MARJA TÄHTINEN

Characterisation of Humoral Immune Responses to HIV-1 Non-Structural Proteins Nef, Rev and Tat in HIV-1 Infected Individuals and in Immunised Animals

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ACADEMIC DISSERTATION

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Supervised by
Professor Kai Krohn
University of Tampere

Reviewed by
Professor Antti Vaheri
University of Helsinki
Professor Timo Vesikari
University of Tampere

Distribution

University of Tampere
Sales Office
P.O. Box 617
33101 Tampere
Finland

Tel. +358 3 215 6055
Fax +358 3 215 7685
taju@uta.fi
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ABBREVIATIONS

aa	:	Amino acid
AIDS	:	Acquired immunodeficiency syndrome
ASX	:	Asymptomatic HIV-seropositive
ARC	:	AIDS-related complex
Arg	:	Arginine
BPV	:	Bovine papilloma virus
BSA	:	Bovine serum albumin
CMV	:	Cytomegalovirus
CTL	:	Cytotoxic T-lymphocyte
Cys	:	Cysteine
DC	:	Dendritic cells
ELISA	:	Enzyme-linked immunosorbent assay
FGF-1	:	Fibroblast growth factor -1
GG	:	Gene gun immunisation
GSH	:	Reduced form of glutathione
Gln	:	Glutamine
Gly	:	Glycine
HIV-1	:	Human immunodeficiency virus type-1
ID	:	Intradermal immunisation
IFN-γ	:	Interferon-γ
IL-1, IL-2	:	Interleukin-1, Interleukin-2
IM	:	Intramuscular immunisation
kb	:	Kilobase
KS	:	Kaposi´s sarcoma
LAS		Lymphadenopathy syndrome
Leu	:	Leucine
LTR	:	Long terminal repeat
MAPK	:	Mitogen-activated protein kinase
MCP-1	:	Monocyte chemoattractant protein -1
MHC	:	Major histocompatibility complex
MIP1-α	:	Macrophage inflammatory protein 1 alpha
MIP1-β	:	Macrophage inflammatory protein 1 beta

MVA	:	Modified vaccinia virus Ankara
Nef	:	Negative factor
NES	:	Nuclear export signal
NK	:	Natural killer cell
NLS	:	Nuclear localisation signal
NMR	:	Nuclear magnetic resonance
PAF	:	Platelet activating factor
PAK	:	p21-activated kinase
PBL	:	Peripheral blood lymphocyte
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
Phe	:	Phenylalanine
Pro	:	Proline
Rev	:	Regulator of expression of virion proteins
RRE	:	Rev-responsive element
Ser	:	Serine
SHIV	:	Chimeric SIV/HIV virus
SIV	:	Simian immunodeficiency virus
TAK	:	Tat-associated kinase
TAR	:	Trans-activation-responsive element
Tat	:	Trans-activating transcriptional protein
TBS	:	Tris-buffered saline
TGFβ-1	:	Transforming growth factor beta-1
Th1, Th2	:	T-helper type 1, T-helper type 2
Thr	:	Threonine
TNFα	:	Tumor necrosis factor alpha
Tyr	:	Tyrosine

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) - a disease clinically characterised by fatal opportunistic infections and malignancies - was first described in 1981 (Gottlieb et al. 1981, Masur et al. 1981). A few years later, the causative retrovirus was isolated (Barré-Sinoussi et al. 1983, Gallo et al. 1984) and denominated first as HTLV-III/LAV, later as human immunodeficiency virus type-1 (HIV-1).

In the year 2000, more than 36 million people in the world are infected by HIV. As this infection is lethal, most of them will die within a decade, although considerable variation exists in the progression time from the non-symptomatic stage to full-blown AIDS. HIV infection is characterised by a depletion of CD4+ cells (Gottlieb et al. 1981, Fahey et al. 1984) leading ultimately to an aberrant CD4/CD8 ratio. In addition to the quantitative defects, CD4+ cells are defective in their capability to proliferate upon antigen or mitogen stimulation (Gottlieb et al. 1981, Lane et al. 1985), and to produce lymphokines (Murray et al. 1984). Defective NK- and B-cell functions can also be seen in infected subjects (Lane et al. 1983, Bonavida et al. 1986). This destruction of the immune system favours opportunistic infections (e.g. Pneumocystis carinii, cytomegalovirus, Candida albicans, Cryptosporidium, Salmonella, Toxoplasma).

Given the scale of the AIDS pandemic and the long time required for development and distribution of an AIDS vaccine, the number of vaccines now in clinical testing is totally inadequate. After more than 15 years of research on HIV, only one vaccine concept is being tested for efficacy in humans (Phase III trials). Therefore, constant efforts to learn more about the immunogenicity of the virus and to develop new potentially useful immunogens to defeat HIV are needed.

The purpose of this thesis was to investigate the humoral immunogenicity of HIV-1 Nef, Rev and Tat proteins and evaluate their applicability for DNA vaccines.

REVIEW OF THE LITERATURE

1. HIV-1 – a Trojan horse among viruses

HIV-1 is a member of the *Retroviridae* family and belongs to the subfamily of the *Lentivirinae*, like maedi visna virus (MVV), caprine arthritis encephalitis virus (CAEV), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV) and simian immunodeficiency virus (SIV). These viruses are non-oncogenic pathogens, and they can persist in the infected host for years causing slowly proceeding diseases.

HIV-1 has several wily ways to remain persistent and avoid the immune responses of the host. Firstly, it infects cells of the immune system and disables their normal function (Laurence 1985, Margolis 1998). Secondly, the HIV-1 genome can stay within a cell for a long, clinically latent period when the delicate balance between virus replication and the immune response to the virus determines both the outcome of the infection and its rate of progression. Furthermore, as activation of HIV-infected T cells or monocyte/macrophages during normal immune responses results in the spread of HIV virions, this virus has developed the ability to use normal immune processes to its own reproductive advantage (Stevenson et al. 1990). Thirdly, through the high antigenic variation caused by the infidelity of reverse transcriptase (Roberts et al. 1988), HIV-1 can evade immune attacks against the outer envelope protein (Watkins et al. 1996). Finally, cell-to-cell fusion seen in HIV infection (Phillips 1994) allows the virus to spread without entering the extracellular fluid thus escaping the effects of antibodies.

1.1. Genomic structure of HIV-1

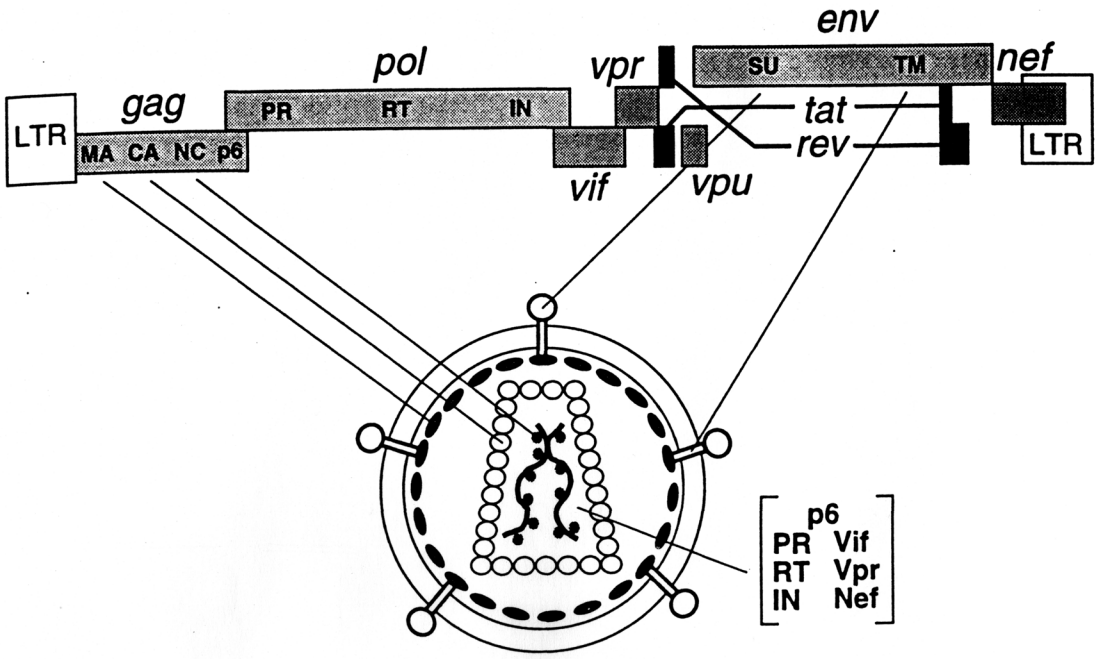
The HIV-1 genome consists of a >9 kb-long RNA molecule encoding nine open reading frames (Ratner et al. 1985, Wain-Hobson et al. 1985) (Fig. 1). Three of these encode the Gag, Pol and Env polyproteins, which are subsequently proteolysed into individual proteins common to all retroviruses. HIV-1 also codes for four accessory proteins with various functions (Vif, Vpr, Vpu and Nef) and two regulatory proteins (Rev and Tat) necessary for virus replication (Frankel et al. 1998, Turner et al. 1999). Two covalently joined molecules of viral genomic RNA are packaged into each viral particle (Fig. 1)

At both ends of the HIV genome there are areas called long terminal repeats (LTR) which contain the promoters and polyadenylation signals for viral transcription (Hughes 1983, Starcich et al. 1985). These areas are also needed for integration of viral DNA into the host cell genome, and they contain regulatory elements mediating the transmission of cellular activation signals (Al-Harthi et al. 1998).

Gag produces four proteins (Mervis et al. 1988): matrix (MA, p17) lines the inner surface of the virion membrane and is important for targeting Gag and Gag-Pol polyproteins to the plasma membrane before viral assembly; capsid (CA, p24) forms the conical core of virion and has important roles in virion assembly and uncoating; nucleocapsid (NC, p9) coats the genomic RNA inside the virion core and targets it to virion assembly, and p6 is responsible for Vpr binding, as well as mediating efficient particle release (Freed 1998, Frankel et al. 1998, Turner et al. 1999).

The pol open reading frame codes for three viral enzymes: protease (PR), reverse transciptase (RT) and integrase (IN). These are necessary for cleavage of viral polyproteins, synthesis of a DNA copy of viral RNA genome and integration of the viral genome into the host cell genome, respectively (Katz et al. 1994, Frankel et al. 1998, Turner et al. 1999).

Figure 1. Organisation of the HIV-1 genome and virion (adapted from Frankel and Young 1998).



Env polyprotein (gp160) can be dissociated into two glycoproteins, gp120 (surface, SU) and gp41 (transmembrane, TM), which together form the outer membrane of the virion. SU is a 515 amino acid long protein, and it contains five highly variable loops (V1-V5); on the average only 66% of SU amino acids are conserved, and in the most variable regions this percent is only 10 (Starcich et al. 1986, Modrow et al. 1987). The main function of SU is to bind to the major receptor of HIV, the CD4 molecule, present on helper T-cells and primary macrophages (McKeating et al. 1989, Wyatt et al. 1998). Upon binding, conformational changes in SU expose a surface able to bind to chemokine receptors CCR5, CXCR4 or CCR3, which serve as essential viral co-receptors (Clapham et al. 1997).

The primary function of TM is to mediate fusion between the viral and cellular membranes (McKeating et al. 1989, Wyatt et al. 1998). The N-terminus of TM is needed for initiation of this process whereas the transmembrane region is important for gp120 anchoring and proceeding of fusion. The intraviral C-terminus interacts with MA. TM contains no highly variable regions: 80% of its amino acids are conserved (Modrow et al. 1987).

The accessory proteins Vif, Vpr and Vpu have various functions. Vif is a protein that enhances the infectivity of virus particles and stability of viral DNA; it also may play a role in virion assembly (Simon et al. 1996, Cohen et al. 1996). Vpr functions by transporting viral component into the nucleus, and it may induce G2 cell cycle arrest preventing the proliferation of HIV-infected cells (Cohen et al. 1996, Frankel et al. 1998). Both Vif and Vpr are located in the virion particle. Vpu promotes degradation of intracellular CD4 molecules, thus helping newly synthesized Env molecules to transfer to the cell membrane for virion assembly. Vpu can also down-regulate MHC class proteins and stimulate virion release (Jabbar 1995, Frankel et al. 1998).

The structure and function of Nef, Rev and Tat proteins, which play the leading part in this thesis, are reviewed in more detail in the following three chapters.

1.2. Accessory protein Nef – a story of missing function

The Nef gene found from HIV-1 is unique to primate immunodeficiency viruses. It codes for a 27-kDa protein which is post-translationally modified by myristoylation

and phosphorylation (Arya et al. 1986). The myristoylation site in the N-terminus of Nef is well conserved (Shugars et al. 1993), indicating the important role of myristoylation in enabling Nef to link the cell membrane and cytoskeleton (Kaminchik et al. 1994, Fackler et al. 1997). With myristoylation, Nef is able to bind to CD4, which leads to down-regulation of this receptor molecule (Harris et al. 1994). Myristoylation is also needed for virion incorporation of Nef (Welker et al. 1998), but the role and significance of Nef in the viral particles is not currently known. Nef may be phosphorylated at several Ser or Thr residues by protein kinase C (Bodeus et al. 1995, Coates et al. 1997). Phosphorylation has been suggested to play a role in the ability of Nef to regulate transcription factors NF-kB and AP-1 (Bandres et al. 1994), and in the ability to down-regulate CD4 molecules (Luo et al. 1997); it probably also has other regulatory effects on Nef function.

Nef is the most abundant viral protein during the early phase of HIV-1 gene expression. In infected cells, it is expressed as a 27-kDa protein capable of forming dimers and trimers, which may be the active form of Nef *in vivo* (Kienzle et al. 1993, Arold et al. 2000, Liu et al. 2000). In addition, a 25-kDa isoform translated from an internal start codon 57 bases downstream from the initiation site can often be seen (Kaminchik et al. 1991). Due to the lack of a myristoylation site, this protein is found in a soluble cytoplasmic form. Some authors have also claimed that the carboxy terminus of Nef could be exposed on the outer surface of virus-infected cells, where it may enhance the cytotoxic response against CD4+ cells as well as target CD4 memory population (Fujii et al. 1996a, Otake et al. 2000). The Nef protein released from destroyed cells is cytotoxic to uninfected CD4+ cells and, as a matter of fact, the sera of HIV-1 infected individuals have been reported to contain significant amounts of soluble Nef, indicating that Nef may participate in helper-T cell destruction in vivo (Fujii et al. 1996b). Extracellular Nef may also stimulate latently infected cells into productive HIV infection (Fujinaga et al. 1995).

Virus particles contain 5-70 molecules of core-bound Nef (Pandori et al. 1996, Welker et al.1996, Kotov et al. 1999). Nef has been shown to enchance the phosphorylation of MA during virion maturation (Swingler et al. 1997). The majority of Nef proteins in virions are present as 20-kDa proteins that are formed from the full-length molecule by cleavage between amino acids 57-58 during the maturation of the

virion (Gaedigk-Nitschko et al. 1995). Viral protease cleaves the C-terminal core domain from the membrane-associated N-terminus, but the significance of this process is not known.

The function of Nef has been obscure. In early studies, it was suggested that Nef acts as a negative factor in virus replication by repressing the transcription from the LTR (Ahmad et al. 1988, Niederman et al. 1989), hence it received its acronym "negative factor". Further studies revealed that although variations in experimental conditions may give conflicting results, Nef is able to induce virus replication through T-cell activation in primary quiescent CD4 cells (De Ronde et al. 1992, Miller et al. 1994, Spina et al. 1994), is needed for maintaining high virus load in persistent virus infections (Kestler et al. 1991), is the major disease determinant in transgenic mice (Hanna et al. 1998) and may contribute to the neuropathogenesis seen in AIDS-patients (Koedel et al. 1999). These effects are enabled by at least four activities associated with Nef: induction of CD4 and MHC class I down-regulation, enhancement of viral infectivity and alterations in cellular signalling pathways.

In CD4-positive cells, Nef is able to down-regulate the expression of CD4 mainly by enhancing its endocytosis as well as lysosomal degradation, which leads to a decrease in the half-life of this protein, whereas the synthesis and intracellular transport of CD4 molecules are not affected by Nef (Aiken et al. 1994, Anderson et al. 1994, Rhee et al. 1994, Bandres et al. 1995). A dileucine-based sorting signal (aa 160-165, E/DXXXLL) in Nef is used to address cellular sorting machinery (Craig et al. 1998). Nef functions in a multistep process, first by dissociating the CD4-$p56^{lck}$ complex that leads to the exposition of an endocytosis motif present in the intracellular domain of CD4 (Rhee et al. 1994, Bandres et al. 1995, Kim et al. 1999). Thereafter, Nef connects CD4 to clathrin-containing adaptor complexes, which function as vesicle coat components in different membrane traffic pathways (Greenberg et al. 1997, Bresnahan et al. 1998, Piguet et al. 1998). Finally, Nef targets internalized CD4 molecules to degradation by connecting CD4 to β-COP protein present in endosomes (Piguet et al. 1999). Various other cellular proteins, including vacuolar ATPase, phospatidylinositol-3-kinase and p35 thioesterase may be involved in Nef-induced CD4 endocytosis (Liu et al. 1997, Lu et al. 1998, Kim et al. 1999). In addition to the

process described above, Nef is also able to suppress the function of a novel protein Naf1, which increases the cell surface CD4 expression (Fukushi et al. 1999). At least when strongly overexpressed in transient transfection systems, extracellular CD4 molecules have been shown to be deleterious for budding virions, either by inhibiting HIV-1 progeny virion release by binding to Env proteins (Ross et al. 1999) or by decreasing the amount of Env incorporated, thus making the virions less infective (Lama et al. 1999). Hence, the purpose of Nef-induced CD4 down-regulation appears to be to enhance the budding or infectivity of virus particles.

The Nef-induced MHC class I and CD4 endocytoses are separate processes: the domains involved in the MHC class I down-regulation consists of an N-terminal alpha-helix (aa 17-26), an acidic stretch (aa 62-66) and a Pro-rich seqment (aa 69-78) of Nef, whereas CD4 binding and down-regulation is mediated through several amino-acids in the core region of Nef (most importantly: aa WLE 57-59, GGL 95-97, RR 105-106, L110, D123, EE 154-155, DD 174-175) (Aiken et al. 1996, Grzesiek et al. 1996, Wiskerchen et al. 1996, Greenberg et al. 1998, Mangasarian et al. 1999, Akari et al. 2000, Liu et al. 2000). In addition, Nef targets the MCH I protein to the trans-Golgi network by connecting the cytoplasmic tail of MHC I to the PACS-1 dependent protein-sorting pathway (Piguet et al. 2000). Decreasing the amount of MHC class I molecules on the cell surface is one mechanism that HIV-1 uses to escape the CTL response directed against virus-infected cells (Collins et al. 1998).

Nef protein enhances the infectivity of virions in a producer cell-dependent manner (Tokunaga et al. 1998a), suggesting that interactions with cellular factors are needed for this process. One possible counterpart may be the Hck tyrosine kinase, as virus particles produced in cells expressing mutated Hck are significantly less infectious (Tokunaga et al. 1998b). In Nef, the Pro-rich sequence (aa 69-78), a conserved RR motif (aa 105-106) and the dileucine motif (aa 160-165) are needed for optimal viral infectivity (Wiskerchen et al. 1996, Craig et al. 1998). The proteolytic cleavage of Nef is not required for the enhanced infectivity (Chen et al. 1998), but association with the core may play some role in infectivity as chimeric viruses containing mutated Nef from different alleles connnected to gag-pol regions from different ones showed variations in infectivity (Ono et al. 2000).

Nef has several effects on cellular signalling pathways. First, through its well conserved proline-rich $(PxxP)_4$ motif it is capable of binding to SH3 domains of several Scr family tyrosine kinases, including Hck, Lyn, Lck, Fyn (Saksela et al. 1995, Collette et al. 1996a, Lee et al. 1996). This interaction with Nef may either enhance the kinase activity of the enzyme (Hck), have no effect (Lyn, c-Src) or suppress their function (Fyn, Lck) (Collette et al. 1996a, Greenway et al. 1996, Briggs et al. 2000). The Hck kinase activation caused by Nef has been reported to cause malignant transformation of fibroblasts (Briggs et al. 1997); similar transformation and association with tyrosine kinase has been earlier reported by Du et al. (1995). Secondly, Nef associates with serine kinases, including various isoforms of protein kinase C (Smith et al. 1996, Ambrosini et al. 1999), members of the mitogen-activated-protein-kinase (MAPK) pathways (Greenway et al. 1995, Hodge et al. 1998, Li et al. 2000, Manninen et al. 2000) and PAK2 (Renkema et al. 1999). Nef may also have effect on PAKs indirectly by activating their regulators (Lu et al. 1996, Fackler et al. 1999). Thirdly, Nef may alter Ca^{2+} homeostasis in a variety of cells (Foti et al. 1999, Zegarra-Moran et al. 1999, Manninen et al. 2000). Finally, the direct binding of Nef to an important component of T-cell receptors may lead to activation of T-cells without antigen stimulation, apoptosis due to Fas-ligand induction and T-cell receptor downregulation (Bell et al. 1998, Xu et al. 1999).

Several other features of Nef may also participate in the enhancement of HIV-1 replication and the pathogenesis caused by it. Within a few hours after infection of cells, Nef stimulates the reverse transcription of proviral DNA (Aiken et al. 1995, Chowers et al. 1995, Schwarz et al. 1995). Also, in infected macrophages the production of MIP1-α and MIP1-β is induced by Nef – a phenomenon which leads to chemotaxis and activation of resting T lymphocytes and permits productive HIV-1 infection (Koedel et al. 1999, Swingler et al. 1999). In addition, Nef may impair the Th1/Th2 cytokine balance by repressing the synthesis of Th1 type cytokines (Luria et al. 1991, Collette et al. 1996b, Haraguchi et al. 1998), although conflicting results showing increased IFN-γ or IL-2 expression caused by Nef also exists (Koedel et al. 1999, Quaranta et al. 1999, Wang et al. 2000).

Taken together, the data gained from several years of intense research has revealed that instead of being a relatively weak negative repressor, Nef protein is an active protein which enhances replication and infectivity of HIV and plays a role in many cellular events taking place in infected cells.

1.3. Regulatory protein Rev – from nucleus to cytoplasm

HIV-1 Rev (regulator of expression of virion proteins) is a 19-kDa phosphoprotein coded by a gene overlapping with *tat* in the +1 reading frame (Sodroski et al. 1986). This protein is essential for HIV replication as it functions by activating the nuclear export of unspliced viral mRNAs (Feinberg et al. 1986), which are needed as templates for translation of Gag and Pol genes, as the precursor RNA for production of diverse subgenomic mRNAs and as viral genome molecules incorporated into new virus particles.

HIV-1 Rev can be divided into two discrete functional domains. The Arg-rich domain consisting of amino acids 34-50 (isolate HXB) contains both the nuclear localisation signal (NLS) (Malim et al. 1989, Böhnlein et al. 1991) and the RNA-binding region (Daly et al. 1989, Böhnlein et al. 1991, Malim et al. 1991a). It is flanked on both sides by sequences that are needed for multimerisation of the protein (Malim et al. 1991a). The Leu-rich domain, spanning residues 75-83, functions as the effector domain able to activate cellular proteins intrinsic to nuclear mRNA transport (Venkatesh et al. 1990, Malim et al. 1991b); in addition, it contains the nuclear export signal (NES) (Fischer et al. 1995, Wen et al. 1995). Rev is phosphorylated at several serine residues (Cochrane et al. 1989, Meggio et al. 1996). However, there is discrepancy over the effect of phosphorylation, as disruption of some phosphorylation sites does not appear to affect Rev function (Cochrane et al. 1989) or seems to be linked to Rev down-regulation (Meggio et al. 1996).

Rev achieves its effect by binding to a *cis*-acting target, the Rev-responsive element (RRE) – a 351-nucleotides-long complex RNA structure that resides within the env intron and is therefore present in all unspliced or single spliced mRNAs (Mann et al. 1994). The specific sequence in RRE involved in primary Rev binding is surprisingly limited: NMR studies, in vitro genetic selection assays and crystal structure analysis have revealed a 34-nucleotides-long RNA hairpin structure binding to Rev amino

acids 34-50, and identified the points of contact between Rev and RRE (Bartel et al. 1991, Battiste et al. 1996, Hung et al. 2000). The structure in this area is distorted by the formation of two non-Watson-Crick purine-purine base pairs which allow the α-helical RNA-binding domain of Rev to enter the major groove and contact specific nucleotides. The rest of RRE is needed for maximal rev reactivity: it ensures appropriate folding and presentation of the high-affinity Rev-binding site and facilitates the oligomerisation of Rev (Mann et al. 1994, Van Ryk et al. 1999).

