of CD4+ helper and CD8+ effector T lymphocytes.

Initially, we considered that we might have a chance to use SIV tat as an antigen carrier to deliver HIVA and other HIV-1 proteins into target cells for the induction or boost of antigen specific CTL and antibody responses in our future works. Therefore, various baculovirus clones containing the open reading frame of HIVA or other HIV-1 proteins were produced (not shown). These immunogens were expressed in the baculovirus expression system, since gene sequences of these immunogens are GC rich (>60 %) and contain a large amount of rare codons which are not suitable for the bacteria expression system, and posttranslational modifications in insect cells are similar to that in mammalian cells which might not affect the protein structure too much. However, this project was not continued, as SIV tat was found incompetent for the purpose we proposed. In the mean while, the gag protein domain of HIVA termed P24P17 was produced in the baculovirus expression system **(Fig. ox1,2)** for ELISA analyses to detect antibody responses of vaccine volunteers. From 18 of DNA, 8 of MVA and 9 of DNA/MVA vaccine volunteers, only one volunteer from DNA/MVA group was confirmed producing antibodies specific to **P24P17 (Fig. ox3)**. This might be in agreement with the general knowledge that antibody responses were not generally induced by DNA-based vaccine

regimens. However, this assay to measure the development of Gag-specific antibodies could be potentially used to facilitate the identification of HIV infections in high risk individuals.

2. Use SIV tat protein as an antigen carrier to induce CTL responses.

The development of vaccines for acquired immunodeficiency syndrome (AIDS) has been proven an unexpected difficult challenge for scientists. Even though HIV-1 has been known to cause this disease for over two decades, a vaccine is not available. AIDS epidemic continues to rage despite advances on the therapeutic approaches. The expense and possible side effects have precluded the application of anti-viral drugs, an universal treatment for AIDS. At present development of a safe, inexpensive, and effective vaccine still appears to be the best opinion to prevent the spread of this disease. Slower rates of progression to disease are correlated with lower levels of virus, and the reduction of virus is in part due to virus-specific cell-mediated and humoral immune responses in infected individuals (Goudsmit, 1992; Kalams, 2003). Responses of cytotoxic T lymphocytes (CTL) are believed to play a key role in controlling viremia (Hanke and McMichael, 1999; McMichael and Hanke, 2002). These cells can recognize fragments of viral proteins that were presented by class-I MHC molecules on surfaces of antigen presenting cells or virus infected cells. After receiving proper signals, CTLs can reduce the viral production by releasing factors that cause the lysis of infected cells or inhibit virus production in non-lytic manners (Henkart, 1994; Griffiths, 1995). Ideally, if CTLs are specific to early viral proteins, infected cells will be negatively affected before a significant number of progeny virions are released, and thus the control of infection is achieved. Rather than using DNA or live viral vectors, it could be a useful alternative approach to induce CTL responses by using proteins or peptides to carry candidate protein antigens into target cells.

HIV-1 tat protein has been successfully used to transfer exogenous proteins into target cells, and this membrane transversing capability was shown to be mediated by the arginine rich sequence of tat protein (Fawell, et al. 1994; Park,

et al. 2002; Schwarze, et al. 1999). The idea is that if protein antigens are directly transferred into cytosol of target cells by tat protein, antigens will be mainly processed in the class-I presentation pathway. In the previous work at St Andrews, the tat protein of SIVmac32H(J5) was genetically fused with glutathione S-transferase (GST) and expressed in the E. coli expression system, but the yield of fusion proteins was poor (Hanke, 1993). However, after changing the codons of three consecutive arginine residues of SIV tat protein to highly used codons, high levels of protein expression were achieved (Diassiti, 2002).

SIV tat is able to bind to cell surfaces

To examine whether SIV tat protein is capable of delivering exogenous proteins into target cells, SIV tat was expressed as a fusion form called GTHP (composed of GST, SIV tat, histidine and Pk tags) in E. coli. Following the standard purification procedure, cultured cells were incubated with purified proteins and the distribution of proteins was examined by immunfluorescent staining **(Fig. tat5)**. The SIV tat protein domain was observed to cause accumulation of this fusion protein on cell surfaces, but the distribution inside cells was not observed in this test. These results were consistent with observations in previous works (Diassiti, 2002). These immuno-fluorescence data may indicate that SIV tat protein was not able to transverse cell membranes in the same fashion as has been reported for HIV tat protein. Another explanation is that SIV tat protein might be of great affinity to cell membranes, forcing GTHP fusion proteins to accumulate on cell surfaces rather than transverse into cells. This membrane binding activity was mapped to a region (containing 30 amino acid residues) which is highly rich in the content of basic residues similar to that of HIV tat protein **(Fig. tat7)**. The membrane-binding domain of SIV tat protein was not clearly shown to transfer an attached protein into target cells, but was shown to bind to cell surfaces.

