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Immune Dysregulation in HIV-1 Infected Lymphoid Tissue

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ABSTRACT

Lymphoid compartments are major sites for HIV-1 replication. We evaluated cytokines, chemokines and immunological effector function at the single cell level in lymphoid tissues in chronically HIV-1 infected patients. HIV-1 infected lymphoid tissue was characterized by an extensive proinflammatory activation (IL-1α, IL-1β and IL-12) and vigorous Th1 type cytokine expression (IL-2 and IFN-γ) while Th2 cytokines remained low (IL-4 and IL-10). Treatment with highly active anti-retroviral therapy (HAART) resulted in a significant reduction of proinflammatory as well as of Th1 type of cytokine expression in lymphoid tissue. However, the pool of HIV-1 DNA containing cells (1-5%) remained virtually unchanged even after more than one year of HAART while the initial 3-8-fold increase of $CD8^+$ T cells was normalized. We hypothesized that lack of elimination of HIV-1 infected cells was due to impaired cytolytic effector function in activated $CD8^+$ T cells that normally are mediated by either Fas-L/Fas interaction or perforin/granzyme A (grA). $CD8^+$ T cells in HIV-1 infected lymphoid tissue were found to have upregulated Fas-L and grA expression while perforin expression was not concomitantly induced. This was however not true for $CD8^+$ T cells obtained from acutely EBV infected patients, which instead showed upregulation of both perforin and grA expression. Our data indicated a defective cytolytic activity in $CD8^+$ T cells at local sites of HIV-1 replication within lymphoid tissue. To elucidate the molecular mechanism involved in inhibition of perforin expression in HIV-1 infection, we initiated short term *in vitro* cultures using freshly isolated peripheral blood cells from HIV-1 seronegative donors. Exogenous addition of Nef protein was found to mediate downregulation of perforin in $CD8^+$ T cells. Sequence alignments and molecular modeling of different Nef proteins suggested that the so-called disordered loop corresponding to residues 148-178 was a likely contributor to the observed Nef-mediated downregulation of perforin expression. Macaques infected with a *nef*-deleted virus displayed high perforin expression within lymphoid tissues in contrast to macaques infected with the wild-type virus, which suggested a role for Nef as perforin modulator *in vivo*.

Furthermore, cytokine mediated regulation of chemokine receptor expression (CCR5-CXCR4) was studied in placenta tissue from transmitting (TT) and non-transmitting (TNT) HIV-1 infected women. We found upregulation of CCR5 combined with IL-2 expression both at the protein and mRNA level in placenta from TT women. This was associated with an increase in the number of *gag-pol* mRNA expressing cells. In contrast, the placenta from TNT women was characterised by upregulation of Th2 type (IL-4, IL-10) cytokine expression. We found an association between Th2 type of cytokine (IL-4) expression and Leukemia Inhibitor Factor (LIF) production in placenta from TNT tissue while LIF expression was low in placenta from TT women. Therefore, we investigated whether LIF secretion may play a role in HIV-1 inhibition. LIF inhibited HIV-1 replication in a co-receptor independent manner. This inhibition was dependent upon the expression of LIF-Rβ (gp130) on the surface of HIV-1 susceptible cells. The identification of LIF as an inhibitor of HIV-1 replication may lead to the development of a novel anti-retroviral treatment.

Keywords: HIV-1, HAART, cytokines, chemokines, lymphoid tissue, perforin, Nef, LIF, placenta, chemokine receptor

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ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals;

I Jan Andersson, Thomas E. Fehniger, Bruce K. Patterson, John Pottage, Michelle Agnoli, Paul Jones, Homira Behbahani and Alan Landay. Early reduction of immune activation in lymphoid tissue following highly active HIV therapy. AIDS, 12:F123-F129, 1998.

II Homira Behbahani, Alan Landay, Bruce K. Patterson, Paul Jones, John Pottage, Michelle Agnoli, Jan Andersson and Anna-Lena Spetz. Normalization of immune activation in lymphoid tissue following highly active antiretroviral therapy. Journal of Acquired Immune Deficiency Syndromes, 25:150-156, 2000.

III Jan Andersson, Homira Behbahani, Judy Lieberman, Elizabeth Connick, Alan Landay, Bruce Patterson, Anders Sönnerborg, Karin Loré, Stefania Uccini and Thomas E. Fehniger. Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection. AIDS, 13:1295-1303, 1999.

IV Homira Behbahani, Adane Achour, Annelie Tjernlund, Laura Leitner, Donald L. Sodora, Jakob Nilsson, Dong Zhang, Bruce K. Patterson, Judy Lieberman, Jan Andersson and Anna-Lena Spetz. HIV-1 Nef downregulates perforin expression in $CD8^+$ T cells providing a potential mechanism for impaired cytolytic function. Sumbitted.

V Homira Behbahani, Edwina Popek, Patricia Garcia, Jan Andersson, Anna-Lena Spetz, Alan Landay, Zareefa Flener and Bruce K. Patterson. Up-regulation of CCR5 expression in the placenta is associated with human immunodeficiency virus-1 vertical transmission. American Journal of Pathology, 157:1811-1818, 2000.

VI Bruce K. Patterson, Homira Behbahani, William J. Kabat, Yvonne Sullivan, Maurice R.G. O'Gorman, Alan Landay, Zareefa Flener, Nadia Khan, Ram Yogev and Jan Andersson. Leukemia inhibitory factor inhibits HIV-1 replication and is upregulated in placentae from nontransmitting women. The Journal of Clinical Investigation, 107:287-295, 2001.

CONTENTS

ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
Ab	antibody
bp	base pairs
BSS	Earl's balanced salt solution
CCR	CC chemokine receptor
CD	cluster of differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CXCR	CXC chemokine receptor
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing nonintegrin
DNA	deoxyribonucleic acid
env	envelope gene
EBV	Epstein Barr virus
ER	Endoplasmatic reticulum
FAM	5-carboxyfluorescien
Fas-L	Fas ligand
FDC	Follicular Dendritic cell
FITC	fluorescein isothiocyanate
gag	group associated gene
GM-CSF	Granulocyte macrophage-colony stimulating factor
grA	granzyme A
HIV-1	Human immunodeficiency virus type 1
HAART	Highly Active Anti-Retroviral Therapy
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
I-κB	Inhibitory kappa B
LC	Langerhans cell
LT	Lymphoid tissue
LTR	Long terminal repeats
mAb	monoclonal antibody
M-CFS	Macrophage-colony stimulating factor
MDM	Monocyte-derived macrophages
MHC	Major Histocompatibility Complex
MIP	Macrophage inflammatory protein

Nef	negative regulatory element
NF-κB	nuclear factor kappa B
NK	Natural killer cell
PE	phycoeryterin
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pol	polymerase gene
RANTES	Regulated on T cell activation, normal T cell expressed and secreted
RT	Reverse transcriptase
Rev	regulator of virion proteins
RNA	ribonucleic acid
SEA	Staphylococcal Enterotoxin A
SIV	Simian immunodeficiency virus
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tat	Transactivator
TAMRA	5′-carboxy-tetramethylrodamine
TCR	T cell receptor
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
WHO	World Health Organizations

INTRODUCTION

Acquired Immune Deficiency Syndrome (AIDS) was first reported in 1981 with the description of *Pneumocystis carinii* pneumonia in five homosexual men in Los Angeles (Gottlieb *et al.,* 1981; Masur *et al.,* 1981; Siegal *et al.,* 1981). Subsequently, Human Immunodeficiency Virus type 1 (HIV-1) was first isolated from an infected individual in Europe at Pasteur Institute in Paris in 1983 (Barre-Sinoussi *et al.,* 1983) and later in USA as the causative agent of AIDS (Gallo *et al.,* 1984). This virus was found to gradually destroy the immune system by depletion of CD4 positive T lymphocytes.

The global epidemic

In the end of 2000 (report, 2000), the Joint United Nation Programme on HIV-1/AIDS (UNAIDS) and the World Health Organization (WHO) estimated that over 36,1 million people were living with HIV-1/AIDS and about 21.8 million people had died from AIDS. The incidence of infection is highest in Sub Sahara Africa and rising in low-income countries. Only 0.1% of patients receive anti-AIDS treatment in these countries (report, 1999). The number of children under 15 who have lived or are living with HIV-1 since the start of the epidemic in the late 1970s has reached approximately 4.8 million and 3.6 million of them have already died. The majority is from Sub-Saharan Africa. Epidemiological estimation revealed that about 600 000 infants became infected with HIV-1 during 2000, and more than 90% were children born to HIV-1 infected mothers. Children younger than 15 years of age showed 50% higher risk of dying of HIV-1opportunistic infections or other HIV-1 related illnesses such as diarrhea and respiratory tract infections (report, 1999).

Since then, there has been an enormous drive to develop vaccines and therapies to prevent the global spread of HIV-1 infection. An understanding of how HIV-1 causes damage to the immune system and circumvents elimination is necessary for the development of vaccine and new therapeutic strategies. Therefore, the study of lymphoid tissue from HIV-1 infected individuals gives us crucial insight into immune response during disease progression. In this thesis, the cytokines, chemokines and cytolytic effector molecule expression in HIV-1 infected lymphoid tissue were studied. In addition, a novel role for a soluble endogenous factor causing inhibition of HIV-1 replication in placental tissue was reported.

The virus

HIV-1 is classified as a human Lentivirus and belongs to the *Retroviridiae* family. It is an enveloped virus and its cell-derived lipid membrane gives a spherical outer shape (Gelderblom *et al.,* 1987). HIV-1 contains two copies of a positive charged single stranded RNA genome, approximately 9.2kbp in length. The encoded proteins are divided into three classes; *gag, pol, and env*, which are flanked on both sides by long terminal repeats (LTRs). The *gag* encodes for the matrix, capsid and nucleocapsid proteins while *pol* codes for reverse transcriptase (RT), protease and integrase. The *env* gene encodes the envelope glycoproteins (gp120 and gp41) which are important for virus binding to the target receptors (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984b). In addition to the three structural genes, the HIV-1 provirus contains six additional open reading frames (ORF) which encode for accessory (*vpu, vpr, vif*, and *nef*) and regulatory proteins (*tat* and *rev*) (Levy, 1993). The Vpu increases the release of HIV-1 from the surface of an infected cell and degrades CD4 in the endoplasmatic reticulum (ER) (Strebel *et al.,* 1988; Willey *et al.,* 1992). The Vpr plays a role in the nuclear localization of viral nucleic acids contained within the preintegration complex and affects cell differentiation (Zhao *et al.,* 1994). It has been reported that the Vif protein plays an important role for proviral DNA synthesis (von Schwedler *et al.,* 1993). The Nef is a 27-kD myristoylated protein that is the first viral protein to accumulate to detectable levels in a cell following HIV-1 infection (Kim *et al.,* 1989). HIV-1 Nef was found to downregulate CD4 (Garcia and Miller, 1992) and MHC class I molecule expression (Schwartz *et al.,* 1996). Tat is a transcriptional transactivator protein that is essential for HIV-1 replication (Ruben *et al.,* 1989). The Rev protein facilitates transport of unspliced and single spliced mRNA from the nucleus to the cytoplasm (Zapp and Green, 1989).

It is generally believed that the HIV-1 virus originates from Africa. In 1959, the first documented HIV-1 seropositive serum sample was collected in central Africa (Nahmias *et al.,* 1986). The identification of the African monkey viruses, i.e. the simian immunodeficiency viruses (SIVs) provided additional evidence that the HIV-1 may originated from Africa (Kanki *et al.,* 1985; Fultz *et al.,* 1986; Murphey-Corb *et al.,* 1986; Tsujimoto *et al.,* 1988). A previous study suggested that certain species of chimpanzee, the *Pan troglodytes troglodytes* might be the common natural reservoir for HIV-1 (Gao *et al.,* 1999). The simian virus that infects chimpanzees (SIVcpz) shows 80% homology to HIV-1 (Peeters *et al.,* 1989).

Natural history of HIV-1 infection

This infectious disease begins with a meeting between virus particles and an unprimed immune system. Clinically, HIV-1 infection can be divided into three phases; acute, chronic and AIDS.

Acute phase

A decrease of $CD4^+$ T cells occurs during acute initial infection (Lang *et al.,* 1989). The CD4 protein is the first molecule (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984b; Maddon *et al.,* 1986) to bind HIV-1 through the viral gp120 envelope protein in combination with a chemokine receptors (Steimer *et al.,* 1991). The HIV-1 virus replicates in cells expressing CD4 antigen on their surface. Aside from T cells, other cells including monocytes, macrophages, dendritic cells (DC) (Cameron *et al.,* 1996; Ayehunie *et al.,* 1997) and Langerhans cells (LC) in the epidermis (Ayehunie *et al.,* 1995) are susceptible to HIV-1 infection. Moreover, non-dividing cells, such as follicular dendritic cells (FDC) and brain microglial cells (He *et al.,* 1997), can be targets for virus entry and infection (Klatzmann *et al.,* 1984a; Gartner *et al.,* 1986; Ho *et al.,* 1986; Salahuddin *et al.,* 1986; Patterson and Knight, 1987). Nevertheless, $CD4^+$ T cells seem to be the major target cells (Haase, 1999). The onset of acute viral infection is accompanied with a rise of free virus and viral antigens in blood caused by the production of up to 10^{10} (billion) viral particles per day (Piatak *et al.,* 1993; Ho *et al.,* 1995). Classical clinical symptoms in infected individuals include a brief illness, fever, night sweats, headache and swollen lymph nodes resembling infectious mononucleosis (Cooper *et al.,* 1985; Ho *et al.,* 1985b; Clark *et al.,* 1991; Daar *et al.,* 1991; Henrard *et al.,* 1995; Quinn, 1997; Schacker *et al.,* 1998). In addition, tonsillar enlargement, and splenomegaly are prominent features observed in HIV-1 infected lymphoid tissue (LT) as the earliest clinical descriptions of acute HIV-1 infection (Cooper *et al.,* 1985). Within a few weeks, the level of virus in the blood declines. This coincides with the development of an immunological response to HIV-1 (Mellors *et al.,* 1996; Mellors *et al.,* 1997). HIV-1 specific cytolytic T lymphocytes (CTLs) appear (Roos *et al.,* 1992; Borrow *et al.,* 1994; Mackewicz *et al.,* 1994) and increase in frequency in the peripheral blood (Koup *et al.,* 1994; Pantaleo *et al.,* 1994; Schmitz *et al.,* 1999). Neutralizing antibodies (Cooper *et al.,* 1988; Cooper and Lacey, 1988; Cooper and Jeffers, 1988; Ariyoshi *et al.,* 1992; Moore *et al.,* 1995) that have a key role of immune responses to most viral infections, appear after onset of the cellular immune response in primary infection (Koup *et al.,* 1994; Pellegrin *et al.,* 1996). Following initial replication in target cells, the virus disseminates via the blood stream into different organs such as brain, spleen and peripheral lymphoid tissue. At this time replication has reached a plateau (steady state). Generation of primary immune activity results in a significant drop in viral load to a more stable viral set point (Mackewicz *et al.,* 1994; Musey *et*

al., 1997). The quality of innate and adaptive immune activity at the set point may account for differences in viral virulence and disease progression (Reimann *et al.,* 1994; Deacon *et al.,* 1995; Mellors *et al.,* 1996; Mellors *et al.,* 1997; Rosenberg and Walker, 1998; Phair, 1999; Pitcher *et al.,* 1999; Lyles *et al.,* 2000).

Chronic phase

In general, a chronic stage or asymptomatic phase will be established between primary infection and the development of clinical immunodeficiency (AIDS). A majority of infected individuals remain asymptomatic after primary infection but eventually develop immunodeficiency 4-12 years after seroconversion (Lifson *et al.,* 1988; Jason *et al.,* 1989; Munoz *et al.,* 1989; Rutherford *et al.,* 1990). This asymptomatic period is associated with two main pathophysiological characteristics; gradual loss of $CD4^+$ T cells and persistent viral replication in the peripheral lymphoid tissues (Pantaleo *et al.,* 1991; Embretson *et al.,* 1993; Pantaleo *et al.,* 1993b; Haase *et al.,* 1996; Cavert *et al.,* 1997; Chun *et al.,* 1997a; Wong *et al.,* 1997b; Finzi *et al.,* 1999; Furtado *et al.,* 1999; Hockett *et al.,* 1999; Zhang *et al.,* 1999). The number of infected cells in the peripheral lymphoid tissue is correlated with plasma viral load (Pantaleo *et al.,* 1991; Embretson *et al.,* 1993; Pantaleo *et al.,* 1993a; Harris *et al.,* 1997; Hockett *et al.,* 1999). Approximately 5% of infected individuals preserve immune function and normal $CD4^+$ T cell levels for more than a decade and are termed long-term non-progressors (LTNP) (Learmont *et al.,* 1992; Sheppard *et al.,* 1993; Buchbinder *et al.,* 1994; Greenough *et al.,* 1994; Keet *et al.,* 1994; Deacon *et al.,* 1995; Schrager and Fauci, 1995).