Rev binds to RRE as a monomer (Cook et al. 1991, Malim et al. 1991), but additional Rev molecules then bind and multimerise so that eight or more Revs may be bound to a single RRE (Daly et al. 1993, Mann et al. 1994, Van Ryk et al. 1999). This oligomerisation stabilises the Rev-binding site in RRE (Charpentier et al. 1997) and is essential for Rev function (Malim et al. 1991a). Thus, a hypothesis has been proposed that the latent phase of HIV infection may be a consequence of insufficient amount of Rev protein leading to delay in the expression of viral antigens (Malim et al. 1991a). Multimerisation can occur also in the absence of RRE (Olsen et al. 1990, Cole et al. 1993).

Rev has been shown to shuttle rapidly between nucleus and cytoplasm (Meyer et al. 1994), and this shuttling cycle is dependent on cellular import and export pathways (Görlich et al. 1996). The Arg-rich NLS of Rev is recognised in the cytoplasm by a transport receptor, importin-β, which interacts directly with nuclear pore complexes (Henderson et al. 1997) mediating the stepwise transport of Rev-importin-β complex into the nucleus. Following translocation, the interaction of importin-β with a nuclear protein, RanGTPase, induces the disassembly and release of Rev into nucleoplasm (Henderson et al. 1997). Because the Arg-rich NLS of Rev also functions as the RNA binding domain, dissociation results in Rev becoming available for binding to RRE. After binding and multimerisation, the Leu-rich NES regions in Rev are still exposed and capable to bind multiple copies of an export receptor, CRM1/exportin-1 connected to RanGTP (Fornerod et al. 1997, Neville et al. 1997). Additional proteins (e.g. Rev interacting protein/Rev activation domain-binding protein Rip/Rab, nucleoporin-like protein1, Nup98, Nup159, Nup214), which are classified as nucleoporins due to the presence of typical Phe-Gly repeats, may also interact with

this export complex (Stutz et al. 1996, Neville et al. 1997, Farjot et al. 1999, Floer et al. 1999, Zolotukhin et al. 1999) or may be necessary in leading the complex out of the nucleus. Once in the cytoplasm, Rev is released from the complex, perhaps through displacement by Rip/Rab (Floer et al. 1999) and can be imported again for further transport cycles.

A number of additional proteins are also capable of interacting with HIV-1 Rev. Two of them are related to cellular splicing (Tange et al. 1996, Powell et al. 1997): binding of the Rev/RRE complex to them inhibits splicing, thus giving Rev an additional method to protect full-length viral mRNA. Rev also binds to a eucaryotic initiation factor 5A (eIF5A) (Ruhl et al. 1993); as binding is necessary for Rev-mediated transport (Bevec et al. 1996) and eIF5A can bind also to CRM1 (Rosorius et al. 1999), this protein may be part of the export complex. Rev also binds to protein B23 (Fankhauser et al. 1991), a nucleolar phosphoprotein whose activities are proposed to play a role in ribosome assembly. Protein B23 inhibits the aggregation of Rev (Szebeni et al. 1999).

An additional interesting feature associated with Rev is the finding that the human endogenous retrovirus K (HERV-K) family can also encode a sequence-specific nuclear export factor binding to CRM1 and to a viral RNA target (Yang et al. 1999, Boese et al. 2000). This HERV-K RNA sequence is also recognised by HIV-1 Rev thus providing evidence for an evolutionary link between HIV-1 and a group of endogenous retroviruses that first entered the human genome approximately 30 million years ago.

1.4. Regulatory protein Tat – a multipotent troublemaker

All lentiviruses encode small RNA-binding proteins which regulate the transcription of the viral genome from the LTR of the virus (Tang et al. 1999). In HIV-1, this nuclear transcriptional activator protein (Tat) is essential for virus replication (Dayton et al. 1986, Fisher et al. 1986), and it acts as an elongation factor (Kao et al. 1987) binding to a 59-residue-long transactivation-responsive region (TAR) present in the 5´ end of the nascent RNA molecule (Dingwall et al. 1989). TAR folds a specific stem loop structure, the configuration of which is essential for transactivation, as mutations destabilising the TAR stem by disrupting base-paring abolish Tat-stimulated

transcription (Selby et al. 1989). The binding of Tat to TAR activates a protein kinase complex (Tat-associated kinase, TAK) which hyperphosphorylates the carboxy-terminus of RNA polymerase II thus leading to a stabilised form of polymerase able to proceed transcription (Karn 1999). As Tat can bind to several transcription factors including TFIID, TFIIB, TFIIH and SP1 (Jeang et al. 1993, Kashanchi et al. 1994, Parada et al. 1996, Veschambre et al. 1997) it may also have some effect to the initiation step of transcription (Laspia et al. 1989). For efficient binding and transcriptional activity in vivo, Tat protein needs to be acetylated by histone acetyltransferases p300 and p300/CBP-associating factor (Kiernan et al. 1999). In addition, even though Tat can form also dimers and trimers, only the monomeric form of Tat is the relevant functional form (Tosi et al. 2000).

HIV-1 infected or tat gene-transfected cells can release Tat via a leaderless secretory pathway into the culture supernatant (Ensoli et al. 1990, Chang et al. 1997) where uninfected cells can take it up; this may lead to trans-activation of various cellular genes (Frankel et al. 1988, Ensoli et al. 1993, Demirhan et al. 1999a). Biologically significant amounts of Tat have also been detected in the sera of HIV-1 infected individuals (Westendorp et al. 1995).

In field isolates of HIV-1, Tat is a 14-kDa protein coded by two exons responsible for residues 1-72 and 73-> , respectively (Sodroski et al. 1985). In laboratory-passaged virus strains (e.g. LAI, HXB2, pNL4-3), a single nucleotide change in amino acid 87 creates a stop codon leading to the expression of a truncated, 86-amino-acid-long protein (Myers et al. 1996). According to the nature of its amino acid sequence, Tat protein can roughly be divided into several domains (Kuppuswamy et al. 1989, Bayer et al. 1995) with various functional and pathogenic characteristics.

An acidic N-terminal region (aa 1-20) forms a stable structure sandwiched between glutamine-rich and core regions (Bayer et al. 1995). It has been shown to bind to T-cell activation marker CD26 and inhibit its dipeptidyl peptidase IV activity (Wrenger et al. 1997), which is necessary for regulation of immune responses (Kähne et al. 1999). Thus, the amino terminus may be responsible for the reported Tat-mediated inhibition of antigen- and mitogen-induced proliferation of PBMCs and T-cell clones (Viscidi et al. 1989, Benjouad et al. 1993, Chirmule et al. 1995, Zagury D et al. 1998),

as well as for the detected impairment of T-cell functions in HIV-infected individuals (Miedema et al. 1988). Also, the amino terminus enhances viral reverse transcription by an as yet unknown method (Ulich et al. 1999).

The Cys-rich region (aa 21-37) contains seven highly conserved cysteine molecules, most of which are essential for virus replication (Sadaie et al. 1990). This region is responsible for the intramolecular disulfide bond formation of Tat (Koken et al. 1994), and it induces HIV replication and participates in TAR-dependent trans-activation (Boykins et al. 1999). Furthermore, it can bind to cell surface reseptors on monocytes and has chemotactic activity on them (Albini et al. 1998a); it also triggers angiogenesis (Boykins et al. 1999), which in part enhances the formation of KS attributed to Tat (Ensoli et al. 1990, 1994).

The core region (aa 38-48) composes a conserved and rigid α-helical structure shown to enhance the binding of Tat to TAR sequence in LTR (Churcher et al. 1993, Bayer et al. 1995). Together with the Cys-rich region, it has chemotactic activity on monocytes (Albini et al. 1998a). Amino acids 1-48 all together have been suggested to circumscribe the minimal activation domain of Tat (Carroll et al. 1991).

The basic region (aa 49-59) is also highly conserved and contains an RKKRRQRRR motif needed for TAR RNA binding (Dingwall et al. 1989, Weeks et al. 1990) and nuclear localisation through a novel import pathway (Hauber et al. 1989, Truant et al. 1999). In addition, this domain can act as a chemo-attractant for dendritic cells and monocytes (Benelli et al. 1998), and it is involved in the uptake of protein from extracellular space (Chang et al. 1997, Vives et al. 1997), in angiogenesis leading to KS (Albini et al. 1996) and in neurotoxic effects of Tat (Sabatier et al. 1991, Weeks et al. 1995).

The Gln-rich region (aa 60-76) forms a rigid structure that pairs with three nucleotides in the TAR loop, thus providing an additional motif in Tat to recognise TAR RNA (Loret et al. 1992).

The C-terminal region (aa 77-101) of Tat was initially considered functionally dispensable as full transactivation capacity was observed with a truncated Tat protein coded by the first exon (Sodroski et al. 1985). However, this C-terminal part has many non-transcriptional effects: it contains an RGD sequence shown to act as a chemo-attractant for dendritic cells and monocytes (Benelli at al. 1998), and it is involved in the integrin-mediated cell adhesion of Tat (Brake et al. 1990b), neuron disorganisation (Kolson et al. 1993, Orsini et al. 1996) and impairment of dendritic cell function (Zocchi et al. 1997). Furthermore, the Tat second exon can hyperactivate human PBLs by inducing IL-2 production (Ott et al. 1997) and bind to a human translation elongation factor leading to the reduced translation of cellular, but not viral, mRNAs (Xiao et al. 1998). The second exon is also needed for Tat-induced apoptosis in T-cell lines and primary CD4 T cells (Bartz et al. 1999).

In addition to the activities described above, Tat has several cellular functions not yet located to any particular domain of the protein. Extracellular Tat can trigger an intracellular signalling cascade by activating various kinases, including c-Jun N-terminal and Src kinases (Ganju et al. 1998, Kumar et al. 1998), phosphatidylinositol 3-kinase (Borgatti et al. 1997), mitogen-activated protein kinases (Ganju et al. 1998, Oshima et al. 2000) and protein kinase C (Borgatti et al. 1998). Some of these effects may be mediated by a T-cell receptor binding protein, p56lck (Manna et al. 2000). Tat can also block L-type Ca^{2+} channels (Poggi et al. 1998) thus leading to impairment of several functions dependent on Ca^{2+} entry. In addition, Tat may cause a significant decline in total intracellular GSH content, which leads to a condition of oxidative stress (Opalenik et al. 1998, Choi et al. 2000). In dopaminergic rat cells, Tat inhibits the expression of tyrosine hydroxylase, the rate-limiting enzyme for the dopamine biosynthetic pathway, as well as release of dopamine into the culture medium (Zauli et al. 2000).

The expression of many cytokines is modulated by Tat: upregulation of TNF, IL-1, IL-2, IL-6, IL-8, IL-10, MCP-1, PAF, TGFβ-1 and FGF-1 by Tat has been detected in various cell lines (Buonaguro et al. 1992, Westerdorp et al. 1994, Blazevic et al. 1996, Conant et al. 1998, Ott et al. 1998, Sawaya et al. 1998, Del Sorbo et al. 1999, Nath et al. 1999), whereas levels of MIP1-α and IL-12 are downregulated by Tat (Sharma et

al. 1996, Ito et al. 1998, Poggi et al. 1998). Extracellular Tat also enhances the expression of chemokine receptors CCR5 and CXCR4 on lymphocytes or monocytes/macrophages, thus rendering bystander cells more susceptible to infection with M- or T-tropic viruses (Huang et al. 1998, Secchiero et al. 1999). In addition, Tat shares homology with CC chemokines and is able to activate chemokine receptors CCR2 and CCR3 by binding to them (Albini et al. 1998b).

2. Humoral immune response against HIV

Primary virus infection often induces detectable antibody and cytotoxic T-lymphocyte (CTL) responses as well as activation of innate immune cells. CTL responses occur early (in 7-10 days) and decrease after resolving virus infection, usually within 2-3 weeks post-infection (Whitton et al. 1996). The antibody response usually peaks later than CTL (in 2-4 weeks), but detectable levels may be found for a lifetime (Whitton et al. 1996). Humoral and cellular responses have different but symbiotic tasks in defending the host in virus infection: antibodies reduce the load of extracellular infectious units, thereby decreasing the number of infected cells that T cells have to deal with, and T cells kill cells soon after infection ensuring that the amount of virus released is minimised thus easing the work of antibodies (Whitton et al. 1996).

2.1. Antibodies against structural proteins of HIV

Antibodies to various HIV-1 proteins appear usually within 1-4 weeks, but cases of seroconversion have been described up to six months after infection (Ranki et al. 1987b, Horsburgh et al. 1989). The appearance of HIV-spesific antibodies is slow as compared to other virus infections. The most immunogenic proteins are gp120, gp41 and p24, and antibodies recognising them may be found during all clinical stages of the infection. Although cell-mediated immune response is needed to destroy HIV-infected cells, humoral immunity may also play a critical role in preventing and modulating infections. Chimpanzees vaccinated with gp160 or V3 peptides were protected against an intraclade challenge, and antibody titer to the V3 loop of gp120 as well as neutralising antibody titers were shown to be correlates of protection (Girard et al. 1995). Furthermore, protection in non-human primates was achieved using passive immunisation of immunoglobulins collected from the serum of HIV-infected individuals or monoclonal antibodies (Prince et al. 1991, Emini et al. 1992, Shibata et al. 1999, Baba et al. 2000, Mascola et al. 2000). Protection was efficient

whether monkeys were challenged intravenously or intravaginally. Similarly, studies in mice constituted with human peripheral blood mononuclear cells exhibiting severe combined immunodeficiency syndrome showed that pre- and post-exposure protection against HIV infection was achieved using immunisations of murine or human monoclonal antibodies (Safrit et al. 1993, Gauduin et al 1997). All studies were performed with antibodies directed against gp120 or gp41. As the tested monoclonal antibodies were specific for the principal neutralisation determinants of HIV, neutralisation is propably needed for protection.

2.2. Neutralising antibodies

Virus neutralising antibodies are antibodies capable of reducing infectivity in vitro in the absence of other inactivating factors. Their HIV-neutralising capacity can be measured by assessing syncytium formation or p24 production in cells infected with antibody treated virus. Neutralising antibodies can be found in the sera of HIV-infected individuals in various disease categories and in the sera of healthy, seropositive homosexuals (Robert-Guroff et al. 1985, Weiss et al. 1985). However, the antibody titers in these individuals, as well as in vaccinated animals, are often low, and cross-clade neutralisation of primary isolates is weak (Poignard et al. 1996). Furthermore, the emergence of mature antibodies with heterologous neutralising activity requires time (Moog et al. 1997, Cole et al. 1998). Instead, certain monoclonal antibodies have strong neutralising activity against a broad spectrum of primary isolates (Poignard et al. 1996).

Consistent with other retroviral systems (Steeves et al. 1974, Grant et al. 1983, Clapham et al. 1984), the envelope glycoproteins are the targets for neutralising antibodies (Rusche et al. 1987, Weiss et al. 1988). A multitude of mechanisms are used in neutralisation: antibodies may decrease virus infectivity before virion attachment to CD4-cells, probably by perturbing some property of the envelope that is required for entry (McDougal et al. 1996), antibodies specific for gp120-CD4 binding site inhibit virion attachment (Ugolini et al. 1997) and antibodies against gp41 act at post-attachment step of infection (Sattentau et al. 1995, Ugolini et al. 1997). Antibodies may also neutralise various virions at later steps by inhibiting uncoating and transription of the virus (Dimmock 1984, Virgin et al. 1994), and in fact, antibodies against internal structures have also been shown to mediate neutralisation

in HIV infection (Sarin et al. 1986, Papsidero et al. 1989, Ferns et al. 1991). In these cases, neutralisation may be a consequence of sequence homology between the internal protein and membrane-associated or secreted proteins (Sarin et al. 1986, Buratti et al. 1997)

2.3. Antibody-dependent cellular cytotoxicity (ADCC)

Antibodies may participate in the killing of infected cells by bridging infected cells to non-immune effector cells (eg. NK cells of macrophages), which are not antigen-specific. Antibodies capable of inducing this antibody-dependent cellular cytotoxicity (ADCC) can be found in the majority of HIV-infected individuals, but their titers decrease as the disease progress (Rook et al. 1987, Goudsmit et al. 1988). However, ADCC may be critical to the control of viral replication in acute infection (Connick et al. 1996), as well as to the maintenance of the latent phase in non-progressors (Baum et al. 1996). In addition to ADCC, antibody-dependent complement-mediated cytotoxicity has been shown in HIV-infected individuals (Sullivan et al. 1996), and recent studies have revealed that an antigenic domain of Nef exposed on the surface of HIV-1-infected cells may be one component that induces this complement-mediated cytotoxicity (Yamada et al. 1999).

ADCC needs three components: target cells that express components of the pathogen on their surface, IgG-class antibodies recognising the pathogen and effector cells bearing the Fc gamma receptor (Ahmad et al. 1996). In HIV-infected individuals, Env proteins are the main targets of ADCC (Evans et al. 1989), although antibodies against p24 also correlate with ADCC (Rook et al. 1987).

At present, there is not much evidence about the participation of Nef-, Rev- or Tat-specific antibodies in the classical mechanisms of humoral dependent virus destruction (e.g. neutralisation and ADCC), yet these antibodies have several effects both in vitro and in vivo. These are described in the following sections.

2.4. Antibodies against HIV-1 Nef

Nef-specific antibodies arise early in the infection and may occasionally precede the occurrence of antibodies toward HIV-1 structural proteins (Ranki et al. 1987a, Ameisen et al. 1989). Nef is highly immunogenic: around 70% of HIV-1-infected

individuals have antibodies against it (Franchini et al. 1987, De Ronde et al. 1988, Sabatier et al. 1989, Cheingsong-Popov et al. 1990, Chen et al. 1999), and high initial levels of Nef-specific antibodies early after infection are associated with a lack of rapid progression to AIDS, thus indicating that Nef antibodies may serve as a clinical marker of disease progression (O´Shea et al. 1991, Reiss et al. 1991, Chen et al. 1999, Yamada et al. 1999). However, conflicting results showing no clear correlation with viral latency or with disease progression also exist (Franchini et al. 1987, Kirshhoff et al. 1991, Cheingsong-Popov et al. 1990, Ranki et al. 1990). These conflicting results may be a consequence of several factors: some of the studies were done using longitudial follow-up and others by evaluating the antibody titers in groups of patients at various disease stages, the amount of sera tested varied (14 versus 267), and the extent and appearance of antibodies during the course of infection also varied considerably between individual patients or animals.

Table 1. B-cell epitopes of Nef

	Human epitopes (aa)
Ameisen et al. (1989)	45 - 69, 148 - 161
Sabatier et al. (1989)	1 - 66, 171 - 205
Kienzle et al. (1991)	1 - 33
Schneider et al. (1991)	26 - 44, 51 - 67, 122 - 135, 143 - 176
Yamada et al. (1999)	87 - 101
	Mouse epitopes (aa)
Schneider et al. (1991)	11 - 24, 28 - 43, 60 - 73, 78 - 103
Ovod et al. (1992)	21 - 41, 31 - 50, 51 - 71, 61 - 80, 151 - 170, 161 - 180
Otake et al. (1994)	1 - 33, 148 - 157, 158 - 206, 192 - 206
	Primate epitopes (aa)
Bahraoui et al. (1990)	17 - 35, 52 - 66, 65 - 146, 185 - 205
Putkonen et al. (1998)	165 - 210
	Single-chain antibody epitopes
Chang et al. (1998)	194 - 206

Nef-antibody assays are often hampered by false positive results, and certain Nef-monoclonals can stain tissue samples taken from uninfected individuals. These non-HIV-derived positive results probably arise from immunological cross-reactivity between Nef and some cellular/viral proteins or contaminants of the antigen (Ranki et al. 1990, Cheingsong-Popov et al. 1990, Parmentier et al. 1992, Scheider et al. 1993). In fact, Nef has been shown to be homological at least with IL-2 receptor and various protein kinases (Samuel et al. 1987), with human thyrotropin receptor (Burch et al. 1991), with a human spumaretroviral gene (Maurer et al. 1987) and with certain neuroactive proteins interacting with K+ channels (Werner et al. 1991). Therefore, many investigators have tried to identify actual Nef-specific B-cell epitopes that are recognised only by the sera of HIV-infected individuals (Ameisen et al. 1989, Sabatier et al. 1989, Kienzle et al. 1991, Schneider et al. 1991, Yamada et al. 1999), by sera of chimpanzees or cynomolgus monkeys immunised with HIV-antigens or plasmid DNA (Bahraoui et al. 1990, Putkonen et al. 1998) or by monoclonal or single-chain antibodies (Schneider et al. 1991, Ovod et al. 1992, Otake et al. 1994, Chang et al. 1998). Overlapping synthetic peptides of varying lengths as well as fusion proteins were used for this purpose. Several linear antigenic regions were characterised (Table 1), and many of the human epitopes were shown to totally or partially overlap with the mouse and non-human primate epitopes. In general, all parts of Nef protein contain antigenic regions capable of inducing antibody synthesis in vivo (Table 1).

2.5. Antibodies against HIV-1 Rev

The immunogenicity of Rev has been demonstrated both in HIV-infected humans and immunised animals (Chanda et al. 1988, Dairaku et al. 1989, Reiss et al. 1989a, Reiss et al.1989b, Devash et al. 1990, Kreusel et al. 1993, Pardridge et al. 1994, Ranki et al. 1994, Orsini et al. 1995, Hinkula et al. 1997). A large variation in the prevalence of Rev antibodies is seen between different human studies: according to Dairaku et al. (1989) Rev antibodies can be seen in all HIV-seropositive individuals, whereas other groups have detected Rev antibodies in less than 40% of seropositive individuals (Chanda et al. 1988, Reiss et al. 1989b, Devash et al. 1990). In longitudinal studies, Rev antibodies could be found either persistently, transiently or intermittently in the follow-up period (Reiss et al. 1989b), but a lower prevalence of Rev antibodies was

seen in sera from patients with AIDS, compared with patients with ARC and symptom-free HIV-1 infected individuals (Reiss et al. 1989b, Devash et al. 1990).

Interestingly, both intracellular single-chain anti-Rev antibodies and chemically modified (cationized) antibodies capable of entering cells via endocytosis can significantly inhibit HIV-1 replication in various cell lines and in PBMC (Pardridge et al. 1994, Wu et al. 1996). Inhibition is seen with antibodies recognising functionally important domains of the protein (eg. amino acids 34-50 containing both the nuclear localisation signal and the RNA-binding region, amino acids 70-84 containing the activation domain and the nuclear export signal), but also with an antibody recognising amino acids 96-109 having no known function (Pardridge et al. 1994, Wu et al. 1996).

In Rev, antigenic epitopes recognised by human sera, mouse monoclonal antibodies, rabbit sera or single-chain antibodies have been mapped with synthetic peptides or protein footprinting analysis (Dairaku et al. 1989, Kreusel et al. 1993, Pardridge et al. 1994, Ranki et al. 1994, Orsini et al. 1995, Pilkington et al. 1996, Wu et al. 1996, Henderson et al. 1997, Jensen et al. 1997) (Table 2). The Arg-rich region of Rev, containing both the nuclear localisation signal (NLS) and the RNA-binding sequence, and the Leu-rich activation domain are clearly the most antigenic regions. Also, the carboxy-terminus of the protein is immunogenic, at least in mice.

Table 2. B-cell epitopes of Rev

	Human epitopes (aa)
Dairaku et al. (1989)	33 - 48
	Mouse epitopes (aa)
Kreusel et al. (1993)	69 - 82
Ranki et al. (1994)	33 - 48
Orsini et al. (1995)	38 - 44, 90 - 116
Henderson et al. (1997)	32 - 50, 72 - 91, 102 - 116
Jensen et al. (1997)	5 - 15, 65 - 85, 95 - 105
	Rabbit epitope (aa)
Pardridge et al. (1994)	35 - 50
	Single-chain antibody epitopes (aa)
Pilkington et al. (1996)	52 - 64, 75 - 88
Wu et al. (1996)	70 - 84, 96 - 109

2.6. Antibodies against HIV-1 Tat

Several reports have demonstrated the immunogenicity of Tat in HIV-1 infected humans (Aldovini et al. 1986, Barone et al. 1986, Franchini et al. 1987, Krone et al. 1988, McPhee et al. 1988, Reiss et al. 1989b, Demirhan et al. 1999b) and in mice immunised with recombinant protein (Brake et al. 1990a, Ranki et al. 1994, Tosi et al. 2000) or plasmid-DNA (Hinkula et al. 1997). In HIV-infected patients, the reported prevalence of antibodies to Tat varies from less than 30 to 100 percent, and longitudinal studies reveal that the antibody response is either constantly or occasionally detectable. Interestingly, significantly lower levels of Tat antibodies were seen in patients with Kaposi´s sarcoma (Demirhan et al. 1999b), and there is a correlation of low or absent antibody response to Tat with p24 antigenemia and progression to AIDS (Reiss et al. 1991, Rodman et al. 1993, Re et al. 1995, Zagury J-F et al. 1998, Cohen et al. 1999), indicating that a humoral response against Tat may have protective effects. Conflicting results show no significant differences in Tat

antibodies in various disease stages and even increased levels of Tat antibodies in AIDS (Aldovini et al. 1986, Franchini et al. 1986, McPhee et al. 1988).