SIV tat can be used as an adjuvant

To investigate effects of SIV tat protein on the immunogenicity of GTHP fusion protein, BALB/c mice were injected with soluble GTHP or GHP proteins. Blood was collected for ELISA analyses in which GHP was used as target

antigen to measure the production of antigen specific antibodies IgG1 and IgG2a **(Fig. tat8)**. The titer of IgG1 in mice injected with GTHP (>6.4k) is much higher than that of mice injected with GHP (>800), indicating that the higher level of antibody production was contributed by SIV tat protein. The titer of IgG2a in mice injected with GTHP was detected (>1.6k), while the production of IgG2a was not detected in mice injected with GHP. This result suggested that immunization with SIV tat fusion proteins might lead immune responses toward Th1 responses, since IgG2a has been reported to be an indicator for Th1 responses in mice (Coffman, et al. 1993). In addition, given adjuvants were not present in these injection regimens, SIV tat protein appeared to act as an adjuvant to improve antibody production and Th1 responses. Production of IFN-γ could be also promoted, since IFN-γ can be secreted by the Th1 subset and promote IgG2a production by B cells. This adjuvant effect might require the full length of SIV tat protein or just part of it. If the later is the case, it will be useful to map the critical region that could be used to carry various protein antigens for the vaccine development. Those truncation forms of SIV tat protein we made for mapping the membrane-binding domain of SIV tat would be useful for this test.

Soluble vs. insoluble SIV tat fusion protein

Since the nature of proteins and the condition of storage may affect the solubility of purified proteins, it could be interesting to test whether protein solubility can affect the adjuvant effect of SIV tat. When mice were injected with insoluble GTHP, the production of IgG1 (>25.6k) and IgG2a (>12.8k) was significantly reduced, compared with the injection with soluble GTHP (IgG1: >>51.2k; IgG2a: >51.2k) **(Fig. tat9)**. This suggested that it might be better to preserve antigens in the soluble form, when SIV tat protein is used as an antigen carrier in vaccine formula. The insoluble GTHP protein was observed not binding on cell membranes of cultured cells (data not shown), indicating that the membrane-binding property of SIV tat protein was compromised when proteins were aggregated. Furthermore, the membrane-binding property might increase the immunogenicity of protein antigens and thus enhance immune responses. However, the membrane-binding property might somehow partially recover in

animal bodies, so the production of IgG1 and IgG2a was detected when mice were injected with insoluble GTHP.

Alum salts do not affect SIV tat on the induction of Th1 responses

Alum salt is the only licensed adjuvant for immunization in human (O'Hagan, 1997), and has been wildly used for enhancing antibody production but not for cytotoxic immune responses (Gupta, 1998; Gupta, and Siber, 1995). It would be of interest to investigate whether alum salts can affect soluble SIV tat fusion proteins on the induction of antibody and Th1 responses. Since protein antigens can be either co-precipitated with or adsorbed on alum salts, thus it is worth to compare the effect of these two forms of alum-antigens on the outcome of immunization **(Fig. tat11)**. Significant differences on the production of IgG1 (>>51.2k) were not observed between these two immunization groups, but this level of antibody titer is obviously higher than that (>51.2k) in mice injected with soluble protein alone **(Fig. tat9)**. This suggested that preparation procedures of alum-antigens will not affect the effect of alum salts on enhancing antibody production, in agreement with the adjuvant property of alum salts (Cox, and Coulter, 1997). The IgG2a titer of mice injected with antigens prepared in the adsorption procedure (>51.2k) was similar to that of mice injected with soluble antigens alone (>51.2k), but was significant higher than that of mice injected with antigens prepared in the co-precipitation procedure (>12.8k) **(Fig. tat9, 11)**. Therefore, it could be concluded that alum salts can barely affect SIV tat protein on the induction of IgG2a, when antigens were prepared in the adsorption procedure. However, when antigens were prepared in the co-precipitation procedure, the production of IgG2a was significantly diminished by alum salts. Generally, the combination of alum salts and SIV tat protein fusion proteins in the adsorption procedure was shown to enhance antibody production without affecting the activation of Th1 responses. It would be potentially valuable in future works to investigate whether antibody and Th1 responses can be induced by alum-antigens which contain SIV tat and protein antigens in the separate unit.

Measure CTL responses induced by SIV tat fusion protiens

SIV tat protein has been shown to mediate the induction of Th1 responses which are in favor of activating CTL responses (Parronchi, et al. 1992), and thus SIV tat protein seems highly possible to induce CTL responses. To confirm the generation of CTL responses, mice (BALB/c) were injected with CTL-epitope fused GTHP fusion proteins (GTIHP or GTSHP), and lymphocytes were subjected to CTL assays **(Fig. tat18)**. Unfortunately, there was no significant CTL response detected in these two immunization groups, even though these two proteins have been confirmed to bind to cultured cells **(Fig. tat16)** and to induce production of IgG2a in mice **(Fig. tat17)**. This might indirectly suggest that SIV tat is not able to transfer exogenous protein antigens into target cells, and therefore antigens were not presented on class-I MHC molecules to activate CTL responses.