There are several factors, which can be involved in disease progression. Viral characteristics were shown to have a critical role in long-term non-progressive infection in some individuals. This was first described by Learmont *et al.* from patients who were infected with attenuated virus and remained immunologically stable despite a decade of infection (Learmont *et al.,* 1992). Animal studies by Kestler *et al*. showed that monkeys inoculated with SIV with deletions in the *nef* gene had no signs of disease and maintained low viral burden along with normal $CD4^+$ T cell counts (Kestler *et al.,* 1991). These studies showed that *nef*-deleted or HIV-1 isolates with mutant *nef* could be associated with slow progression or long-term non-progression (Lefrere *et al.,* 1997). Recently, it has been reported that $CD4^+$ T cells acutely infected with *nef*-deleted virus disappear after exposure to CTL *in vitro*, in contrast to *nef*-competent HIV-1 infected cells, which are less efficiently cleared (Collins *et al.,* 1998).

Among genetic host factors, a polymorphism in co-receptors correlated with delayed rate of HIV-1 disease progression. For instance, a mutation in the chemokine receptor gene, CCR5Δ32, has been shown to provide strong protection from infection with

HIV-1 (Dean *et al.,* 1996; Huang *et al.,* 1996; de Roda Husman *et al.,* 1997; Michael *et al.,* 1997a; Michael *et al.,* 1997b; Zimmerman *et al.,* 1997; McDermott *et al.,* 1998; Mummidi *et al.,* 1998). This deletion result in the production of a defective, truncated CCR5 protein, which remained in the cytoplasm of the cells. These cells of infected individuals are resistant to R5- using HIV-1 isolates but remain susceptible to RX4-isolates (Berger *et al.,* 1998). This genetic background is associated with significantly lower HIV-1 RNA plasma levels.

AIDS phase

A rapid decline of $CD4^+$ T cells (Connor *et al.,* 1993; de Wolf *et al.,* 1997) and HIV-1 specific $CD8^+$ T cells (Champagne *et al.,* 2001) combined with an increasing HIV-1 viral load are correlated with the onset of symptoms and progression to AIDS (Mellors *et al.,* 1996; Ferbas, 1998; Ogg *et al.,* 1998). As the CD4 count falls below 200 cells/µl, opportunistic infections begin to occur. AIDS is associated with opportunistic infections such as *Candida albicans*, *Peumocystis carinii pneumonia* and lung disease caused by *Mycobacterium tuberculosis* (CDC, 1993; CDC, 1994).

Treatment

The AIDS epidemic has been a constant driving force for the discovery of new substances that inhibit HIV-1 replication. The development of safe antiviral treatment has resulted in significant reduction of mortality and morbidity in HIV-1 infected patients to whom treatment is available.

The first anti-retroviral drug to be approved for treatment of HIV-1 infection, was zidovudine (AZT) in 1987. Zidovudine is a nucleoside analogue with reverse transcriptase inhibitory capacity (NRTI) and the AZT mono-therapy became the standard of HIV-1 therapy for several years. Later the addition of another NRTI drug, lamivudine, combined with AZT, resulted in widespread delay in progression to AIDS and death. However, it was not until the introduction of protease inhibitor (PI) and triple-drug regimens in 1996 that a sufficient anti-retroviral effect was achieved (Gulick *et al.,* 1997). The protease inhibitors were shown to inhibit the late step of viral replication when viral protein precursors need to be cleaved by virus specific protease. As, the HIV-1 RT and protease enzymes are essential for complete viral replication, these enzymes became ideal targets for selective drugs. The development of other drug combinations led to the suppression of HIV-1 replication and HIV-1 RNA to undetectable levels. This sustained reduction in viral replication was shown to improve immune function, delay progression of disease and prolong survival (Autran *et al.,* 1997; Finzi *et al.,* 1997; Palella *et al.,* 1998).

However, despite the successful suppression of HIV-1 replication for many months and years, a long-lived reservoir of infected cells persist. This provides the need for continuation of long-term therapy in infected patients (Pomerantz *et al.,* 1990; Finzi *et al.,* 1999; Furtado *et al.,* 1999; Martinez *et al.,* 1999). Estimation of latently infected resting $CD4^+$ T cells suggested that more than 60 years of anti-viral therapy would be required for virus eradication (Siliciano and Siliciano, 2000). Although several studies reported that highly active anti-retroviral therapy (HAART) reduce plasma viral burden to below the level of detection and discontinuation of therapy led to rapid viral rebound in most patients (Chun *et al.,* 1997b; Finzi *et al.,* 1997; Wong *et al.,* 1997b). The development of potent anti-retroviral therapy lead to immune reconstitution and restoration of functional immune responses. Therefore, addition of therapeutic vaccines during effective HAART therapy may be a way to restore HIV-1 immunity. This may allow discontinuation of HAART, which would be an important achievement since a big problem with HAART is the development of severe side effects (Cavert *et al.,* 1997; Perrin and Telenti, 1998).

HIV-1 infected lymphoid tissue

HIV-1 is commonly transmitted through sexual contact and the very early stages of infection establish a persistent infection in LT. Virus is produced and stored at this site and slowly depletes the immune system of $CD4^+$ T cells, setting the stage for AIDS.

In fact, HIV-1 was first isolated from a lymph node (Barre-Sinoussi *et al.,* 1983) and was detected several years later by electron microscopy and immunoperoxidase antigen staining in LTs (Tenner-Racz *et al.,* 1985; Biberfeld *et al.,* 1987). Later, the more sensitive *in situ* hybridization technique for the detection of HIV-1 RNA in tissue provided an important information on the distribution of HIV-1 virus in tissues (Emilie *et al.,* 1990; Fox *et al.,* 1991; Spiegel *et al.,* 1992). The use of quantitative RT-PCR and analysis of viral burden indicated that viral replication was far greater in LT compared to peripheral blood mononuclear cells (PBMC) from the same individual (Pantaleo *et al.,* 1993a).

The predominant route of HIV-1 transmission involves entry across the mucosal associated lymphoid tissue (MALT) or across the genital mucosa during sexual contact (Royce *et al.,* 1997). In 1987, the epidermal LC cells were reported to be first target for HIV-1 infection (Braathen *et al.,* 1987). In addition, studies with rhesus macaques have reported that DCs initially become infected shortly after vaginal inoculation through sexual mucosal transmission of SIV (Spira *et al.,* 1996). These studies showed that the residing DCs or LC cells within epithelial surfaces can be the initial target

cells after mucosal exposure to the virus. These cells express the CD4 molecule on their surfaces, which serve as receptors for viral gp120. Later discoveries revealed that HIV-1 entry into cells also requires chemokine co-receptors (Deng *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996). HIV-1 transmission frequently involves virus strains that use CXCR4 or CCR5, which are the principal coreceptors for the virus. Furthermore, elegant studies (Geijtenbeek *et al.,* 2000a; Geijtenbeek *et al.,* 2000b) have elucidated that the initial events of HIV-1 infection may not require productive infection of the LCs. One recent study showed that a C-type lectin, dendritic cell-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), was abundantly expressed on DCs and suggested to mediate the first contact between HIV-1 and the DCs (Geijtenbeek *et al.,* 2000a; Geijtenbeek *et al.,* 2000b). DC-SIGN may bind the virus particle in the periphery and thereafter carry the virus to regional lymphoid tissues where the fusion of the viral envelope with the $CD4^+$ T helper cell membrane occurs.

Dissemination of HIV-1 virus in lymphoid tissue

Hence HIV-1 may be captured intracellularly by DCs or carried by DC-SIGN to lymph nodes via afferent lymphatics. The fusion of the viral envelope with the T helper cell membrane occurs in regional extrafollicular areas of LTs. There, DCs initiate activation of naïve T cells and promote primary activation (Banchereau and Steinman, 1998). Activated $CD4^+$ T cells are vital for facilitation of the humoral and cellular immune responses against virus infection. The expansion of antigen specific cytokine expressing $CD4^+$ and $CD8^+$ T cells in HIV-1 infection is important for control and elimination of infected cells. During primary infection an extensive HIV-1 replication occurs in HIV-antigen specific $CD4^+$ T cells in gut-associated lymphoid tissue (GALT), which include the tonsils, adenoids and appendix, and other related lymphoid tissue such as lymph nodes and spleen, reaching viral loads 10 to 1000-fold higher than peripheral blood (Pantaleo *et al.,* 1991). More than 90% of $CD4^+$ productively infected T cells are found in lymph nodes during primary infection (Haase, 1999). Later on, a fraction of the productively $CD4^+$ T cells with integrated HIV-1 DNA (Chun *et al.,* 1995) become latently infected memory $CD4^+$ T cells and provide persistent virus in the tissue. However, these latently HIV-1 infected cells may circumvent HIV-1 specific CTL and be a major reservoir for propagation of HIV-1in tissues (Brodie *et al.,* 2000). In the lymph node, activated $CD4^+$ T cells interact with B cells resulting in proliferation of B cells in follicles. This results in HIV-1 specific antibody responses. Once T cells are activated, they are much more susceptible to HIV-1 infection than resting inactivated T cells (Pantaleo *et al.,* 1993b). *In situ* PCR has shown that the majority of HIV-1 infected cells are $CD4^+$ T cells that contain HIV-1 DNA, found in the follicular mantle of germinal complex (GC) and in the paracortex of lymphoid regions (Embretson *et al.,* 1993). In addition, trapping of viral particles has been detected in FDC in the germinal center of

lymph nodes (Haase *et al.,* 1996). These cells bind immune complexes consisting of virus and HIV-1 specific antibodies, which can explain why high incidences of HIV-1 RNA are usually detected in the germinal center of lymph nodes. In addition, quantitative techniques based on *in situ* hybridization and *in situ* image analysis has demonstrated that the majority of viral RNA during acute phase of disease is present on the surfaces of FDCs. These cells may not produce virus but have a remarkable ability to store infectivity of HIV-1 particles as immune complexes with neutralizing antibodies (Heath *et al.,* 1995). Hence the pool of latent FDCs provide another way for virus escape since HAART can not affect this resting reservoir.

Cytokines and HIV-1 infection

Cytokine network

Due to the involvement of several cytokines in the homeostatic regulation of the immune responses and their ability to influence (i.e. induce or suppress) HIV-1 expression, it is of interest to determine the degree and pattern of cytokine expression during disease progression. The mobilization and activation of immune competent cells supported by local cytokine expression may contribute to the propagation of virus replication. Cytokines are small structural proteins or glycoproteins, which have multiple sources, targets and functions. They regulate host defenses against infection and injury in a paracrine and autocrine fashion. Activated T cells and macrophages are major cytokine producers. Based on *in vitro* and *in vivo* experimental data, immunoregulatory cytokines can be subdivided into three different groups; Th1 cytokines (IFN-γ, IL-2, TNF-β and IL-15), involved in cellular immune responses and Th2 cytokines (IL-4, IL-5, IL-6, IL-10 and IL-13) responsible for induction of humoral immune responses and the Th0 cytokine (TGF-β). Furthermore, macrophages and DCs are specialized to produce proinflammatory (IL-1α/β, TNF-α, IL-12, IL-18) or anti-inflammatory (IL-1ra, IL-6, IL-10, TGF-β) cytokines. Cytokine produced by Th1 and Th2 phenotypes can regulate the production of each other. It has been shown that the IFN-γ inhibits the production of Th2 type of cytokines (O'Garra and Murphy, 1994) but not of Th1 type. In addition, IL-10 via the effect on antigen-presenting cells, inhibits the production of IL-12, IFN-γ and other cytokines (Fiorentino *et al.,* 1991). Furthermore, the Th1 cytokines inhibit IL-4 effects on B cells. IL-4 is important for antibody maturation (Mosmann *et al.,* 1986) (Figure 1).

Cytokines involved in HIV-1 replication

Cytokines play a major role in enhancement of HIV-1 replication in LT (Chun *et al.,* 1998). The observed increase in cytokine production in HIV-1 infected LT may stimulate viral replication as well as induce adaptive HIV-1 specific immune responses. Expansion

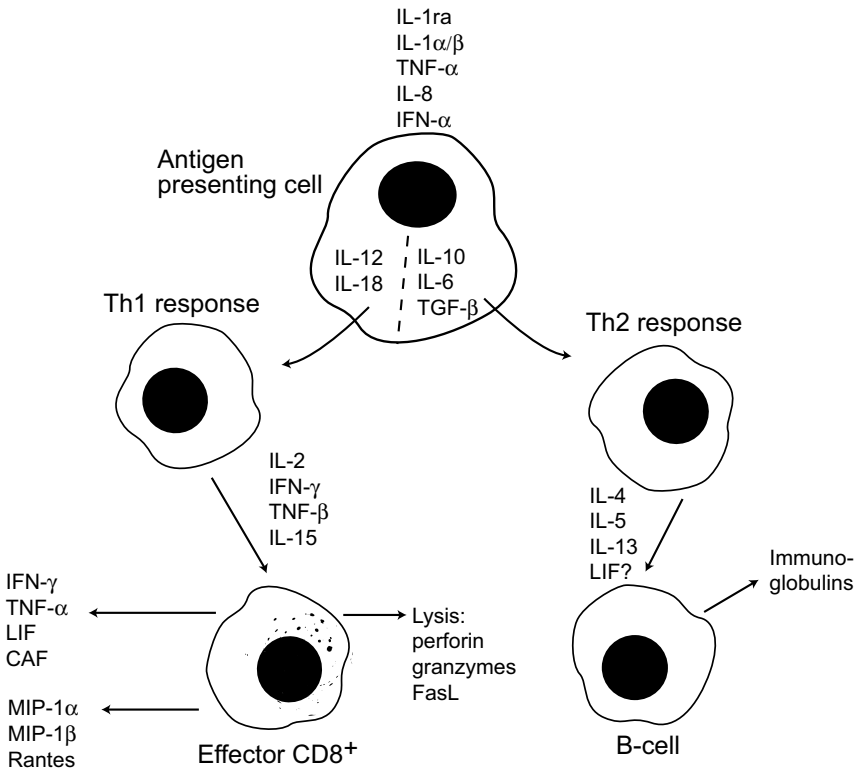

Figure 1. This figure illustrates regulation of Th1 and Th2 type of cytokine mediated immune responses. Activation of antigen presenting cells, such as macrophages result in production of proinflammatory cytokines (TNF-α, IL-1α/β IL-6 and IL-8). In addition, later on these cells produce IL-1ra, IL-10 and TGF-β cytokines which downregulate inflammation. Furthermore, they produce IFN-α that has an inhibitory role on HIV-1 viral replication. IL-12 and IL-18 produced by antigen presenting cells, induce Th1 responses while IL-10, IL-6 and TGF-β induce Th2 type cytokines. The Th1 cytokine producing cells induce macrophage activation and formation of cytotoxic T cells. These cells predominantly eliminate target cells by production and release of perforin, granzymes and Fas-L. In addition, CTLs can inhibit HIV-1 replication by release of soluble factors including chemokines. LIF was recently identified as a novel exogenous inhibitory factor. In contrast to Th1 type of cytokines, the Th2 type of response involves IL-4, IL-5 and IL-13, which regulate B cell activation and immunoglobulin production.

and activation of $CD4^+$ T cells in LTs is dependent on transcription factors, effector molecules, cytokines and co-stimulatory cell surface proteins, including cytokine receptors and adhesion molecules (Crabtree, 1989). Among the cytokines, IL-1α/β, IL-2, IL-3, TNF-α, IL-6 and IL-7 have been shown to stimulate HIV-1 replication *in vitro* (Rosenberg and Fauci, 1989; Rosenberg and Fauci, 1990; Rosenberg and Fauci, 1991; Biswas *et al.,* 1992; Poli and Fauci, 1992; Chun *et al.,* 1998). In contrast, other cytokines such as IL-4, TGF-β, IFN-γ (Novak *et al.,* 1990; Lazdins *et al.,* 1991; Poli *et al.,* 1991; Rosenberg and Fauci, 1991) may suppress HIV-1 infection.

Among the cytokines, the interferons are a group of proteins and glycoproteins, which have a common property of inhibiting virus replication. IFN-α and IFN-β produced by DCs, lymphocytes and fibroblasts respectively, are able to interfere with viral replication in target cells (Pomerantz and Hirsch, 1987; Ullum *et al.,* 1997; Bot *et al.,* 1998). IFN-α appears at an early stage during virus infection, before the development of cell-mediated and humoral immunity. A loss of IFN-α production has been associated with high HIV-1 RNA levels and AIDS (Ho *et al.,* 1985a; Yamamoto *et al.,* 1986; Koyanagi *et al.,* 1988; Poli *et al.,* 1989; Williams and Colby, 1989; Soumelis *et al.,* 2001). IFN-γ is the hallmark of effector function for the Th1 subsets of T cells that are involved in cell-mediated cytotoxicity (Mosmann and Coffman, 1989). A rise of IFN-γ production was detected in plasma, LTs, PBMCs and cerebrospinal fluid of HIV-1 infected persons (Sonnerborg *et al.,* 1988; Graziosi *et al.,* 1994; Fauci, 1996; Fauci *et al.,* 1996; Appay *et al.,* 2000). However, in more advanced patients, fewer cells produce IFN-γ in response to HIV-1 antigen exposure. The number of antigen specific IFN-γ producing PBMC cells initially increases with HAART therapy (Ullum *et al.,* 1997; Bailer *et al.,* 1999; Huang *et al.,* 2000). The reduced IL-2 production in PBMC has also long been recognized in HIV-1 disease (Fan *et al.,* 1993; Kinter and Fauci, 1996; Klein *et al.,* 1997; Sousa and Victorino, 1998).