In vitro, relatively low levels of extracellularly-added anti-Tat antibodies clearly inhibited HIV-1 replication in various cell lines and in PBMC cultures in a concentration-dependent manner (Steinaa et al. 1994, Re et al. 1995, Tosi et al. 2000), and were able to counteract the HIV-1 induced immunosuppression of T cells, as well as the HIV-1 induced generation of suppressive T cells (Lachgar et al. 1996, Zagury D et al. 1998). Also, Tat-specific antibodies were shown to be responsible for minimisation of chronic plasma viremia in vaccinated rhesus macaques (Goldstein et al. 2000). Thus, extracellular Tat antibodies may inhibit the paracrine activation pathway shown to be one feature of the extracellular Tat protein (Ensoli et al. 1993). Furthermore, lipidated anti-Tat antibodies capable of entering the cells, as well as intracellular single-chain antibodies, can also inhibit Tat-mediated transactivation of HIV-1 LTR, replication of HIV-1 or intracellular trafficking of Tat (Mhashilkar et al. 1995, Cruikshank et al. 1997, Poznansky et al. 1998).

Tat-reactive IgM-class antibodies have been found in the sera of non-HIV-infected individuals (Rodman et al. 1992, Rodman et al. 1993), and certain monoclonal antibodies against Tat react with various tissues from uninfected individuals (Parmentier et al. 1992), suggesting that this protein contains epitopes closely reminiscent of a cellular protein capable of inducing natural antibodies. Tat shares homology with CC chemokines, but no other cellular homoloques of Tat have been described so far.

Several antigenic epitopes have been localised using mouse monoclonal antibodies, human sera, cynomolgus monkey sera or single-chain antibodies (Krone et al. 1988, McPhee et al. 1988, Brake et al. 1990, Ranki et al. 1994, Mhashilkar et al. 1995, Pilkington et al. 1996, Putkonen et al. 1998, Demirhan et al. 1999b, Tosi et al. 2000) (Table 3). In Tat, the amino terminus as well as the Arg-rich region containing the TAR-binding motif are most often recognised by specific antibodies. Interestingly, sera from patients having KS differ from the sera of asymptomatic patients not only in the lower prevalence of Tat-specific antibodies, but also in a different epitope recognition pattern (Demirhan et al. 1999b). The antibody profile in KS sera was

limited to only a few epitopes, and none of the sera exhibited antibodies against the TAR-binding region, which was always recognised by sera from asymptomatic patients.

Table 3. B-cell epitopes of Tat

	Human epitopes (aa)
Krone et al. (1988)	Amino terminus
McPhee et al. (1988)	71 - 83
Demirhan et al. (1999)	11 - 24, 36 - 50, 41 - 54, 46 - 60, 52 - 60, 56 - 70
	Mouse epitopes (aa)
Brake et al. (1990)	5 - 22, 44 - 63
Ranki et al. (1994)	1 - 16
Tosi et al. (2000)	1 - 9, 52 - 55, 81 - 86
	Primate epitope (aa)
Putkonen et al. (1998)	31 - 65
	Single-chain antibody epitopes (aa)
Mhashilkar et al. (1995)	2 - 21
Pilkington et al. (1996)	22 - 33

2.7. Humoral immune dysfunctions in HIV infection

The most prominent dysfunctions seen in HIV infection are the polyclonal or monoclonal activation of B-cells, hyperimmunoglobulinemia affecting all isotypes, elevated autoantibody titers, poor response to antigens or mitogens and the development of B-cell lymphomas (Lane et al. 1983, Knowles et al. 1988, Terpstra et al. 1989, Daniel et al. 1996, Lopalco et al. 2000). Some of these abnormalities can precede the CD4+ T-cell defects or can be seen in purified B-cell cultures indicating that they are at least partly intrinsic to B-cells (Pahwa et al. 1986, Terpstra et al. 1989).

Several mechanisms are involved in B-cell dysfunction. Firstly, the gradual destruction of CD4+ helper T cells will lead to impairment of B-cell function as well. Secondly, biased or restricted antibody V-region gene usage may limit the available repertoire of antibodies against HIV-1 and opportunistic infections (Berberian et al. 1991, Muller et al. 1993). Thirdly, the elevated level of B-cells bearing the IL-6 receptor in HIV-infected subjects, together with enhanced production of IL-6 (Boue et al. 1992, van der Meijden et al. 1998), can contribute to the hypergammaglobulinemia. Finally, the decreased level of CD70, a TNF-related trans-membrane protein induced by the activation of lymphocytes, on B-cells from HIV-infected subjects is involved in the low IgG production after T-cell dependent antigen stimulation (Wolthers et al. 1997). Changes in the expression of other cytokines and cytokine reseptors may also contribute to the destruction of humoral response.

3. Vaccination studies with Nef, Rev or Tat

3.1. The clinical importance of immune response against Nef, Rev and Tat

The regulatory proteins Rev and Tat, as well as the accessory protein Nef, belong to proteins expressed early in the life cycle of the virus, soon after virion entry into the cell. Thus, an immune response directed against them may prevent later steps in the HIV life cycle and inhibit the release of progeny virus particles. In fact, an immune response against these early proteins has been shown to correlate with protection or attenuation of the disease (O´Shea et al. 1991, Reiss et al. 1991, Rodman et al. 1993, De Maria et al. 1994, Langlade-Demoyen et al. 1994, Re et al. 1995, Van Baalen et al. 1997, Chen et al. 1999, Yamada et al. 1999). The facts that HIV-1 strains with low pathogenicity have deletion in these proteins (Deacon et al. 1995, Iversen et al. 1995, Yamada et al. 2000), and that a shift from predominantly spliced regulatory viral mRNA patterns to a predominantly unspliced pattern is associated with disease progression (Michael et al. 1995) further underline the essential role of these proteins, and the beneficial effects an immune response against them may provide.

3.2. Previous vaccination strategies

Early vaccination studies mainly focused on using gp160 subunits (recombinant proteins, peptides) and viral, bacterial or DNA vectors carrying env-genes as the immunising antigen. With these vaccine candidates, an antibody response, as well as cell-mediated immune responses, may follow, but in humans neither total protection

nor suppression of disease, virologic measures or progression to AIDS has been observed (Connor et al. 1998, Evans et al. 1999, Goebel et al. 1999, Sandström et al. 1999). One possible mechanism for this may be the ability of HIV-1 to rapidly mutate and generate new quasispecies differing on the envelope sequences.

Other widely tested vaccine candidates have been the live attenuated virus constructs, which contain either mutated or totally deleted HIV or SIV genes (Daniel et al. 1992, Quesada-Rolander et al. 1996, Igarashi et al. 1997, Cranage et al. 1997). In animals vaccinated with attenuated SIV, immune responses may be elicited owing to the endogenous expression of native SIV proteins and/or antigen presentation in the native replication site of virus. The live attenuated vaccines prepared from SIV have provided the most long-lasting and impressive protection against SIV so far (Hulskotte et al. 1998): protection is seen against heterologous SIV strains and against challenge via intravenous, mucosal or oral route. However, the replication-competent viral vaccines raise safety concerns for clinical trials in humans, especially because deleted vaccine strains can evolve into fast-replicating variants by multiplication of remaining sequence motifs (Berkhout et al. 1999), and because SIV constructs with multiple gene deletions can be pathogenic in new-born monkeys (Baba et al. 1995). Furthermore, the level of protection may be dependent on the time-point of challenge (Hulskotte et al. 1998).

As the accessory and regulatory proteins Nef, Rev and Tat are more conserved than Env, they have become important targets for the design of vaccines. All three proteins have been used in immunisation studies either as recombinant proteins, peptides or corresponding genes cloned in various bacterial, viral or DNA vectors.

3.3. Antigen-specific immune responses in vaccinated individuals

Nef has been shown to induce antibodies in animals immunised either with recombinant protein (Bahraoui et al. 1990, Schneider et al. 1991, Ovod et al. 1992, Otake et al. 1994) or DNA plasmids with or without protein boosting (Hinkula et al.1997, Putkonen et al 1998, Moureau et al. 1999, Billaut-Mulot et al. 2000). In humans, the antibody responses after DNA vaccination were of low magnitude (Calarota et al. 1999), but a Nef-lipopeptide immunisation induced antibodies in the majority of vaccinees (Gahery-Segard et al. 2000). Nef raised proliferative and/or

CTL responses in animals immunised with various DNA plasmids (Asakura et al. 1996, Hinkula et al. 1997, Putkonen et al. 1998, Ayyavoo et al. 2000, Billaut-Mulot et al. 2000) and bacterial vectors (Winter et al. 1995). A Nef-specific cell-mediated response was also detected in humans immunised with DNA plasmids (Calarota et al. 1999), lipopeptides (Gahery-Segard et al. 2000) and viral vectors (Evans et al. 1999). However, the DNA immunisation of treatment-naive HIV-infected patients caused no significant decrease in viral load or increase in the CD4+ lymphocyte counts (Calarota et al. 1999), nor were DNA-primed/protein-boosted monkeys having both antibody and proliferative responses against Nef, Rev and Tat protected against intravenous challenge with SHIV (Putkonen et al. 1998), a chimeric SIV expressing env, rev and tat genes of HIV-1.

Also, Rev induced antibody production in immunised mice and rabbits (Kreusel et al. 1993, Pardridge et al. 1994, Ranki et al. 1994, Orsini et al. 1995, Henderson et al. 1997, Hinkula et al. 1997, Jensen et al. 1997), but in DNA-vaccinated HIV-infected patients, as well as in DNA-immunised and protein-boosted cynomolgus monkeys, antibody response against Rev was low (Putkonen et al. 1998, Calarota et al. 1999). Transient Rev-specific CTL and proliferative responses were induced by DNA vaccination in these patients and in seronegative volunteers receiving an env/rev DNA vaccine (Boyer et al. 2000). A transient proliferative response, but no CTL activity was induced in monkeys. As earlier indicated, these responses provided neither protection nor reduction in the viral load or enhancement in CD4+ lymphocyte counts. However, recent studies in cynomolgus monkeys showed that vaccination with SIVmac Rev and Tat genes cloned in recombinant Semliki Forest virus (SFV) and recombinant vaccinia virus (MVA) could protect monkeys against challenge with SIV (Osterhaus et al. 1999), but the immunological parameters correlating with this protection are still unclear.

Immunisation with Tat-protein or peptides induced high levels of Tat-antibodies in various animals (Brake et al. 1990a, Ranki et al. 1994, Cafaro et al. 1999, Goldstein et al. 2000, Pauza et al. 2000, Tosi et al. 2000); also DNA immunisation raised antibody production, albeit often at lower levels if no protein boosting was given (Hinkula et al. 1997, Putkonen et al. 1998, Caselli et al.1999). The Tat antibodies detected in the sera of immunised animals were capable of neutralising the effect of extracellular Tat on

HIV-1 replication (Cafaro et al. 1999, Caselli et al. 1999). In seronegative humans, immunisation with chemically modified Tat protein induced high levels of circulating antibodies (Gringeri et al. 1999), but DNA vaccination caused no enhancement in antibody titers in HIV-patients having a very low Tat-antibody response before immunisation (Calarota et al. 1999). A T-cell proliferative response to Tat protein has been detected in humans (Calarota et al. 1999, Gringeri et al. 1999), monkeys (Putkonen et al. 1998, Cafaro et al. 1999, Pauza et al. 2000) or mice (Caselli et al. 1999) immunised either with Tat protein or DNA constructs. Low level CTL responses towards Tat have been reported in DNA-vaccinated humans (Calarota et al. 1999) and in protein-immunised monkeys (Cafaro et al. 1999).

Even though the first DNA vaccination studies using plasmids containing non-structural genes showed no protection upon challenge (Putkonen et al. 1998), the recent vaccination studies using non-structural whole proteins seem promising. Protection was seen in the majority of cynomolgus monkeys immmunised with Tat-protein (Cafaro et al. 1999) and a significant attenuation of disease (e.g. lower vRNA copies and p27 levels in plasma, lower chemokine receptor expression on circulating CD4+ cells, higher CD4 count) was detected in rhesus macaques immunised with chemically modified Tat-protein (Pauza et al. 2000). Furthermore, live viral vectors carrying Rev and Tat genes protected cynomolgus monkeys (Osterhaus et al. 1999). However, the immunological responses needed for protection are still unclear. Some studies showed that both the humoral and cell-mediated responses were needed for attenuation of infection (Pauza et al. 2000), while in other reports the neutralisation titers did not correlate with protection (Cafaro et al. 1999). Future studies investigating the role of various immunological parameters (including cytokine levels) in protection, as well as new ways to improve immunogenicity of vaccines, still wait to be done.

AIMS OF THE PRESENT STUDY

The regulatory proteins Rev and Tat, as well as the accessory protein Nef, belong to proteins expressed early in the life cycle of the virus, soon after virion entry into the cell. Thus, an intracellularly acting immune response directed against them may prevent later steps in the HIV life cycle and inhibit the release of progeny virus particles. Furthermore, as these proteins are more conserved than highly variable membrane proteins of HIV-1, they are possible candidates for vaccine development, and possible targets for specific immunotherapy in HIV-1 infected patients. The present work was undertaken to study the antigenicity of these proteins, as well as the usefulness of these proteins as vaccine components.

The specific aims of this study were:

1. To investigate the prevalence of antibodies against these proteins in HIV-1-infected individuals.

2. To map the B-cell epitopes of Nef, Rev and Tat recognised by human sera.

3. To generate DNA vaccine constructs containing genes for Nef, Rev or Tat.

4. To test the immunogenicity of these constructs in mice, and analyse the humoral immune response raised against Nef, Rev and Tat.

MATERIALS AND METHODS

1. Study population

The HIV-positive sera used in Study I, II and III were collected from voluntary German or Finnish HIV-infected individuals. In Study I, HIV-negative but Nef-positive sera were also analysed. These sera were collected from homosexual men belonging to the HIV-risk group or from voluntary dermatological patients. Sera from healthy laboratory workers and medical students were used as negative controls.

In Study IV and V, Balb/c mice (5-8 weeks old) were used in immunisation experiments. Mice receiving only the plain vector without HIV-1 genes cloned in were used as negative controls.

2. Detection of humoral immune response

The antibodies against whole Nef, Rev or Tat proteins were detected either with Western blotting or ELISA. In these assays, recombinant proteins (isolate BRU) were used as antigens. Nef was obtained either from Prof. V. Erfle (GSF, Germany), from Dr. M-P. Kieny and Dr. J-P. Lecocq (Transgene, France) or from Dr. M. Harris (MRC AIDS Directed Programme Reagent Project, United Kingdom). Recombinant Rev and Tat proteins were from Intracell, American Biotechnologies Inc or from AIDS Research and Reference Reagent Program, NIAID, NIH.

2.1. Western blotting

In Studies I, II, III, IV and V antibodies to Nef, Rev or Tat protein were detected by Western blotting (Towbin et al. 1979) as follows: purified recombinant protein was boiled in sample buffer containing 1% SDS and 1% 2-mercaptoethanol, then run on 10% or 12.5% polyacrylamide gel and subsequently transferred onto 0.45 μm nitrocellulose paper. Strips were first blocked with 2% BSA-1% normal goat serum in 5% nonfat milk-TBS, and thereafter incubated with diluted sera (1:100) overnight. After each incubation step, unbound proteins were removed by washing strips three times with TBS - 0.05% Tween-20 and twice with water. Binding of immunoglobulins was detected by incubating strips with biotinylated anti-human IgG (Vector) followed by horseradish peroxidase conjugated avidin (Vector). As the substrate, 4-chloro-1-naphtol (Sigma) was used.

2.2. Protein ELISA

In the protein ELISA (Study III, IV and V) Nunc Maxi-Sorp plates were coated in an overnight incubation with Nef, Rev or Tat protein in PBS (50-100 ng/well). Plates were blocked with 1% BSA - 1% normal rabbit serum in phosphate-buffered saline (PBS) and incubated with diluted (1:100) human or mouse sera overnight. After washings, plates were incubated 2 h with diluted (1:500) secondary antibody, peroxidase conjugated anti-mouse IgG (DAKO) or anti-mouse IgG1, IgG2a, IgG2b, IgG3 or IgM (Caltag). Colour intensity produced from the substrate (2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in phosphate-citrate buffer was measured at 405 nm using Labsystems Multiscan Plus ELISA-plate reader.

When analysis of the HIV-infected human sera was performed (Study III), serum was defined to be positive if its absorbance was higher than background + 3 standard deviations (SD). At the time point when analysis of DNA-immunised mouse sera was performed (Studies IV and V), polyclonal sera from protein-immunised mice were available to be used as a positive control mouse serum, and a more quantitative value (EIU) for each serum could be obtained using the following formula: $EIU = (OD_{\text{mouse serum}} - OD_{\text{normal mouse serum}}) \times 100/(OD_{\text{positive control mouse serum}} - OD_{\text{normal mouse serum}})$ (Miettinen et al. 1989). An EIU value above 10 was considered positive. In Study V, the IgG1/IgG2a ratio for each Nef-antibody positive mouse was calculated from the corresponding absorbance values measured against whole protein.

3. Characterisation of epitopes

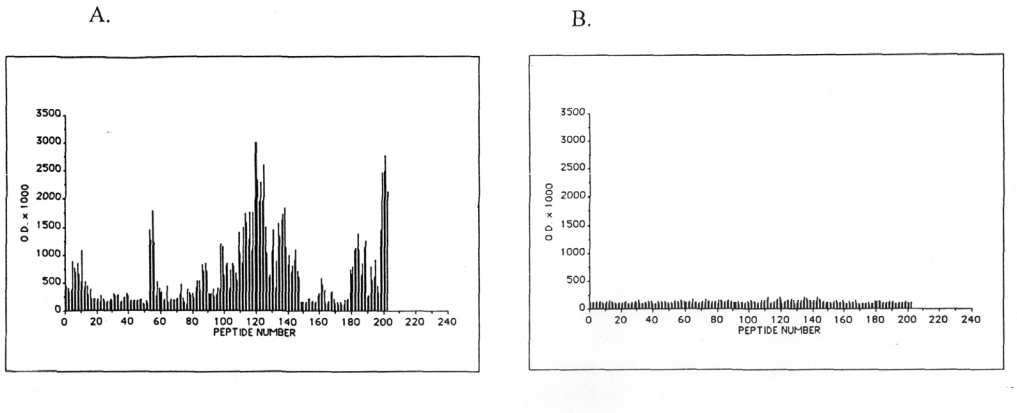
3.1. Epitopes recognised by human sera: Pepscan method

The epitope characterisations of human antibodies (Study I, II and III) were done using the Pepscan method (Geysen et al. 1984). In this method, overlapping peptides spanning the whole sequence of the protein of interest are synthesised on tips of polyethylene pins which are arranged in the shape of a 96-well microtiter plate, and the binding of antibodies is detected with ELISA. In peptide synthesis, preactivated Fmoc amino acids and corresponding Fmoc chemistry were used according to the instructions given by the manufacturer of the Epitope Scanning Kit (Cambridge Research Biochemicals). The size of the peptides varied: a rough epitope mapping was done using 8-9 amino acid long peptides (Study I and III), whereas further

epitope characterisation was done using smaller, down to 3 residues long peptides (Study II).

After synthesis, pins were blocked by incubating them in 1%ovalbumin - 1 %BSA - 1% Tween-20 in PBS for 1 h at room temperature. After blocking, pins were incubated with diluted (1:500-1:1200) serum samples for 2 h at room temperature (Study I) or overnight at +4 °C (Study II and III). To remove unbound proteins after each antibody-incubation step, the pin plates were washed by shaking them heavily three times for 5 min in 1% Tween-20 in PBS and once in water. Pins were then incubated for 1 h at room temperature with the secondary antibody, peroxidase-conjugated rabbit anti-human IgG in blocking media, washed and substrate (2,2'-azino-bis-3-ethylbenzthiazolinesulphonic acid (ABTS) in phosphate-citrate buffer) was added for 30-45 min. The photometric determination was carried out in an ELISA reader at 405 nm. Bound antibodies were removed from the pins between each test by sonicating pin plates once for 30 minutes in a water bath containing 1% SDS - 0.1% 2-mercaptoethanol in 0.1 M NaH_2PO_4, twice for 10 minutes in water and finally for 3 minutes with methanol. Typical reactivity patterns of patient and control sera against synthesised Nef peptides are shown in Figure 2.

Figure 2. ELISA results of sera against peptides derived from HIV-1 Nef protein. A) Serum from an HIV-1-infected man. B) Serum from a negative control.



In order to verify that the epitopes found with peptides bound to polyethylene pins are also recognised by human sera when expressed as soluble peptides, peptides representing epitope sequences were synthesised on a Zinsser Analytics SMPS-350 Multipeptide Synthesiser using conventional Fmoc chemistry (Fmoc amino acids, di-isopropylcarbodiimide [DIC], hydroxy-benzotriazole [HOBt] and Wang-resin) (Fields et al. 1990), and a peptide ELISA was performed (Study III). Assays to measure the reactivity of the sera against each peptide were performed similarly as the protein ELISA, except that the amount of antigen in each well was 5-50 μg. Also, the background value for each serum was subtracted from the peptide absorbances given by that particular serum. The absorbance values of the patient group and the negative control group were compared using the Mann-Whitney *U*-test.

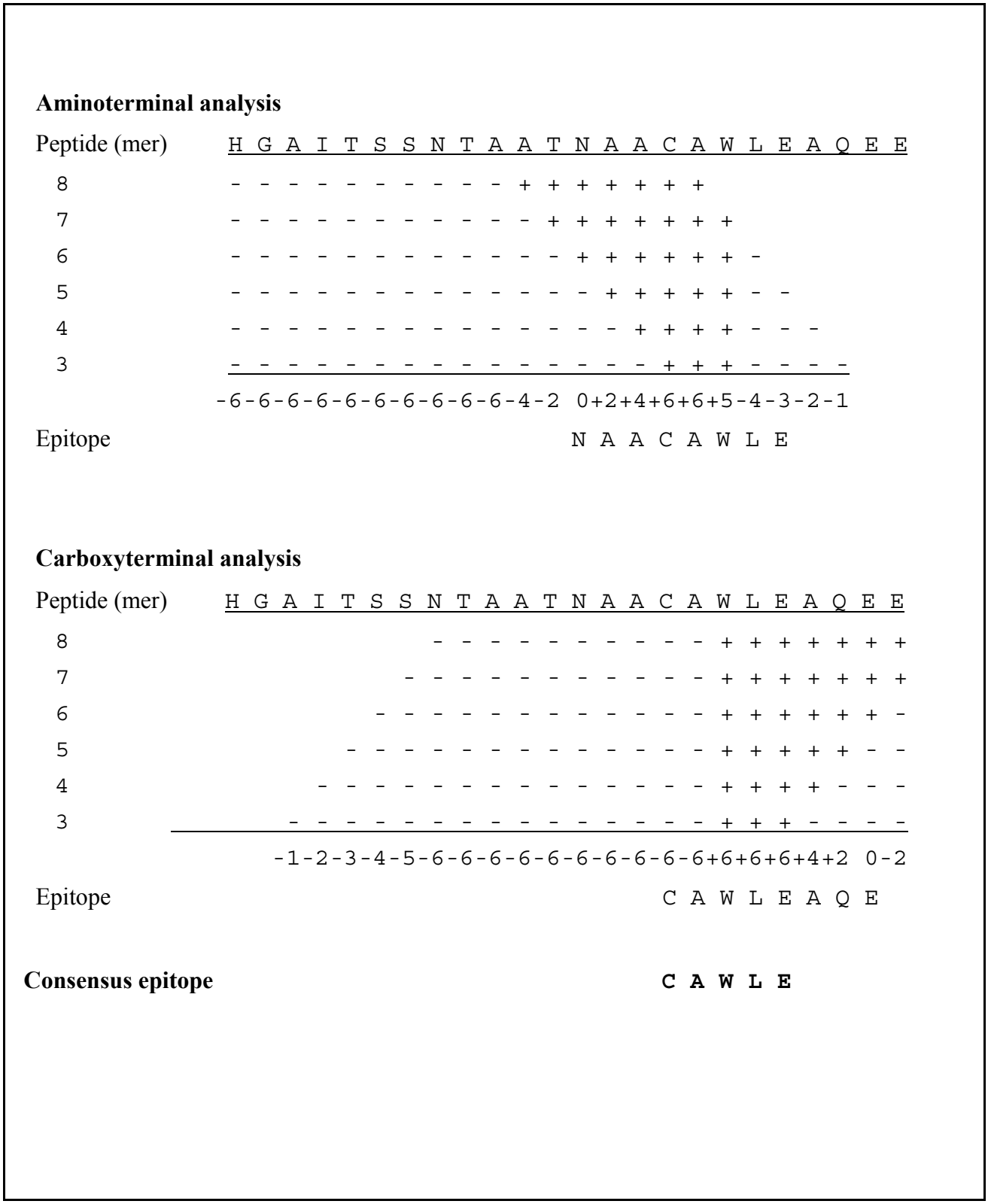
3.2. Epitopes recognised by mouse sera: Peptide ELISA

At the time point when epitope characterisation using DNA-immunised mouse sera was done (Study V), Pepscan plates were too old and worn out to be reused, so the epitope searching was done with peptide ELISA using 15-17 amino acid long, soluble peptides scanning the whole amino acid sequence of Nef (isolate BRU). The peptides were received from the European Vaccine against AIDS (EVA) reagent repository. The protocol followed protein ELISA (Chapter 2.2.), except that the amount of antigen coated per well was 500 ng. Nine sera from mice immunised with a gene gun (GG), intradermally (ID) or intramuscularly (IM) giving high EIU values against whole Nef protein (titers 1:5000 – 1:10 000) were selected for epitope mapping. Their reactivity against each peptide was indicated with an ELISA value (EIUpep) calculated similarly as EIU values in Chapter 2.1. An EIUpep value above 10 was considered positive and to represent an epitope recognised by mouse serum.