There is another explanation that the CTL epitope fused in the GTSHP fusion protein might be a minor epitope, so CTL responses were not detected. However, following the standard CTL assay, this epitope was confirmed a dominant epitope **(Fig. tat18-4)**. It has been reported that HIV tat protein can induce the production of IL-2 (Ehret, et al. 2001) which is a cytokine known to drive helper T cells toward Th1 responses. Therefore, SIV tat protein might posses similar function to activate Th1 responses, so the production of IgG2a was observed in mice injected with SIV tat fusion proteins.

Although SIV tat was not shown promisingly to induce CTL responses, in fact SIV tat was shown significantly to enhance the immunogenicity of an attached protein antigen and activate Th1 responses. Therefore, SIV tat could be potentially used as an adjuvant in the protein vaccine formula focusing on production of antibody responses.

3. Use rAd to boost CTL responses primed by SIV tat fusion proteins.

In the previous results, CTL responses were barely induced, if at all, by the immunization with GTSHP, but the production of IgG2a was obvious which

was an indicator for Th1 responses. Since factors (such as IFN-γ) generated in Th1 responses can help activate CTL responses, weak CTL responses might be induced following the immunization with GTSHP. Normally, strong CTL responses were not induced by DNA based vaccines, and additional immunizations with live viral vectors were required for the induction of strong CTL responses. A live viral vector could be used to test whether GTSHP could similarly prime CTL responses. Based on the safety concern, recombinant adenovirus was used in this approach, since the desired gene transferred by this virus expressed transiently in target cells without the requirement of integration and this virus can not replicate in normal cells. Therefore, recombinant adenovirus Ad/GTSHP (carrying the gene of GTSHP) were generated and used to inject mice which have been immunized with GTSHP proteins. In theory, following the injection of Ad/GTSHP, GTSHP protein will be produced in target cells, processed by the proteasome system, and presented by class-I MHC molecules on target cell surfaces. Antigen specific cytotoxic T lymphocytes will recognize the presented epitope and receive stimuli from immune factors generated in Th1 responses which were activated by the injection of GTSHP proteins. Therefore, antigen specific CTL responses were successfully activated by this prime-boost immunization regime.

However, in practice the induction of CTL responses was not observed following this approach **(Fig. Ad9)**, even though this recombinant virus was confirmed able to infect target cells and express GTSHP **(Fig. Ad8)**, and IgG2a antibodies were generated after the injection of GTSHP proteins (data not shown). Recombinant adenovirus has been successfully applied to induce antigen specific CTL responses in mice and non-human primates for the development of HIV vaccines (Pinto, et al. 2003; Shiver, and Emini, 2004; Shiver, et al. 2002). However, when mice received one dose or even two doses of Ad/GTSHP, CTL responses were not induced **(Fig. Ad9)**. Together with these results and the confirmation that the CTL epitope cloned in the GTSHP construct is a dominant epitope in BALB/c mice **(Fig. tat18-4)**, presentation of this epitope in class-I MHC molecules might be suspected somehow fail, so epitope specific CTL responses were not observed. It is known that residues flanking a CTL epitope strongly influence its liberation efficiency by the

proteasome system (Holzhutter, et al. 1999; Theobald, et al. 1998), and might combine with a CTL epitope to generate a junction epitope. The presence of such a junction epitope could create undesired immunodominance effects, redirecting the immune response to irrelevant epitopes and in some cases suppressing the induction of responses to the desired epitpes (Perkins, et al. 1991). Not only epitopes for CTLs but also epitopes for Th cells are required to achieve successful CTL responses (Livingston, et al. 2002; Von Herrath, et al. 1996). Ubiquitination of proteins results in proteolysis by proteasomes. Since peptide cargos of MHC class I molecules are derived from a proteasome-mediated proteolysis pathway, ubiquitination will increase peptide presentation by the host cells (Rodriguez, et al. 1998; Velders, et al. 2001). Therefore, the existence of ubiquitination conserve sequence would be another item required to be check on GTSHP construct. Interestingly, when mice were injected with Ad/GTSHP the base values of specific lysis on CTL assays were obviously higher than that of mice injected with GTSHP proteins **(Fig. Ad9-2,-4)**. This non-specific killing effect might be caused by NK cells which could be activated after the immunization with Ad/GTSHP.