Cytokines involved in the immune response to HIV-1 infection

The IL-1α and IL-β are cytokines produced in the early phase of inflammation exerting multiple effect on innate and adaptive immune responses. Over expression of IL1-α and IL-1β contribute to the pathogenesis of many diseases. Both primary HIV-1 infection and the asymptomatic phase are characterised by profound proinflammatory cytokine expression (Andersson *et al.,* 1998; Chun *et al.,* 1998). The increase of proinflammatory cytokines has been detected in PBMC from HIV-1 infected individuals as well as in HIV-1 infected LT (Graziosi *et al.,* 1994; Andersson *et al.,* 1998). Proinflammatory cytokines can initiate signals for maturation and migration of DCs to

the regional lymph nodes and spleen (Barratt-Boyes *et al.,* 1997) and induce HIV-1 replication in latently infected resting $CD4^+$ memory T cells (Fauci *et al.,* 1996; Weissman *et al.,* 1996; Moriuchi *et al.,* 2000).

The most crucial cytokine to sway the immune system towards a Th1 profile is IL-12. IL-12 plus IL-18 possess both proinflammatory and immuneregulatory activity. Decreased production of IL-12 has previously been found in PBMCs from HIV-1 infected persons (Chehimi *et al.,* 1994; Benyoucef *et al.,* 1998; Vanham *et al.,* 1999). Moreover, IL-12 and IL-18 facilitate the secretion of IFN-γ which is considered beneficial for its antiviral activity (Miedema *et al.,* 1988; Wu *et al.,* 1993). The observed impairment of HIV-1 antigen specific Th1 responses in PBMC in HIV-1 infection is suggested to be related to a deficient production of IL-12 (Murray *et al.,* 1984; Bailer *et al.,* 1999; Hirsch *et al.,* 1999; Torre *et al.,* 1999). In addition, the suppression of IL-12 production by IL-10 from monocyte-derived macrophages (MDM) in HIV-1-infected patients demonstrates another possibility for impaired induction of Th1 and CTL responses (Chougnet *et al.,* 1996). The interaction between CD40 on DCs and CD40L on $CD4^+$ T cells with subsequent induction of IL-12/IFN-γ production is thought to be important in both HIV-1 specific CD8 cytotoxiciy and humoral responses (Trinchieri, 1997). Thus, reduced CD40L expression during HIV-1 infection may have a strong impact on T cell dependent IL-12 production, critical for the establishment of a Th1 response (Ma and Montaner, 2000). Another important mediator of the inflammatory response is TNF-α (Beutler, 1995) identified as an important factor in HIV-1 disease pathogenesis. TNF-α can accelerate HIV-1 replication *in vitro* (Matsuyama *et al.,* 1989) and stimulate the release of other cytokines (Sherry and Cerami, 1988; Lenardo and Baltimore, 1989). A correlation between enhanced TNF-α production and HIV-1 replication has been reported in several studies (Hober *et al.,* 1989; Hober *et al.,* 1992; Kong *et al.,* 1996; Alonso *et al.,* 1997; Lee *et al.,* 1997; Bergamini *et al.,* 1999; Lore *et al.,* 1999). The molecular mechanism of TNF-α influence on HIV-1 replication was identified as activation of the cellular transcription factor NF-κB in both T cells (Osborn *et al.,* 1989) and macrophages (Griffin *et al.,* 1989). Upon activation, NF-κB disassociates from an inhibitory molecule (I-κB) which allows migration of NF-κB to the nucleus. Binding sites for NF-κB also exist within long-term repeat (LTR) of the transcription site region of the HIV-1 genome. This binding triggers or potentiates HIV-1-expression (Siebenlist *et al.,* 1994). Indeed, TNF-α, together with IL-2 and IL-6, were shown to support viral replication *in vitro* in PBMC of HIV-1 infected individuals and in latently infected $CD4^+$ T cells (Chun *et al.,* 1998).

A switch from HIV-1 antigen induced Th1 type of cytokine expression to IL-4 and IL-10 have been shown to be associated with HIV-1 disease progression in some studies

(Zagury *et al.,* 1998) but decreases in others (Graziosi *et al.,* 1994; Maggi *et al.,* 1994). IL-4 and IL-10 together with IL-5 and IL-13 can enhance humoral immunity, inhibit cell-mediated immunity, and result in a protective humoral immunity for pathogens removed in mucosal sites. Progression of HIV-1 infection has been associated with imbalances of cytokine production leading to a switch from a Th1 to a Th2 profile (Clerici *et al.,* 1994; Wang *et al.,* 1994; Shearer and Clerici, 1996; Doherty *et al.,* 1997). In the late stage of disease, HIV-1 infected individuals are characterized by increased HIV-1 antigen induction of Th2 cytokines with concomitant loss of IL-2 and IFN-γ production in PBMCs. The decreased expression of *in vitro* induced IL-2 production, has been linked to the possible reduced renewal capacity of the T cells (Fleury *et al.,* 1998) as well as to the increased susceptibility to apoptosis (Adachi *et al.,* 1996). IL-2 plus IL-15 has an important role for CD8 T cell growth and survival. Support for protective role of cytokines was further demonstrated in murine retrovirus–induced AIDS-like syndrome (Wang *et al.,* 1994; Doherty *et al.,* 1997). The presence of *in vitro* induced HIV-1 specific Th1 cytokines expression may be correlated to prevention of primary HIV-1 infection in HIV-1 seronegative individuals exposed to HIV-1 (Clerici and Shearer, 1993; Clerici and Shearer, 1994). Thus, both *in vivo* and *in vitro* data indicates that the HIV-1 infection is associated with imbalance of inflammatory and immunoregulatory cytokines. *In vivo* cytokines are mainly produced in the lymphoid compartments. Therefore, the investigation of cytokine dysregulation in LT may shed further insights into the immuopathogentic mechanism operating in HIV-1 infection.

Chemokine receptors and their role in HIV-1 infection

As mentioned before, HIV-1 uses the CD4 molecule as the main receptor for infection of the cells. In addition to CD4, HIV-1 requires a co-receptor for entry into target cells. These co-receptors were shown to belong to the chemokine receptor family. Chemokine receptors are large molecule with seven transmembrane receptors (7-TMR) and are members of the G protein-coupled receptor family.

In 1995, the discovery of an inhibitory effect on some viral strains by the chemokines led to identification of chemokine receptors as co-receptors for HIV-1 (Cocchi *et al.,* 1995). This led to the cloning of a gene that encodes a chemokine receptor-like protein (Feng *et al.,* 1996). This protein when expressed on non-human cell lines together with $CD4^+$ T cells could allow not only the binding of T tropic viruses but also their envelope fusion. This molecule was termed ”fusin” and was later renamed CXCR4. This phenotype was originally observed with isolates that had been adapted in the laboratory to replicate in T cell lines and was subsequently observed with some clinical isolates. Viruses that infected activated T cells or transformed T cell lines, were

designated T tropic (syncytium-inducing or X4-using) isolates and have been isolated from AIDS individuals (Schuitemaker *et al.,* 1992; Connor *et al.,* 1997). In general HIV-1 X4-using isolate uses the α-chemokine receptor CXCR4 as the major co-receptor.

The chemokines, RANTES (Schall *et al.,* 1988), macrophage inflammatory protein-1α (MIP-1α) (Sherry *et al.,* 1988), and MIP-1β (Sherry *et al.,* 1988) were, however, shown bind to an additional co-receptor (Samson *et al.,* 1996). The latter co-receptor called CCR5, together with CD4 was identified as a major receptor for macrophage tropic strains (Alkhatib *et al.,* 1996; Choe *et al.,* 1996). The HIV-1 strains that infected primary macrophages more efficiently than T cell lines; were designated M tropic (nonsyncytium-inducing, R5-using). HIV-1 R5-isolates use the β-chemokine receptor CCR5 as their co-receptor. It was determined that R5 viruses predominate in most individuals shortly after infection or during asymptomatic phase (Zhu *et al.,* 1993; van't Wout *et al.,* 1994).

CCR5 and CXCR4 are both expressed on known cell and tissue targets of HIV-1, consistent with roles in disease transmission and progression. In particular, CCR5 expression has been documented on cells that may be important targets in the establishment of initial infection, including $CD4^+$ T cells (Raport *et al.,* 1996; Bleul *et al.,* 1997; Wu *et al.,* 1997), monocyte/macrophages (Combadiere *et al.,* 1996; Rottman *et al.,* 1997; Zhang *et al.,* 1998), DCs (Granelli-Piperno *et al.,* 1996; Ayehunie *et al.,* 1997; Rubbert *et al.,* 1998), LC cells (Dittmar *et al.,* 1997; Zaitseva *et al.,* 1997), and the mucosa of rectum and colon (Zhang *et al.,* 1998) as well as vagina and cervix (Zaitseva *et al.,* 1997; Patterson *et al.,* 1998; Zhang *et al.,* 1998). CXCR4 is expressed in many of the same cells and tissues as CCR5, however, at lower levels in the genital tract (Granelli-Piperno *et al.,* 1996; Raport *et al.,* 1996; Ayehunie *et al.,* 1997; Bleul *et al.,* 1997; Dittmar *et al.,* 1997; Rottman *et al.,* 1997; Zaitseva *et al.,* 1997; Patterson *et al.,* 1998; Zhang *et al.,* 1998). Several reports suggest an important role for CCR5 during initial viral transmission (van't Wout *et al.,* 1994) and for CXCR4 and/or possibly other coreceptors in late stages of disease progression.

In addition to CCR5 and CXCR4, other chemokine receptors have been identified to act as viral entry receptors for HIV-1, HIV-2 and SIV (Choe *et al.,* 1996). However, the majority of primary viral strains, from various parts of the world, were shown to use CXCR4 or CCR5 as co-receptors (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996; Oberlin *et al.,* 1996; Simmons *et al.,* 1996; Zhang *et al.,* 1996; Bjorndal *et al.,* 1997; Scarlatti *et al.,* 1997).

Soluble factors inhibiting HIV-1 infection

HIV-1 protection mediated by $CD8^+$ T cells is not only due to their ability to lyse the virally infected cells by the granule exocytosis pathway or by death domain-containing receptors (Henkart, 1994). The importance of soluble molecules released from $CD8^+$ T cells to suppress viral replication has been suggested previously (Walker *et al.,* 1986; Brinchmann *et al.,* 1990; Mackewicz *et al.,* 1991). The secretion of soluble factors including cytokines such as IFN-γ, CD8 derived antiviral factor (CAF), IL-16 (Zhou *et al.,* 1997), chemokines (RANTES, SDF-1and MIP-1α/β) (Cocchi *et al.,* 1995) were shown to interfere with HIV-1 replication (Levy *et al.,* 1996).

Chemokines, initially called ”chemotactic cytokines”, are small soluble proteins (8-14 kDa) and usually containing four conserved cystein residues. They are secreted by activated leukocytes and stromal cells, including endothelial cells upon inflammatory stimuli (Oppenheim, 1993; Baggiolini *et al.,* 1994; Schall and Bacon, 1994; Rollins, 1997). Chemokines are involved in chemotaxis, activation of integrins and adhesion, lymphocytes and DCs trafficking. They play an important role in inflammation and in immune responses (Baggiolini, 1998; Luster, 1998; Cyster, 1999; Locati and Murphy, 1999). The CC or β-chemokines represent the largest group and generally attract monocytes, lymphocytes, basophils and eosinphils but not neutrophils. As mentioned before, RANTES, MIP-1α/β produced by $CD8^+$ T cells suppress HIV-1 replication in PBMCs and inhibit entry of macrophage tropic (R5) but not T cell tropic (X4) HIV-1 infection *in vitro* (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Dragic *et al.,* 1996; Dolei *et al.,* 1998).

Stromal cell derived factor-1 (SDF-1) was shown to have an important role in B-cell development (Bleul *et al.,* 1996; Nagasawa *et al.,* 1996; Oberlin *et al.,* 1996). The CXC chemokine SDF-1 (Berson *et al.,* 1996; Feng *et al.,* 1996), a ligand for CXCR4, inhibits HIV-1 infection in cells expressing CXCR4 and CD4. Previously a novel human CC chemokine, monocyte-derived chemokine (MDC), was identified (Godiska *et al.,* 1997). MDC is produced by macrophages and DCs and is chemotactic for monocytes, monocyte-derived dendritic cells and IL-2 activated NK cells. MDC suppressed infection of CD8-depleted peripheral blood cells by a broad panel of R5- and R-4 using HIV-1 isolates (Pal *et al.,* 1997).

Chemokines were shown to mediate blocking of HIV-1 entry via neutralization of different chemokine receptors (Brandt *et al.,* 2002). The complex of viral gp120 envelope and CD4 (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984b) on the host cell surface, binds to co-receptors (Wu *et al.,* 1996), which in turn, expose the N-terminal

fusogenic sequence of gp41. Chemokines disrupt this process by inducing co-receptor internalization into endosomes and prevent the formation of gp120-CD4 complex. However, in contrast to their HIV-1 suppressive activities, certain chemokines have also been shown to exert enhancement of viral replication (Schmidtmayerova *et al.,* 1996; Dolei *et al.,* 1998; Kelly *et al.,* 1998; Kinter *et al.,* 1998; Gordon *et al.,* 1999).

Among the cytokines, IFN-γ, IL-2, IL-4, IL-10, M-CSF and GM-CSF have been reported to modulate CCR5 and CXCR4 expression in primary HIV-1 infected monocytes or MDM (Kutza *et al.,* 1998; Wang *et al.,* 1998). CCR3, CCR8 and CXCR4 are preferentially expressed on Th2 cells while CCR5 is more abundant on the Th1 cell surface of Th1 specific T cells (Sallusto *et al.,* 1997; Bonecchi *et al.,* 1998; Sallusto *et al.,* 1998).

CAF (Mackewicz *et al.,* 1994) is secreted from $CD8^+$ T cells of HIV-1-infected patients. The CAF-associated antiviral activities have been reported to correlate with delayed disease progression in HIV-1 infected patients that control viral replication in a MHC class I unrestricted way (Walker *et al.,* 1991). CAF inhibits HIV-1 RNA transcription (Mackewicz *et al.,* 1995) via STAT1 activation, leading to interferon regulatory–1 (IRF-1) induction and inhibition of gene expression regulated by the HIV-1 LTR (Chang *et al.,* 2002). However, viral entry, reverse transcription and pro-viral integration are unaffected by CAF. Furthermore, it inhibits the replication of HIV-1 strains that use CXCR4 and not CCR5 for entry. IL-16 secreted by activated $CD8^+$ T cells was also shown to inhibit HIV-1 and SIV replication in infected $CD4^+$ T cells, monocytes and eosinophils (Maciaszek *et al.,* 1997). The IL-16-mediated inhibition of HIV-1 was not at the level of viral entry or reverse transcription, but at messenger RNA expression (Zhou *et al.,* 1997).

HIV-1 specific cytotoxic T cell responses

CTLs are major effector cells in most viral infections (Yap et al., 1978; Askonas and Taylor, 1987; Carmichael et al., 1993). Presentation of viral proteins to $CD8^+$ CTL generally requires synthesis of antigens within the cell that results in binding of peptides (8-10 amino acids) (Townsend et al., 1985; Falk et al., 1990) to MHC class I molecule peptide-binding cleft (Zinkernagel and Doherty, 1975; Bjorkman et al., 1987; Townsend and Bodmer, 1989). Most viruses that establish persistent infection have evolved mechanisms to avoid the immune response such as escaping MHC class I restricted $CD8^+$ T cells (McLaughlin-Taylor et al., 1994; Mackewicz et al., 1995; Elliott et al., 1997; Silins et al., 1997). For instance, herpes simplex virus (HSV) establishes resistance to CTL recognition via a cytosolic HSV protein, which inhibits peptide transport from

cytosol into ER. This result in prevention of the peptide loading of MHC class I molecules for presentation to $CD8^+$ T cells (York et al., 1994). Similarly, human cytomegalovirus (HCMV) has evolved strategies to avoid recognition of CTLs. For example, CMV downregulates HLA-A and -B expression on the surface of infected cells (Ploegh, 1998). Furthermore, CMV interferes with MHC class I pathway using genes from the unique short (US) region of CMV such as US6 that prevents peptide loading of the MHC class I molecules by inhibiting TAP-mediated peptide translocation into the ER (Park et al., 2002). In HIV-1 infection, the Nef protein downregulates MHC class A and B, which impairs the HIV-1 specific $CD8^+$ T cells from identification of infected cells (Cohen et al., 1999).

HIV-1 specific $CD8^+$ T cell mediated cytotoxicity is commonly found in high numbers during the asymptomatic phase of infection (Yasutomi *et al.,* 1993; Reimann *et al.,* 1994) and deteriorate during disease progression to AIDS (Hoffenbach *et al.,* 1989; Pantaleo *et al.,* 1990; Carmichael *et al.,* 1993; Klein *et al.,* 1995). Moreover, circulating antiviral CTLs have declined when opportunistic infections begin (Hoffenbach *et al.,* 1989) rendering them incapable of eliminating virus-producing cells (Goulder *et al.,* 1997; Pantaleo *et al.,* 1997; Soudeyns and Pantaleo, 1997). Virus specific CTLs were shown to suppress HIV-1 replication *in vitro* by direct cytotoxicity (Walker *et al.,* 1987; Cocchi *et al.,* 1995). HIV-1 specific CTL responses have been observed as early as a few days from onset of symptomatic acute phase before neutralizing antibody responses could be detected (Koup *et al.,* 1994; Pellegrin *et al.,* 1996). The appearance of CTLs seems to be concomitant with decreasing viremia in infected patients. Studies have showed that after approximately 4-7 days of infection, CTLs appear and reduce viral infected cells in blood and lymph nodes of SIV-infected rhesus monkeys (Yasutomi *et al.,* 1993; Reimann *et al.,* 1994).