3.3. Analysis of minimisation results: Matrix method

In Study II, each epitope was scanned using 3-8 mer peptides. This resulted in tens (or even over one hundred) of absorbance results each giving information about the same epitope. To be able to handle these data, a matrix method was developed (Fig. 3).

Figure 3. Matrix analysis of one patient serum against 3-8 mer peptides scanning Nef epitope 2.



In the matrix, the x-axis shows the sequence of the analysed region and the y-axis the size (mer) of the studied peptides. The matrix was filled in by giving the reaction with each peptide a positive or negative sign (+, -) as follows: a reaction was considered positive if the absorbance given by the peptide was two times higher than the absorbance of background. The background for each serum against each epitope was calculated as the mean of the lowest 40% of ELISA values given by peptides from the area of the epitope in question. For a positive reaction, a + sign was placed into the matrix at the point corresponding to the length and starting point of the peptide (aminoterminal analysis), or at the point corresponding to the length and end point of the peptide (carboxyterminal analysis). A – sign was correspondingly given to peptides yielding negative results. At the bottom of the matrix, arithmetical sum values of signs for each amino acid are given. The analyses (aminoterminal/carboxy-terminal) were performed in duplicate to ensure that minimal reactive sequences were detected irrespective of their situation in the amino- or carboxyterminus of the peptide.

On the basis of these values, an epitope was determined to be the region where the sum values exceeded zero. This diminished the effect of an incidental positive reaction due to unspecific binding. As the shortest analysed peptide was three amino acids long, an additional two amino acids at either the amino or the carboxy terminus had to be considered as part of the epitope, too. Finally, as an epitope should react irrespective of its position in a peptide, we deduced the final or consensus epitope to be the region that was found antigenic in both aminoterminal and carboxyterminal analyses. This precondition excluded some sequences that were positive when situated in the carboxy terminus but not in the amino terminus of the peptide. It also prevented the edges of the matrix containing only few values from giving false results.

3.4. Homology search and variability index

Homological sequences to the identified epitopes were searched from Swiss-Prot protein bank by using the homology search program Fasta (European Bioinformatics Institute, EBI). For each stretch of epitope, the amount of mismatch amino acids was selected to allow 25% dishomology. A variability index for each amino acid inside the epitopes was calculated according to the reported sequences of various isolates

(Myers et al. 1990). Ten different isolates were compared to the BRU isolate used in Studies II and III, and the amount of deviant amino acids was summed up to give the variability index.

4. DNA immunisation experiments

4.1. Construction of DNA vectors

The DNA vaccines used in mouse immunisation experiments (Study IV and Study V) were vectors based on bovine papilloma virus (BPV) transcription elements, and they contained the origin of BPV, together with the E1 and E2 genes (pBN-vectors) or E2 alone (pCG-vector). These plasmids were developed and kindly provided by Prof. M. Ustav, Tartu University, Estonia. The products of the E1 and E2 genes are proteins necessary and sufficient for self-replication of the plasmid, as well as for transcriptional activation (Ustav et al. 1991). pCG-plasmids devoid of E1 are not able to replicate but they show enhanced and prolonged expression of cloned genes.

The nef, rev and tat genes (isolate BRU) cloned under CMV or RSV promoters were derived from corresponding pC-vectors kindly provided by Dr. B. Cullen. The cloning and other molecular biology works (DNA purification, transfections, restriction enzyme digestions, PCR etc.) were done according to appropriate, well-known methods (Sambrook et al. 1989) as described in Study IV and Study V. Plasmids were purified using QIAGEN plasmid purification columns, and cloned genes were sequenced using the Perkin-Elmer ABI 310 automatic sequencer to confirm the correct sequences of genes. Replication of pBN-based vectors was confirmed in a replication assay performed in Tartu University, Estonia.

The expression of the cloned genes in cell lines transfected with the plasmids was confirmed with Western blotting and immunohistochemical staining (Studies IV, V). In addition, the Rev and Tat proteins produced by these vectors were shown to be functionally active in co-transfection assays with Rev-defective SIV, with Gag-encoding plasmid pNLgagSty330 carrying the rev responsive element (RRE) sequence, or with plasmid pNLCATw carrying the LTRs from HIV-1. These assays were done either in Stockholm, Sweden or at Tampere University. They are described in detail in Study V.

4.2. Immunisation protocol

Balb/c mice were used for all immunisation experiments. Each mouse received six injections within two weeks, the total amount of DNA administered being 6 μg for gene gun immunisation (GG) and 150 μg for intramuscular (IM) and intradermal immunisation (ID). For gene gun immunisations, plasmid was precipitated onto 1 μm gold particles following the procedure in the Helios Gene Gun Instruction Manual (Bio Rad Laboratories). Each cartridge contained 0.5 mg gold and 1 μg DNA. Mice were immunised on shaved abdominal skin using a helium discharge pressure of 300 psi. For intradermal or intramuscular immunisations, plasmid DNA was dissolved in sterile saline (25 μg / 20 μl) and injected on dorsal skin or m. quadriceps femoris, respectively.

4.3. Analysis of immune responses

In experiments, where the immunogenicity of the vectors was tested (Study IV and Study V), mice were sacrified four weeks after the last immunisation, serum samples were taken for Western blotting and ELISA, and splenocytes were harvested for the CTL and proliferation assays. In experiments, where the duration of the immune response was examined (Study V), first mice were sacrified after four weeks and the rest were followed up to six months sacrifying two mice/month. Western blotting and ELISA were performed as described in Chapters 2.1. and 2.2. Cell-mediated immune responses were performed in our research group according to the methods described in Study IV and V.

RESULTS

1. Prevalence of Nef-, Rev- and Tat-specific antibodies in HIV-infected persons

Before the start of Study I, the prevalence of antibodies against the accessory protein Nef among Finnish HIV-infected individuals was evaluated at Tampere University and Helsinki University using Western blotting (Ranki et al. 1990). Nef-specific antibodies were found in 54% (29/54) of individuals at any time during a prospective follow up. However, Nef antibodies were also found in 5% (7/141) of HIV-risk group individuals, in 5% (5/93) of non-risk dermatological patients and in 11% (4/37) of uninfected, healthy blood donors. From each of these groups, the most positive sera

showing the strongest bands in Western blotting were chosen for epitope mapping (Study I and Study II).

The prevalence of antibodies against the regulatory proteins Rev and Tat were evaluated in Study III using ELISA and Western blotting. Rev- and Tat-specific antibodies were found in 12% (10/83) and 18% (15/83) of infected individuals, respectively. All HIV-1-negative control sera were negative in the ELISA-assay and in the Western blot. At the early stages of HIV infection (ASX, LAS), anti-Rev antibody positivity was found in 12% (6/52) of the patients, and at later stages of infection (ARC, AIDS) 15% (4/26) of patients had anti-Rev antibodies. Likewise, the anti-Tat antibody positivity was found in 17% (9/52) and 23% (6/26) of patients, respectively.

2. Characterisation of human B-cell epitopes in HIV-1 Nef

The preliminary study (Study I) determined the antigenic regions in Nef recognised by sera from HIV-positive/Nef-positive patients, from HIV-negative/Nef-positive risk group individuals and from HIV-negative/Nef-positive non-risk dermatological patients or controls. This rough characterisation was done using 9-mer peptides spanning the whole sequence of three different Nef isolates (BRU, SF2, MAL) and the polyethylene pin method (Geysen et al. 1984). As the size of the epitopes found with these 9-mer peptides exceeded the average size of linear epitopes, which is generally thought to be only from 4 to 6 amino acids (Geysen et al. 1988), the specific immunogenic structures within these epitopes and flanking amino acids were further characterised using shorter (3-8 mer) long peptides representing the sequence of isolate BRU (Study II).

Nine antigenic regions were recognised by sera from HIV-1 infected individuals in Study I. The majority of sera (6/10) recognised at least 2 of the epitopes, which consisted of amino acids 8-16 (Epitope 1; SSVVGWPTV), 53-60 (Epitope 2; NAACAWLEA), 80-90 (Epitope 3a; TYKAAVDLSHF), 97-101 (Epitope 3b; LEGLI), 115-127 (Epitope 3c; YHTQGYFPDWQNY), 136-147 (Epitope 3d; PLTFGWCYKLVP), 158-169 (Epitope 4; KGENTSLLHPVS), 180-191 (Epitope 5; VLEWRFDSRLA), 197-206 (Epitope 6; ELHPEYFKNC) (numbering and sequence according to isolate BRU). These epitopes were very similar in all three isolates; only

epitope 4 seemed to be specific for the MAL-isolate. Interestingly, also the majority (4/6) of sera from HIV-negative individuals belonging to the HIV-risk group recognised most of these epitopes, whereas sera from the HIV-negative non-risk group usually did not. The only non-risk group individual, whose serum strongly recognised the Nef-specific epitopes, was a heterosexual man with no known HIV exposure, but with a newly recognised autoimmune thyroiditis.

The minimisation of the previously found epitopes (Study II) showed that epitopes 2, 3d and 6 consisted of linear antigenic stretches (CAWLE, LTFGWC, PEYF, respectively) whereas epitopes 1, 3a, 3b, 3c, and 5 displayed a more complex reaction pattern showing reactivity concentrated into several small, down to three aa long regions. In the MAL-isolate specific epitope 4 clear minimal antigenic stretches could not be found. Three stretches in epitope 1 (WSK,VGW,TVRERMRR), two stretches in epitope 3a (PLRPM, SHFLK), two stretches in epitope 3b (SQRRQD, DLW), three stretches in epitope 3c (IYHT, QGYFPDWQN, GVR) and two stretches in epitope 5 (EVLEWRFDSR, VAR) were identified. A homology search against sequences in the Swiss-Prot protein bank revealed that the middle part of the epitope 3c (QGYFPDWQN), epitope 3d (LTFGWC) and the beginning of epitope 5 (EVLEWRFDSR) seem to be genuinely Nef-specific, whereas several homological proteins were found to other antigenic parts of the protein. Eight of the found B-cell epitopes (1, 3a, 3b, 3c, 3d, 4, 5, 6) overlapped totally or partly with the identified T-cell epitopes of Nef (Koenig et al. 1990, Cullmann et al. 1991, Hadida et al. 1992, Goulder et al. 1997, Lieberman et al. 1997, Sandberg et al. 2000). Analysis of the variability index showed that many of the epitopes were situated in an area with low variation.

3. Characterisation of human B-cell epitopes in HIV-1 Rev and Tat

The characterisation of Rev and Tat epitopes was done using overlapping, 8-mer peptides representing the sequences of isolate BRU and patient sera shown to contain Nef-specific antibodies. In Rev, the two most frequently recognised epitopes were near the amino terminus of the protein within amino acids 12-20 (LIRTVRLIK) and 38-49 (RRNRRRRWRERQ). The third epitope was mapped around amino acids 55-62 (ISERILGT) and the fourth around amino acids 78-83 (LERLTL). These latter two

epitopes were not constantly recognised by all tested sera, but the reactivity, especially against the fourth epitope, could be very high.

In Tat, four antigenic regions were also found: the most frequently recognised epitopes, showing also the highest absorbances, were situated in the centre of the protein and consisted of amino acids 21-37 (ACTNCYCKKCCFHCQVC) and 39-58 (ITKALGISYGRKKRRQRRRA). In addition, a frequently recognised area was loacated around amino acids 6-10 (PRLEP) in the amino terminus of Tat. Some sera also recognised a short sequence in the carboxy terminus of the protein around amino acids 74-82 (TSQSRGDPT).

Using an ELISA based on soluble synthetic epitope-derived peptides, and sera from HIV-infected and control individuals, the recognition of Rev epitopes 1, 2 and 4, as well as Tat epitopes 1, 2 and 3, was shown to be either highly specific ($p < 0.001$) or specific ($p < 0.05$) for HIV-infected individuals. Using the variability index, these epitopes were shown to contain long conserved amino acid sequences. Furthermore, many of them overlap totally or partially with known T-cell epitopes or predicted HLA class I peptide-binding motifs of Rev and Tat (Blazevic et al. 1993 and 1995, van Baalen et al. 1997), as well as with the functionally important regions of these proteins.

4. Humoral immune responses induced by DNA immunisation in mice

In Study IV, Balb/c mice were immunised with various DNA vectors carrying the Nef gene of HIV-1. These vectors were in in vitro assays shown to express the corresponding proteins. Four weeks after the last immunisation mice were sacrified and the humoral response was analysed.

The majority of mice (14/16) immunised with the replicating pBN-CMV-Nef or pBN-RSV-Nef constructs developed antibodies within four weeks; a response was detected already within two weeks in a few mice immunised with pBN-CMV-Nef. The EIU values in these mice were highly variable, ranging from 16 up to 100. In contrast, only a few (2/12) mice immunised with the non-replicating pCG-Nef vector had a weak antibody response against Nef after four weeks, and no response in any mice

was seen in two weeks. The majority of mice also developed a CTL response against Nef, and this response was not dependent on the vector used for vaccination.

In Study V, the immunogenicity of one of the previously tested vectors (pBN-RSV-Nef) was further evaluated using different DNA delivery methods (e.g. GG, ID, IM). In addition, the immunogenicity of two other HIV-1 genes (rev and tat) given in that pBN-vector were characterised. Also, to assess the Th-type of the response, antibody subclasses in mice immunised with various methods were analysed, and Nef-specific epitopes recognised by these antibodies were characterised.

In the Nef GG group, all mice developed specific antibodies within four weeks after the last immunisation; the EIU values for Nef gene gun immunised mice varied between 25-78 (mean 51). Antibody response could be detected up to six months with no decrease in EIU values. In the Nef IM group 50%, and in the Nef ID group 75% of mice showed antibodies against this protein. EIU values in the IM group varied between 24-75 (mean 43) and in the ID group between 12-100 (mean 56). The titers of Nef-positive sera varied between 1:100 – 1:10 000, and a clear correlation between titer and EIU value was seen. In the Rev GG group and the Tat GG group no antibodies were detected after four weeks. The IgG1/IgG2a antibody ratio was much higher in the Nef GG group than in the Nef IM group, showing that gene gun immunisation raised mainly a Th2 type response distinguished by IgG1 class antibodies (Del Prete et al. 1991). Intramuscular immunisation caused a Th1-type response where IgG2a class antibodies prevailed. The response in the ID group seemed to have a mixed character. In gene gun immunised mice IgG1-class antibodies remained elevated throughout the six months follow-up period, indicating that no class switching took place later on. Other antibody subclasses tested were not significantly elevated in any of the Nef groups. None of the control mice had Nef-, Rev- or Tat-specific antibodies.

In addition to humoral responses, immunisation with Nef-vector induced cell-mediated responses in mice vaccinated with various methods. Rev vector was also efficient in raising both proliferative and CTL responses. Tat vector was a poor immunogen in all respects.

5. Charaterisation of B-cell epitopes in Nef recognised by DNA-immunised mouse sera

Three sera from each immunisation group having high EIU values against the whole Nef protein were selected for epitope mapping done with soluble synthetic peptides. EIUpep values for each peptide and each serum were measured; these values reflected the intensity of colour produced by antibodies binding to Nef peptides as compared to binding to the whole Nef protein. With some sera, peptides were recognised even better than the whole protein probably due to steric hindrance caused by other portions of the protein. The N-terminus of Nef contained two antigenic regions that were recognised by almost all antibodies irrespective of the immunisation method. These areas were situated between amino acids 9-24 (SVIGWLTVRERMRRAE) and amino acids 49-64 (AATNAACAWLEAQEEE). In addition, peptide 118-133 (QGYFPDWQNYTPGPGV) was found to be considerably antigenic. Interestingly, even though all the sera belonging to the GG group showed clear humoral response against the whole Nef protein, only one out of three recognised well linear epitopes represented by peptides.

DISCUSSION

We have analysed the humoral immune response against Nef, Rev and Tat - all proteins interfering not only with the life cycle of the virus but also with several cellular processes essential for the host cell. The reason for our interest was that an antibody response against these early proteins could be useful for diagnostic purposes, for inducing a sterilising immunity against HIV or for inducing an immune response capable of slowing down an ongoing HIV infection.

1. Prevalence of Nef-, Rev and Tat-specific antibodies in HIV-infected individuals

All three non-structural proteins have been shown to be immunogenic in vivo, but large variations ranging from less than 30 to 100 percent exist in the reported prevalence of antibodies against these proteins. This probably reflects the great diversity in antibody assays used by different scientists: variation is seen in the actual method (ELISA vs. Western blotting), in method parameters (incubation times, buffers, blocking media, cut-off values), in antigens used (recombinant proteins with varying lengths, peptides, fusion proteins), and in study populations (stage of disease,

age, route of infection) etc. However, Nef has constantly been shown to be the most immunogenic of these three proteins. This is somewhat surprising, as one would expect to see the best response against the extracellular Tat protein and not against the Nef protein, which is mainly intracellular and has probably only its carboxy terminus exposed on the cell surface. One explanation for the strong Nef response may come from gp160 studies of Parren et al. (1997), which showed that the antibody response in HIV-1 infection is principally elicited by viral debris rather than virions. In addition, as Nef protein is almost twice as big as Rev and Tat, this characteristic provides Nef with more antigenic regions capable of inducing both cellular and humoral responses.

We have also shown that the prevalence of Nef antibodies among HIV-infected individuals was higher than the prevalence of Rev and Tat antibodies. In our studies, however, the prevalences of all three antibodies were generally lower than other groups have reported. This may be a consequence of the method used: we used Western blotting for antibody detection, as our aim was to further characterise the linear epitopes recognised by positive sera. In Western blotting the antigen is denatured and only antibodies recognising linear epitopes react. Thus, sera recognising only conformational epitopes were not detected in our assay.

A slight increase was detected in Rev and Tat antibody prevalence between individuals at early stages of infection (ASX, LAS) and individuals having ARC or AIDS (Study III). However, as the amount of antibody-positive patients was small, this finding was not statistically significant. In addition, as our study was not a longitudinal one, no conclusions about the relationship of antibody response and disease progression can be drawn.

2. B-cell epitopes of Nef, Rev and Tat proteins

False-positive results are often seen in HIV-antibody assays, not only when antibodies against Nef, Rev and Tat proteins are measured but also in antibody assays for structural proteins. These cross-reactions may arise from previous infections, homological sequences in cellular proteins and from antibodies to endogenous retroviral gene products, which have been found in a number of infectious, chronic, and malignant diseases (Lower et al. 1986). Therefore, our preliminary idea was that

the actual HIV-derived antigen-specific stretches not reacting with sera from non-HIV–infected individuals could be found by mapping the B-cell epitopes of Nef, Rev and Tat. Furthermore, as non-structural proteins are expressed soon after the virus enters the cell and before structural proteins appear (Ranki et al. 1994), antibody responses against these epitopes were thought to be possible diagnostic markers of an ongoing HIV infection in individuals belonging to HIV-risk group, but being seronegative in conventional HIV tests.

Even though several studies had revealed immunogenic areas that were recognised by animal sera in Nef, Rev and Tat, the characterisation of human epitopes had gained less attention. Especially poorly characterised were Rev and Tat proteins. Our epitope mapping using relatively short peptides scanning the whole protein sequences were among the first ever done, and untill now not many new reports have come out (Tables 1-3).

Studies I, II and III revealed several antigenic epitopes in Nef, Rev and Tat. In Nef, the majority of sera from HIV-positive individuals, as well as HIV-negative individuals belonging to the HIV-risk group, recognised several of the identified nine epitopes, whereas false-positive sera from the HIV-negative non-risk group did not. Similarly in Rev and Tat, three epitopes were shown to be specifically recognised only by HIV-infected individuals. Thus, these epitopes seem to be genuinely HIV-specific and potentially useful as markers of latent infection. The only non-risk group individual, whose serum strongly recognised the Nef-specific epitopes, was a heterosexual man with no known HIV exposure, but a newly recognised autoimmune thyroiditis. As Nef is homological to human thyrotropin receptor, and as antibodies generated against a thyrotropin receptor peptide representing this homological region are cross-reactive with Nef protein (Bursch et al. 1991), the reactivity against Nef seen with this patient´s serum propably is a consequence of the autoimmune disease. This observation was done in 1990 when retroviruses in autoimmune thyroid diseases were unknown; since then HTLV-I and HTLV-II have been connected to these diseases (Yokoi et al. 1995, Akamine et al. 1996), and a putative nef mRNA has been isolated from HTLV-I infected cells (Orita et al. 1993). Our observation also revealed, that by characterisation of the epitopes, most false-positive responses can be

eliminated, but cross-reaction with antibodies induced perhaps by other retroviruses may still exist.

In Nef, epitope 1 overlaps with human epitopes found by Kienzle et al. (1989), epitope 2 with human epitopes found by Ameisen et al. (1989), Sabatier et al. (1989) and Schneider et al. (1991) and epitope 3a with epitope found by Yamada et al. (1999); epitope 3b has not been shown to be immunogenic by others; epitope 3c overlaps with one epitope recognised by Schneider et al. (1991); epitope 3d is shown by others to be immunogenic in animals; epitope 4 overlaps with epitope found by Schneider et al. (1991); epitopes 5 and 6 overlap with epitopes found by Sabatier et al. (1989). Most of these epitopes are also immunogenic in mice or non-human primates. In Rev, Dairaku et al. (1989) had shown that amino acids 33-48 are immunogenic in humans, and we also found an epitope at this region. In addition, we characterised three more epitopes not previously shown to be immunogenic in humans, but later shown to be immunogenic in mice. In Tat, three of the recognised epitopes overlap with found human and animal epitopes, and the fourth (aa 21-37) overlaps with a recently developed single-chain antibody. Taken together, as several reports confirm our findings, the epitopes we have found represent areas immunogenic in vivo.

The minimisation procedure performed in Study II revealed an interesting phenomenon: epitopes found by longer peptides may actually consist of one linear region or of several, almost adjacent amino acid stretches. Futhermore, these closely situated stretches may give a positive result to a long peptide mainly consisting of the intervening, non-immunogenic amino acids and thus give distorted information about an epitope. As seen in Table 4, when the minimal epitopes of Nef were characterised, one-stretch epitopes (eg. 2, 3d, 6) were shown to be situated clearly in the middle of the previously detected longer epitopes, whereas in two- or three-stretch epitopes (eg. 1, 3a, 3b, 3c, 5) antigenic amino acids were also scattered in the flanking regions of them. Taking together, the matrix analysis allows the borders of the epitopes to be defined more exactly, so in epitope mapping the use of peptides of varying lengths is more informative.