4. Transfer SV5 v protein into target cells.

SIV tat protein has been shown to carry fusion proteins to bind cultured cell surfaces, but the ability to transverse cell membrane remains unclear. Therefore, to examine this ability, a functional assay was introduced in which the SV5 v protein was genetically fused with SIV tat protein and expressed in the bacterial system. Since SV5 v protein can cause protease-mediated degradation of stat1 protein which leads to block interferon signaling in host cells (Didcock, et al. 1999), this phenomena might be observed if SIV tat is authentically able to transverse cell membranes. When cultured cells were incubated with tat/v fusion proteins, cell surfaces were evenly covered with tat/v proteins **(Fig. tv3)** but the degradation of stat1 was not detected **(Fig. tv4)**. The function of v protein might be compromised in the tat/v fusion construction, so the gene of tat/v was cloned into a mammalian expression vector to examine the function of tat/v protein. Transiently expressed in cultured cells, tat/v was shown functional to block

interferon signaling **(Fig. tv6)**, which suggested that the function of v protein can be hardly affected by the fusion of tat protein. The v domain of tat/v might be not functional in the bacterial expression system, so further examination would be required to confirm the function of v protein produced by bacteria. The HIV tat protein was well known to transverse cell membranes from HIV infected cells to uninfected cells, and this ability was mainly contributed by the arginine rich domain (YGRKKRRQRRR) (Ensoli, et al. 1993). Since SIV tat contains a similar arginine rich domain (RKRRRTPKKAK), we might expect that when SIV tat are transiently expressed in cultured cells they can spread to the surrounding cells. However, there was no obvious difference between the distribution of tat/v and v proteins in trnasfected cells **(Fig. tv7)**. Since the green fluorescent protein (GFP) has been widely and successfully applied to demonstrate the membrane-penetration activity of HIV tat protein and peptides, GFP was genetically fused with tat/v to examine the SIV tat protein. This fusion protein was shown to bind to cultured cell surfaces rather than locate inside target cells **(Fig. tv9)**, and the degradation of stat1 in target cells was not observed either **(Fig. tv10)**. Together, these results described above all seem to suggest that SIV tat can not deliver an attached protein into target cells in the same fashion as HIV tat performed.

The intercellular transportation property of other viral proteins is also questioned in several groups. The arginine rich region (YGRKKRRQRRR) of HIV tat was reported to deliver an attached protein into cultured cells (Nagahara, et al. 1998), but in our previous work a recombinant protein containing this sequence and SV5 v protein was not shown to translocate into cultured cells (data not shown). Furthermore, this sequence was believed not to deliver IκB protein into cultured cells (R. T. Hay, personal communication). Other group also argue that this sequence has no detectable transmembrane activity when attached to a full-length protein synthesized de novo (Leifert, et al. 2002). The herpes simples virus protein VP22 is secreted from infected cells and imported to the nuclei of adjacent cells. However, it was strongly suggested that the ability of VP22 fusion proteins to enter cells may be due to the artificial effect during fixation for immunocytochemistry (Melan and Sluder, 1992; Lundberg and Johansson, 2001; Lundberg, et al. 2003).

5. Transfer SV5 v gene into target cells.

SV5 v protein has been known to cause proteasome-mediated degradation of stat1 protein, therefore leading to block interferon signaling in host cells. Furthermore, it has been reported that the size of virus plaques and the production of interferon sensitive virus were increased in v-expressing cells (Young, et al. 2003). Therefore, if SIV tat is of capability to transverse cell membranes, the tat/v fusion protein would be a potentially useful reagent to help isolation and generation of interferon sensitive viruses, enhancing vaccine developments. Although this approach was dampened by the incapacity of SIV tat, transient expression of v proteins in target cells would be a feasible approach to reach the goal described above. Therefore, the recombinant adenovirus Ad/v (carrying SV5 v gene) was generated, and used to infect cultured cells. Encouragingly, SV5 v genes were efficiently transferred into cultured cells, and the degradation of stat1 was associated with the expression of v proteins **(Fig. tv12)**. It would be convenient using Ad/v to infect various cell lines for isolation and generation of various virus types, since the time consuming process for generating v-expressing cell lines will be avoided. In gene therapy practices, it appears that T cell dependent, antigen-specific immunity limits the duration of transduced gene expression following inoculation of naïve animals and largely prevents gene expression following secondary administration (Barr, et al. 1995; Dai, et al. 1995; Engelhardt, et al. 1994). Ad/v might be applied to interrupt interferon responses which might be able to modulate or block host immune responses transiently. Administration of Ad/v might only results in transient immunosuppression and minimized effects on pre-existing immunity, in favor of the achievement of gene therapy. Furthermore, Ad/v could be potentially used to establish a drug screening system to identify drugs against infection with interferon sensitive viruses.

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Appendix

Information in this part was adapted from Diassiti (Diassiti, 2002).