The first HIV-1 specific CTLs were identified by limiting dilution assay which revealed HIV-1 specific memory T cells (Koup *et al.,* 1991; Carmichael *et al.,* 1993; Klein *et al.,* 1995). The use of tetramer staining has indicated a high frequency of circulating $CD8^+$ T cells specific for immunodominant HIV epitopes (Moss *et al.,* 1995; Altman *et al.,* 1996; Sykulev *et al.,* 1996; Borrow *et al.,* 1997; Collins *et al.,* 1998). However, despite high frequencies of HIV-1 specific precursor CTLs and the existence of soluble factors with suppressive function, the question arises as to why these cells cannot provide better elimination of infected cells.

There are several proposal for the escape routes of CTLs. Phenotypic analysis suggests that HIV-1 specific $CD8^+$ T cells have not been fully differentiated into mature effector cells (Andersson *et al.,* 1999; Appay *et al.,* 2000; Champagne *et al.,* 2001). Mice infected with lymphocytic choriomeningitis virus (LCMV) have shown overwhelming virus infection resulting in exhaustion of the antiviral CTL response (Moskophidis *et*

al., 1993). Similarly, massive antigenic stimulation after HIV-1 infection can also be responsible for HIV-1 specific CTL exhaustion (McMichael and Rowland-Jones, 2001). $CD4^+$ T cells are important to maintain effective antiviral $CD8^+$ CTLs. The loss of HIV-1 specific $CD4^+$ T cell counts may alter $CD8^+$ T cell maturation and function and subsequently reduce HIV-1 specific antiviral CTL responses. The $CD4^+$ T cells secrete either IL-2 or IL-15, important growth factors for CD8 cell survival or express molecules whose ligation is directly or indirectly required for CTL differentiation. For example, ligation of CD40L on CD4 T cells activates DCs to provide signals for development of $CD8^+$ CTLs (Bennett *et al.,* 1998; Borrow *et al.,* 1998; Schoenberger *et al.,* 1998). In addition, MDM from HIV-1 infected individual were shown to produce less IL-12 and more IL-10 (Chougnet *et al.,* 1996). Low IL-12 expression combined with disruption of CD40/CD40L interaction may generate CTL dysfunction. Moreover, the acquisition of escape mutations is another strategy employed by virus to avoid recognition by CTLs (Lieberman *et al.,* 2001; McMichael and Rowland-Jones, 2001). However, despite well-controlled viral replication after HAART (Cavert *et al.,* 1997; Gulick *et al.,* 1997; Hammer *et al.,* 1997; Wong *et al.,* 1997a), antiviral HIV-1 specific CTLs fail to provide lifelong protection in the vast majority of patients (Appay *et al.,* 2002). CTLs use two principle mechanisms for killing of target cells; granule exocytosis and Fas/Fas-L pathway (Figure 2).

Granule exocytosis pathway

One important mechanism is the granule-exocytosis pathway. The secretory lysosomes of CTLs (Henkart, 1994) contain a pore forming molecule, called perforin, and a collection of serine proteases termed granzymes that together cause cell death (Smyth and Trapani, 1995). This granule-exocytosis pathway, critical for the immune defense against most viral infections, is dependent on the presence of perforin (Kagi *et al.,* 1995) and plays a role in both innate (NK-cells) and adaptive defense (CTL) against pathogens (Tschopp and Nabholz, 1990). The Ca^{2+} dependent perforin-mediated pathway is antigen specific, and therefore only targets cells displaying specific antigen bound to MHC class I (Podack *et al.,* 1985; Hayes *et al.,* 1989; Heusel *et al.,* 1994). Mice genetically deficient in perforin are characterized by increased susceptibility to viral infections such as LCMV. They also display defective allograft rejection (Kagi *et al.,* 1994) and reduced surveillance against tumors (van den Broek *et al.,* 1996) as well as absence of NK cell cytolytic function (Kagi *et al.,* 1994). Perforin deficiency has also been identified in patients characterized by a major immune dysregulation called familial hemophogocytic lymphohistiocytosis (FHL), that invariably leads to premature death (Stepp *et al.,* 1999). The importance of perforin-mediated pathway in the clearance of HIV-1 infected cells has also been reported. This was shown by lysis of HIV-1 infected primary T cells via perfroin-dependent granule mediated pathway (Shankar *et al.,* 1998). The synergistic role of perforin and granzymes is essential for

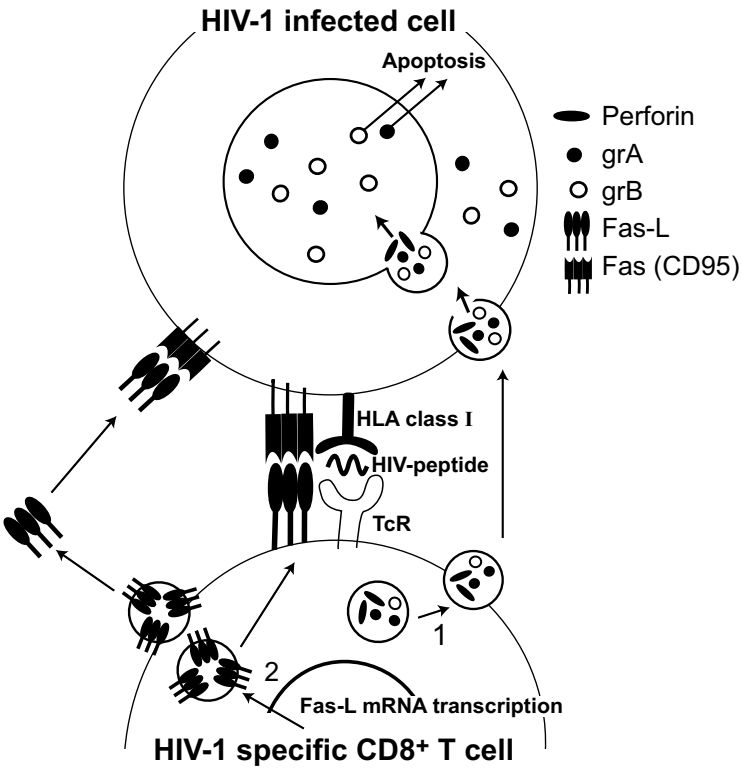

Figure 2. This figure illustrates mechanisms for elimination of HIV-1 infected cells. **1.** The granule exocytosis pathway results in a rapid release of a pore forming protein, perforin and granzymes into the space between the effector and the target cell. The perforin action is calcium dependent and allows perforin to undergo conformational changes. Perforin inserts into the target membrane, oligomerizes and forms pores. Granzymes can thereafter enter directly through the perforin pores. After entrance to the target cells, the granzymes initiate either caspase-dependent or caspase-independent death pathways resulting in DNA fragmentation and cell death. **2.** The Fas and Fas-L pathway utilizes trimer form of Fas-L expressed on the surface of the effector cell to crosslink Fas on the target cell. This pathway is also initiated by TCR antigen recognition, which results in Fas-L mRNA expression and *de novo* synthesis of the trimeric Fas-L membrane protein in the endoplasmic reticulum. This protein can be transported to the cell membrane either with the granules, where it can be released via vesicles or with subsequent TCR stimulus to the plasma membrane via vesicles. Trimeric surface Fas-L crosslinks Fas on the target cell, thereby triggering caspase activation via Fas-associated death domain protein (FADD) and caspase-8. The soluble form of Fas-L, a new alternative splicing of Fas-L, can block cell associated Fas-L mediated apoptotic process.

the exocytosis-mediated lysis of the target cells (Shi *et al.,* 1997). Granzymes are structurally, functionally and genetically related to other hematopoietic serine proteinases (Smyth and Trapani, 1995). Granzyme expression is restricted to activated T cells, γ/δ T cells and Natural killer cells (NK-cells) (Masson and Tschopp, 1985; Garcia-Sanz *et al.,* 1987; Ebnet *et al.,* 1991). Most $CD8^+$ T cells express different types of granzymes that generate similar activation of caspase mediated cell death (Trapani *et al.,* 1999).

Fas and Fas-Ligand pathway

Another pathway for induction of apoptosis involves engagement of death domain-containing receptor Fas (CD95) with the Fas ligand (FasL) on the effector cells (Nagata, 1996). Upon binding Fas multimerizes, resulting in recruitment of Fas-associated death domain protein (FADD) and triggering of the caspase cascade (Medema *et al.,* 1997). This allows the death signal to be transduced to the interior of the target cell (Lowin *et al.,* 1994). During HIV-1 infection there is extensive programmed cell death that probably occurs in both infected and uninfected cells (Gougeon *et al.,* 2000). Much of this apoptosis is mediated by Fas-FasL and cytokine interactions. The HIV-1 protein Tat and gp120 have been shown to induce Fas expression on $CD4^+$ T cells *in vitro* and an elevation of Fas expression has been reported on $CD4^+$ T cells in HIV-1-infected individuals (Banda *et al.,* 1992; Oyaizu *et al.,* 1994; Katsikis *et al.,* 1995; Westendorp *et al.,* 1995). In addition, the Nef protein is involved in the upregulation of Fas-L on target cells (Xu *et al.,* 1999). Nef disrupts the process of cell death both by protecting the infected cells from apoptosis (Wolf *et al.,* 2001) and by killing of surrounding cells that might seek to destroy by stimulating apoptosis (Geleziunas *et al.,* 2001). Furthermore, the intracellular Nef protein was shown to increase the availability of the anti-apoptotic BcL-2 protein resulting in host cell survival and increased viral replication (Wolf *et al.,* 2001).

HIV-1 infection in children

The first child with HIV-1 infection was described in early 80-ies. Disease progression differs considerably among HIV-1 infected children (Scott *et al.,* 1989; Tovo *et al.,* 1992). At birth, few children have signs of infection, but by one year 80-90% will manifest some symptoms of infection. Approximately 20% of vertically HIV-1 infected infants develop AIDS within the first months of life; the remainder show slower disease progression (De Rossi *et al.,* 1996). One-third of the children (30%) have no or mild symptoms of disease by the age of 6 years (Tovo *et al.,* 1992). The explanation for differences in disease progression in HIV-1 infected children is still controversial. In 1996, a collaborative study discussed that viral factors, host immune defense, timing

of transmission, viral load and host genetic factors might be involved in transmission and disease progression. A correlation between $CD4^+$ count and an increased number of HIV-1 specific cytotoxic T cell precursor cells was found in infected infants with slow disease progression (Martin *et al.,* 1996).

Vertical transmission from mother to child

Vertical transmissions of HIV-1 occur in approximately 8 to 39% of pregnancies, in the absence of antiviral treatment (Ryder *et al.,* 1989). The risk of mother- to child transmission has decreased in European countries and US to less than 2%, due to broad usage of anti-retroviral therapy during pregnancy. Transmission rates are still higher in African and low-income countries where this care is not always available. Transmission from mother to child can occur *in utero* (Backe *et al.,* 1992), at the time of delivery (Sprecher *et al.,* 1986) and after birth (Jovaisas *et al.,* 1985). Approximately 10% of all perinatally infected children become infected in early pregnancy (Brossard *et al.,* 1995).

Numerous factors have been proposed to influence maternal–fetal transmission. Factors associated with vertical transmission can be divided into maternal factors such as maternal viral load (Weiser *et al.,* 1994; Garcia *et al.,* 1999; Shaffer *et al.,* 1999), maternal neutralizing antibodies (St Louis *et al.,* 1993; Khouri *et al.,* 1995; Ugen *et al.,* 1997) or maternal HIV-1 specific CTL activity (Jin *et al.,* 1998). Furthermore, maternal placenta interface factors, such as Fas-L (Menu *et al.,* 1989; Hunt *et al.,* 1997; Uckan *et al.,* 1997) or tumor necrosis factor-related apoptosis-inducing ligand/ Apo-2 L expression (Phillips *et al.,* 1999), *chorioamnionitis* (Ladner *et al.,* 1998; Wabwire-Mangen *et al.,* 1999) or HIV-1 suppressive activity (Bourinbaiar and Lee-Huang, 1995; Lunardi-Iskandar *et al.,* 1998) all have an important influence on the efficacy of transmission.

Placental tissue

The placenta is a fetal organ with a fetal and a maternal side. The fetal portion consists of *chorion*, and the maternal portion of the *decidua basalis*. The *chorionic* plate divides into primary, secondary and tertiary or *chorionic villi*. The *decidua basal* forms a compact layer called the *basal plate* that is the maternal component of the placenta. This organ is a barrier between maternal and fetal exchanges of products across the *placental barrier.* The fetal blood arrives through the two branches that supply *chorionic villi* and then return to the fetus via the *umbilical vein*. The maternal blood crosses the basal plate permitting exchanges of oxygen and metabolites between maternal and foetal blood. Subsequently, the maternal blood is drained via maternal venous system. *Chorionic villi* have a simple structure and are arranged as a double layer of trophoblasts.

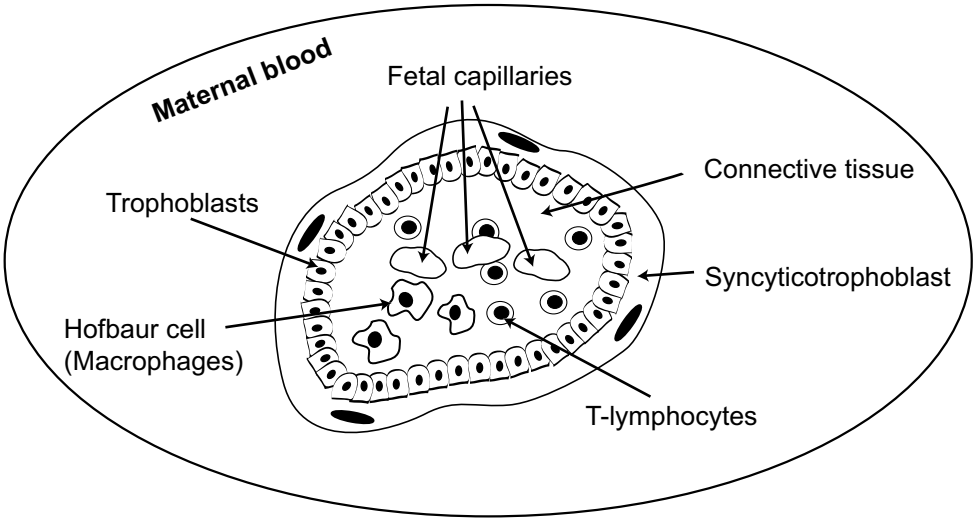

Figure 3. This illustration shows a schematic view of a cut section of a chorionic villus corresponding to the placental barrier.

The placental macrophages (*Hofbauer cells*), lymphocytes fibroblasts and fetal capillaries are scattered within layers of *trophoblasts*. The inner layer is made up of cuboidal cells called *cytotrophoblasts*, which generate and maintain the outer layer. The outer layer of *trophoblastic* cells is termed *syncytiotrophoblasts* and is a continuous multinucleated epithelium. This layer has a direct contact with maternal blood. The placental barrier consist of two layers of *trophoblasts, stroma*, *chorionic villous* and epithelium of the fetal villous vessel. The thickness and the total surface area are of great importance in the maternal-fetal exchange (Cunningham *et al.,* 1993) (Figure 3).

The placental size is generally normal in HIV-1infected pregnant women as observed by microscopic examination (Jauniaux *et al.,* 1988). The only histopathological abnormality has been found in placenta of HIV-1 infected mothers, an acute inflammatory process termed, *Chorioamnionitis* (Jauniaux *et al.,* 1988; Schwartz *et al.,* 2000). In other viral infections such as cytomegalovirus and rubella virus, the virus reaches to the placenta from the maternal blood (transplacental) and causes inflammation of the placental villi. The structural disruptions in the syncytiotrophoblasts

or other mechanical brakes in the placenta barrier have been proposed for transplacental passage of HIV-1 or other infectious agents (Leach *et al.,* 1996).

The role of co-receptor expression for vertical transmission

It seems that R5-using isolate variants initiate HIV-1 infection after sexual, parenteral and vertical transmission (van't Wout *et al.,* 1994). Numerous studies comparing maternal HIV-1 isolates to those transmitted to their children reveal transmission of a selected population of HIV-1 isolates (Scarlatti *et al.,* 1993; Briant *et al.,* 1995; Menu *et al.,* 1999). The differences in host cell preference can be attributed to the differing ability of HIV-1 to use alternative co-receptors. Based on several experiments, investigators speculate that a preferential selection of R5-using viruses occurs during viral transmission. Several studies have compared maternal HIV-1 isolates to those transmitted to their children (Scarlatti *et al.,* 1993; Briant *et al.,* 1995; Ugen *et al.,* 1997). However, all studies agree that some form of selection occurs during vertical transmission. In general, HIV-1 virus penetrates the trophoblast layer in placenta and encounters cells expressing HIV-1 co-receptors such as lymphocytes and Hofbaur cells (macrophage-like cells). Placental macrophages support HIV-1 replication of laboratory strains of both R5- and R4-using isolates (Lewis *et al.,* 1990; McGann *et al.,* 1994).