Table 4. Minimisation of Nef epitopes

Epitope number	Epitopes found with 9-mer peptides (aa)	Epitopes found with 3-8-mer peptides (aa)
1	8 – 16	5 - 7, 11 - 13, 15 – 22
2	53 – 60	56 – 60
3a	80 – 90	75 – 79, 88 – 92
3b	97 – 101	103 – 107, 111 – 113
3c	115 – 127	114 -117, 118 – 126, 132 – 132
3d	136 –147	137 –141
4	158 – 169	ND*
5	180 – 191	179 – 188, 195 – 197
6	197 – 206	200 - 203

* Not done

3. Applicability of recognised epitopes

As antibodies against HIV-1 structural proteins may appear as late as several months after the infection, the epitopes of Nef, Rev or Tat and an antibody response against them were thought to be useful as an early indication of HIV-1 infection. However, subsequent studies revealed that antibodies against non-structural proteins are only seldom seen before antibodies against structural proteins. Furthermore, as shown in Study I, by using epitopes most false-positive responses could be eliminated, but cross-reaction with antibodies perhaps induced by other retroviruses still existed. Thus, the diagnostic value of these epitopes remains limited.

On the other hand, many of these epitopes overlap partly or totally with known T-cell epitopes. This makes them suitable to be used in a vaccine capable of inducing both humoral and cell-mediated responses. Several neutralising and/or protective peptide

vaccines have been developed against viruses, eg. foot-and-mouth disease, hepatitis B, human rhinovirus, human respiratory syncytial virus (DiMarchi et al. 1986, McCray et al. 1987, Emini et al. 1989, Bastien et al. 1999). Previously these peptide vaccines needed to be conjugated to carrier proteins, but recently multiepitopic vaccines have been delivered with DNA immunisation methods (Fomsgaard et al. 1999, Hanke et al. 1999, Ishioka et al. 1999). In addition to raising both humoral and cell-mediated responses, vaccines based on identified epitopes are safer, as these relatively short amino acid chains are not capable of inducing all the harmful effect of Nef, Rev and Tat described in detail in Chapters 1.2, 1.3 and 1.4.

Many of the identified epitopes overlap with functionally important areas of Nef, Rev and Tat. Thus, antibodies binding to these sites may prevent the correct function of these proteins, and in such way participate in virus neutralisation. Several reports, which show that antibodies against Nef, Rev and Tat have protective effects in HIV-infected individuals (O´Shea et al. 1991, Reiss et al. 1991, Re et al. 1995, Zagury J-F et al. 1998, Chen et al. 1999, Cohen et al. 1999, Yamada et al. 1999,) and can inhibit replication in in vitro assays (Pardridge et al. 1994, Steinaa et al. 1994, Mhashilkar et al. 1995, Re et al. 1995, Wu et al. 1996, Cruikshank et al. 1997, Poznansky et al. 1998, Tosi et al. 2000), support the opinion of their beneficial effects. Furthermore, passive immunisation of immunoglobulins recognising envelope structures have been shown to be protective in primates (Prince et al. 1991, Emini et al. 1992, Shibata et al. 1999, Baba et al. 2000, Mascola et al. 2000); passive immunotherapy of Nef, Rev or Tat antibodies has not been tested, but is worth consideration. Such a therapy could be especially efficient against Tat, which has been shown to be functionally active extracellularly, but might also work against Nef and Rev, which are present in cell debris.

In Nef, antigenic sites overlapping with functionally important areas are in epitope 1 region amino acids 17-26 and partially in epitope 3a region amino acids 69-78 necessary for MCH I down-regulation (Mangasarian et al. 1999); in epitope 2 region amino acids 57-59 (WLE) responsible for CD4 binding during down-regulation (Grzesiek et al. 1996); in epitope 3a region PXXP motif binding to SH3 domains Src kinases (Saksela et al. 1995); in epitope 3b region RR motif needed for CD4 down-regulation and optimal infectivity (Aiken et al. 1996, Wischerchen et al. 1996, Craig

et al. 1998); in epitope 3c region D123 required for oligomerisation of Nef (Liu et al. 2000); in epitope 3d region amino acids 132-147 regulating productive HIV infection from latency (Fujinaga et al. 1995) and in epitope 4 region endocytosis motif (ENTSLL) needed for CD4 down-regulation and optimal viral infectivity (Wischerchen et al. 1996, Breshnahan et al.1998, Craig et al. 1998,).

In Rev, the epitope between amino acids 38-49 is located inside the domain responsible for nuclear localisation and RNA-binding (Malim et al. 1989). The carboxy-terminal epitope between amino acids 78-83 overlaps with the region shown to contain the NES (Fuscher et al. 1995, Wen et al. 1995) and to be the effector domain able to activate cellular proteins intrinsic to nuclear mRNA transport (Venkatesh et. al. 1990, Malim et al. 1991b).

In Tat, the amino terminal epitope aa 6-10 overlaps with the region shown to be associated with the inhibition of antigen- and mitogen-induced proliferation of cells (Wrenger et al. 1997, Viscidi et al. 1989, Benjouad et al. 1993, Chirmule et al. 1995, Zagury D et al. 1998) and the increment of viral reverse transcription (Ulich et al. 1999). The epitope between amino acids 21-37 overlaps with the region responsible for intramolecular disulfide bond formation of Tat (Koken et al. 1994), for attracting and binding to monocytes (Albini et al. 1998a), for inducing HIV replication and participating in TAR-dependent trans-activation and for triggering angiogenesis (Boykins et al. 1999). The epitope in aa 39-58 overlaps with minimal activation domain, with amino acids needed for binding to TAR and inducing nuclear localisation (Dingwall et al. 1989, Hauber et al. 1989, Carrol et al. 1991), with the region involved in the uptake of protein from extracellular space and in angiogenesis and neurotoxic effects of Tat (Sabatier et al. 1991, Weeks et al. 1995, Albini et al. 1996, Chang et al. 1997, Vives et al. 1997). The carboxy-terminal epitope of Tat in aa 74-82 contains the RGD adhesion sequence shown to be involved in integrin-mediated cell adhesion of Tat (Brake et al. 1990b), to be responsible for aggregation of neurons and astrocytes, and to act as a chemo-attractant for dendritic cells and monocytes (Kolson et al. 1993, Benelli et al. 1998) impairing also their function (Zocchi et al. 1997).

4. DNA vaccination and humoral response

In Studies IV and V it was shown that with DNA immunisation, antibodies against Nef were elicited, but the percentage of responding mice was clearly dependent on the vector used for immunisation. The best humoral response was achieved with the replicating-competent pBN-plasmids supposed to produce the cloned antigen for a prolonged period. This vector was also capable of inducing cell-mediated responses in mice, and immune responses were elicited using different immunisation methods. However, even though we showed in in vitro assays that pBN-plasmids carrying either rev or tat genes produced the corresponding functionally active proteins, the in vivo immune response raised by them was different from the one raised against Nef, with the lack of an antibody response being the most striking difference. Rev was able to induce cell-mediated responses, but Tat was inefficient also in this respect. Our results were consistent with previous DNA vaccination studies with a CMV-based vector, where it was shown that in the sera of Balb/c mice immunised with HIV-1 regulatory genes, antibodies against Nef were found frequently but antibodies against Rev and Tat were almost absent (Hinkula et al. 1997). Also, DNA vaccination studies in humans and primates showed stronger antibody response against Nef than against Rev or Tat (Putkonen et al. 1998, Calarota et al. 1999).

There may be several reasons for the lack of an antibody response against Rev and Tat. Firstly, small quantities of antigen (<1 ng) are sufficient to elicit cellular responses in genetic immunisation, whereas at least a 40-fold higher amount of the same antigen is required to elicit a high titer antibody response (Barry et al. 1997). In the case of our plasmids, the Nef construct may be superior to Rev and Tat constructs in this respect. Secondly, natural Rev and Tat proteins are less immunogenic than Nef also in HIV-infected individuals, which may be the consequence of the bigger size of Nef or the different localisation of these three proteins within the cell.

Several parameters associated with the antigen affect the efficiency of DNA vaccines to induce humoral response. The cellular localisation of the DNA vaccine antigen has a significant effect on the magnitude but not on the subclass of antibody responses: humoral response is suboptimal when antigen is cytoplasmic, better with membrane-associated antigens and best with secreted antigens (Boyle et al. 1997, Drew et al. 2000). In this respect one would expect Tat to be a better antigen than Nef. However,

it was recently shown that when a signal sequence mediating antigen secretion was added to a plasmid used for DNA immunisation, the immune response was enhanced for other proteins tested but not for Tat (Svanholm et al. 1999). This may be due to the strong nuclear localisation signal within Tat that prevents the protein from reaching the subcellular secretory pathway, thus weakening its antigenic presentation. Furthermore, the immunosuppressive function of Tat (Viscidi et al. 1989, Benjouad et al. 1993, Chirmule et al. 1995, Zagury D et al. 1998) also extends into the inhibition of antibody production (Cohen et al. 1999).

Depending on the immunisation methods used, different cells may be responsible for taking up the injected plasmid, and antigen processing and presentation in these cells may vary, leading to humoral and cellular immune responses towards different epitopes. In Study V it was shown that regardless of whether the pBN-Nef plasmid is delivered by gene gun or is injected through intradermal or intramuscular route, the antibodies directed against Nef recognise two main epitopes between amino acids 9-24 and 49-64 (SVIGWLTVRERMRRAE and AATNAACAWLEAQEEE, respectively). These epitopes overlap with the epitopes found in HIV-infected individuals (Study I and II) and with the epitopes found in mice immunised with corresponding whole protein (Ovod et al. 1992). Furthermore, in Study V, the antibody subclasses were characterised in order to see whether any of the immunisation routes would favour either Th-type 1 or 2 response. The results of the antibody subclass analysis divided the responses into separate Th1 (IM) and Th2 (GG) pathways, and the detected IgG1 and IgG2a subclass pattern remained stable throughout the follow-up period. This is consistent with previous findings (Pertmer et al. 1996), where it was shown that in DNA immunisation, the antibody subclass pattern is already fixed at the primary immunisation and can not be modulated even by successive immunisations using other methods.

Taking together, studies IV and V showed that Nef is a potent antigen when introduced in a DNA vaccine. As Rev and Tat in DNA vaccines have repeatedly been shown to be poor inducers of humoral response, modifications improving the antigenicity of them may be necessary. Furthermore, as each of these three proteins may have severe negative effects on transfected or bystander cells, the safety of

vaccines should be improved by mutations targeted to functionally important regions of these proteins or by using multiepitopic amino acid sequences as antigens.

SUMMARY AND CONCLUSIONS

HIV-1 is a retrovirus causing the gradual destruction of immune system in infected individuals, and ultimately leads to the lethal acquired immune deficiency syndrome (AIDS). In addition to the structural proteins, HIV-1 codes for accessory and regulatory proteins necessary for replication of the virus, for maximal infectivity and for various cellular events enhancing the spreading of the virus. As the accessory and regulatory proteins Nef, Rev and Tat are more conserved than Env, they have become important targets for the design of vaccines. This thesis has concentrated on the humoral immune responses elicited by the accessory protein Nef and the regulatory proteins Rev and Tat.

The results show that of these three proteins, Nef is the most immunogenic in infected individuals. It contains nine antigenic regions scattered throughout the protein. Both Rev and Tat contain four antigenic regions specifically recognised by sera from HIV-1 infected individuals. Many of the identified human B-cell epitopes either totally or partially overlap with B-cell epitopes recognised by animal sera, with known T-cell epitopes and with functionally important areas of the proteins.

New DNA vaccine constructs carrying the whole Nef, Rev or Tat genes were tested for immunogenicity in mice. The pBN-Nef construct was the most immunogenic, capable of inducing both humoral and cell-mediated responses that were detectable throughout the follow-up period. Furthermore, an immune response was elicited regardless of whether the plasmid was delivered by gene gun or injected through intradermal or intramuscular route. The Rev construct elicited only a cell-mediated response, and the Tat-construct was a poor immunogen in all respects. Thus, in future vaccine studies, modifications/methods improving their antigenicity may be necessary. Furthermore, as each of these three proteins may have severe negative effects on transfected or bystander cells, the safety of vaccines should be improved by mutations targeted to functionally important regions of these proteins or by using multiepitopic amino acid sequences as antigens. The epitopes characterised in this

study are good candidates for this; as they overlap known T-cell epitopes, they might induce both humoral and cell-mediated responses.

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Marja Tähtinen

REFERENCES

Ahmad N and Venkatesan S (1988): Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science 241: 1481-1485.

Ahmad A and Menezes J (1996): Antibody-dependent cellular cytotoxicity in HIV infections. FASEB J 10: 258-266.

Aiken C, Konner J, Landau NR, Lenburg ME and Trono D (1994): Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 sytoplasmic domain. Cell 76: 853-864.

Aiken C and Trono D (1995): Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J Virol 69: 5048-5056.

Aiken C, Krause L, Chen Y-L and Trono D (1996): Mutational analysis of HIV-1 Nef: Identification of two mutants that are temperature-sensitive for CD4 downregulation. Virology 217: 293-300.

Akamine H, Takasu N, Komiya I, Ishikawa K, Shinjyo T, Nakachi K and Masuda M (1996): Association of HTLV-I with autoimmune thyroiditis in patients with adult T-cell leukaemia (ATL) and in HTLV-I carriers. Clin Endocrinol 45: 461-466.

Akari H, Arol S, Fukumori T, Okazaki T, Strebel K and Adachi A (2000): Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J Virol 74: 2907-2912.

Albini A, Benelli R, Presta M, Rusnati M, Ziche M, Rubartelli A, Paglialubga G, Bussolino F and Noonan D (1996): HIV-tat protein is a heparin-binding angiogenic growth factor. Oncogene 12: 289-297.

Albini A, Benelli R, Giunciuglio D, Cai T, Mariani G, Ferrini S and Noonan DM (1998a): Identification of a novel domain of HIV tat involved in monocyte chemotaxis. J Biol Chem 273: 15895-15900.

Albini A, Ferrini S, Benelli R, Sforzini S, Giunciuglio D, Aluigi MG, Proudfoot AE, Alouani S, Wells TN, Mariani G, Rabin RL, Farber JM and Noonan DM (1998b): HIV-1 Tat protein mimicry of chemokines. Proc Natl Acad Sci USA 95: 13153-13158.

Aldovini A, Debouck C, Feinberg MB, Rosenberg M, Arya SK, Wong-Staal F (1986): Synthesis of the complete trans-activation gene product of human T-lymphotropic virus type III in Eschericia coli: Demonstration of immunogenicity in vivo and expression in vitro. Proc Natl Acad Sci USA 83: 6672-6.

Al-Harthi L and Roebuck KA (1998): Human immunodeficiency virus type-1 transcription: role of the 5´-untranslated leader region (review). Int J Mol Med 1: 875-881.

Ambrosini E, Slepko N, Kohleisen B, Shumay E, Erfle V, Aloisi F and Levi G (1999): HIV-1 Nef alters the expression of betaII and epsilon isoforms of protein kinase C and activation of the long terminal repeat promoter in human astrocytoma cells. Glia 27: 143-151.

Ameisen J-C, Guy B, Chamaret S, Loche M, Mouton Y, Neyrinck J-L, Khalife J, Leprevost C, Beaucaire G, Boutillon C, Gras-Masse H, Maniez M, Kieny M-P, Laustriat D, Berthier A, Mach B, Montagnier L, Lecocq J-P and Capron A (1989): Antibodies to the NEF protein and to NEF peptides in HIV-1 infected seronegative individuals. AIDS Res Hum Retroviruses 5: 279-291.

Anderson SJ, Lenburg, M, Landau NR and Garcia JV (1994): The cytoplasmid domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef. J Virol 68: 3092-3101.

Arold S, Hoh F, Domerque S, Birck C, Delsue MA, Jullien M and Dumas C (2000): Characterization and molecular basis of the oligomeric structure of HIV-1 nef protein. Protein Sci 9: 1137-1148.

Arya SK and Gallo RC (1986): Three novel genes of human T-lymphotropic virus type III: immune reactivity of their products with sera from acquired immune deficiency syndrome patients. Proc Natl Acad Sci USA 83: 2209-2213.

Asakura Y, Hamajima K, Fukushima J, Mohri H, Okubo T and Okuda K (1996): Induction of HIV-1 Nef-specific cytotoxic T lymphocytes by Nef-expressing DNA vaccine. Am J Hematol 53: 116-117.

Ayyavoo V, Kudchodkar S, Ramanathan MP, Le P, Muthumani K, Megalai NM, Dentchev T, Santiago-Barrios L, Mrinalini C and Weiner DB (2000): Immunogenicity of a novel DNA vaccine cassette expressing multiple human immunodeficiency virus (HIV-1) accessore genes. AIDS 14: 1-9.

Baba TW, Jeong YS, Pennick D, Bronson R, Greene MF and Ruprecht RM (1995): Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macagues. Science 267: 1820-1825.

Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Aychunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu Y, Wright JE, Chou TC and Ruprecht RM (2000): Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal-simian-human immunodeficiecny virus infection. Nat Med 6: 200-206.

Bahraoui E, Yagello M, Billaud J-N, Sabatier J-M. Guy B, Muchmore E, Girard M and Gluckman J-C (1990): Immunogenicity of the human immunodeficiency virus (HIV) recombinant nef gene product. Mapping of T-cell and B-cell epitopes in immunized chimpanzees. AIDS Res Hum Retroviruses 6: 1097-1098.

Bandres JC, Luria S and Ratner L (1994): Regulation of human immunodeficiency virus Nef protein by phosphorylation. Virology 201: 157-161.

Bandres JC, Shaw AS and Ratner L (1995): HIV-1 Nef protein downregulation of CD4 surface expression: relevance of the lck binding domain of CD4. Virology 207: 338-341.

Barone AD, Silva JA, Ho DD, Gallo RC, Wong-Staal F, Chang NT (1986): Reactivity of E coli derived trans-activating protein on human lymphotropic virus type III with sera from patients with acquired immune deficiency syndome. J Immunol 137: 669-673.

Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L (1983): Isolation of a T-lymphocyte retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220: 868-871.

Barry MA, and Johnston A (1997): Biological features of genetic immunisation. Vaccine 15: 788-791.

Bartel DP, Zapp ML, Green MR and Szostak JW (1991): HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. Cell 67: 529-536.

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

Bastien N, Trudel M and Simard C (1999): Complete protection of mice from respiratory syncytial virus infection following mucosal delivery of synthetic peptide vaccine. Vaccine 17: 832-836.

Battiste JL, Mao H, Rao NS, Tan R, Muhandiram DR, Kay LE, Frankel AD and Williamson JR (1996): Alpha helix-RNA major groove recognition in an HIV-1 rev peptide-RRE RNA complex. Science 273: 1547-1551.

Baum LL, Cassutt KJ, Knigge K, Khattri R, Margolick J, Rinaldo C, Kleeberger CA, Nishanian P, Henrard DR and Phair J (1996): HIV-1 gp 120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. J Immunol 157: 2168-2173.

Bayer P, Kraft M, Ejchart A, Westendorp M, Frank R and Rösch P (1995): Structural studies of HIV-1 Tat protein. J Mol Biol 247: 529-535.

Bell I, Ashman C, Maughan J, Hooker E, Cook F and Reinhart TA (1998): Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) zeta chain leads to TCR down-modulation. J Gen Virol 79: 2717-2727.

Benelli R, Mortarini R, Anichini A, Giunciuglio D, Noonan DM, Montalti S, Tacchetti C and Albini A (1998): Monocyte-derived dendritic cells and monocytes migrate to HIV-Tat RGD and basic peptides. AIDS 12: 261-268.

Benjouad A, Mabrouk K, Moulard M, Gluckman JC, Rochat H, Van Rietschoten J and Sabatier JM (1993): Cytotoxic effect on lymphocytes of Tat from human immunodeficiency virus (HIV-1). FEBS Lett 319: 119-124.

Berberian L, Valles-Ayoub Y, Sun N, Martinez-Maza O and Braun J (1991): A VH clonal deficit in human immunodeficiency virus-positive individuals reflect a B-cell maturation arrest. Blood 78: 175-179.

Berkhout B, Verhoef K, van Wamel JLB and Back NKT (1999): Genetic instability of live, attenuated human immunodeficiency virus type 1 vaccine strains. J Virol 73: 1138-1145.

Bevec D, Jaksche H, Oft M, Wohl T, Himmelspach M, Pacher A, Schebesta M, Koettnitz K, Dobrovnik M, Csonga R, Lottspeich Fand Hauber J (1996): Inhibition of HIV-1 replication in lymphocytes by mutant of the Rev cofactor eIF-5A. Science 271: 1858-1860.

Billaut-Mulot O, Idziorek T, Ban E, Kremer L, Dupre L, Loyens M, Riveau G, Locht C, Capron A and Bahr GM (2000): Interleukin-18 modulates immune responses induced by HIV-1 Nef DNA prime/protein boost vaccine. Vaccine 19: 95-102.

Blazevic V, Ranki A, Mattinen S, Valle S-L, Koskimies S, Jung G and Krohn KJE (1993): Helper T-cell recognition of HIV-1 Tat synthetic peptides. J Aquir Immune Defic Syndr 6: 881-890.

Blazevic V, Ranki A and Krohn KJE (1995): Helper and cytotoxic T-cell responses of HIV type 1-infected individuals to synthetic peptides of HIV type 1 Rev. AIDS Res Hum Retroviruses 11: 1335-1341.

Blazevic V, Heino M, Lagerstedt A, Ranki A and Krohn K (1996): Interleukin-10 gene expression induced by HIV-1 Tat and Rev in the cells of HIV-1 infected individuals. J Acquir Immune Defic Syndr 13: 208-214.

Bodeus M, Marie-Cardine A, Bougeret C, Ramos-Morales F and Benarous R (1995): In vitro binding and phosphorylation of human immunodeficiency virus type 1 Nef protein by serine/threonine protein kinase. J Gen Virol 76: 1337-1344.

Boese A, Sauter M and Mueller-Lantzsch N (2000): A rev-like NES mediates cytoplasmic localization of HERV-K cORF. FEBS Lett 468: 65-67.

Bonavida B, Katz J and Gottlieb M (1986): Mechanism of defective NK cell activity in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. I. Defective trigger on NK cells for NKCF production by target cells, and partial restoration by IL 2. J Immunol 137: 1157-1163.

Borgatti P, Zauli G, Colamussi ML, Gibellini D, Previati M, Cantley LL and Capitani S (1997): Extracellular HIV-1 Tat protein activates phosphatidylinositol 3- and Akt/PKB kinases in CD4+ T lymphoblastoid Jurkat cells. Eur J Immunol 27: 2805-2811.

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

Boyle JS, Koniaras C and Lew AM (1997): Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. Int Immunol 9: 1897-1906.

Boue F, Wallon C, Goujard C, Barre-Sinoussi F, Galanaud P and Delfraissy JF (1992): HIV induces IL-6 production by human B lymphocytes. Role of IL-4. J Immunol 148: 3761-3767.

Boyer JD, Cohen AD, Vogt S, Schumann K, Nath B, Ahn L, Lacy K, Bagarazzi ML, Higgins TJ, Baine Y, Ciccarelli RB, Ginsberg RS, MacGregor RR and Weiner D (2000): Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of β-chemokines. J Infect Dis 181: 476-483.

Boykins RA, Mathieux R, Shankavaram UT, Gho YS, Lee SF, Hewlett IK, Wahl LM, Kleinman HK, Brady JN, Yamada KM and Dhawan S (1999): Cutting edge: a short polypeptide domain of HIV-1 Tat protein mediates pathogenesis. J Immunol 163: 15-20.

Brake DA, Goudsmit J, Krone WJA, Schammel P, Appleby N, Meloen RB et al. (1990a): Characterization on murine monoclonal antibodies to the Tat protein from human immunodeficiency virus type 1. J Virol 64: 962-965.

Brake DA, Debouck C and Biesecker G (1990b): Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type 1 transactivation protein, tat. J Cell Biol 111: 1275-1281.

Bresnahan PA, Yonemoto W, Ferrell S, Williams-Herman D, Geleziunas R and Greene WC (1998): A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. Curr Biol 8: 1235-1238.

Briggs SD, Sharkey M, Stevenson M and Smithgall TE (1997): SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1. J Biol Chem 272: 17899-17902.

Briggs SD, Lerner EC and Smithgall TE (2000): Affinity of Src family kinase SH3 domains for HIV Nef in vitro does not predict kinase activation by Nef in vivo. Biochemistry 39: 489-495.