1. DNA sequence of cloning vector pGEX-2T.

1 acgttatcga ctgcacggtg caccaatgct tctggcgtca ggcagccatc ggaagctgtg
61 gtatggctgt gcaggtcgta aatcactgca taattcgtgt cgctcaaggc gcactcccgt
121 tctggataat gtttttgcg ccgacatcat aacggttctg gcaaatattc tgaaatgagc
181 tgttgacaat taatcatcgg ctcgtataat gtgtggaatt gtgagcggat aacaatttca
241 cacaggaaac agtattcatg tcccctatac taggttattg gaaaattaag ggccttgtgc
301 aacccactcg acttcttttg gaatatcttg aagaaaaata tgaagagcat ttgtatgagc
361 gcgatgaagg tgataaatgg cgaaacaaaa agtttgaatt gggtttggag tttcccaatc
421 ttccttatta tattgatggt gatgttaaat taacacagtc tatggccatc atacgttata
481 tagctgacaa gcacaacatg ttgggtggtt gtccaaaaga gcgtgcagag atttcaatgc
541 ttgaaggagc ggttttggat attagatacg gtgtttcgag aattgcatat agtaaagact
601 ttgaaactct caaagttgat tttcttagca agctacctga aatgctgaaa atgttcgaag
661 atcgtttatg tcataaaaca tatttaaatg gtgatcatgt aacccatcct gacttcatgt
721 tgtatgacgc tcttgatgtt gttttataca tggacccaat gtgcctggat gcgttcccaa
781 aattagtttg ttttaaaaaa cgtattgaag ctatcccaca aattgataag tacttgaaat
841 ccagcaagta tatagcatgg cctttgcagg gctggcaagc cacgtttggt ggtggcgacc
901 atcctccaaa atcggatctg gttccgcgtg gatccccggg aattcatcgt gactgactga
961 cgatctgcct cgcgcgtttc ggtgatgacg gtgaaaacct ctgacacatg cagctcccgg
1021 agacggtcac agcttgtctg taagcggatg ccgggagcag acaagcccgt cagggcgcgt
1081 cagcgggtgt tggcgggtgt cggggcgcag ccatgaccca gtcacgtagc gatagcggag
1141 tgtataattc ttgaagacga aagggcctcg tgatacgcct atttttatag gttaatgtca
1201 tgataataat ggtttcttag acgtcaggtg gcacttttcg gggaaatgtg cgcggaaccc
1261 ctatttgttt atttttctaa atacattcaa atatgtatcc gctcatgaga caataaccct
1321 gataaatgct tcaataatat tgaaaaagga agagtatgag tattcaacat ttccgtgtcg
1381 cccttattcc cttttttgcg gcattttgcc ttcctgtttt tgctcaccca gaaacgctgg
1441 tgaaagtaaa agatgctgaa gatcagttgg gtgcacgagt gggttacatc gaactggatc
1501 tcaacagcgg taagatcctt gagagttttc gccccgaaga acgttttcca atgatgagca
1561 cttttaaagt tctgctatgt ggcgcggtat tatcccgtgt tgacgccggg caagagcaac
1621 tcggtcgccg catacactat tctcagaatg acttggttga gtactcacca gtcacagaaa
1681 agcatcttac ggatggcatg acagtaagag aattatgcag tgctgccata accatgagtg
1741 ataacactgc ggccaactta cttctgacaa cgatcggagg accgaaggag ctaaccgctt
1801 ttttgcacaa catgggggat catgtaactc gccttgatcg ttgggaaccg gagctgaatg
1861 aagccatacc aaacgacgag cgtgacacca cgatgcctgc agcaatggca acaacgttgc
1921 gcaaactatt aactggcgaa ctacttactc tagcttcccg gcaacaatta atagactgga
1981 tggaggcgga taaagttgca ggaccacttc tgcgctcggc ccttccggct ggctggttta
2041 ttgctgataa atctggagcc ggtgagcgtg ggtctcgcgg tatcattgca gcactggggc
2101 cagatggtaa gccctcccgt atcgtagtta tctacacgac ggggagtcag gcaactatgg
2161 atgaacgaaa tagacagatc gctgagatag gtgcctcact gattaagcat tggtaactgt
2221 cagaccaagt ttactcatat atactttaga ttgatttaaa acttcatttt taatttaaaa
2281 ggatctaggt gaagatcctt tttgataatc tcatgaccaa aatcccttaa cgtgagtttt
2341 cgttccactg agcgtcagac cccgtagaaa agatcaaagg atcttcttga gatccttttt