The role of strain selection in vertical transmission of HIV-1 across the placenta during pregnancy is highly relevant for disease progression. Moreover, placental cytokines may drive chemokine receptor expression to protect against transmission by selecting for isolates demonstrated in numerous sequencing studies as less likely to be transmitted. Several studies of cytokine expression in the placenta have revealed production of a variety of type 1, type 2 and proinflammatory cytokines (Chougnet *et al.,* 1996; Marzi *et al.,* 1996; Reuben *et al.,* 1996; Deneys and De Bruyere, 1997; Lee *et al.,* 1997; Piccinni *et al.,* 1998). The cytokine milieu of the placenta and the hormonal-cytokine network at the maternal-fetal interface has a crucial role in preventing fetal allograph rejection and supporting implantation. Thus, the association of cytokine expression in the placental milieu to viral transmission selection may indicate additional factors involved in HIV-1 vertical transmission.

AIMS OF THIS STUDY

The overall purpose of this study was to investigate immunological responses in HIV-1 infected lymphoid tissue. An understanding of how HIV-1 generates damage to the immune system and circumvents elimination is vital for the development of vaccine and new therapeutic strategies. This formed the aims of the current investigation:

To evaluate immune activation within HIV-1 infected lymphoid tissue before and after introduction of highly active anti-retroviral therapy (HAART).

To evaluate the $CD8^+$ T cell-mediated cytolytic effector molecule expression of perforin and granzyme A, in lymphoid tissue and blood in chronically HIV-1 infected patients.

To investigate the molecular mechanism involved in HIV-1-mediated down-regulation of perforin *in vitro*.

To quantify CCR5 and CXCR4 expression as well as local cytokine production in placenta tissue from HIV-1 transmitting and non-transmitting women.

To characterize a novel HIV-1 suppressive molecule, which may be involved in inhibition of HIV-1 replication in placental tissue.

MATERIALS AND METHODS

Ethical approval of study cohort

All studies have been examined and approved by the Ethic Committee of Karolinska Institutet. The HIV-1 seronegative biopsies were obtained after institutional review board approval from Huddinge University Hospital. The macaque studies were undertaken at the California Regional Primate Research Center in Davis, California. All animals were maintained in accordance with NIH guidelines and appropriate approvals of the local Animal Care and Use Committees were obtained.

Study participants

Paper I and II

Three HIV-1 seropositive asymptomatic anti-retroviral treatment naïve individuals were recruited for this study. The $CD4^+$ blood counts were greater than 400×10^6 cells/L blood. Biopsies were obtained prior to and 4, 12 and 56 weeks after therapy with zidovudine, lamivudine (3TC) and indinavir. The mean HIV-1 RNA level in plasma was 11300 copies/ml (mean value) before HAART and reduced to lower detection limit (<50 copies/ml) after therapy (Roche, Nutley, NJ).

Paper III

Cryopreserved lymph nodes and tonsil biopsies were obtained from 16 HIV-1 infected individuals without anti-retroviral treatment. Plasma HIV-1 viremia was measured by branch-DNA assay (Chiron, Emeryville, CA).

Paper IV

Cryopreserved lymph nodes and spleen were obtained from two macaques infected with a *nef* deleted SIV-EGFPΔ*nef* ($1\times10^{4.5}$ $TCID_{50}$) virus and two macaques were inoculated with wild-type SIVmac251 (1×10^5 $TCID_{50}$).

Paper V and VI

Twenty-three HIV-1 infected women were enrolled; sixteen samples were collected from non-transmitting women (TNT) and seven from transmitting women (TT). Placenta samples were obtained from women at the time of normal vaginal delivery after consent. Transmitting women were matched with TNT women for maternal CD4 count, maternal viral load, placenta gestational age, and age. All women were off anti-retroviral therapy during pregnancy. Only women with the w/w CCR5 genotype were selected for this study. In addition, term placentae from HIV-1 seronegative women devoid of inflammation or other pathology were used as normal controls.

Methods

Quantification of HIV-1 DNA in tissues (Paper I and II)

HIV-1 *gag* DNA was detected by fluorescent *in situ* 5´-nuclease assay (FISNA). The tissues were fixed overnight in molecular biology grade Streck Tissue Fixative (MBF). The PCR was performed in cell suspensions with primers and a fluorescent probe against *gag*. A special Taq DNA polymerase was used in the PCR reaction to cleave *in situ* a 5' reporter dye (FAM) from the hybridised probe. The reporter dye was thereby disassociated from the 3' quenching dye (TAMRA) which remained within the cells. The signals were evaluated and quantified for the presence of HIV-1 *gag* DNA by an ACAS 570 laser confocal microscope.

Quantification of HIV-1 RNA in tonsils (Paper I, II and V)

HIV-1 RNA was detected by *in situ* hybridization in tissues fixed and permeabilized in Permeafix (Ortho Diagnostics). Following washing with PBS and 2× SSC the sections were dehydrated through graded alcohol and air-dried. Thereafter, the tissues were rehydrated with 50 µl hybridization buffer containing a cocktail of 5-carboxyfluorescein-labeled oligonucleotides specific for HIV-1 *gag-pol* mRNA (MolPATH, Inc. Frankfort, Michigan, USA). The probe was hybridized to the target sequence. Thereafter the tissue sections were washed with 2× SSC, Triton X-100 at 42°C. Multi-parameter analysis of cell surface molecules and HIV-1 *gag-pol* mRNA was performed on an ACAS laser confocal microscope (Meridian Instruments, Okemos, MI).

PBMC preparation and fixation (Paper III, IV)

PBMC from HIV-1 seronegative healthy blood donors and HIV-1$^+$ individuals were isolated from Heparinizied-blood by centrifugation on Ficoll-Hypaque (Pharmacia, Upsala, Sweden) density gradient. Subsequently cells were washed with sterile phosphate buffered saline (PBS) and counted. After indicated periods of stimulation time, the cells were transferred to adhesion slides (Bio-Rad Lab, Munich, Germany) and allowed to adhere to the slides for 10 min. Excess cells were washed away with PBS. The slides were fixed in 2% formaldehyde in PBS at pH 7.4 for 10 min at room temperature. Following three additional washes with PBS, the cells were air dried and stored at –20°C until use.

HIV-1 infected tissues preparation and fixation (Paper I, II, III, V and VI)

Cryopreserved HIV-1 infected biopsies, embedded in OCT-compound (Tissue-TEK, Mites, Elkhart, IN), were cut in 8 µm thick sections and mounted on HTC glass slides (Novakemi, Sweden) and fixed with 2% formaldehyde (Sigma, Sweden) in PBS for 15 minutes at room temperature. Slides were then washed with balanced salt solution (BSS) (Gibco Ltd, Paisley, UK) and stored at -20°C until staining.

Cell culture (Paper IV)

Isolated PBMC were resuspended in culture medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), L-glutamine plus penicillin and streptomycin (Gibco, Paisly, UK). Subsequently, the cells were stimulated with purified superantigen SEA, recombinant Nef or Nef peptides. Cultured cells were harvested after indicated periods of stimulation time, transferred to adhesion slides (Bio-Rad Lab, Munich) and fixed in 2% formaldehyde in PBS.

Detection of cell markers, cytokines, chemokines and effector molecules in tissue and PBMC by immunohistochemistry (Paper I, II, III, IV, V and VI)

Prior to immunohistochemical staining, the harvested cells or sectioned tissues were blocked by FCS (1%) to prevent unspecific staining. Sections/cells were permeabilized with 0.1 % saponin (Riedelde Haen AG, Seelze, Germany) dissolved in 1x Earl's balanced salt solution (BSS) (Gibco Ltd, Paisley, UK) during staining procedure. After peroxidase quenching and serum blocking, the sections were incubated with primary antibodies over night or for one hour at 37°C. Biotinylated secondary antibodies were applied followed by incubation with avidin-biotin hors-radish peroxidase complex (Vectastain Elite Kit). A colour reaction was developed by addition of 3′-diaminobezidine tetrahydrochloride (DAB). The sections were counterstained with hematoxylin. The specificity of cytokine staining was ensured by incubation of recombinantly produced cytokines to block specific cytokine staining (at a concentration of 20-50 μg/ml, to its corresponding cytokine-specific antibody 2-5μg/ml at $^{+}$4°C overnight).

Two colour staining for co-localization (Paper III)

For double labelling experiments, the tissues and cells were first incubated with the cell surface specific mAbs. The cells were thereafter labelled with secondary biotinylated antibodies followed by avidin-biotin alkaline phosphatase APC complex (Vectastain APC Kit, Vector Lab). A blue alkaline phosphatase substrate was used to develop a blue colour reaction. The cells were subsequently stained for effector molecules using the technique described above.

Quantification of cell surface markers, cytokine, chemokine and effector molecules expression by *in situ* imaging (Paper I, II III, IV, V and VI)

The immunohistochemically-stained cells and tissue sections were examined in a Leica RXM microscope, equipped with a 3CCD colour camera and analysed by a Quantiment 550 IW image analyser. A PC program directed the image processor with a special software routine, developed for this application (Bjork *et al.,* 1996). The cells were enumerated by the image analysis system by setting the colour and morphology of the

hematoxylin counterstained cells as a standard. The frequency of cytokine, chemokine, cell surface phenotype markers and effector molecule expressing cells was assessed by examination of at least 1 000 cells. In tissue stained sections, the frequency of positively stained cells was expressed as a percentage of total tissue area (Bjork *et al.,* 1996; Andersson *et al.,* 1998). Only lymphoid tissues were included in the analysis, epithelium was excluded from the analysis. For cultured cells, the number of cytokine and effector molecule expressing were instead analyzed by counting the positive cells out of the total number of cells in each digital image manually combined with *in situ* image analysis for evaluating the total number of cells. Cytokine, surface marker and effector molecule expressing cells were identified by a characteristic staining pattern of protein accumulated in the Golgi-stacks, surface markers and with granule characteristic distributions of the cells.

Intracellular detection of effector molecule expressing cells by flow cytometry (Paper IV)

The cultured cells were fixed using the Caltag Laboratories (Burlingame, CA) Fix and Perm Kit according to the manufacturer's protocol. The staining procedure was performed in the presence of brefeldin A (Sigma-Stockholm, Sweden) at a final concentration of 10ug/ml. The permeabilization of the cell membrane was carried out by the inclusion of saponin in the reagents. The cells were analyzed in a Becton Dickinson FACS Calibur flow cytometer using CellQuest software. The negative control samples were incubated with antibodies and isotype control monoclonal antibodies specific for mouse IgG2b and mouse IgG1.

Alignment of amino acid sequences and molecular modeling (Paper IV)

Sequences of HIV-1 variants from the HIV-1 sequence Database at the Los Alamos National Laboratory (http://HIV-1-web.lanl.gov/) were aligned with the Multalin software package (http://prodes.toulouse.inra.fr/multalin/multalin.html). Co-ordinates of the molecular model of the full length NL4-3 Nef model were kindly provided by Dr Geyer (UCSF Cancer Research Institute, San Francisco, California). The substitutions of interest were introduced using the O program and figures were created using the programs Bobscript.

Preparation of RNA extraction from tissue (Paper V)

Tissue samples were homogenized for RNA extraction, snap-frozen in OCT embedding compound, or fixed in Streck tissue Fixative (Streck Laboratories, Omaha,NE). RNA was extracted from biopsy specimens by homogenizing fresh biopsies with TriReagent using diethyl pyrocarbonate-treated, autoclaved, disposable homogenizes. After homogenization RNA was purified as per the manufacturer's protocol. RNA pellets were resuspended in 1x transcription buffer (Promega, Madison, WI) with RQ1 RNase-

free DNase. DNA was digested to remove contaminating DNA. The mixture was extracted once with phenol:chloroform:isoamyl:alcohol, and once with chloroform:isoamyl:alcohol. The aqueous layer was removed and the RNA was precipitated in 3 volumes of ethanol and 1/40 volume of 3 mol/L of sodium acetate overnight at -20°C.

Messenger mRNA quantification using real-time RT-PCR (Paper V and VI)

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed by preparing a reaction mix containing specific primers, internally-conserved fluorogenic probes, and rTth polymerase. The reaction mix was then added directly to extracted RNA in RNase, DNase-free water (Ambion, Austin, TX). Input RNA was normalized using glyceraldehyde-3-phosphate dehydrogenase mRNA quantification (PE Applied Biosystems). Reverse transcription and thermal amplification were performed using the following linked profile: reverse transcription 30 minutes at 60°C, cDNA denaturation 5 minutes at 95°C, and 40 cycles of denaturation (95°C for 15 seconds) and annealing/extension (60°C for 1 minute) in a 7700 sequence detection system (PE Applied Biosystems). Duplicate standard curves with copy number controls ranging from 10 copies to 10^5 copies were run with each optical 96-well plate (PE Applied Biosystems). In addition, no template controls were included with each plate. Primer and probe sequences have been previously described.

Immunophenotyping/Fluorescence *in situ* Hybridization (I-FISH) (Paper V and VI)

Tissue frozen in OCT embedding compound (Fisher Scientific, Pittsburgh, PA) was cut at 5 μm thickness and adhered to silanized slides (PE Applied Biosystems) followed by simultaneous immunophenotyping/fluorescence *in situ* hybridization as previously described (Collins *et al.,* 2000). Briefly, the sections were air-dried, rehydrated in PBS, and labeled with optimized concentrations of phycoerythrin-conjugated antibodies specific for the cell type of interest. HIV-1 mRNA was detected in the tissues using the ViroTect (Invirion, Frankfort, MI). The probe was hybridized to the target sequence, in a GeneAmp 1000 slide cycler (PE Applied Biosystems). The quantification of cell surface receptors and HIV-1 *gag-pol* mRNA was performed on a laser confocal microscope (Olympus, Melville, NY). Quantification of viral copies was performed using Metamorph software and fluorescent equivalents bead standards (Flow Cytometry Standards, San Juan, PR) and the formula:

$$\frac{\text{Total pixels (HIV-1}^{+}\text{cell-background)}}{\text{Cell}} \times \frac{\text{FITC equivalents}}{\text{pixel}} \times \frac{0.007 \text{ copies}^*}{\text{FITC equivalent}} = \frac{\text{HIV-1 copies}}{\text{cell}}$$

*Based on 142 fluorescein equivalents per HIV-1 copy.

Real-time quantitative PCR for HIV-1 *gag* and U5/R DNA (Paper VI)

Quantitative, kinetic DNA PCR was performed in suspension with primers (Schmidtmayerova *et al.,* 1998) and an internally conserved sequence-specific, fluorogenic probe labeled at the 5' end with FAM and at the 3' end with TAMRA to DNA extraction. Thermal amplification was performed as described previously (Collins *et al.,* 2000).

The following primers (Schmidtmayerova *et al.,* 1998) and probes were used:
LTR U5/R—sense-5'-GGCTAACTAGGGAACCCACTG-3'
Antisense-5'-CTGCTA*GAG*ATTTTCCACACTGAC-3',
Probe 5'-FAM-TGTGTGCCCGTCTGTTGTGTG-TAMRA-3';
LTRU3/*gag*—sense-5'-CAGATATCCACTGACCTTTGG-3'
Antisense-5'-GCTTAATACTGACGCTCTCGCA-3',
Probe 5'-FAM-*GAG*GCTTAAGCAGTGGGTTC-TAMRA-3'.

***In vitro* assays for anti-HIV-1 activity** (Paper VI)

Replication end-point concentration assays were performed using a tissue culture ID50 (TCID50) for different HIV-1 isolates to infect phytohemagglutinin-stimulated (PHA-stimulated) PBMCs as described (McLeod *et al.,* 1992). Varied concentrations of recombinant LIF were used before addition of virus to stimulated PBMC. All strains were tested in quadruplicate wells in three separate experiments. The mean p24 antigen produced in positive control PBMCs (without addition of LIF) averaged 843 ± 412 pg/ml, including all isolates tested.

Cell-proliferation assays (Paper VI)

PBMCs were isolated from heparinized blood and placed in quadruplicate wells in the presence or absence of LIF at 0, 0.1, 1.0, and 10.0 pg/ml. PHA was used at 0.25 µg/ml and 1 µCi [3H] thymidine was added per well after 72 hours of culture. The cultures were harvested and counted 18 hours later.

Explant placenta organ culture (Paper VI)

Fresh placenta tissue was obtained after informed consent was given. The placenta was cut into small pieces, and the fragments were washed extensively in PBS to remove any contaminating blood. Thereafter, the fragments were placed in MEM media with Earle's salts and antibiotic/antimycotic solution as described previously (Amirhessami-Aghili and Spector, 1991). Placenta fragments were pre-treated with rLIF, IL-4 (R&D Systems), or polyclonal anti-LIF (R&D Systems), and infected with HIV-1 Bal or the HIV-1 primary isolate. After infection, total HIV-1 DNA and RNA were extracted using TriReagent per manufacturer's instructions. HIV-1 DNA quantification was performed using real-time PCR as described previously.