Buonaguro L, Barillari G, Chang HK, Bohan CA, Kao V, Morgan R, Gallo RC and Ensoli (1992): Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. J Virol 66: 7159-7167.

Buratti E, Tisminetzky SG, D´Agaro P and Baralle FE (1997): A neutralizing monoclonal antibody previously mapped exclusively on human immunodeficiency virus type 1 gp41 recognizes an epitope in p17 sharing the core sequence IEEE. J Virol 71: 2457-2462.

Burch HB, Nagy EV, Lukes YG, Cai WY, Wartofsky L and Burman KD (1991): Nucleotide and amino acid homology between the human thyrotropin receptor and the HIV-1 Nef protein: identification and functional analysis. Biochem Biophys Res Commun 181: 498-505.

Böhnlein E, Berger J and Hauber J (1991): Functional mapping of the human immunodeficiency virus type 1 Rev RNA binding domain: new insights into the domain structure of Rev and Rex. J Virol 65: 7051-7055.

Cafaro A, Caputo A, Fracasso C, Maggiorella MT, Goletti D, Baroncelli S, Pace M, Sernicola L, Koanga-Mogtomo ML, Betti M, Borsetti A, Belli R, Åkerblom L, Corrias F, Butto S, Heeney J, Verani P, Titti F, and Ensoli B (1999): Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. Nat Med 5: 643-650.

Calarota SA, Leandersson A-C, Bratt G, Hinkula J, Klinman DM, Weinhold KJ, Sandström E and Wahren B (1999): Immune responses in asymptomatic HIV-1 infected patients after HIV-DNA immunisation followed by highly active antiretroviral treatment. J Immunol 162: 2330-2338.

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

Caselli E, Betti M, Grossi MP, Balboni PG, Rossi C, Boarini C, Cafaro A, Barbanti-Brodano G, Ensoli B and Caputo A (1999): DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. J Immunol 162: 5631-5638.

Chanda PK, Ghrayeb J, Wong-Staal F (1988): Non-random distribution of antibodies to the TRS protein of human immunodeficiency virus in infected people with different clinical status. AIDS Res Hum Retroviruses 4: 11-16.

Chang HC, Samaniego F, Nair BC, Buonaguro L and Ensoli B (1997): HIV Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS 11: 1421-1431.

Chang AH, Hoxie JA, Cassol S, O´Shaughnessy M and Jirik F (1998): Construction of single-chain antibodies that bind an overlapping epitope of HIV-1 Nef. FEBS Lett 441: 307-312.

Charpentier B, Stutz F and Rosbash M (1997): A dynamic view of the HIV-1 Rev-RRE interaction. J Mol Biol 266: 950-962.

Cheingsong-Popov R, Panagiotidi C, Ali M, Bowcock S, Watkins P, Aronstam A, Wassef M and Weber J (1990): Antibodies to HIV-1 nef (p27): Prevalence, significance and relationship to seroconvercion. AIDS Res Hum Retroviruses 6: 1099-1105.

Chen YL, Trono D and Camaur D (1998): The proteolytic cleavage of human immunodeficiency virus type 1 Nef does not correlate with its ability to stimulate virion infectivity. J Virol 72: 3178-3184.

Chen YM, Lin RH, Lee CM, Fu CY, Chen SC and Syu WJ (1999): Decreasing levels of anti-Nef antibody correlate with increasing HIV type 1 viral loads and AIDS disease progression. AIDS Res Hum Retroviruses 15: 43-50.

Chirmule N, Than S, Khan SA and Pahwa S (1995): Human immunodeficiency virus Tat induces functional unresponsiveness in T cells. J Virol 69: 492-498.

Choi J, Liu RM, Kundu RK, Sangiorgi F, Wu W, Maxson R and Forman HJ (2000): Molecular mechanism of decreased glutathione content in human immunodeficiency cirus type 1 Tat-transgenic mice. J Biol Chem 275: 3693-3698.

Chowers MY, Pandori MW, Spina CA, Richmann DD and Guatelli JC (1995): The growth advantage conferred by HIV-1 nef is determined at the level of viral DNA formation and is independent of CD4 downregulation. Virology 212: 451-457.

Churcher MJ, Lamont C, Hamy F, Dingwall C, Green SM, Lowe AD, Butler JG, Gait MJ and Karn J (1993): High affinity binding of TAR RNA by the human immunodeficiency virus type-1 tat protein requires basepairs in the RNA stem and amino acids flanking the basic region. J Mol Biol 230: 90-110.

Clapham P, Nagy K and Weiss RA (1984): Pseudotypes of human T-cell leukemia virus types 1 and 2: neutralization by patients´ sera. Proc Natl Acad Sci USA 81: 2886-2889.

Clapham PR and Weiss RA (1997): Immunodeficiency viruses. Spoilt for choice of co-receptors. Nature 388: 230-231.

Coates K, Cooke SJ, Mann DA and Harris MPG (1997): Protein kinase C mediated phosphorylation of HIV-1 nef in human cell lines. J Biol Chem 272: 12289-12294.

Cochrane AW, Golub E, Volsky D, Ruben S and Rosen CA (1989): Functional significance of phosphorylation to the human immunodeficiency virus Rev protein. J Virol 63: 4438-4440.

Cohen EA, Subbramanian RA and Gottlinger HG (1996): Role of auxiliary proteins in retroviral morphogenesis. Curr Top Microbiol Immunol 214: 219-235.

Cohen SS, Li C, Ding L, Cao Y, Pardee AB, Shevach EM and Cohen DI (1999): Pronounced acute immunosuppression in vivo mediated by HIV Tat challenge. Proc Natl Acad Sci USA 96: 10841-10847.

Cole JL, Gehman JD, Shafer JA and Kuo LC (1993): Solution oligomerization of the rev protein of HIV-1: implications for function. Biochemistry 32: 11769-11775.

Cole KS, Murphey-Corb M, Narayan O, Joang SV, Shaw GM and Montelaro RC (1998): Common themes of antibody maturation to simian immunodeficiency virus, simian-human immunodeficiency virus and human immunodeficiency virus type 1 infections. J Virol 72: 7852-7859.

Collette Y, Dutartre H, Benziane A, Ramos-Morales J, Benarous R, Harris M and Olive D (1996a): Physical and functional interaction of Nef with Lck. HIV-1 Nef induced T-cell signalling defects. J Biol Chem 271: 6333-6341.

Collette Y, Chang HL, Cerdan C, Chambost H, Algarte M, Mawas C, Imbert J, Burny A and Olive D (1996b): Specific Th1 cytokine down-regulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene-induced expression. J Immunol 156: 360-370.

Collins KL, Chen BK, Kalams SA, Walker BD and Baltimore D (1998): HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature 391: 397-401.

Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, Gallo RC and Major EO (1998): Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. Proc Natl Acad Sci USA 95: 3117-3121.

Connick E, Marr DG, Zhang XQ, Clark SJ, Saag MS, Schooley RT and Curiel TJ (1996): HIV-specific cellular and humoral immune responses in primary HIV infection. AIDS Res Hum Retroviruses 12: 1129-1140.

Connor RI, Korber BT, Graham BS, Hahn BH, Ho DD, Walker BD, Neumann AU, Vermund SH, Mestecky J, Jackson S, Fenamore E, Cao Y, Gao F, Kalams S, Kunstman KJ, McDonald D, McWilliams N, Trkola A, Moore JP and Wolinsky SM (1998): Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines.J Virol 72: 1552-1576.

Cook KS, Fisk GJ, Hauber J, Usman N, Daly TH and Rusche JR (1991): Characterization of HIV-1 REV protein: binding stoichiometry and minimal RNA substrate. Nucleic Acids Res 19: 1577-1583.

Craig HM, Pandori MW and Guatelli JC (1998): Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. Proc Natl Acad Sci USA 95: 11229-11234.

Cranage MP, Whatmore AM, Sharpe SA, Cook N, Polyanskaya N, Leech S, Smith JD, Rud EW, Dennis MJ and Hall GA (1997): Macaques infected with live attenuated SIVmac are protected against superinfection via the rectal mucosa. Virology 229:143-154.

Cruikshank WW, Doctrow SR, Falvo MS, Huffman K, Maciaszek J, Viglianti G, Raina J, Kornfeld H and Malfoy B (1997): A lipidated anti-Tat antibody enters living cells and blocks HIV-1 viral replication. J Acquir Immune Defic Syndr Hum Retrovirol 14: 193-203.

Cullman B, Gomard E, Kieny M-P, Guy B, Dreyfus F, Saimot A-G, Sereni D, Sicard D, Levy J-P (1991): Six epitopes reacting with human cytotoxic $CD8^+$ T cells in the central region of the HIV-1 NEF protein. J Immunol 146: 1560-1565.

Dairaku M, Tsuchie H, Ushijima H, Aoki I, Okuda K, Honda M et al. (1989): Incidence and titer of anti-Rev antibody in HIV infection. AIDS 3: 765-766.

Daly TJ, Cooks KS, Gray GS, Maione TE and Rusche JR (1989): Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element in vitro. Nature 342: 816-819.

Daly TJ, Doten RC, Rennert P, Auer M, Jaksche H, Donner A, Fisk G and Rusche JR (1993): Biochemical characterization of binding of multiple HIV-1 Rev monomeric proteins to the Rev responsive element. Biochemistry 32: 10497-10505.

Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK and Desrosiers RC (1992): Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science 258: 1938-1941.

Daniel V, Susal C, Weimer R, Zipperle S, Kropelin M, Zimmermann R, Huth-Kuhne A and Opelz G (1996): Association of T cell dysfunction with the presence of IgG autoantibodies on CD4+ lymphocytes in haemophilia patients; results of a 10-year study. Clin Exp Immunol 104: 4-10.

Dayton AI, Sodroski JG, Rosen CA, Chun Goh W and Haseltine WA (1986): The trans-activating gene of the human T cell lymphotropic virus type III is required for replication. Cell 44: 941-947.

Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A and Chatfield C (1995): Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science 270: 988-991.

Del Prete GF, De Carli M, Ricci M and Romagnani S (1991): Helper activity for immunoglobulin synthesis of TH1 and TH2 human T cell clones: The help of Th1 clones is limited by their cytolytic capacity. J Exp Med 174: 809-813.

Del Sorbo L, DeMartino A, Biancone L, Bussolati B, Conaldi PG, Toniolo A and Camussi G (1999): The synthesis of platelet-activating factor modulates chemotaxis of monocytes induced by HIV-1 Tat. Eur J Immunol 29: 1513-1521.

De Maria A, Cirillo C and Moretta L (1994): Occurrence of human immunodeficiency virus type 1 (HIV-1)-specific cytolytic T cell activity in apparently uninfected children born to HIV-1-infected mothers. J Infect Dis 170: 1296-1299.

Demirhan I, Chandra A, Hasselmayer O and Chandra P (1999a): Intercellular traffic of human immunodeficiency virus type 1 transactivator protein defined by monoclonal antibodies. FEBS Lett 445: 53- 56.

Demirhan I, Chandra A, Hasselmayer O, Biberfeld P and Chandra P (1999b): Detection of distinct patterns of anti-tat antibodies in HIV-infected individuals with or without Kaposi´s sarcoma. J Acquir Immune Defic Syndr 22: 364- 368.

De Ronde A, Reiss P, Dekker J, De Wolf F, Van den Hoek A, Wolps T, Debouck C and Goudsmit J (1988): Seroconversion to HIV-1 negative regulation factor. Lancet 2: 574.

De Ronde A, Klaver B, Keulen W, Smit L and Goudsmit J (1992): Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes. Virology 188: 391-395.

Devash Y, Reagan K, Wood D, Turner J, Parrington M, Kang CY (1990): Antibodies against AIDS proteins. Nature 345: 581.

DiMarchi R, Brooke G, Gale C, Cracknell V, Doel T, Mowat N (1986): Protection of cattle against foot-and-mouth disease by a synthetic peptide. Science 232: 639-641.

Dimmock NJ (1984): Mechanisms of neutralization of animal viruses. J Gen Virol 65: 1015-1022.

Dingwall C, Ernberg I, Gait MJ, Green SM, Heapy S, Karn J, Lowe AD, Singh M, Skinner MA and Valerio R (1989): Human immunodeficeincy virus 1 Tat protein binds transactivation-responsive region (TAR) RNA in vitro. Proc Natl Acad Sci USA 86: 6925-6929.

Drew DR, Lightowlers M and Strugnell RA (2000): Humoral immune response to DNA vaccines expressing secreted, memrane bound and non-secreted forms of the Tania ovis 45W antigen. Vaccine 18: 2522-2532.

Du Z, Lang SM, Sasseville VG, Lackner AA, Ilyinskii PO, Daniel MD, Jung JU and Desrosiers RC (1995): Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. Cell 82: 665-764.

Emini EA, Larson V, Eichberg J, Conard P, Garsky VM, Lee DR, Ellis RW, Miller WJ, Anderson CA and Gerety RJ (1989): Protective effect of a synthetic peptide comprising the complete preS2 region of the hepatitis B virus surface protein. J Med Virol 28: 7-12.

Emini EA, Schleif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, Putney SD, Matsushita S, Cobb KE and Jett CM (1992): Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature 355: 728-730.

Ensoli B, Barillari G, Salahuddin SZ, Gallo RC and Wong-Staal F (1990): Tat protein of HIV-1 stimulates growth of cells derived from Kaposi´s sarcoma lesions of AIDS patients. Nature 345: 84-86.

Ensoli B, Buonaguro G, Barillari , Fiorelli V, Gendelman R, Morgan RA, Wingfield P and Gallo RC (1993): Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein of cell growth and viral transactivation. J Virol 67: 277-287.

Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang HK, Brady JN and Gallo RC (1994): Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi´s sarcoma. Nature 371: 674-680.

Evans LA, Thomson-Honnebier G, Steimer K, Paoletti E, Perkus ME, Hollander H and Levy JA (1989): Antibody-dependent cellular cytotoxicity is directed against both the gp120 and gp41 envelope proteins of HIV. AIDS 3: 273-276.

Evans TG, Keefer MC, Weinhold KJ, Wolff M, Montefiori D, Gorse GJ, Graham BS, McElrath MJ, Clements-Mann ML, Mulligan MJ, Fast P, Walker MC, Excler JL, Duliege AM and Tartaglia J (1999): A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with rgp120 elicits broad and durable CD8+ cytotoxic T lymphocyte responses in seronegative volunteers. J Infect Dis 180: 290-298.

Fackler OT, Kienzle N, Kremmer E, Boese A, Schramm B, Klimkait T, Kücherer C and Mueller-Lantzsch N (1997): Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization. Eur J Biochem 247: 843-851.

Fackler OT, Luo W, Geyer M, Alberts AS and Peterlin BM (1999): Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. Mol Cell 3: 729-739.

Fahey JL, Prince H, Weaver M, Grooplman J, Visscher B, Schwartz K and Detels R (1984): Quantitative changes in T helper or T suppressor/cytotoxic lymphocyte subsets that distinguis acquired immune deficiency syndrome from other immune subset disorders. Am J Med 76: 95-100.

Fankhauser C, Izaurralde E, Adachi Y, Wingfield P and Laemmli UK (1991): Specific complex of human immunodeficiency virus type 1 rev and nucleolar B23 proteins: dissociation by the Rev response element. Mol Cell Biol 11: 2567-2575.

Farjot G, Sergeant A and Mikaelian I (1999): A new nucleoporin-like protein interacts with both HIV-1 Rev nuclear export signal and CRM-1. J Biol Chem 274: 17309-17317.

Feinberg MB, Jarrett RF, Aldovini A, Gallo RC and Wong-Staal F (1986): HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell 46: 807-817.

Ferns RB, Partridge JC, Tisdale M, Hunt N and Tedder RS (1991): Monoclonal antibodies define linear and conformational epitopes pf HIV-1 pol gene product. AIDS Res Hum Retroviruses 7: 307-313.

Fields GB and Noble RL (1990): Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Peptide Protein Res 35: 161-214.

Fisher AG, Feinberg MB, Josephs SF, Harper ME, Marselle LM, Reyes G, Gonda MA, Aldovini A, Debouck C and Gallo RC (1986): The trans-activator gene of HTLV-III is essential for virus replication. Nature 320: 367-371.

Fischer U, Huber J, Boelens WC, Mattaj IW and Luhrmann R (1995): The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell 82: 475-483.

Floer M and Blobel G (1999): Putative reaction intermediates in Crm1-mediated nuclear protein export. J Biol Chem 274: 16279-16286.

Fomsgaard A, Nielsen HV, Kirkby N, Bryder K, Corbet S, Nielsen C, Hinkula J and Buus S (1999): Induction of cytotoxic T-cell responses by gene gun DNA vaccination with minigenes encoding influenca A virus HA and NP CTL-epitopes. Vaccine 18: 681-691.

Fornerod M, Ohno M, Yoshida M and Mattaj (1997): CRM1 is an export receptor for leucine-rich nuclear export signal. Cell 90: 1051-1060.

Foti M, Cartier L, Piguet V, Lew DP, Carpentier JL, Trono D and Krause KH (1999). The HIV nef protein alters Ca(2+) signaling in myelomonocytic cells through SH3-mediated protein-protein interaction. J Biol Chem 274: 34765-34772.

Franchini G, Robert-Guroff M, Aldovini A, Kan NC, Wong-Staal F (1987): Spectrum of antibodies against five HTLV-III antigens in infected individuals: Correlation of antibody prevalence with clinical status. Blood 69: 437-441.

Frankel AD and Pabo CO (1988): Cellular uptake of the Tat protein from human immunodeficiency virus. Cell 55: 1189-1193.

Frankel AD and Young JAT (1998): HIV-1: Fifteen proteins and an RNA. Annu Rev Biochem 67: 1-25.

Freed EO (1998): HIV-1 gag proteins: diverse functions in the virus life cycle. Virology 251: 1-15.

Fujii Y, Otake K, Tashiro M and Adachi A (1996a): Human immunodeficiency virus type 1 Nef protein on the cell surface is cytocidal for human CD4+ T cells. FEBS Lett 393: 105-108.

Fujii Y, Otake K, Tashiro M and Adachi A (1996b): Soluble Nef antigen of HIV-1 is cytotoxic for human CD4+ T cells. FEBS Lett 393: 93-96.

Fujinaga K, Zhong Q, Nakaya T, Kameoka M, Meguro T, Yamada K and Ikuta K (1995): Extracellular Nef protein regulates productive HIV-1 infection from latency. J Immunol 155: 5289-5298.

Fukushi M, Dixon J, Kimura T, Tsurutani N, Dixon MJ and Yamamoto N (1999): Identification and cloning of a novel cellular protein Naf1, Nef-associated factor 1, that increases cell surface CD4 expression. FEBS Lett 442: 83-88.

Gaedigk-Nitschko K, Schon A, Wachinger G, Erfle V, Kohleisen B (1995): Cleavage of recombinant and cell derived human immunodeficiency virus 1 (HIV-1) Nef protein by HIV-1 protease. FEBS Lett 357: 275-278.

Gahery-Segard H, Pialoux G, Charmeteau B, Sermet S, Poncelet H, Raux M, Tartar A, Levy J-P, Gras-Masse H and Guillet J-G (2000): Multiepitopic B- and T-cell responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine. J Virol 74: 1694-1703.

Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, White G, Foster P and Markham PD (1984): Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224: 500-503.

Ganju RK, Munshi N, Nair BC, Liu ZY, Gill P and Groopman JE (1998): Human immunodeficiency virus Tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases and components of focal adhesion in Kaposi´s sarcoma cells. J Virol 72: 6131-6137.

Gauduin MC, Parren PW, Weir R, Barbas CF, Burton DR and Koup RA (1997): Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. Nat Med 3: 1389-1393.

Geysen HM, Meloen RH and Barteling SJ (1984): Use of peptide synthesis to probe viral antigens to a resolution of on single amino acid. Proc Natl Acad Sci USA 81: 3998-4002.

Geysen HM, Mason TJ and Rodda SJ (1988): Cognitive features of continuous antigenic determinants. J Mol Rec 1: 32-39.

Girard M, Meignier B, Barre-Sinoussi F, Kieny MP, Matthews T, Muchmore E, Nara PL, Wei Q, Rimsky L, Weinhold K and Fultz PN (1995): Vaccine-induced protection of chimpanzees against infection by a heterologous human immunodeficiency virus type 1. J Virol 69: 6239-6248.

Goebel FD, Mannhalter JW, Belshe RB, Eibl MM, Grob PJ, de Gruttola V, Griffiths PD, Erfle V, Kunschak M and Engl W (1999): Recombinant gp160 as a therapeutic vaccine for HIV-infection: results of a large randomized, controlled trial. European Multinational IMMUNO AIDS Vaccine Study Group. AIDS 13: 1461-1468.

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

Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA and Saxon A (1981): Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men. N Engl J Med 305: 1425-1431.

Goudsmit J, Ljunggren K, Smit L, Jondal M and Fenyo EM (1988): Biological significance of the antibody response to HIV antigens expressed on the cell surface. Arch Virol 103: 189-206.

Goulder PJ, Reid SW, Price DA, O'Callaghan CA, McMichael AJ, Phillips RE and Jones EY (1997): Combined structural and immunological refinement of HIV-1 HLA-B8-restricted cytotoxic T lymphocyte epitopes. Eur J immunol 27: 1515-1521.

Grant CK, Ernisse BJ, Jarrett O and Jones FR (1983): Feline leukemia virus envelope gp70 of subgroups B and C defined by monoclonal antibodies with cytotoxic and neutralizing functions. J Immunol 131: 3042-3048.

Greenberg ME, Bronson S, Lock M, Neumann M, Pavlakis GN and Skowronski J (1997): Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down regulation. EMBO J 16: 6964-6976.

Greenberg ME, Iafrate AJ and Skowronski J (1998): The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MCH complexes. EMBO J 17: 2777-2789.

Greenway A, Azad A and McPhee D (1995): Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines. J Virol 69: 1841-1850.

Greenway A, Azad A, Mills J, McPhee D (1996): Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. J Virol 70: 6701-6708.

Gringeri A, Santagostino E, Muca-Perja M, Le Buanec H, Bizzini B, Lachgar A, Zagury J-F, Rappaport J, Burny A, Gallo RC and Zagury D (1999): Tat toxoid as a component of a preventive vaccine in seronegative subjects. J Acq Imm Defic Syndr and Hum Rotrovir 20: 371-375.

Grzesiek S, Stahl SJ, Wingfield PT and Bax A (1996): The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. Biochemistry 35: 10256-10261.

Görlich D and Mattaj IW (1996): Nucleocytoplasmic transport. Science 271: 1513-1518.

Hadida F, Parrot A, Kieny MP, Sadat-Sowti B, Mayaud C, Debre P and Autran B (1992): Carboxyl-temrinal and central regions of human immunodeficiency virus-1 NEF recognized by cytotoxic T lymphocytes from lymphoid organs. An in vitro limiting dilution analysis. J Clin Invest 89: 53-60.

Hanke T, Samuel RV, Blanchard TJ, Neumann VC, Allen TM, Boyson JE, Sharpe SA, Cook N, Smith GL, Watkins DI, Cranage MP and McMichael AJ (1999): Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA-prime-modified vaccinia Ankara boost vaccination regimen. J Virol 73: 7524-7532.

Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S and Jolicoeur P (1998): Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell 95: 163-175.

Haraguchi S, Cianciolo GJ, Good RA, James-Yarish M, Brigino E and Day NK (1998): Inhibition of interleukin-2 and interferon-gamma by HIV-1 Nef encoded synthetic peptide. AIDS 12: 820-823.

Harris MP and Neil JC (1994): Myristoylation-dependent binding of HIV-1 Nef to CD4. J Mol Biol 241: 136-142.

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

Henderson BR and Percipalle P (1997): Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localization signal mediates specific binding to human importin-beta. J Mol Biol 274: 693-707.

Hinkula J, Svanholm C, Schwartz S, Lundholm P, Brytting M, Engström G, Benthin R, Glaser H, Sutter G, Kohleisen B, Erfle V, Okuda K, Wigzell H and Wahren B (1997): Recognition of prominent viral epitopes induced by immunization with human immunodeficiency virus type 1 regulatory genes. J Virol 71: 5528-5539.

Hodge DR, Dunn KJ, Pei GK, Chakrabarty MK, Heidecker G, Lautenberger JA and Samuel KP (1998): Binding of c-Raf1 kinase to a conserved acidic sequence within the carboxyl-terminal region of the HIV-1 Nef protein. J Biol Chem 273: 15727-15733.