2401 ttctgcgcgt aatctgctgc ttgcaaacaa aaaaaccacc gctaccagcg gtggtttgtt
2461 tgccggatca agagctacca actcttttte cgaaggtaac tggcttcagc agagcgcaga
2521 taccaaatac tgtccttcta gtgtagccgt agttaggcca ccacttcaag aactctgtag
2581 caccgcctac atacctcgct ctgctaatcc tgttaccagt ggctgctgcc agtggcgata
2641 agtcgtgtct taccgggttg gactcaagac gatagttacc ggataaggcg cagcggtcgg
2701 gctgaacggg gggttcgtgc acacagccca gcttggagcg aacgacctac accgaactga
2761 gatacctaca gcgtgagcta tgagaaagcg ccacgcttcc cgaagggaga aaggcggaca
2821 ggtatccggt aagcggcagg gtcggaacag gagagcgcac gagggagctt ccagggggaa
2881 acgcctggta tctttatagt cctgtcgggt ttcgccacct ctgacttgag cgtcgatttt
2941 tgtgatgctc gtcaggggggg cggagcctat ggaaaaacgc cagcaacgcg gccttttac
3001 ggttcctggc cttttgctgg ccttttgctc acatgttctt tcctgcgtta tcccctgatt
3061 ctgtggataa ccgtattacc gcctttgagt gagctgatac cgctcgccgc agccgaacga
3121 ccgagcgcag cgagtcagtg agcgaggaag cggaagagcg cctgatgcgg tattttctcc
3181 ttacgcatct gtgcggtatt tcacaccgca taaattccga caccatcgaa tggtgcaaaa
3241 cctttcgcgg tatggcatga tagcgcccgg aagagagtca attcagggtg gtgaatgtga
3301 aaccagtaac gttatacgat gtcgcagagt atgccggtgt ctcttatcag accgtttccc
3361 gcgtggtgaa ccaggccagc cacgtttctg cgaaaacgcg ggaaaaagtg gaagcggcga
3421 tggcggagct gaattacatt cccaaccgcg tggcacaaca actggcgggc aaacagtcgt
3481 tgctgattgg cgttgccacc tccagtctgg ccctgcacgc gccgtcgcaa attgtcgcgg
3541 cgattaaatc tcgcgccgat caactgggtg ccagcgtggt ggtgtcgatg gtagaacgaa
3601 gcggcgtcga agcctgtaaa gcggcggtgc acaatcttct cgcgcaacgc gtcagtgggc
3661 tgatcattaa ctatccgctg gatgaccagg atgccattgc tgtggaagct gcctgcacta
3721 atgttccggc gttatttctt gatgtctctg accagacacc catcaacagt attattttct
3781 cccatgaaga cggtacgcga ctgggcgtgg agcatctggt cgcattgggt caccagcaaa
3841 tcgcgctgtt agcgggccca ttaagttctg tctcggcgcg tctgcgtctg gctggctggc
3901 ataaatatct cactcgcaat caaattcagc cgatagcgga acgggaaggc gactggagtg
3961 ccatgtccgg ttttcaacaa accatgcaaa tgctgaatga gggcatcgtt cccactgcga
4021 tgctggttgc caacgatcag atggcgctgg gcgcaatgcg cgccattacc gagtccgggc
4081 tgcgcgttgg tgcggatatc tcggtagtgg gatacgacga taccgaagac agctcatgtt
4141 atatcccgcc gttaaccacc atcaaacagg attttcgcct gctggggcaa accagcgtgg
4201 accgcttgct gcaactctct cagggccagg cggtgaaggg caatcagctg ttgcccgtct
4261 cactggtgaa aagaaaaacc accctggcgc ccaatacgca aaccgcctct ccccgcgcgt
4321 tggccgattc attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag
4381 cgcaacgcaa ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg
4441 cttccggctc gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc
4501 tatgaccatg attacggatt cactggccgt cgttttacaa cgtcgtgact gggaaaaccc
4561 tggcgttacc caacttaatc gccttgcagc acatcccccc ttcgccagct ggcgtaatag
4621 cgaagaggcc cgcaccgatc gcccttccca acagttgcgc agcctgaatg gcgaatggcg
4681 ctttgcctgg tttccggcac cagaagcggt gccggaaagc tggctggagt gcgatcttcc
4741 tgaggccgat actgtcgtcg tcccctcaaa ctggcagatg cacggttacg atgcgcccat
4801 ctacaccaac gtaacctatc ccattacggt caatccgccg tttgttccca cggagaatcc
4861 gacgggttgt tactcgctca catttaatgt tgatgaaagc tggctacagg aaggccagac
4921 gcgaattatt tttgatggcg ttggaatt
//

KEY: **Forward Primer**, **Multiple Cloning Site**, **Reverse Primer**.