Explant thymus organ culture (Paper VI)

Fresh pediatric thymus tissue was dissected into pieces containing 3–6 million cells and cultured as described previously (Bonyhadi *et al.,* 1995). The appropriate tissue fragments were treated with 1 pg/ml LIF (BDPharMingen) for 1 hour before infection. The tissue fragments were transferred into sterile tubes containing undiluted virus. Later the fragments were transferred to nucleopore filters atop gel-foam boats saturated in media (YSSL media containing, human serum, streptomycin, penicillin G, 1x MEM vitamin solution (Life Technologies Inc., Rockville, Maryland, USA) in six-well plates with a maximum of 16 pieces per raft. The tissue fragments were cultured fragments at 37°C, for varying periods of time. At appropriate time intervals, tissue fragments were removed and passed through a 37-µm mesh with PBS, pH 7.4. Cells in suspension were used for immuno-phenotyping and dual immunophenotyping/ultrasensitive fluorescence *in situ* hybridization (UFISH).

Ultrasensitive fluorescence *in situ* hybridization (UFISH) (Paper VI)

Tissues were snap-frozen in OCT freezing compound (Fisher Scientific Co, USA). Cryostat-cut tissue sections (5-µm thick) were adhered to silanized slides, air-dried, fixed briefly in cold acetone, and rehydrated in PBS, pH 7.4. UFISH was performed using ViroTect (Invirion, USA) as described previously (Collins *et al.,* 2000). Image analysis was performed on a dual-laser confocal microscope (Olympus America Inc., Melville, New York, USA).

Statistical analysis

Paper III and IV

Correlation between quantitative variables was evaluated by Spearman co-efficient of correlation. A logarithmic transformation of viral plasma RNA values was used in the data analysis. Two-sided Student's test was used for statistical significance of correlation; a *PT* value of less than 0.05 was considered significant. In paper IV, a Wilcoxon signed-rank test and the non-parametric Mann-Whitney was used for comparisons of study parameters. Values of $P<0.05$ were considered to be significant.

Paper V and VI

Comparison between transmitting and non-transmitting placentae was performed using either the Mann-Whitney rank sum test or paired t-test. Comparisons which yielded a two-tailed *P* value of less than 0.05 were considered to be significant.

RESULTS

HAART normalizes immune activation in lymphoid tissue

Effect of HAART on HIV-1 burden in peripheral blood and tonsils (Paper I and II)

HIV-1 infected patients in the asymptomatic phase were selected for evaluation of immune reconstitution in lymphoid tissues following treatment with HAART. In order to study the effects of HAART on HIV-1 RNA, we measured the viral RNA in plasma before and after treatment. HIV-1 RNA quantification showed a marked reduction of HIV-1 RNA (1-3 $\log_{10}$) (<400copies/ml) in the plasma after 4 weeks of therapy in three patients studied. Furthermore, one year of treatment with HAART resulted in maintained reduction of HIV-1 RNA, to <50 copies/ml, which was below the limit for detection. This result in agreement with previous reports indicated reduced HIV-1 RNA in plasma after one year HAART therapy (Cavert *et al.,* 1997; Finzi *et al.,* 1997).

Furthermore, in order to investigate the effect of HAART on HIV-1 DNA containing cells in tonsillar tissue, we investigated the incidence of HIV-1 DNA containing cells in tonsils by fluorescence *in situ* 5´-nuclease assay (FISNA). The incidence of cellular pro-viral DNA containing cells in the tissues was 6.2% (mean value) before treatment, which was reduced to 4.2 % (mean value) after 4 week of HAART in all patients. In addition, the frequency of HIV-1 DNA containing cells in tissues was further reduced in two patients after 56 weeks of therapy. The content of HIV-1 DNA remained high in one patient (paper II). These results revealed a large pool of tonsil cells that remained HIV-1 DNA positive, despite effective therapy.

The effect of HAART on T cell subsets

Decreased number of $CD8^+$ T cells after HAART in blood and tonsils

Since a gradual decline in the total number of $CD4^+$ T cells is the strongest predictive marker for indication of HIV-1 progression throughout the course of infection, we studied $CD4^+$ T cell counts in peripheral blood and tissue from three infected patients prior to and after HAART therapy. The absolute number of $CD4^+$ T cells in blood, as determined by flow cytometry, increased after 4 weeks of therapy (paper I). Furthermore, after one year of HAART, the absolute numbers of $CD4^+$ T cells remained high or even increased in peripheral blood. We also examined the $CD8^+$ counts in blood and the frequencies of $CD8^+$ cells in tissue. In contrast to $CD4^+$ T cells, the frequency of $CD8^+$ T cells in peripheral blood was moderately reduced after four weeks and normalized after one year of treatment. Additionally, we investigated the expression of $CD4^+$ and $CD8^+$ T cells in tonsillar biopsies of these patients. In comparison to controls, the tonsils of HIV-1 positive subjects showed five to eight

fold higher level of $CD8^+$ T cells, which HAART therapy did not diminish after 4 weeks of treatment (paper I). However, the frequency of $CD8^+$ T cells after 56 weeks of treatment reached normal levels in infected tissue as compared to control tissues. Furthermore, the documented significant decrease in the proportion of $CD8^+$ within T cells led to a progressive increase of the CD4/CD8 ratio at 56 week after start of treatment in both blood and tissue.

HIV-1 infection is associated with a persistent hyperactivation in lymphoid tissue

The effect of HAART on cytokine expression profile in lymphoid tissue

In order to evaluate immune activation prior to and after anti-retroviral therapy, we studied cytokine and chemokine receptor expressing cells in HIV-1 infected LTs. Computerized *in situ* image analysis was used to study local cytokine production, chemokine receptor expression and cellular distribution in tonsil sections and healthy controls. Analysis by *in situ* image demonstrated a 10 to 15-fold elevation of IFN-γ and IL-2 expressing cells prior to HAART as compared to HIV-1 seronegative healthy individuals. After 4 weeks, a reduction in both IFN-γ and IL-2 expression levels was observed. The same HIV-1 infected patients were examined after 12 and 56 weeks of treatment to further study the effects of HAART on immune responses. The frequency of IFN-γ expressing cells, which was reduced at week 4, was comparable to seronegative controls by week 56.

We also assessed the expression of IL-4 and IL-10 in tonsillar sections. Untreated HIV-1 infected patients showed a lower prevalence of IL-4 and IL-10 expressing cells. We observed reduced expression of IL-4 and IL-10 after four weeks of treatment. These levels then remained unchanged at week 12 and 56. In general, a dominance of type 1 cytokines compared to type 2 cytokines was observed in HIV-1 infected tonsils before treatment. Furthermore, HIV-1 infected tonsils were characterized by an extensive pro-inflammatory cytokine expression (IL-1α, IL-β, TNF-α and IL-12) prior to therapy compared with controls. Quantification by *in situ* image analysis showed reduction of proinflammatory cytokines after 4 weeks of therapy. However, the expression of IL-1α remained elevated in one of the three patients at week 56. This patient was the only one with persistent HIV-1 replication.

Overall, this pilot study of cytokine expressing cells in tissue before and after therapy implies that HIV-1 replication may induce proinflammatory and Th1 cytokine synthesis at the local site.

Expression of chemokine receptor reduced after 4 week of therapy

The expression of HIV-1 co-receptors, CCR5 and CXCR4, which initially were elevated four to six times over controls values, were reduced by 40% after 4 weeks of treatment with HAART. The expression of these co-receptors was not studied at week 12 and 56. Thus, the high expression of CCR5 and CXCR4 may be induced by local cytokine expression of the lymphoid site before treatment (Patterson *et al.,* 1998). However, reduced expression of these HIV-1 co-receptors after therapy may diminish ability for viral entry and in turn, reduce the number of newly infected cells.

Myelomonocytic cells activation after HAART

We measured three myeloid antigens, CD68, Mac 328 (L1-antigen) and CD1a as indication of the incidence of activated macrophages and DCs. We found two to three fold higher expression in all HIV-1 positive patients prior to therapy. The frequency of antigen presenting, $CD1a^+$ and $CD68^+$ cells were also estimated in tonsillar tissues at 12 and 56 weeks of therapy. We found a decreased frequency of $CD1a^+$ DCs, which was reduced to control levels at week 56 of therapy. The frequency of $CD68^+$ cells declined in two patients but remained high in one patient.

Cytolytic effector molecule expression in $CD8^+$ T cells in HIV-1 infected lymphoid tissues

In the first two papers, we showed that HIV-1 infected LT returned to a resting state after potent anti-retroviral therapy. With regard to these finding we addressed the question of why high expression of IFN-γ, IL-2 and IL-12 combined with increased interdigitating DCs ($CD1a^+$ cells) before therapy failed to eliminate HIV-1 infected cells. In addition, the persistence of HIV-1 DNA containing cells after therapy indicated an inadequate HIV-1 specific CTL response. Therefore, we hypothesized that a functional block in cytolytic molecule expression in the HIV-1 specific effector $CD8^+$ T cells might be one explanation for why expanded CTLs failed to control the infection. We investigated the expression of perforin and granzyme A (grA) at the single cell level in LT and blood from 16 patients infected with HIV-1 (stage A1-C) who were not taking anti-retroviral drugs.

Perforin is expressed in mature effector $CD8^+$ T cells and NK-cells in conjunction with grA or granzyme B. In order to identify the phenotype of perforin and grA-expressing cells within LT, we first performed two-colour staining for identification of either NK or $CD8^+$ perforin- or grA-producing cells. The quantification by *in situ* image showed that >80% of the granular perforin- or grA-expressing cells in LT were $CD8^+$ T cells. In contrast, a few of the total perforin- or grA-expressing cells were of the $CD56^+CD3^-$ phenotype (2-4%). We next assessed the expression of perforin and

grA in LT from 13 chronically infected patients. Our data showed that these patients, who were not receiving anti-retroviral therapy, lacked perforin-expressing cells ($p<0.02\%$) but had sustained upregulation of grA expressing $CD8^+$ T cells (paper III). However, much higher levels of perforin- and grA-expressing cells were evident in LT from patients with acute infectious mononucleosis compared with biopsies from HIV-1 infected patients ($p<0.001$). We demonstrated a significant increase in $CD8^+$ T cells in HIV-1 infected LT compared to control tissue. Statistical estimations indicated no association between the incidence of perforin expression in LT and $CD8^+$ T cells in the HIV-1 infected individuals. However, we found a positive correlation between grA as well as perforin expression in LT from infectious mononucleosis and HIV-1 seronegative control tissues within $CD8^+$ T cells in tissue.

Cytolytic granule expression in blood from HIV-1 patients and controls

We studied also the expression of perforin and grA- expressing cells in PBMC in order to investigate if the deficit in perforin expression seen in tonsil and LT biopsies, may occur in blood. We analysed the PBMC from seven HIV-1 infected patients included in the tissue study plus additional four HIV-1 positive volunteers. Expression of perforin and grA effector molecules was analysed by *in situ* imaging. We found a significantly higher cellular expression of perforin and grA in PBMC compared with that seen in LT ($p<0.001$) in HIV-1 infected patients. A relative increase in both grA- and perforin-expressing cells in blood was observed in HIV-1 positive subjects compared with healthy control. However the expression of these effector molecules in PBMC from six primary EBV-infected subjects were significantly higher compared to PBMC from HIV-1 individuals ($p<0.01$). The maintenance of perforin expression was not related to HIV-1 disease state. No correlation was found between the percentage of perforin expressing cells and $CD4^+$ cell counts in blood ($p<0.05$). These analyses did not however reveal if the expression of cytolytic effector molecules was in HIV-1 specific CTLs. A later study showed a significant lower expression in T cells binding to HIV-1 tetramers. Hence, perforin expression appears to be impaired during HIV-1 infection, which may be an important clue to explain why CTLs fail to eradicate HIV-1.

Potential mechanism for downregulation of perforin expression during HIV-1 infection

Nef-Mediated Downregulation of Perforin In vitro (Paper IV)

As described above, we found an impaired perforin expression in $CD8^+$ T cells infiltrating HIV-1 infected LT. This indicated defective cytolytic activity in $CD8^+$ T cells at local sites of HIV-1 replication within LT. Therefore we designed *in vitro* studies to evaluate the molecular mechanism involved in inhibition of perforin

expression in HIV-1 infection. The effect of HIV-1 Nef protein on perforin and grA expression was examined.

We measured perforin expression in freshly isolated PBMC from healthy donors after four hours incubation with a recombinant Nef protein derived from a HIV-1_{IIIB} strain ($rNef_{IIIB}$) by *in situ* image of immunohistochemically stained cells. PBMCs from HIV-1 seronegative donors were incubated for 4 hours with culture medium only as control. A significant reduction of perforin expressing cells was observed in $rNef_{IIIB}$-treated PBMC compared to cells cultured in medium only ($p<0.001$). The viability of cells after 4 hours incubation with $rNef_{IIIB}$ was not affected, as assessed by Trypan blue exclusion or flow cytometry of Annexin V and PI-stained cells. Furthermore, the frequency of cells expressing the cytolytic effector molecule grA was also down regulated after 4 hours of incubation with $rNef_{IIIB}$ ($p<0.03$). The down-regulation of perforin was detected in the presence $rNef_{IIIB}$ while addition of the control protein β-galactosidase did not affect the frequency of perforin expressing cells. Kinetic analyses demonstrated that the observed perforin down-regulation was still detectable after 24 hours of incubation ($p<0.05$). The addition of the anti-Nef neutralizing monoclonal antibody (mAb) abrogated the observed phenomenon, confirming the specificity of $rNef_{IIIB}$ in the downregulation of perforin.

Perforin Down-regulation following Exposure to HIV-1_{Ba-L} but not HIV-1_{IIIB} with truncated Nef or the Nef-deleted Isolate HXB-2

HIV-1_{IIIB} contains a mixture of viruses, some of which have truncated mutations in the *nef* gene. To characterize further the Nef-mediated downregulation of perforin, we incubated lymphocytes with cell-free virus HIV-1_{Ba-L}, HIV-1_{IIIB} (with truncated *nef*) or a *nef*-deleted isolate (ΔNef_{HXB-2}) for four hours *in vitro*. The dose of the different HIV-1 strains was standardized in order to generate HIV-1 p24 protein concentration of 0.4pg/ml. *In situ* image analyses revealed a significant down-regulation of perforin and grA expression levels in PBMC exposed to HIV-1_{Ba-L} compared to control cells ($P<0.03$). In contrast, PBMC exposed to HIV-1_{IIIB} with truncated *nef* or the Nef-deleted isolate did not display any perforin downregulation. Subsequently, we used additional recombinant Nef distinct from $rNef_{IIIB}$ to compare its affect on perforin expression. Moreover, incubation with rNef from the HIV-1_{ELI} isolate, instead upregulated perforin expression. These data implied remarkable differences in the capacity of different Nef proteins to suppress perforin expression depending on the amino acid sequence of Nef.

Sequence alignment and molecular modeling of Nef suggest that the so-called disordered loop (residues 148-178) is the main region of interest for perforin downregulation

Sequence alignments of different Nef protein were used to identify the possible region

of the *nef* gene involved in perforin down-regulation. The Ba-L and $rNef_{IIIB}$ sequences, which resulted in perforin downmodulation, are very similar in sequence from residues 1-190, while the truncated HIV-1_{IIIB} sequence terminates at residue 123 and the ELI strain *nef* has numerous non-conservative amino acid substitutions in the region between residues 144-194 (paper IV). These differences suggest that residues 144-190 may be important for perforin downmodulation. In addition, the structural model of the previously published full length Nef (Geyer *et al.,* 2001) was used as a template to identify the regions of potential interest for the downregulation of perforin activity. The substitutions described above are located in three residue clusters; two of which are positioned on the loop consisting of residues 148-178 and the third being localized in the central folded core domain (Arold and Baur, 2001; Geyer *et al.,* 2001). The first cluster (residues 151, 152, 156, 157, 174 and 179) is positioned in the negatively charged central part of the disordered loop. The second cluster (residues 169 and 170, paper IV) is localized at the tip of the same loop. Finally residues 144, 186, 192 and 194 compose the third cluster located in the Nef core domain. However, detailed mutational analyses will be required to pinpoint which of these regions is important for down-modulating perforin, but the restriction of the effect to only some strains of HIV-1 suggests that it is specific.

Nef-mediated downregulation of perforin expression after Staphylococcal enterotoxin A (SEA) stimulation

We next addressed whether Nef protein inhibits perforin expression after activation with the superantigen SEA that is capable of activating high frequencies of T cells in a Vβ-specific manner. SEA stimulation increased perforin expression and resulted in induction of IFN-γ production. The ability of PBMC to produce perforin and grA upon SEA stimulation in the presence or absence of $rNef_{IIIB}$ was measured. The observed induction of perforin and grA following SEA stimulation was however inhibited in the presence of $rNef_{IIIB}$ ($p<0.001$) and ($p<0.002$), respectively.

We further investigated whether the observed downregulation of perforin occurred in $CD3^+CD8^+$ T cells and/or $CD3^-$ NK cells using flow cytometry. The cells were stained after incubation with brefeldin A (BfA) to increase the sensitivity of the intracellular effector molecules. Similar to the *in situ* image analysis results, the percentage of $CD3^+CD8^+$ $perforin^+$ T cells after SEA stimulation increased while addition of $rNef_{IIIB}$ reduced induction of $CD3^+CD8^+$ $perforin^+$ T cells. The double staining demonstrated that the Nef-mediated reduction of perforin expression occurred in $CD3^+CD8^+$ T cells, while we were unable to detect changes in perforin expression in the $CD56^+$ NK cells population. The immune activation of $CD8^+$ T cells by SEA during the short stimulation period thus resulted in activation of memory effector cells and not induction of perforin expression in naïve $CD8^+$ T cells. The latter process has been shown to require 8-12 cell divisions which takes 3-7 days of culture.