Horsburgh CR Jr, Ou CY, Jason J, Holmberg SD, Longini IM Jr, Schable C, Mayer KH, Lifson AR, Schochetman G, Ward JW et al (1989): Duration of human immunodeficiency virus infection before detection of antibody. Lancet 2: 637-640.

Huang L, Bosch I, Hofmann W, Sodroski J and Pardee AB (1998): Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. J Virol 72: 8952-8960.

Hughes SH (1983). Synthesis, integration and transcription of the retroviral provirus. pp. 23-49 in: Vogt PK and Koprowski H (eds) Current topics in microbiology and immunology 103, Springer-Verlag, Heidelberg.

Hulskotte EGJ, Geretti A-M and Osterhaus ADME (1998): Towards an HIV-1 vaccine: lessons from studies in macaque models. Vaccine 16: 904-914.

Hung LW, Holbrook EL and Holbrook SR (2000): The crystal structure of the rev binding element of HIV-1 reveals novel base pairing and conformational variability. Proc Natl Acad Sci USA 97: 5107-5112.

Igarashi T, Ami Y, Yamamoto H, Shibata R, Kuwata T, Mukai R, Shinohara K, Komatsu T, Adachi A and Hayami M (1997): Protection of monkeys vaccinated with vpr- and/or nef-defective simian immunodeficiency virus strain mac/human immunodeficiency virus type 1 chimeric viruses: a potential candidate live-attenuated human AIDS vaccine. J Gen Virol 78: 985-989.

Ishioka GY, Fikes J, Hermanson G, Livingston B, Crimi C, qin M del Guergio MF, Oseroff C, Dahlberg C, Alexander J, Chesnut RW and Sette A (1999): Utilization of MHC class I transgenic mice for development of minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes. J Immunol 162: 3915-3925.

Ito M, Ishida T, He L, tanabe F, Rongge Y, Miyakawa Y and Terunuma H (1998): HIV type 1 Tat protein inhibits interleukin 12 production by human peripheral blood mononuclear cells. AIDS Res Hum Retroviruses 14: 845-849.

Iversen AKN, Shpaer EG, Rodrigo AG, Hirsch MS, Walker BD, Sheppard HW, Merigan TC and Mullins JI (1995): Persistence of attenuated rev genes in a human immunodeficiency virus type 1-infected asymptomatic individual. J Virol 69: 5743-5753.

Jabbar MA (1995): The human immunodeficiency virus type 1 Vpu protein: roles in virus release and CD4 downregulation. Curr Top Microbiol Immunol 193: 107-120.

Jeang KT, Chun R, Lin NH, Gatignol A, Glabe CG and Fan H (1993): In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor. J Virol 67: 6224-6233.

Jensen TH, Jensen A, Szilway AM and Kjems J (1997): Probing the structure of HIV-1 Rev by protein footprinting of multiple monoclonal antibody-binding sites. FEBS Letters 414: 50-54.

Kaminchik J, Bashan N, Itach A, Sarver N, Gorecki M, and Panet A (1991): Genetic characterization of human immunodeficiency virus type 1 nef gene products translated in vitro and expressed in mammalian cells. J Virol 65: 583-588.

Kaminchik J, Margalit R, Yaish S, Drummer H, Amit B, Sarver N, Gorecki M and Panet A (1994): Cellular distribution of HIV type 1 Nef protein: idetification of domains in Nef required for association with membrane and detergent-insoluble cellular matrix. AIDS Res Hum Retroviruses 10: 1003-1010.

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

Karn J (1999): Tackling Tat. J Mol Biol 293: 235-254.

Kashanchi F, Piras G, Radonovich MF, Duvall JF, Fattaey A, Chiang CM, Roeder RG and Brady JN (1994): Direct interaction of human TFIID with the HIV-1 transactivator tat. Nature 367: 295-299.

Katz RA and Skalka AM (1994): The retroviral enzymes. Annu Rev Biochem 63: 133-173.

Kestler HW 3d, Ringler DH, Mori K, Panicali DL, Sehgal PK, Daniel MD and Desrosiers RC (1991): Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65: 651-662.

Kienzle N, Bröker M, Harthus H-P, Enders M, Erfle V, Buck M and Müller-Lantzsch (1991): Immunological study of the nef protein from HIV-1 by polyclonal and monoclonal antibodies. Arch Virol 118: 29-41.

Kienzle N, Freund J, Kalbitzer HR and Mueller-Lantzsch N (1993): Oligomerization of the Nef protein from human immunodeficiency virus (HIV) type 1. Eur J Biochem 214: 451-457.

Kiernan RE, Vanhulle C, Schiltz L, Adam E, Xiao H, Maudoux F, Calomme C, Burny A, Nakatani Y, Jeang KT, Benkirane M and Van Lint C (1999): HIV-1 tat transcriptional activity is regulated by acetylation. EMBO J 18: 6106-6118.

Kim YH, Chang SH, Kwon JH and Rhee SS (1999): HIV-1 Nef plays an essential role in two independent processes in CD4 down-regulation: dissociation of the CD4-p56(lck) complex and targeting of DC4 to lysosomes. Virology 257: 208-219.

Kirschhoff F, Voss G, Nick S, Stahl-hennig C, Coulibaly C, Frank R, Jentsch KD and Hunsmann G (1991): Antibody response to the negative regulatory factor (nef) in experimentally infected macaques: correlation with viremia, disease progression, and seroconversion to structural viral proteins. Virology 183: 267-272.

Knowles DM, Chamulak GA, Subar M, Burke JS, Dugan M, Wernz J, Slywotzky C, Pelicci G, Dalla-Favera R and Raphael B (1988): Lymphoid neoplasia associated with the acquired immunodeficiency syndrome (AIDS). The New York University Medical Center experience with 105 patients (1981-1986). Ann Intern Med 108: 744- 753.

Koedel U, Kohleisen B, Sporer B, Lahrtz F, Ovod V, Fontana A, Erfle V and Pfister HV (1999): HIV type 1 Nef protein is a viral factor for leukocyte recruitement into the central nervous system. J Immunol 163: 1237-1245.

Koenig S, Fuerst TR, Wood LV, Woods RM, Suzich JA, Jones GM, De La Cruz V, Davey RT, Venkatesa S, Moss B, Biddison WE and Fauci AS (1990): Mapping of the fine specificity of a cytolytic T cell Response to HIV-1 nef protein. J Immunol 145: 127-135.

Koken SE, Greijer AE, Verhoef K, van Wamel J, Bukrinskaya AG, Berkhout B (1994): Intracellular analysis of in vitro modified HIV Tat protein. J Biol Chem 269: 8366-8375.

Kolson DL, Buchhalter J, Collman R, Hellmig B, Farrell CF, Debouck C and Gonzales-Scarano F (1993): HIV-1 Tat alters normal organization of neurons and astrocytes in primary rodent brain cell-cultures: RGD sequence dependence. AIDS Res Hum Retroviruses 9: 677-685.

Kotov A, Zhou J, Flicker P and Aiken C (1999): Association of Nef with the human immunodeficiency virus type 1 core. J Virol 73: 8824-8830.

Kreusel J, Aepinus C, Lang S, Beninga J, Prenzel S, Fleckenstein B (1993): Mouse monoclonal antibodies recognize the activation domain of HIV-1 Rev transactivator. Mol and Cell Probes 7: 111-19.

Krone WJA, Debouck C, Epstein L, Heutink P, Meloen R, Goudsmit J (1988): Natural antibodies to HIV-Tat epitopes and expression of HIV-1 genes in vivo. J Med Virol 26: 261-270.

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

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

Kähne T, Lendeckel U, Wrenger S, Neubert K, Ansorge S and Reinhold D (1999): Dipeptidyl peptidase IV: a cell surface peptidase involved in regulating T cell growth (review). Int J Mol Med 4: 3-15.

Lachgar A, Bernard J, Bizzini B, Astgen A, Le Coq H, Fouchard M, Chams V, Feldman M, Burny A and Zagury JF (1996): Repair of the in vitro HIV-1-induced immunosuppression and blockade of the generation of functional suppressive CD8 cells by anti-alpha interferon and anti-Tat antibodies. Biomed Pharmacother 50: 13-18.

Lama J, Mangasarian A and Trono D (1999): Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. Curr Biol 9: 622-631.

Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, and Fauci AS (1983): Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N Engl J Med 313: 79-84.

Lane HC, Depper JM, Greene WC, Whalen G, Waldmann TA and Fauci AS (1985): Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome. Evidence for a selective defect in soluble antigen recognition. N Engl J Med 313: 79-84.

Langlade-Demoyen P, Ngo-Giang-Huong N, Ferchal F, and Oksenhendler K (1994): Human immunodeficiency virus (HIV) nef-specific cytotoxic T lymphocytes in noninfected heterosexual contact of HIV-infected patients. J Clin Invest 93: 1293-1297.

Laspia MF, Rice AP and Mathews MB (1989): HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. Cell 59: 283-292.

Laurence J (1985): The immune system in AIDS. Sci Am 253: 84-93.

Lee CH, Saksela K, Mirza UA, Chait BT and Kuriyan J (1996): Crystal structure and the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. Cell 85: 931-942.

Li X, Multon MC, Henin Y, Schweighoffer F, Venot C, Josef J, Zhou C, LaVecchio J, Stuckert P, Raab M, Mhashilkar A, Tosque B and Marasco WA (2000): Grb3-3 is up-regulated in HIV-1 infected T-cells and can potentiate cell activation through NFATc. J Biol Chem 6 : 30925-30933.

Lieberman J, Fabry JA, Fong DM, Parkerson GR 3rd (1997): Recognition of a small number of diverse epitopes dominates the cytotoxic T lymphocytes response to HIV type 1 in an infected individual. AIDS Res Hum Retroviruses 13: 383-392.

Liu LX, Margottin F, Le Gall S, Schwartz O, Selig L, Benarous R and Benichou S (1997): Binding of HIV-1 Nef to a novel thioesterase enzyme correlates with Nef-mediated CD4 down-regulation. J Biol Chem 272: 13779-13785.

Liu LX, Heveker N, Fackler OT, Arold S, Le Gall S, Janvier K, Peterlin BM, Dumas C, Schwartz O, Benichou S and Benarous R (2000): Mutation of a conserved residue (D123) required for oligomerization of human immunodeficiency virus type 1 Nef protein abolishes interaction with human thioesterase and results in impairment of Nef biological functions. J Virol 74: 5310-5319.

Lopalco L, Pastori C, Cosma A, Burastero SE, Capiluppi B, Boeri E, Beretta A, Lazzarin A and Siccardi AG (2000): Anti-cell antibodies in exposed seronegative individuals with HIV type 1-neutralizing activity. AIDS Res Hum Retroviruses 16: 109-115.

Loret EP, Georgel P, Johnson WC Jr and Ho PS (1992): Circular dichroism and molecular modelling yield a structure for the complex of human immunodeficiency virus type 1 trans-activation response RNA and the binding of region of Tat, the trans-acting transcriptional activator. Proc Natl Acad Sci USA 89: 9734-9738.

Lower R, Lower J and Kurth R (1996): The viruses in all of us: charasteristics and biological significance of human endogenous retrovirus sequences. Proc Natl Acad Sci USA 93: 5177-5184.

Lu X, Wu X, Plemenitas A, Yu H, Sawai ET, Abo A and Peterlin BM (1996): CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1. Curr Biol 6: 1677-1684.

Lu X, Yu H, Liu SH, Brodsky FM and Peterlin BM (1998): Interactions between HIV-1 Nef and vacuolar ATPase facilitate the internalization of CD4. Immunity 8: 647-656.

Luo T, Downing JR and Garcia JV (1997): Induction of phosphorylation of human immunodeficiency virus type 1 Nef and enhancement of CD4 downregulation by phorbol myristate acetate. J Virol 71: 2535-2539.

Luria S, Chambers I and Berg P (1991): Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin-2 mRNA. Proc Natl Acad Sci USA 88: 5326-5330.

Malim MH, Bohnlein S, Hauber J and Cullen B (1989): Functional dissection of the HIV-1 Rev trans-activator--derivation of a trans-dominant repressor of Rev function. Cell 58: 205-214.

Malim MH and Cullen BR (1991 a): HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. Cell 65: 241-248.

Malim MH, McCarn DF, Tiley LS and Cullen BR (1991 b): Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J Virol 65: 4248-4254.

Mangasarian A, Piguet V, Wang JK, Chen YL and Trono D (1999): Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. J Virol 73: 1964-1973.

Mann DA, Mikaelian I, Zemmel RW, Green SM, Lowe AD, Kimura T, Singh M, Butler PJ, Gait MJ and Karn J (1994): A molecular rheostat. Co-operative rev binding to stem I of the rev-responsive element modulates human immunodeficiency virus type-1 late gene expression. J Mol Biol 241: 193-207.

Manna SK and Aggarwal BB (2000): Differential requirement for p56lck in HIV-tat versus TNF-induced cellular responses: effects on NF-kappaB, activator protein-1, c-Jun N-terminal kinase, and apoptosis. J Immunol 164: 5156-5166.

Manninen A, Renkema H and Saksela K (2000): Synergistic activation of NFAT by HIV-1 nef and the Ras/MAPK pathway. J Biol Chem 275: 16513-16517.

Margolis L (1998): HIV: from molecular recognition to tissue pathogenesis. FEBS Lett 433: 5-8.

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpentier CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL and Lewis MG (2000): Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 6: 207-210.

Masur H, Michelis MA, Greene JB, Onorato I, Vande Stouwe RA, Holtzman RS, Wormser G, Brettman L, Lange M, Murray HW and Cunningham-Rundles S (1981): An outbreak of community-acquired Pneumocystis carinii pneumonia. N Engl J Med 305: 1431-1438.

Maurer B and Flügel RM (1987): The 3´-orf protein of human immunodeficiency virus 2 shows sequence homology with the bel3 gene of the human spumaretrovirus. FEBS Lett. 222: 286-288.

McCray J and Werner G (1987): Different rhinovirus serotypes neutralized by antipeptide antibodies. Nature 329: 736-738.

McDougal JS, Kennedy MS, Orloff SL, Nicholson JK and Spira TJ (1996): Mechanism of human immunodeficiency virus type 1 (HIV-1) neutralization: irreversible inactivation of infectivity by an anti-HIV-1 antibody. J Virol 70: 5236-5245.

McKeating JA and Willey RL (1989): Structure and function of the HIV envelope. AIDS 3: 35-41.

McPhee DA, Kemp BE, Cumming S, Stapleton D, Gust ID and Doherty RR (1988): Recognition of envelope and Tat protein synthetic peptide analogs by HIV positive sera or plasma. FEBS Lett 233: 393-396.

Meggio F, D'Agostino DM, Ciminale V, Chieco-Bianchi L and Pinna LA (1996): Phophorylation of HIV-1 Rev protein: implication of protein kinase CK2 and pro-directed kinases. Biochem Biophys Res Commun 226: 547-554.

Mervis RJ, Ahmad N, Lillehoj EP, Raum MG, Salazar FH, Chan HW and Venkatesan S (1988): The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of posttranslational modifications, and evidence for alternative gag precursors. J Virol 62: 3993-4002.

Meyer BE and Malim MH (1994): The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. Genes Dev 8: 1538-1547.

Mhashilkar AM, Bagley J, Chen SY, Szilvay AM, Helland DG and Marasco WA (1995): Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by single chain intrabodies. EMBO J 14: 1542-1551.

Michael NL, Mo T, Merzouki A, O´Shaughnessy M, Oster C, Burke DS, Redfield RR, Birx DL and Cassol SA (1995): Human immunodeficiency virus type 1 cellular RNA load and splicing patterns predict disease progression in a longitudinally studied cohort. J Virol 69: 1868-1877.

Miedema F, Chantal-Petit AJ, Terpstra FG, Schattenkerk JK, de Wolf F, Al BJ, Roos M, Lange JM, Danner SA, Goudsmit J and Schellekens TA (1988): Immunological abnormalities in human immunodeficiency virus (HIV) infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper cell depletion occurs. J Clin Invest 82: 1908-1914.

Miettinen A, Hakkarainen K, Grönroos P, Heinonen P, Teisala K, Aine R, Sillantaka I, Saarenmaa K, Lehtinen M, Punnonen R and Paavonen J (1989): Class specific antibody response to gonococcal infection. J Clin Pathol 42: 72-76.

Miller MD, Warmerdam MT, Gaston I, Greene WC and Feinberg MB (1994): The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages J Exp Med 179: 101-113.

Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, Wolf H (1987): Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J Virol 61: 570-578.

Moog C, Fleury HJ, Pellegrin I, Kirn A and Aubertin AM (1997): Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficien y virus type 1-infected individuals. J Virol 71: 3734-3741.

Moureau C, Moynier M, Kavsan V, Montagnier L and Bahraoui E (1999): Specificity of anti-Nef antibodies produced in mice immunized with DNA encoding the HIV-1 nef gene product. Vaccine 18: 333-341.

Muller S, Wang H, Silverman GJ, Bramlet G, Haighwood N and Kohler H (1993): B-cell abnormalities in AIDS: stable and clonally-restricted antibody response in HIV-1 infection. Scand J Immunol 38: 327-334.

Murray HW, Rubin BY, Masur H and Roberts RB (1984): Impaired production of lymphokines and immune (gamma) interferon in the acquired immunodeficiency syndrome. N Engl J Med 310: 883-889.

Myers G, Rabson AB, Berzofsky JA, Smith TF, Wong-Staal F (1990): Human Retrovirus and AIDS: A Compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.

Myers G, Korber BT, Foley BT, Jeang K-T, Mellors JW and Wain-Hobson S (1996): Human Retrovirus and AIDS: A Compilation and analysis of nucleic acid and amino acid sequences, pp. III-11 to III-26. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.

Nath A, Conant K, Chen P, Scott C and Major EO (1999): Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. J Biol Chem 274: 17098-17102.

Neville M, Stutz F, Lee L, Davis LI and Rosbash M (1997): The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr Biol 7: 767-775.

Niederman TM, Thielan BJ and Ratner L (1989): Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc Natl Acad Sci USA 86: 1128-1132.

Olsen HS, Cochrane AW, Dillon PJ, Nalin CM and Rosen CA (1990): Interaction of the human immunodeficiency virus type 1 Rev protein with a structured region in env mRNA is dependent on multimer formation mediated through a basic strectch of amino acids. Genes Dev 4: 1357-1364.

Ono T, Iwatani Y, Nishimura A, Ishimoto A and Sakai H (2000): Functional association between the nef gene product and gag-pol region of HIV-1. FEBS Lett 466: 233-238.

Opalenik SR, Ding Q, Mallery SR and Thompson JA (1998): Glutathione depletion associated with the HIV-1 TAT protein mediates the extracellular appearance of acidic fibroblast growth factor. Arch Biochem Biophys 351: 17-26.

Orita S, Sato S, Aono Y, Minoura N, Yamashita T, Hinuma Y and Igarashi H (1993): Identification of novel singly spliced pX mRNA transcipts common to all human leukemia virus type 1-related retroviruses. Virus Genes 7: 197-204.

Orsini MJ, Thakur AN, Andrews WW, Hammarskjöld ML and Rekosh D (1995): Expression and purification of the HIV type 1 Rev protein produced in Eschericia coli and its use in the generation of monoclonal antibodies. AIDS Res Hum Retroviruses 11: 945-953.

Orsini MJ, Debouck CM, Webb CL and Lysko PG (1996): Extracellular human immunodeficiency virus type 1 Tat protein promotes aggregation and adhesion of cerebellar neurons. J Neurosci 16: 2546-2552.

O'Shea S, Rostron T, Hamblin AS, Palmer SJ and Banatvala JE (1991). Quantitation of HIV: correlation with clinical, virological and immunological status. J Med Virol 35: 65-69.

Oshima T, Flores SC, Vaitaitis G, Coe LL, Joh T, Park JH, Zhu Y, Alexander B and Alexander JS (2000): HIV-1 Tat increases endothelial solute permeability through tyrosine kinsae and mitogen-activated protein kinase-dependent pathways. AIDS 14: 475-482.

Osterhaus ADME, van Baalen CA, Gruters RA, Schutten M, Siebelink CHJ, Hulskotte EGJ, Tijhaar EJ, Randall RER, van Amerongen G, Fleuchaus A, Erfle V, and Sutter G (1999): Vaccination with Rev and Tat against AIDS. Vaccine 17: 2713-2714.

Otake K, Fujii Y, Nakaya T, Nishino Y, Zhong Q, Fujinaga K, Kameoka M, Ohki K, and Ikuta K (1994): The carboxyl-terminal region of HIV-1 Nef protein is a cell surface domain that can interact with CD4+ T cells. J Immunol 153: 5826-5837.

Otake K, Ohta M, Minowada J, Hatama S, Takahashi E, Ikemoto A, Okuyama H and Fujii Y (2000): Extracellular Nef of HIV-1 can target CD4 memory T population. AIDS 14: 1662-1664.

Ott M, Emiliani S, Van Lint C, Herbein G, Lovett J, Chirmule N, McCloskey T, Pahwa S and Verdin E (1997): Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the CD28 pathway. Science 275: 1481-1485.

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

Ovod V, Lagerstedt A, Ranki A, Gombert FO, Spohn R, Tähtinen M, Jung G and Krohn KJE (1992): Immunological variation and immunohistochemical localization of HIV-1 Nef demonstrated with monoclonal antibodies. AIDS 6: 25-34.

Pahwa S, Pahwa R, Good RA, Gallo RC and Saxinger C (1986): Stimulatory and inhibitory influences of human immunodeficiency virus on normal B lymphocytes. Proc Natl Acad Sci USA 83: 9124-9128.

Pandori MW, Fitch NJ, Craig HM, Richman DD, Spina CA and Guatelli JC (1996): Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. J Virol 70: 4283-4290.

Papsidero LD, Sheu M and Ruscetti F (1989): Human immunodeficiency virus type-1 neutralizing monoclonal antibodies which react with p17 core protein: characterization and epitope mapping. J Virol 63: 267-272.

Parada CA and Roeder RG (1996): Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain. Nature 384: 375-378.

Pardridge WM, Bickel U, Buciak J, Yang J and Diagne A (1994): Enhanced endocytosis and anti-human immunodeficiency virus type I activity of anti-Rev antibodies after cationization. J Inf Dis 169: 55-61.

Parmentier HK, van Wichen DF, Gmelig Meyling FHJ, Goudsmit J and Schuurman H-J (1992): Epitopes of human immunodeficiency virus regulatory proteins tat, nef and rev are expressed in normal human tissue. Am J Pathol 141: 1209-1216.

Parren PWHI, Gauduin M-C, Koup RA, Poignard P, Fisicaro P, Burton DR and Sattentau QJ (1997): Relevance of the antibody response against human immunodeficiency virus type 1 envelope to vaccine design. Immunology Letters 57: 105-112.

Pauza CD, Trivedi P, Wallace M, Ruckwardt TJ, Le Buanec H, Lu W, Bizzini B, Burny A, Zagury D, and Gallo R (2000): Vaccination with Tat toxoid attenuates disease in simian/HIV-challenged macaques. Proc Natl Acad Sci USA 97: 3515-3519.

Pertmer TM, Roberts TR and Haynes JR (1996): Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. J Virol 70: 6119-6125.

Phillips DM (1994): The role of cell-to-cell transmission in HIV infection. AIDS 8: 719-731.

Piguet V, Chen YL, Mangasarian A, Foti M, Caroentier JL and Trono D (1998): Mechanism of nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. EMBO J 17: 2472-2481.

Piguet V, Gu F, Foti M, Demaurex N, Gruenberg J, Carpentier JL and Trono D (1999): Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes. Cell 97: 63-73.

Piguet V, Wan L, Borel C, Mangasarian A, Demareux N, Thomas G and Trono D (2000): HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. Nat Cell Biol 2: 163-167.

Pilkington GR, Duan L, Zhu M, Keil W and Pomerantz RJ (1996): Recombinant human Fab antibody fragments to HIV-1 Rev and Tat regulatory proteins: direct selection from a combinatorial phage display library. Mol Immunol 33: 439-450.

Poggi A, Rubartelli A and Zocchi MR (1998): Involvement of dihydropyridine-sensitive calcium channels in human dendritic cell function. Competition by HIV-1 Tat. J Biol Chem 273: 7205-7209.

Poignard P, Klasse PJ and Sattentau QJ (1996): Antibody neutralization of HIV-1. Review. Immunology Today 17: 239-246.

Powell DM, Amaral MC, Wu JY, Maniatis T and Greene WC (1997): HIV Rev-dependent binding of SF2/ASF to the Rev response element: possible role in Rev-mediated inhibition of HIV RNA splicing. Proc Natl Acad Sci USA 94: 973-978.