2. DNA and amino acid sequences of GST protein.

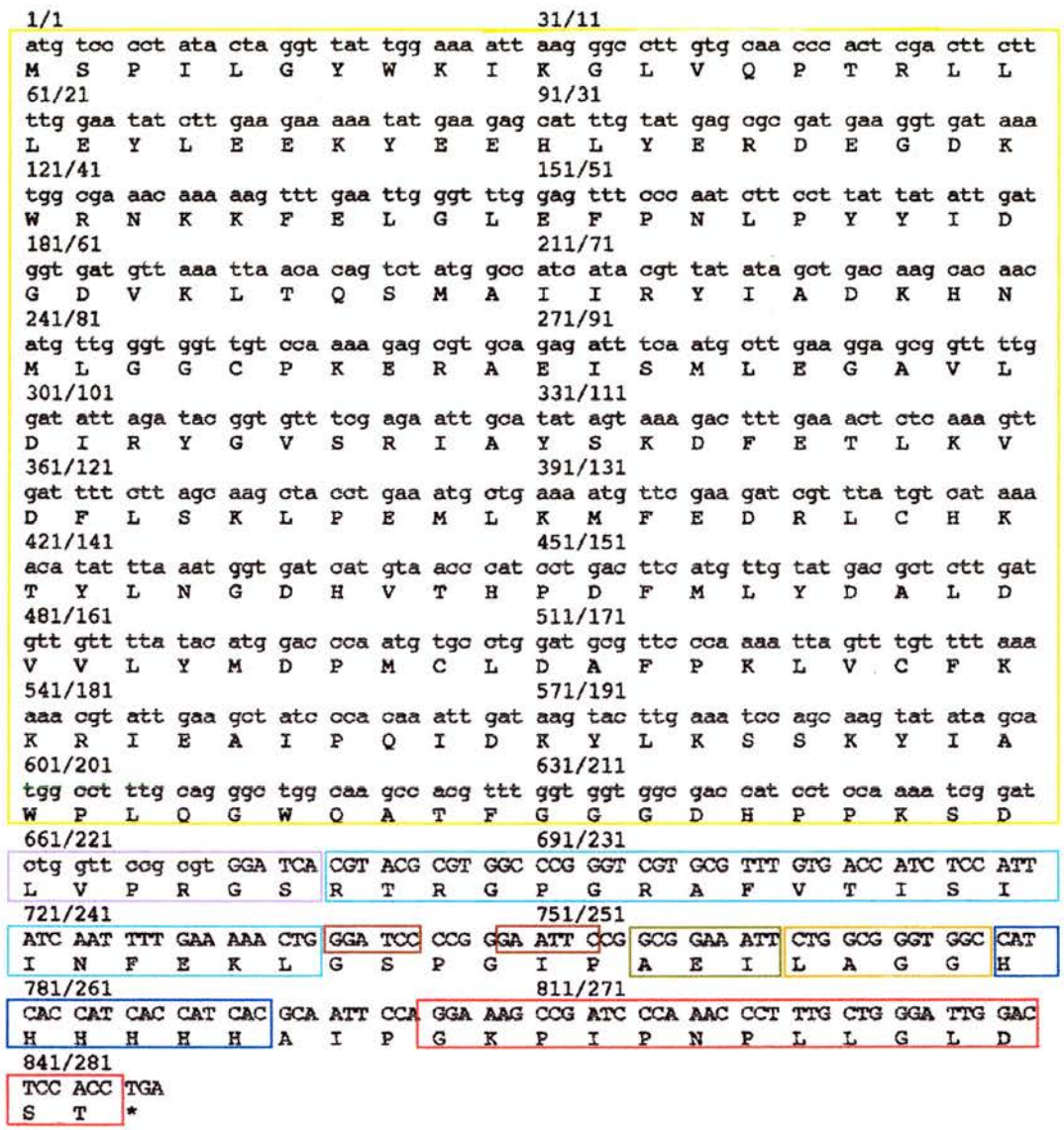
```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K
```

3. DNA and amino acid sequences of protein translation regions of pGHP.

```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                             691/231
ctg gtt ccg cgt GGA TCC CCG GGA ATT CCG GCG GAA ATT CTG GCG GGT GGC CAT CAC CAT
L   V   P   R   G   S   P   G   I   P   A   E   I   L   A   G   G   H   H   H
721/241                                             751/251
CAC CAT CAC GCA ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC
H   H   H   A   I   P   G   K   P   I   P   N   P   L   L   G   L   D   S   T
781/261
TGA
*
```

KEY: GST, pGEX, BamHI, EcoRI, spacer, APCS, HIS, PK.

4. DNA and amino acid sequences of protein translation regions of pGCHP.



KEY: GST, pGEX, CTLs, BamHI, EcoRI, spacer, APCS, HIS, PK.