To verify whether Nef-mediated downregulation of perforin expression was evident at the mRNA level, *in situ* hybridization analyses were performed. An almost 2-fold increase in perforin mRNA positive cells was demonstrated in SEA-stimulated PBMCs after short-term culture by *in situ* hybridisation in two independent experiments. Addition of $rNef_{IIIB}$ abrogated the SEA-mediated induction of perforin mRNA, hence consistent with our protein results.

A role for Nef as perforin-modulator in vivo

To investigate the effect of Nef-deletion *in vivo,* paired animals were infected with SIV-EGFPΔ*nef* mac239 and SIV mac 239 (wild type). SIV viral replication, measured by plasma viral RNA, was attenuated after infection with SIVΔ*nef*-mac239. However, the acute peak viremia, measured by the amount of infectious cell-associated virus, was similar to those obtained after wild-type virus infection (Stahl-Hennig *et al.,* 2002). The highest viral load appeared two weeks post-infection in both animal groups. We then analyzed lymphoid tissues (spleen and lymph nodes) obtained 45 days post-infection for perforin and grA expression at the single cell level. Macaques infected with the wild-type virus displayed low perforin expression (0.2-0.6%) of total cells, while infection with SIVΔ*nef*-mac239 resulted in higher perforin expression (2-5%). However, lymphoid tissues from both groups had similar levels of grA^+ expression, in 2 to 8% of lymphocytes (mean 5%). Notably, perforin-dependent grA nuclear translocation in target cells, detected by morphological criteria (Andersson *et al.,* 2002) was more prevalent in tissues from SIVΔ*nef*-mac239 infected animals than in tissues derived from SIVmac251 infected macaques (paper IV). We concluded from these studies that certain Nef proteins are able to downregulate perforin expression in $CD8^+$ T cells.

Regulation of HIV-1 replication in the placenta

Chemokine receptor expression in the placenta (Paper V)

Many studies suggest that some virus selection occur during vertical transmission. Moreover, few studies have identified that placenta could be involved in this selection process. In paper V, we investigated one possible mechanism for HIV-1 selection involved in mother- to child transmission. The CCR5-using isolates are shown to be predominantly transmitted from mother to child. To investigate the role of chemokine receptor expression in maternal-fetal transmission we studied placental tissue from 16 HIV-1 non-transmitting (TNT) and 7 HIV-1 transmitting (TT) women as well as 4 normal controls. In order to quantify chemokine receptor expression, total placenta RNA was extracted from TT and TNT women as well as normal controls. Real time

RT-PCR was used for quantification of chemokine receptors in these tissues. Quantification by real time RT-PCR showed that the number of CCR5 mRNA copies/ 10^5 cells were increased 2-3-fold ($p<0.01$) and the number of CXCR4 mRNA copies were reduced by half in the TT placentas compared to TNT and normal controls (paper V). In order to quantify CXCR4 and CCR5 protein expressing cells, CXCR4 and CCR5 protein expression were analyzed by *in situ* image in sections stained by immunohistochemistry. Furthermore, quantification by *in situ* image showed that CXCR4 protein expressing cells were present in equivalent numbers in TNT placentas compared to TT placentas. Conversely, CCR5 protein expression was upregulated 3-fold in TT placentas compared to TNT placentas ($p<0.02$). These results provide evidence for predominant expression of CCR5 mRNA and protein in TT placenta tissue, which is dominantly used by R5-using HIV-1 isolates.

Characterization of the cytokine milieu in placenta

It has previously been reported that Th type 1 and Th type 2 cytokines are involved in the regulation of chemokine receptor expression. To determine if the chemokine receptor expression pattern observed in the placenta was driven by locally produced cytokines, real-time RT-PCR quantification and *in situ* image were used to quantify cytokines in placenta tissue. In TNT placentas, we found a significant elevation of Th type 2 cytokine (IL-4 and IL-10) mRNA relative to Th type 1 (IL-2) mRNA expression ($p<0.02$). This cytokine pattern with a dominance of type 2 over type 1 cytokines was observed also in normal controls. The cytokine pattern observed in TT placentas, on the other hand, was reversed with an upregulation of IL-2 and a downregulation of type 2 cytokines compared to TNT placentas or normal control ($p<0.01$).

Furthermore, to confirm translation of cytokine mRNA into protein, tissue sections were stained by immunohistochemistry and the local intracellular expression of cytokines was measured by *in situ* imaging. Type 2 cytokine (IL-4 and IL-10) expressing cells were significantly upregulated in TNT placentas compared to TT placentas ($p<0.02$). Biopsies from TT placentas showed significantly higher frequency of IL-2 expressing cells compared to TNT placentas ($p<0.05$). Thus our results demonstrated that placenta from TNT women maintained a normal type 2 cytokine milieu as compared to placenta from TT women. Thus, a Th type 2 to Th type 1 cytokine pattern shift may cause upregulation of chemokine receptor, CCR5, in TT placenta and facilitate vertical transmission of R5 using HIV-1 strains.

Chemokine receptor expression of placental cells productively infected by HIV-1

To analyze whether there was any selective utilization of chemokine receptors by HIV-1 in infected placenta tissue we used a sensitive *in situ* hybridization technique

simultaneously with immunophenotyping. The transmitting placenta had a statistically significant increase in the number of HIV-1 *gag* and *pol* expressing cells compared to TNT as determined by quantitative laser confocal microscopy (2.3% vs 0.02%) of total cells. More than 99% of cells expressing HIV-1 *gag-pol* were CCR5 positive cells in TT placenta samples in contrast to the TNT placentas (<10%). The great majority of productively HIV-1 infected cells in TNT placenta expressed CXCR4 while a minority of productively HIV-1 infected cells expressed CCR5 in the TNT placenta. This study indicated a selective utilization of CCR5 in transmission of HIV-1 cross the placenta.

Quantification of cytokines and Leukemia Inhibitory Factor (LIF) in placenta from transmitting and non-transmitting women (Paper VI)

To identify potential inhibitory factors in low virus load TNT placentae (paper V), we performed RT-PCR against 47 genes expressed in placentae including cytokines, chemokines, and hormones (paper VI). Consistent with data from paper V, higher IL-4 mRNA and protein expressing cells were found in TNT placentae compared to TT ($p<0.02$). However, levels were not significantly different as compared to expression in normal placentae. Moreover, the Th type 1 cytokine IL-2 mRNA and protein level was upregulated in transmitting placentae compared to both non-transmitting placentae and normal placentae ($p<0.05$).

We quantified the expression of LIF mRNA and protein in TT and TNT placentae as well as controls. LIF has been described as a secreted glycoprotein belonging to the IL-6 family (Stahl *et al.,* 1990; Metcalf, 1991; Metcalf *et al.,* 1991). LIF is expressed throughout normal pregnancy in the placenta. In humans, LIF can be secreted from the endometrial epithelium (Senturk and Arici, 1998), $CD16^{-}CD56^{+}$ NK cells (Saito *et al.,* 1993) and Th2 cells (Piccinni *et al.,* 1998). In the presence of upregulated IL-4 (Piccinni *et al.,* 1998), a significant elevation of LIF mRNA and protein expression was found in TNT placentae compared to TT placenta ($p<0.001$). However, LIF mRNA and protein expression was not significantly different in TNT placenta compared to normal placenta controls. Our results displayed significantly elevated LIF mRNA and protein expression in TNT placentae compared to TT placentae ($p<0.001$).

Identification of LIF as a potent HIV-1 suppressor

In order to test the effects of LIF on HIV-1 replication, we performed triplicate dose-dependent inhibition experiments in quadruplicate wells with a range of LIF concentrations from 0.05 pg/ml to 10 pg/ml. Our results showed that LIF inhibited the replication of CCR5-using (R5) HIV-$1_{Ba\text{-}L}$, the CXCR4-using (X4) HIV-1_{Lai}, the dual tropic (R5X4) HIV-1_{ME46}, and the primary isolates HIV-$1_{33074/33015}$ with an IC_{50}

between 0.3 and 0.7 pg/ml. Thus, the primary isolate HIV-$1_{93US151}$ taken from an infected, newborn child was inhibited by LIF with the lowest IC_{50} of 0.1 pg/ml. Because significant cell growth inhibition would affect HIV-1 production in culture, we assessed the proliferative response of PBMCs to PHA stimulation in the presence or absence of LIF. LIF concentrations that inhibited HIV-1 production had no inhibitory effect on cell proliferation.

LIF inhibits HIV-1 replication but not HIV-1 infection of target cells in vitro

To elucidate the site of LIF action on HIV-1 replication, we first determined the effects of LIF on expression of CD4 and co-receptors CCR5 and CXCR4. PBMC cultured with or without LIF were stained and analyzed using flow cytometry. We saw no effect of LIF on CD4, CCR5, or CXCR4 expression as compared to untreated controls. These data suggest that LIF neither blocks anti-CD4, -CXCR4, or -CCR5 antibody binding sites nor regulates their cell surface expression, as do other cytokines. To determine if LIF inhibits the HIV-1 life cycle at the reverse transcription stage, we quantified early LTR U5/R and late LTR/*gag* reverse transcripts (Bukrinsky *et al.,* 1993) in HIV-1 infected PBMCs with or without LIF treatment. Quantification by RT-PCR demonstrated low levels of both early LTR U5/R and late LTR/*gag* reverse transcripts after infection with different HIV-1 isolates. In addition LIF-untreated controls infected with all different HIV-1 isolates expressed high levels of both early and late reverse transcripts. These data provide insight into the inhibitory pathway and site of action by suggesting that the anti-viral activity of LIF takes place prior to reverse transcription, and so distinguishes it from the CD8 T cell-derived antiviral factor (CAF).

HIV-1 inhibition by LIF required gp130-LIFRβ expression

It has been shown that LIF upregulates Janus kinase (JAK) after binding to the gp130-LIFRβ receptor heterodimer (Davis *et al.,* 1993) and ultimately phosphorylates tyrosine residues on the STAT family of transcription factors (Fujio *et al.,* 1997). To gain insight into the role of gp130-LIFRβ-mediated signal transduction in HIV-1 inhibition, we infected MT-2 cells (gp130-LIFRβ-negative) and PBMCs in the presence and absence of LIF. The MT-2 cells were resistant to the HIV-1 inhibitory effects of LIF. These data indicated that LIF inhibitory affect on HIV-1 infection was dependent upon the expression of gp130-LIFRβ on the surface of HIV-1 susceptible cells.

The effects of LIF in ex vivo cultured placentae and thymus

To test the effects of LIF in an *in vivo* model of HIV-1 infection, we infected tissue fragments of placentae with either a T-tropic isolate, HIV-1_{Ba-L} or a primary isolate (HIV-$1_{93US151}$) in the presence of LIF or neutralizing, polyclonal anti-LIF antibody as a specificity control. In these experiments, we used concentrations of LIF known to

inhibit HIV-1 in PBMCs and concentrations of anti-LIF antibodies known to neutralize >90% of LIF bioactivity. After infection, total DNA was extracted and HIV-1 DNA quantification was performed using real-time PCR. LIF inhibited infection of placental explant tissue by HIV-1_{Ba-L} in a dose dependent manner. Addition of 5μg/ml of neutralizing polyclonal anti-LIF increased infection of the placenta organ culture relative to the LIF-untreated control. These results may reflect additional neutralization of endogenous LIF produced by the placental tissue. To further corroborate our findings in a second *ex vivo* model, thymus tissue grown in explant culture was infected by HIV-1_{Lai} in the presence or absence of LIF. HIV-1 productively infected CD4, CD8 double positive T cells while CD4 or CD8 single positive T cells were inhibited by 1pg/ml of LIF, as determined by sensitive simultaneous immunophenotyping/UFISH. Thus, LIF was able to inhibit HIV-1 in a tissue other than placenta indicating that our previous observation were not dependent on placenta specific factors such as β-HCG (De *et al.,* 1997).

Quantification of HIV-1 DNA and mRNA in placentae

To investigate differences in HIV-1 load in placentae from TT and TNT women, we quantified unspliced HIV-1 *gag* mRNA and DNA in placental tissues. Expression of HIV-1 *gag* mRNA was found in four of five (80%) TT placentae and only one of six (17%) TNT tissue (paper VI). The average number of HIV-1 *gag* mRNA copies (510/ 20,000 GADPH) in the TT placentae were significantly higher than in TNT placentae ($p<0.05$). In contrast, no significant difference was found in the number of HIV-1 DNA^+ cells in placenta tissue from TT versus TNT. These data provide further evidence for the lack of HIV-1 RNA production in TNT placentae and support the possibility of LIF-mediated HIV-1 suppressive *in vivo* activity within these placental tissues.

DISCUSSION

The cytokine and chemokine expression profile before and after HAART in HIV-1 infected lymphoid tissue

When we began these studies the majority of HIV-1 investigations had focused on blood. However, we believed that the direct sampling of productively infected cells from peripheral blood did not reflect what was happening in the HIV-1 infected LT (Andersson *et al.,* 1998; Bucy *et al.,* 1999). T cells in the circulating blood represent only ~1%-2% of the total lymphocyte pool. Thus, the vast majority of potential HIV-1 targets cells are localized in tissues. In 1995, HAART therapy opened a new era in HIV-1 treatment history. Since then, an extensively knowledge has been accumulated with regard to HIV-1 pathogenesis and the relationship between the immune system and HIV-1 within the infected tissue. Studies on the impact of anti-retroviral therapy on the LT may provide important insight into the pathogenesis of HIV-1 infection.

In order to examine the effect of HIV-1 infection on the immune system in LTs we investigated the expression of cytokines, chemokines, anti-viral effector molecules and chemokine receptors in biopsies obtained from infected patients. We developed a method for quantifying intracellular cytokine and chemokine protein expression at the single cell level based on immunohistochemistry followed by computerized *in situ* image analysis (Bjork *et al.,* 1996).There are several different methods for measuring production of cytokines; e.g. Northern blot, semiquantitative reverse transcription PCR (RT-PCR) and *in situ* hybridization. However, the disadvantage of studying cytokines by these methods is that most cytokines are modified at both post-transcriptional and post-translational levels and cytokines carry out their functions mainly after secretion of the protein. Therefore, detection of cytokine mRNA prior to secretion of corresponding protein might not be a reliable marker for biological effect of the released effector molecule. Further, the Northern blotting and *in situ* hybridization methods are qualitative measurements of mRNA. Regarding other techniques that measure cytokines at the protein level both the ELISPOT and intracellular staining by flow cytometry require disruption of tissues and *in vitro* restimulation prior to analysis. This provides information on the potential rather than actual expression of cytokines. The advantage with immunohistochemistry is preservation of morphology and distribution of cytokine expressing cells as well as the possibility to quantify extracellular secretion of cytokines in tissues. Flow cytometry on the other hand has the advantage of allowing multi-colour analysis in a shorter time. The optimal settings would be combine direct *in situ* image with flow cytometric analysis.

Effect of HAART on HIV-1 burden in peripheral blood and tonsils

It is believed that most HIV-1viral production takes place in LT (Embretson *et al.,* 1993; Pantaleo *et al.,* 1993a). HAART dramatically reduces the plasma viral load of HIV-1 infected individuals to undetectable levels (Cavert *et al.,* 1997; Finzi *et al.,* 1997). However even prolonged treatment with HAART for >5 years fails to eradicate HIV-1. Studies have showed that the amount of virus detectable in lymph nodes is 1 to 4 logs higher than that in plasma, raising the question of whether decreasing virus in plasma correlates with HIV-eradication in lymphoid compartments (Hammer *et al.,* 1996; Schacker *et al.,* 1998).

In order to evaluate the effect of HAART on immune reconstitution within HIV-1 infected LT, we examined cellular responses *in situ* in three HIV-1 infected patients in asymptomatic stage. It could be seen that HAART therapy resulted in a significant reduction in plasma HIV-1 RNA (less than 50 copies/ml) and numbers of HIV-1 $mRNA^+$ cells in the lymphoid samples during the year studied. By sensitive *in situ* PCR combined with a fluorescent probe, we detected HIV-1 *gag* DNA in tissues after one year of therapy. However, we could not identify the phenotype of HIV-1 DNA containing cells in tonsil sections from these patients. The prevailing of HIV-1 DNA containing cells may provide a source for viral replication in the lymphoid system tissue despite effective viral suppression by potent anti-retroviral therapy (Cavert *et al.,* 1997; Chun *et al.,* 1997a; Chun *et al.,* 1997b; Wong *et al.,* 1997b; Finzi *et al.,* 1999; Furtado *et al.,* 1999).

Effect of HAART on cytokines involved in the adaptive immune response

Replication of HIV-1 in lymphoid compartments results in increased cytokine production and cellular activation (Graziosi *et al.,* 1996). The observed increase in cytokine production in HIV-1 infected lymphoid tissue may in turn enhance ongoing viral replication. As cellular immunity at the local site of infection is critical for immune control of HIV-1 infection, we therefore quantified the effect of HAART on cytokine expression at the local site of infection.