Poznansky MC, Foxall R, Mhashilkar A, Coker R, Jones S, Ramstedt U and Marasco W (1998): Inhibition of human immunodeficiency virus replication and growth advantage of CD4+ T cells from HIV-infected individuals that express intracellular antibodies against HIV-1 gp120 or Tat. Hum Gene Ther 9: 487-496.

Prince AM, Reesink H, Pascual D, Horowitz B, Hewlett I, Murthy KK, Cobb KE, Eichberg JW (1991): Prevention of HIV infection by passive immunization with HIV immunoglobulin. AIDS Res Hum Retroviruses 7: 971-973.

Putkonen P, Quesada-Rolander M, Leandersson A-C, Schwartz S, Thorstensson R, Okuda K, Wahren B and Hinkula J (1998): Immune responses but not protection against SHIV by gene-gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. Virology 250: 293-301.

Quaranta MG, Camponeschi B, Straface E, Malorni W and Viora M (1999): Induction of interlukin-15 production by HIV-1 nef protein: a role in the proliferation of uninfected cells. Exp Cell Res 250: 112-121.

Quesada-Rolander M, Makitalo B, Thorstensson R, Zhang YJ, Castanos-Velez E, Biberfeld G and Putkonen P (1996): Protection against mucosal SIVsm challenge in macaques infected with a chimeric SIV that expresses HIV type 1 envelope. AIDS Res Hum Retroviruses 12: 993-999.

Ranki A, Weiss S, Valle S-L, Antonen J and Krohn K (1987a): Neutralizing antibodies in HIV (HTLV-III) infection: Correlation with clinical outcome and antibody response against different viral proteins. Clin Exp Immunol 69: 231-939.

Ranki A, Valle SL, Krohn M, Antonen J, Allain JP, Leuther M, Franchini G, Krohn K (1987b): Long latency precedes overt seroconversion in sexually transmitted human-immunodeficiency-virus infection. Lancet 2: 589-593.

Ranki A, Järvinen K, Valle S-L, Nurmilaakso P and Krohn K (1990): Antibodies to recombinant HIV-1 nef protein detected in HIV-1 infection as well as in nonrisk individuals. J Acquir Immune Defic Syndr 3: 348-355.

Ranki A, Lagerstedt A, Ovod V, Aavik E, Krohn KJE (1994): Expression kinetics and subcellular localization of HIV-1 regulatory proteins Nef, Tat and Rev in acutely and chronically infected lymphoid cell lines. Arch Virol 139: 365-78.

Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K, Ivanoff L, Petteway Jr. SR, Pearson MI, Lautenberger JA, Papas TS, Ghrayeb J, Chang NT, Gallo RC and Wong-Staal F (1985): Complete nucleotide sequence of the AIDS virus HTLV-III. Nature 313: 277-284.

Re MC, Furlini G, Vignoli M, Ramazotti E, Roderigo G, De Rosa V et al. (1995): Effect of antibody to HIV-1 tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. J Acquir Immune Defic Syndr Hum Retrovirol 10: 408-416.

Reiss P, de Ronde A, Dekker J, Aramideh M, de Wolf F, Debouck C, Lange J and Goudsmit J (1989a): Seroconversion to HIV-1 Rev- and Tat-gene encoded proteins. AIDS 3: 105-107.

Reiss P, de Ronde A, Lange JMA, de Wolf F, Dekker J, Danner SA, Debouck C and Goudsmit J (1989b): Low antigenicity of HIV-1 Rev: Rev-specific antibody response of limited value as correlate of Rev gene expression and disease progression. AIDS Res Hum Retroviruses 5: 621-628.

Reiss P, de Wolf F, Kuiken CL, de Ronde A, Dekker J, Boucher CA, Debouck C, Lange JM and Goudsmit (1991): Contribution of antibody response to recombinant HIV-1 gene-encoded products Nef, Rev, Tat, and protease in predicting development of AIDS in HIV-1-infected individuals. J Acquir Immune Defic Syndr 4: 165-72.

Renkema GH, Manninen A, Mann DA, Harris M and Saksela K (1999): Identification of the Nef-associated kinase as p21-activated kinase 2. Curr Biol 9: 1407-1410.

Rhee SS and March JW (1994): Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. J Virol 68: 5156-5163.

Robert-Guroff M, Brown M and Gallo RC (1985): HTLV-III-neutralizing antibodies in patients with AIDS and AIDS-related complex. Nature 316: 72-74.

Roberts JD, Bebenek K and Kunkel TA (1988): The accuracy of reverse transcriptase from HIV-1. Science 242: 1171-1173.

Rodman TC, Pruslin FH, To SE and Winston (1992): Human immunodeficiency virus (HIV) Tat-reactive antibodies present in normal HIV-negative sera and depleted in HIV-positive sera. Identification of the epitope. J Exp Med 175: 1247-1253.

Rodman TC, To SE, Hashish H and Manchester K (1993): Epitopes for natural antibodies of human immunodeficiency virus (HIV)-negative (normal) and HIV-positive sera are coincident with two key functional sequences of HIV Tat protein. Proc Natl Acad Sci USA 90: 7719-7723.

Rook AH, Lane HC, Folks T, McCoy S, Alter H and Fauci AS (1987): Sera from HTLV-III/LAV antibody-positive individuals mediate antibody-dependent cellular cytotoxicity against HTLV-III/LAV-infected T cells. J Immunol 138: 1064-1067.

Rosorius O, Reichart B, Kratzer F, Heger P, Dabauvalle MC and Hauber J (1999): Nuclear pore localization and nucleocytoplasmic transport of eIF-5A: evidence for direct interaction with the export receptor CRM1. J Cell Sci 112: 2369-2380.

Ross TM, Oran AE and Cullen BR (1999): Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. Curr Biol 9: 613-621.

Ruhl M, Himmelspach M, Bahr GM, Hammerschmid F, Jaksche H, Wolff B, Aschauer H, Farrington GK, Probst H and Bevec D (1993): Eucaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. J Cell Biol 123: 1309-1320.

Rusche JR, Lynn DL, Robert-Guroff M, Langlois AJ, Lyerly HK, Carson H, Krohn K, Ranki A, Gallo RC and Bolognesi DP (1987): Humoral immune response to the entire human immunodeficiency virus envelope glycoprotein made in insect cells. Proc Natl Acad Sci USA 84: 6924-6928.

Sabatier J-M, Clerget-Raslain B, Fontan G, Fenouillet E, Rochat H, Garnier C, Gluckman J-C, Van Rietschoten J, Montagnier L and Bahraoui E (1989): Use of synthetic peptides for the detection of antibodies against the NEF regulating protein in sera of HIV-infected patients. AIDS 3: 215-220.

Sabatier JM, Vives E, Mabrouk K, Benjouad A, Rochat H, Duval A, Hue B and Bahraoui E (1991): Evidence for neurotoxic ectivity of tat from human immunodeficiency virus type 1. J Virol 65: 961-967.

Sadaie MR, Mukhopadhyaya R, Benaissa ZN, Pavlakis GN and Wong-Staal F (1990): Conservative mutations in the putative metal-binding region of human immunodeficiency virus tat disrupt virus replication. AIDS Res Hum Retroviruses 6: 1257-1263.

Safrit JT, Fung MS, Andrews CA, Braun DG, Sun WN, Chang TW and Koup RA (1993): hu-PBL-SCID mice can be protected from HIV-1 infection by passive transfer of monoclonal antibody to the principal neutralizing determinant of envelope gp120. AIDS 7: 15-21.

Saksela K, Cheng G and Baltimore D (1995): Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J 14: 484-491.

Sambrook J, Fritsch ET and Maniatis T (1989): Molecular Cloning 1-3. A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press.

Samuel KP, Seth A, Konopka A, Lautenberger JA and Papas TS (1987): The 3´-orf protein of human immunodeficiency virus shows structural homology with the phosphorylation domain of human interleukin-2 receptor and the ATP-binding site of the protein kinase family. FEBS Lett 218, 81-86.

Sandberg JK, Leandersson AC, Devito C, Kohleisen B, Erfle V, Achour A, Lev M, Schwartz S, Karre K, Wahren B and Hinkula J (2000): Human immunodeficiency virus type 1 Nef epitopes recognized in HLA-A2 transgenic mice in response to DNA and peptide immunization. Virology 273: 112-119.

Sandström E, Wahren B, Nordic VAC-04 Study Group (1999): Therapeutic immunization with recombinant gp160 in HIV-1 infection: a randomised double-blind placebo-controlled trial. Lancet 353: 1735-1742.

Sarin P, Sun D, Thornton A, Naylor P and Goldstein A (1986): Neutralization of HTLV-III/LAV replication by antiserum to thymosin alpha. Science 232: 1135-1137.

Sattentau QJ, Zolla-Pazner S and Poignard P (1995): Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. Virology 206: 713-717.

Sawaya BE, Thatikunta P, Denisova L, Brady J, Khalili K and Amini S (1998): Regulation of TNFalpha and TGFbeta-1 gene transcription by HIV-1 Tat in CNS cells. J Neuroimmunol 87: 33-42.

Schneider T, Harthus H-P, Hildebrandt P, Niedrig M, Bröker M, Eigelt W, Beck A and Pauli G (1991): Epitopes of the HIV-1 negative factor (nef) reactive with murine monoclonal antibodies and human HIV-1 positive sera. AIDS Res Hum Retroviruses 7: 37-44.

Scheider T, Beck A, Ropke C, Ullrich R, Harthus HP, Broker M and Pauli G (1993): The HIV-1 Nef protein shares an antigenic determinant with T-cell surface protein. AIDS 7: 647-654.

Schwarz O, Marcchal V, Danos O and Heard JM (1995): Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. J Virol 69: 4053-4059.

Secchiero P, Zella D, Caoitani S, Gallo RC and Zauli G (1999): Extracellular HIV-1 tat protein up-regulates the expression of surface CXC-chemokine receptor 4 in resting CD4+ T cells. J Immunol 162: 2427-2431.

Selby MJ, Bain ES, Luciw P and Peterlin BM (1989): Structure, sequence and position of the stem-loop in TAR determine transcriptional elongation by Tat through the HIV-1 long terminal repeat. Genes Dev 3 :547-558.

Sharma V, Xu M, Ritter LM and Wilkie NM (1996): HIV-1 tat induces expression of a new hematopoietic cell-specific transcription factor and downregulates MIP-1 alpha gene expression in activated T-cells. Biochem Biophys Res Commun 223: 526-533.

Shibata R, Igarashi T, Haigwood N, Buckler-White A, Ogert R, Ross W, Willey R, Cho MW and Martin MA (1999): Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med 5: 204-210.

Shugars DC, Smith MS, Glueck DH, Nantermet PV, Seillier-Moiseiwitsch F and Swanstrom R (1993): Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo. J Virol 67: 4639-4650.

Simon JH and Malim MH (1996): The human immunodeficiecy virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. J Virol 70: 5297-5305.

Smith BL, Krushelnycky BW, Mochly-Rosen D and Berg P (1996): The HIV nef protein associates with protein kinase C theta. J Biol Chem 271: 16753-16757.

Sodroski J, Patarca R, Rosen C, Wong-Staal F and Haseltine W (1985): Location of the trans-activating region on the genome of human T-cell lymphotropic virus type III. Science 229: 74-77.

Sodroski J, Goh WC, Rosen C, Dayton A, Terwilliger E and Haseltine W (1986): A second post-transcriptional trans-activator gene required for HTLV-III replication. Nature 321: 412-417.

Spina CA, Kwoh TJ, Chowers MY, Guatelli JC and Richman DD (1994): The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J Exp Med 179: 115-123.

Starcich B, Ratner L, Josephs SF, Okamoto T, Gallo RC and Wong-Staal F (1985): Characterization of long terminal repeat sequences of HTLV-III. Science 227: 538-540.

Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF, Gallo RC et al. (1986): Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell 45: 637-648.

Steeves RA, Strand M and August JT (1974): Structural proteins of mammalian oncogenic RNA viruses: murine leukemia virus neutralization by antisera prepared against purified envelope glycoprotein. J Virol 14: 187-189.

Steinaa L, Soresen AM, Nielsen JO and Hansen JES (1994): Antibody to HIV-1 Tat protein inhibits the replication of virus in culture. Arch Virol 139: 263-271.

Stevenson M, Stanwick TL, Dempsey MP and Lamonica CA (1990): HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J 9: 1551-1560.

Stutz F, Izaurralde E, Mattaj IW and Rosbash M (1996): A role of nucleoporin FG repeat domains in export of human immunodeficiency virus type 1 Rev protein and RNA from the nucleus. Mol Cell Biol 16: 7144-7150.

Sullivan BL, Knopoff EJ, Saifudding M, Takefman DM, Saarloos MN, Sha BE and Spear GT (1996): Susceptibility of HIV-1 plasma virus to complement mediated lysis. Evidence for a role in clearance of virus in vivo. J Immunol 157: 1791-1798.

Svanholm C, Bandholtz L, Lobell A, and Wigzell H (1999): Enhancement of antibody response by DNA immunization using expression vectors mediating efficient antigen secretion. J Immunol Meth 228: 121-130.

Swingler S, Gallay P, Camaur D, Song J, Abo A and Trono D (1997): The Nef protein of human immunodeficiency virus type 1 enhances serine phosphorylation of the viral matrix. J Virol 71: 4372-4377.

Swingler S, Mann A, Jacque J-M, Brichacek B, Sasseville VG, Williams K, Lackner AA, Janoff EN, Wang R, Fisher D and Stevenson M (1999): HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat Med 5: 997-1003.

Szebeni A and Olson MO (1999): Nucleolar protein B23 has molecular chaperone activities. Protein Sci 8: 905-912.

Tang H, Kuhen KL and Wong-Staal F (1999): Lentivirus replication and regulation. Ann Rev Genet 33: 133-170.

Tange TO, Jensen TH and Kjems J (1996): In vitro interaction between human immunodeficiency virus type 1 Rev protein and splicing factor ASF/SF2-associated protein, p32. J Biol Chem 271: 10066-10072.

Terpstra FG, Al BJ, Roos MT, De Wolf F, Goudsmit J, Schellekens PT and Miedema F (1989): Longitudinal study of leukocyte functions in homosexual men seroconverted for HIV: rapid and persistent loss of B cell function after HIV infection. Eur J Immunol 19: 667-673.

Tokunaga K, Kojima A, Kurata T, Ikuta K, Akari H, Koyama AH, Kawamura M, Inubushi R, Shimano R and Adachi A (1998a): Enhancement of human immunodeficiency virus type 1 infectivity by Nef is producer-cell dependent. J Gen Virol 79: 2447-2453.

Tokunaga K, Kiyokawa E, Nakaya M, Otsuka N, Kojima A, Kurata T and Matsuda M (1998b): Inhibition of human immunodeficiency virus type 1 virion entry by dominant-negative Hck. J Virol 72: 6257-6259.

Tosi G, Mezza R, De Lerma B, D´Agostino A, Mazza S, Corradin G, Albini A, Noonan DM, Ferrini S and Accolla RS (2000): Highly stable oligomerization forms of HIV-1 Tat detected by monoclonal antibodies and requirement of monomeric forms for the transactivating function on the HIV-1 LTR. Eur J Immunol 30: 1120-1126.

Towbin H, Staehelin T and Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354.

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

Turner BG and Summers MF (1999): Structural biology of HIV. J Mol Biol 285: 1-32.

Ugolini S, Mondor I, Parren PW, Burton DR, Tilley SA, Klasse PJ and Sattentau QJ (1997): Inhibition of virus attachment to CD4+ target cells is a major mechanism of T cell line-adapted HIV-1 neutralization. J Exp Med 186: 1287-1298.

Ulich C, Dunne A, Parry E, Hooker CW, Gaynor RB and Harrich D (1999): Functional domains of Tat required for efficient human immunodeficiency virus type 1 reverse transcription. J Virol 73: 2499-2508.

Ustav M and Stenlund A (1991): Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J 10: 449-457.

van Baalen CA, Pontesilli O, Huisman RC, Geretti AM, Klein MR, de Wolf F, Miedema F, Gruters RA, and Osterhaus ADME (1997): Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. J Gen Virol 78: 1913-1918.

van der Meijden M, Gage J, Breen EC, Taga T, Kishimoto T and Martinez-Maza O (1998): IL-6 receptor (CD126´IL-6R´) expresssion is increased on monocytes and B lmphocytes in HIV infection. Cell Immunol 190: 156-166.

van Ryk DI and Venkatesan S (1999): Real-time kinetics of HIV-1 Rev – Rev response element interactions. J Biol Chem 274: 17452-17463.

Venkatesh LK and Chinnadurai G (1990): Mutants in a conserved region near the carboxy-terminus of HIV-1 Rev identify functionally important residues and exhibit a dominant negative phenotype. Virology 178: 327-330.

Veschambre P, Roisin A and Jalinot P (1997): Biochemical and fuctional interaction of the human immunodeficiency virus type 1 Tat transactivator with the general transcription factor TFIIB. J Gen Virol 78: 2235-2245.

Virgin HW 4th, Mann Ma and Tyler KL (1994): Protective antibodies inhibit reovirus internalization and uncoating by intracellular proteases. J Virol 68: 6719-6729.

Viscidi RP, Mayur K, Lederman HM and Frankel AD (1989): Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. Science 246: 1606-1608.

Vives E, Brodin P and Lebleu B (1997): A truncated HIV-1 Tat protein basic domain repidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272: 16010-16017.

Wain-Hobson S, Sonigo P, Danos O, Cole S and Alizon M (1985): Nucleotide sequence of the AIDS virus, LAV. Cell 40: 9-17.

Wang JK, Kiyokawa E, Verdin E and Trono D (2000): The Nef protein of HIV-1 associates with rafts and primes T cells for activation. Proc Natl Acad Sci USA 97: 394-399.

Watkins BA, Buge S, Aldrich K, Davis AE, Robinson J, Reitz MS and Robert-Guroff M (1996): Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. J Virol 70: 8431-8437.

Weeks KM, Ampe C, Schultz SC, Steitz TA and Crothers DM (1990): Fragment of the HIV-1 Tat protein specifically bind TAR RNA. Science 249: 1281-1285.

Weeks BS, Lieberman DM, Johnson B, Roque E, Green M, Loewenstein P, Oldfield EH and Kleinman HK (1995): Neurotoxicity of the human immunodeficiency virus type 1 tat transactivator tp PC12 cells requires the Tat amino acid 49-58 basic domain. J Neurosci Res 42: 34-40.

Weiss RA, Clapham PR, Cheingsong-Popov R, Dalgleish AG, Carne CA, Weller IVD and Tedder RS (1985): Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS risk-patients. Nature 316: 69-72.

Weiss RA, Clapham PR, McClure Mo, McKeating JA, McKnight A, Dalgleish AG, Sattentau QJ and Weber JN (1988): Human immunodeficiency viruses: neutralization and receptors. J Acquir Immune Defic Syndr 1: 536-541.

Welker R, Kottler H, Kalbitzer HR and Krausslich HG (1996): Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology 219: 228-236.

Welker R, Harris M, Cardel B and Krausslich HG (1998): Virion incorporation of human immunodeficiency virus type 1 Nef is mediated by a bipartite membrane-targeting signal: analysis of its role in enhancement of viral infectivity. J Virol 72: 8833-8840.

Wen W, Meinkoth JL, Tsien RY and Taylor SS (1995): Identification of a signal for rapid export of proteins from the nucleus. Cell 82: 463-473.

Werner T, Ferroni S, Saermark T, Brack-Werner R, Banati RB, Mager R, Steinaa L, Kreutzberg GW and Erfle V (1991): HIV-1 Nef protein exhibits structural and functional similarity to scorpion peptides interacting with K+ channels. AIDS 5: 1301-1308.

Westendorp MO, Li-Weber M, Frank RW and Krammer PH (1994): Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. J Virol 68: 4177-4185.

Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM and Krammer PH (1995): Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature 375: 497-500.

Whitton JL and Oldstone MBA (1996): Immune response to viruses. In: Fields Virology, Third edition (Eds. Fields BN, Knipe DM, Howley PM et al.) pp.345-374. Lippincott-Raven Publishers, Philadelphia, USA.

Winter N, Lagranderie M, Gangloff S, Leclerc C, Gheorghiu M and Gicquel B (1995): Recombinant BCG strains expressing the SIVmac251nef gene induce proliferative and CTL responses agaisnt nef synthetic peptides in mice. Vaccine 13: 471-478.

Wiskerchen M and Cheng-Mayer C (1996): HIV-1 Nef association with cellular serine kinase correlates with enhanced virion infectivity and efficient proviral DNA synthesis. Virology 224: 292-301.

Wolthers KC, Otto SA, Lens SM, Van Lier RA, Miedema F and Meyaard L (1997): Functional B-cell abnormalities in HIV type 1 infection: role of CD40L and CD70. AIDS Res Hum Retroviruses 13: 1023-1029.

Wrenger S, Hoffmann T, Faust J, Mrestani-Klaus C, Brandt W, Neubert K, Kraft M, Olek S, Frank R, Ansorge S and Reinhold D (1997): The N-terminal structure of HIV-1 Tat is required for suppression of CD26-dependent T cell growth. J Biol Chem 272: 30283-30288.

Wu Y, Duan L, Zhu M, Hu B, Kubota S, Bagasra O and Pomerantz RJ (1996): Binding of intracellular anti-rev single chain variable fragments to different epitopes of human immunodeficiency virus type 1 Rev: variation in viral inhibition. J Virol 70: 3290-3297.

Wyatt R and Sodroski J (1998): The HIV-1 envelope glycoproteins: fusogens, antigens and immunogens. Science 280: 1884-1888.

Xiao H, Neuveut C, Benkirane M and Jeang KT (1998): Interaction of the second coding exon of Tat with human EF-1 delta delineates a mechanism for HIV-1-mediated shut-off of host mRNA translation. Biochem Biophys Res Commun 244: 384-389.

Xu XN, Laffert B, Screaton GR, Kraft M, Wolf D, Kolanus W, Mongolsapay J, McMichael AJ and Baur AS (1999): Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain. J Exp Med 189: 1489-1496.

Yamada T and Iwamoto A (1999): Expression of a novel Nef epitope on the surface of HIV type 1-infected cells. AIDS Res Hum Retroviruses 15: 1001-1009.

Yamada T and Iwamoto A (2000): Comparison of proviral accessory genes between long-term nonprogressors and progressors of human immunodeficiency virus type 1 infection. Arch Virol 145: 1021-1027.

Yang J, Bogerd HP, Peng S, Wiegand H, Truant R and Cullen BR (1999): An ancient family of human endogenous retroviruses encodes a functional homolog of the HIV-1 Rev protein. Proc Natl Acad Sci USA 96: 13404-13408.

Yokoi K, Kawai H,Akaike M, Mine H and Saito S (1995): Presence of human T-lymphotropic virus type II-related genes in DNA of peripheral leukocytes from patients with autoimmune thyroid diseases. J Med Virol 45: 392- 398.

Zagury J-F, Sill A, Blattner W, Lachgar A, Le Buanec H, Richardson M, Rappaport J, Hendel H, Bizzini B, Gringeri A, Carcagno M, Criscuolo M, Burny A, Gallo RC and Zagury D (1998): Antibodies to the HIV-1 Tat protein correlated with nonprogression to AIDS: a rationale for the use of Tat toxoid as an HIV-1 vaccine. J Hum Virology 1: 282-292.

Zagury D, Lachgar A, Chams V, Fall LS, Bernard J, Zagury J-F, Bizzini B, Gringeri A, Santagostini E, Rappaport J, Feldman M, Burny A and Gallo RC (1998): Interferon α and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. Proc Natl Acad Sci USA 95: 3851-3856.

Zauli G, Secchiero P, Rodella L, Gibellini D, Mirandola P, Mazzoni M, Milani D, Dowd DR, Capitani S and Vitale M (2000): HIV-1 Tat-mediated inhibition of the tyrosine hydroxylase gene expression in dopaminergic neuronal cells. J Biol Chem 275: 4159-4165.

Zegarra-Moran O, Rasola A, Rugolo M, Porcelli AM, Rossi B and Galietta LJ (1999): HIV-1 nef expression inhibits the activity of a Ca2+-dependent K+ channel involved in the control of the resting potential in CEM lymphocytes. J Immunol 162: 5359-5366.

Zocchi MR, Poggi A and Rubartelli A (1997): The RGD-containing domain of exogenous HIV-1 Tat inhibits the engulfment of apoptotic bodies by dendritic cells. AIDS 11: 1227-1235.

Zolotukhin AS and Felber BK (1999): Nucleoporins nup98 and nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev. J Virol 73: 120-127.