5. DNA and amino acid sequences of protein translation regions of pGTHP.

```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                             691/231
ctg gtt ccg cgt GGA TCC ATG GAG ACA CCC TTG AGG GAG CAG GAG AAC TCA TTA GAA TCC
L   V   P   R   G   S   M   E   T   P   L   R   E   Q   E   N   S   L   E   S
721/241                                             751/251
TCC AAC GAG CGC TCT TCA TGC ATT TCA GAG GCG GAT GCA ACC ACT CCA GAA TCG GCC AAC
S   N   E   R   S   S   C   I   S   E   A   D   A   T   T   P   E   S   A   N
781/261                                             811/271
CTG GGG GAG GAA ATC CTC TCT CAA CTA TAC CGC CCT CTA GAG GCG TGC TAT AAC ACA TGC
L   G   E   E   I   L   S   Q   L   Y   R   P   L   E   A   C   Y   N   T   C
841/281                                             871/291
TAT TGT AAA AAG TGT TGC TAC CAT TGC CAG TTT TGT TTT CTT AAA AAG GGA TTG GGG ATA
Y   C   K   K   C   C   Y   H   C   Q   F   C   F   L   K   K   G   L   G   I
901/301                                             931/311
TGT TAT GAG CAG TCA CgA AAG cGt cGc cGt ACT cCG AAA AAG GCT AAG GCT AAT ACA TCT
C   Y   E   Q   S   R   K   R   R   R   T   P   K   K   A   K   A   N   T   S
961/321                                             991/331
TCT GCA TcA AAC AAC AgA CCC ATA TCC AAC AGG ACC CGG CAC TGC CAA CCA GAG AAG GCA
S   A   S   N   N   R   P   I   S   N   R   T   R   H   C   Q   P   E   K   A
1021/341                                            1051/351
AAG AAG GAG ACG GTG GAg AAG GCG GTG GCA ACA GCT CCT GGC CTT GGC AGA GGa att cCG
K   K   E   T   V   E   K   A   V   A   T   A   P   G   L   G   R   G   I   P
1081/361                                            1111/371
gcg gaa att ctg gcg ggt ggc cat cac cat cac cat cac GcA ATT gCA GGA AAG CCG ATC
A   E   I   L   A   G   G   H   H   H   H   H   H   A   I   A   G   K   P   I
1141/381                                            1171/391
CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
P   N   P   L   L   G   L   D   S   T   *
```

KEY: GST, pGEX, BamHI, TAT, EcoRI, spacer, APCS, HIS, PK.

6. DNA and amino acid sequences of protein translation regions of pGCTHP.

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1/1                                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                                             691/231
ctg gtt ccg cgt GGA TCA CGT ACG CGT GGC CCG GGT CGT GCG TTT GTG ACC ATC TCC ATT
L   V   P   R   G   S   R   T   R   G   P   G   R   A   F   V   T   I   S   I
721/241                                                             751/251
ATC AAT TTT GAA AAA CTG GGA TCC ATG GAG ACA CCC TTG AGG GAG CAG GAG AAC TCA TTA
I   N   F   E   K   L   G   S   M   E   T   P   L   R   E   Q   E   N   S   L
781/261                                                             811/271
GAA TCC TCC AAC GAG CGC TCT TCA TGC ATT TCA GAG GCG GAT GCA ACC ACT CCA GAA TCG
E   S   S   N   E   R   S   S   C   I   S   E   A   D   A   T   T   P   E   S
841/281                                                             871/291
GCC AAC CTG GGG GAG GAA ATC CTC TCT CAA CTA TAC CGC CCT CTA GAG GCG TGC TAT AAC
A   N   L   G   E   E   I   L   S   Q   L   Y   R   P   L   E   A   C   Y   N
901/301                                                             931/311
ACA TGC TAT TGT AAA AAG TGT TGC TAC CAT TGC CAG TTT TGT TTT CTT AAA AAG GGA TTG
T   C   Y   C   K   K   C   C   Y   H   C   Q   F   C   F   L   K   K   G   L
961/321                                                             991/331
GGG ATA TGT TAT GAG CAG TCA CGA AAG CGT CGC CGT ACT CCG AAA AAG GCT AAG GCT AAT
G   I   C   Y   E   Q   S   R   K   R   R   R   T   P   K   K   A   K   A   N
1021/341                                                            1051/351
ACA TCT TCT GCA TCA AAC AAC AGA CCC ATA TCC AAC AGG ACC CGG CAC TGC CAA CCA GAG
T   S   S   A   S   N   N   R   P   I   S   N   R   T   R   H   C   Q   P   E
1081/361                                                            1111/371
AAG GCA AAG AAG GAG ACG GTG GAG AAG GCG GTG GCA ACA GCT CCT GGC CTT GGC AGA GGA
K   A   K   K   E   T   V   E   K   A   V   A   T   A   P   G   L   G   R   G
1141/381                                                            1171/391
ATT CCT GCG GAA ATT CTG GCG GGT GGC CAT CAC CAT CAC CAT CAC GCA ATT CCA GGA AAG
I   P   A   E   I   L   A   G   G   H   H   H   H   H   H   A   I   P   G   K
1201/401                                                            1231/411
CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
P   I   P   N   P   L   L   G   L   D   S   T   *
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KEY: GST, pGEX, CTLs, BamHI, TAT, EcoRI, spacer, APCS, HIS, PK.