A varying cytokine profiles has been reported in HIV-1 infection from *in vitro* studies (Poli and Fauci, 1992). It should be however noted that many different methods have been used to investigate cytokine expression in HIV-1 infection. Furthermore, most studies analyzed expression of cytokines in blood following *in vitro* re-stimulation. Analyses of cytokine expression in LTs has not been extensively performed. We analyzed the expression of IL-2 and IFN-γ in tonsil sections from three HIV-1 infected patients and seronegative controls. Strong evidence for HIV-1 associated immune activation was found by the high expression of IL-2 and IFN-γ observed in different

regions within the tonsillar biopsies. In addition, extensive expression of IL-12 was found in the tonsils prior to therapy. *In situ* image analysis revealed that IL-2 and IFN-γ decreased to normal levels after one year of therapy suggesting that the level of cellular activation diminished following initiation of potent anti-retroviral therapy. The low frequency of IL-2 detected after therapy might be beneficial considering that IL-2, along with other cytokines, can support viral replication (Chun *et al.,* 1997b) and increase expression of HIV-1 chemokine co-receptors (Patterson *et al.,* 1998). Using the immunostaining technique, we found that the high expression of IL-12 seen prior to therapy was reduced shortly after start of treatment. In line with our results others have reported failure to restore HIV-1 antigen specific production of IL-12 in PBMC after anti-retroviral therapy which may limit efficient priming of naïve $CD4^+$ T cells against HIV-1 infection (Grassi *et al.,* 1999).

High frequency of IL-4 and IL-10-expressing cells have been reported in *in vitro* HIV-antigen stimulated peripheral blood mononuclear cells in the AIDS stage (Clerici and Shearer, 1993). We could not detect many IL-4 and IL-10 cytokine expressing cells in tonsil sections obtained from HIV-1 infected patients in the chronic asymptomatic phase (Graziosi *et al.,* 1996).

Effects of HAART on proinflammatory cytokines

Both primary HIV-1 infection and the asymptomatic phase are characterized by profound proinflammatory cytokine activation. Proinflammatory cytokines such as IL-6, and TNF-α together with IL-2, were shown to support viral replication *in vitro* in PBMC of HIV-1 infected individuals and in latently infected $CD4^+$ T cells (Chun *et al.,* 1998). The observed increase in cytokine production (IL-1, IL-6, IL-2) in HIV-1 infected LT may enhance ongoing viral replication. Macrophages, DCs and B cells are potent producers of IL-1α/IL-1β, which are involved in the primary activation of naïve T cells. It has been suggested that exposure of DCs to HIV-1 may create an important and persistent source of immune activation. Such cytokine production may generate signals to induce HIV-1 replication in latently infected resting CD4 memory T cells. We found that both IL-1α and IL-1β expression was increased in HIV-1 infected tonsils prior to therapy. The expression of IL-1β was normalized after initiation of HAART. IL-1α expression, however, was continuously elevated in the patient who showed high HIV-1 DNA contents in LT. Additional reports demonstrated a reduction of inflammatory cytokines TNF-α and IL-6 following HAART therapy (Lederman *et al.,* 1998; Sloand *et al.,* 1999). The persistence of pro-viral DNA containing cells, despite effective therapy has been documented in earlier reports (Zhang *et al.,* 1999). The persistent high incidence of IL-1α in lymphoid tissue may represent an ongoing inflammatory response or cytokine dysregulation that may contribute to rapid viral

rebound in $CD4^+$ T cells containing pro-viral DNA upon HAART discontinuation.

Interdigitating Dendritic cells in lymphoid tissue

Furthermore, by microscopic examination we found that $CD1a^+$ expressing cells were localized in the extrafollicular area in tonsils where the DC-T cell interaction occurs. There, DCs form clusters with antigen-specific T cells, creating a microenvironment in which immunity can develop (Ingulli *et al.,* 1997). Our results showed that the initial significant increased incidence of $CD1a^+$ DCs was highly reduced after one year of HAART therapy. This may indicate that the treatment resulted in reduced influx of DCs with capacity to phagocytose and present newly formed HIV-1 antigens to naive T cells. DCs have an important role in HIV-1 pathogenesis, delivering the virus to lymphoid sites and efficiently transmitting the virus to neighboring $CD4^+$ T cells. Consistent with our data (prior to therapy) high incidence of DCs was recently found in HIV-1 infected tissues. This study demonstrated a distinct block in co-stimulatory molecule expression (CD80/CD86) on DCs in lymph nodes of patients both in acute and chronic phase of HIV-1 infection (Lore *et al.,* 2002). The lack of adequate co-stimulation has been suggested to lead to a decreased ability to produce IL-2 from $CD4^+$ T cells. Subsequently limited $CD8^+$ T cell maturation (Barker *et al.,* 1999). Thus, incomplete maturation or reduction in frequencies of DCs as a consequence of treatment may reduce this recruitment to LT and limit their capacity to generate HIV-1 specific $CD4^+$ T cell responses.

Taken together, this study was evaluated the direct effects imposed by local HIV-1 replication on the immune system. Our findings indicated a predominant proinflammatory and Th1 cytokine expression profile in HIV-1 infected lymphoid compartments, which returned to a more resting state after HAART treatment. However, these results are obtained from a very limited number of biopsies of asymptomatic HIV-1 infected patients. Further studies with more patients are needed to examine cellular immune responses in HIV-1 infected tissues in more detail.

Impaired expression of cytolytic effector molecules expressed in $CD8^+$ T cells in HIV-1 infected lymphoid tissue

In order to investigate why the immune response fails to prevent progressive immunodeficiency despite the high frequency of HIV-1 specific $CD8^+$ T cells, we analyzed the expression of cytolytic effector molecules in the HIV-1 infected LTs. We hypothesized that a defect in the production or expression of cytolytic effector molecules in T cells and NK cells could result in persistence of HIV-1 replicating cells. We evaluated expression of perforin and grA in LT and peripheral blood isolated from patients with chronic HIV-1 infection. Expression of these cytolytic effector molecules was also measured in LT during acute EBV infection in order to compare their expression in the setting of a viral infection in which cellular immune reactions leads

to viral clearance.

We demonstrated the lack of perforin expression in LT from patients with chronic HIV-1 infection. In contrast, much higher levels of perforin- and grA-expressing cells were evident in LT from patients with acute infectious mononucleosis. A significant increase in $CD8^+$ T cells was evident in the LT from HIV-1 infected patients. However, follicular destruction and $CD8^+$ T cell depletion was evident in patients at the later stage of disease. Furthermore, two-color staining for perforin or grA revealed that >80% of perforin and grA granule expressing cells belonged to the $CD8^+$ T cell lineage. It could be argued that the low perforin expression observed in the cohort study was due to impairment of CTL maturation. Phenotypic analysis of tetramer stained HIV-1 specific $CD8^+$ T cells indicates that circulating viral-specific $CD8^+$ T cells have been previously activated and belong to the memory phenotype (Altman *et al.,* 1996; Ogg *et al.,* 1998; Appay *et al.,* 2000; Shankar *et al.,* 2000; Chen *et al.,* 2001). Differentiation to effector CTLs, which identify naïve CTLs versus effector CTLs, was shown to be associated with down-modulation of CD27 and CD28. The recently activated HIV-1 specific $CD8^+$ T cells no longer expressed CD28, CD27, CD62L and CD45RA (Altman *et al.,* 1996; Ogg *et al.,* 1998; Appay *et al.,* 2000; Shankar *et al.,* 2000; Chen *et al.,* 2001). Furthermore, HIV-1 $tetramer^+$ cells were shown to be CD28 negative and $CD27^+$ in most donors (Trimble and Lieberman, 1998; Appay *et al.,* 2000; Shankar *et al.,* 2000). These HIV-1 specific CD8+ cells did not express high level of perforin in blood from HIV-1 infected patients (Appay *et al.,* 2000). Recent studies have shown that the HIV-1 specific cell pool consists of pre-terminally differentiated $CD45RA^-$ $CCR7^-$ cells, which indicates a skewed maturation of HIV-1 specific memory $CD8^+$ T cells (Champagne *et al.,* 2001). HIV-1 specific CTLs have been observed as early as a few days following the onset of acute symptoms, in general before neutralizing antibody responses (Koup *et al.,* 1994; Pellegrin *et al.,* 1996). CTLs were shown to some degree to be responsible for the initial control of HIV-1 replication (Rowland-Jones and McMichael, 1995). Maturation into competent cytotoxic T cells may be blocked during the initial encounter with antigen because of defects or lack of co-stimulatory molecules in antigen presenting cells (Lieberman *et al.,* 2001).

Moreover, degranulation of $CD8^+$ T cells may be another explanation for low perforin content in our cohort study. This is unlikely since perforin and grA are co-localized to the same granules in the effector cytotoxic T cells and we demonstrated high frequency of grA-expressing cells in the same $CD8^+$ cells, which were lacking perforin-expression. Consistent with this data, lack of perforin expression in HIV-1 specific $CD8^+$ T cells was recently shown in LT from early stage (acute) of HIV-1 infection (Andersson *et al.,* 2002). A relative reduction in the number of $CD8^+$ T cells expressing perforin mRNA in LT in primary HIV-1 infected patients further suggest that low perforin in

granules may be due to maturation arrest and not degranulation (Andersson *et al.,* 2002). We also found that $CD8^+$ T cells expressing perforin mRNA was raised in HIV-1 infected LT after one year of therapy (unpublished observation). However, the cells expressing perforin were still few and constituted less than <0.05% of total cell pool. Altogether, these data revealed that $CD8^+$ T cells in both early and chronic HIV-1 infection displayed low perforin expression, which might explain their inability to efficiently perform their task of killing HIV-1 infected cells.

The induction of the apoptotic process is dependent upon the presence of perforin in conjunction with grA and granzyme B (Tenner-Racz *et al.,* 1993; Bochan *et al.,* 1994; Rukavina *et al.,* 1998). The quantification by *in situ* image analysis revealed significantly higher expression of grA-positive cells in all stage of HIV-1 infection compared to perforin-positive cells in LTs. Our study indicated that at sites of HIV-1 replication a significant upregulation of grA expression occurs within single $CD8^+$ T cells but these cells showed limited expression of perforin. Could this dissociation between grA and perforin expression contribute to impaired cytotoxic T cell activity at sites of viral replication? Further experiments need to be carried out regarding the dissociation between grA and perforin expression in HIV-1 specific $CD8^+$ T cells.

We hypothesized that the local release of HIV-1 proteins could be involved in $CD8^+$ T cells failing to control HIV-1 replication. Extracellular accumulation of HIV-1 proteins such as Tat and Nef, in the absence of neutralizing antibodies, has been shown to upregulate the production of CC-chemokines, IL-1, TNF-α and IL-6 which can in turn induce macrophage-DC and naïve T-cell activation (Lafrenie *et al.,* 1997; Nath *et al.,* 1999; Swingler *et al.,* 1999). Furthermore, Tat can downregulate HLA class II at the surface of HIV-1 infected cells (Kanazawa *et al.,* 2000). On the other hand, Nef down-modulates expression of HLA-A and B molecules, but not HLA-C or HLA-E that may impair the recognition of HIV-1 infected cells by $CD8^+$ T cells (Cohen *et al.,* 1999). Analyses using genetically modified cytotoxic T cell clones that do not require MHC class I-presented antigen showed that diminished antigen-presentation might be a mechanism for impaired cytotoxic activity (Yang *et al.,* 2002).

Downregulation of perforin expression *in vitro*

It is difficult to explain why impaired perforin production occurs in an environment of upregulated Th1 cytokines (IL-2, IFN-γ, IL-12) and β-chemokines (Andersson *et al.,* 1998; Behbahani *et al.,* 2000). As mentioned above, $CD8^+$ T cells have emerged as important players in the control and protection of HIV-1 infection by direct killing of infected cells. However, why $CD8^+$ T cells fail to clear HIV-1 and what mechanisms HIV-1 has evolved to escape from this defense are not fully understood (Lieberman *et al.,* 2001).We hypothesized that extracellular HIV-1 proteins may suppress perforin

expression by CTL. We demonstrated that Nef, an early multifunctional regulatory HIV-1 and SIV protein, might be involved in the downregulation of perforin in $CD8^+$ T cells. Incubation of PBMCs from HIV-1 seronegative donors for 4 hours with recombinant $rNef_{IIIB}$ showed a significant reduction of perforin expressing cells as measured at the single cell level by *in situ* imaging. Furthermore, consistent inhibition of SEA-stimulated perforin expression occurred in the presence of Nef. These findings suggest that exogenous Nef may impair the induction of the cytolytic machinery in activated $CD8^+$ T cells.

It was previously demonstrated by using $CD4^+$ T cells acutely infected with *nef*-deleted or *nef* -competent virus that Nef plays a role in the impairment of cytotoxic T cell activity (Collins *et al.,* 1998). Cells infected with the *nef*-deleted virus were eliminated after exposure to cytotoxic T cells, in contrast to *nef*-competent HIV-1 infected cells, which were cleared less efficiently (Collins *et al.,* 1998). In the present report, reduced effector molecule expression was induced in freshly isolated cells after short exposure to exogenous Nef. Because T cell clones and cell lines grown in high concentrations of IL-2 have artificially elevated levels of perforin expression compared to $CD8^+$ T cells directly analysed *ex vivo*, the effect of Nef on $CD8^+$ T cell perforin may have been overlooked in the past studies with T cell clones and cell lines.

We also studied if Nef-mediated perforin downregulation was a common attribute to several HIV-1 isolates. Four different Nef isolates displayed remarkable differences in their ability to affect perforin expression. Recently, it has been reported that variations in amino acid sequences isolated from patients ranged up to 30% even within the same type of HIV-1 strain (Kang *et al.,* 1998).

The most fascinating aspect of the Nef protein structure is its flexible region, which can mediate many functions (Geyer *et al.,* 2001). In order to identify the structural modification in HIV-1 isolates and recombinant Nef used in our study, sequence alignments of the different proteins were used. The alignment of the Ba-L and $rNef_{IIIB}$ sequences, which result in perforin downmodulation, display a similar sequence from residues 1-190, while the truncated $HIV\text{-}1_{IIIB}$ sequence terminates at residue 123 and the ELI strain *nef* has numerous non-conservative amino acid substitutions in the region between residues 144-194. The alignment of the sequences of Nef molecules used in our study revealed the importance of the C-terminal region in the downregulation of perforin expression. Using a model of full-length Nef (Geyer *et al.,* 2001), formed by the assembly of various Nef portions that have been already structurally determined, we found three main regions differ between perforin downregulating and non-downregulating (Grzesiek *et al.,* 1996; Lee *et al.,* 1996; Arold and Baur, 2001; Geyer *et al.,* 2001). Two of these were located on the so-called disordered 148-178 loop and

the third is localized in the core domain of Nef. The disordered loop (residues 148-178) plays an important role in Nef internalization (Geyer *et al.,* 2001).

However, this study did not provide a mechanism for the inhibition of perforin expression by Nef. The effect of Nef may be indirect. One intriguing possibility is that Nef induces secretion of soluble factors involved in regulation of perforin expression (Smyth *et al.,* 1991; Federico *et al.,* 2001) or at least is dependent upon the presence of antigen presenting cells. This was suggested by our finding (unpublished data) that monocyte-depleted PBMCs failed to show any modulation of perforin while monocytes pre-treated with rNef could confer perforin downregulation in $CD8^+$ T cells. *In vitro* experiments have revealed Nef internalization in human monocytes (Alessandrini *et al.,* 2000) which in turn was proposed to alter the pattern of soluble factors released by monocytes and macrophages (Federico *et al.,* 2001). Extracellular Nef may affect monocytes that consequently induce production of other factors involved in reduction of perforin expression in $CD8^+$ T cells. Studies have shown that perforin reduction by the Nef HIV-1 protein may be a consequence of increasing TGF-β synthesis (Smyth *et al.,* 1991; Simmons *et al.,* 2001) or IL-10 (Brigino *et al.,* 1997). Expression of other soluble factors, particularly TGF-β needs to be further studied. Previously, the role of extracellular Nef and its interaction in calcium and calmodulin pathway has been reported (Brigino *et al.,* 1997). This may lead to change of calcium levels in the cells and modify perfoirn release from the granules of $CD8^+$ T cells. Furthermore, we showed that macaques infected with *nef*-deleted SIVmac239 had high perforin expression in lymphoid tissues compared to macaques infected with wild type SIV. Although SIVΔ *nef*-mac239 replication is attenuated *in vivo*, it has a similar replicative capacity to wild-type SIV in T cells that were co-cultured with mature DCs (Messmer *et al.,* 2000). The observed high perforin expression in *nef*-deleted SIVmac239 was in agreement with a recent study also analysing lymphoid tissue (Stahl-Hennig *et al.,* 2002). The relatively low perforin expression in macaques infected with wild-type virus was however not supported (Stahl-Hennig *et al.,* 2002) but it is in agreement with our previous studies analysing lymphoid tissue from HIV-1 infected individuals (Andersson *et al.,* 1999; Andersson *et al.,* 2002). Sequence analyses of the viral sub-clones used may aid to resolve this apparent discrepancy.

The ability of Nef to down-regulate perforin and grA expression, may be one piece in the puzzle of explaining why $CD8^+$ T cells fail to control HIV-1 replication. Not only does Nef interfere with antigen presentation on infected cells, it may also interfere with the ability of the immunoreactive cells to deliver the lethal hit. Targeting the 148-178 loop of Nef may be useful in developing new therapies to augment the cellular immune response to the virus.

A possible mechanism associated with HIV-1 vertical transmission

It is apparent that HIV-1 can be transmitted from mother to child throughout pregnancy. To examine transmission from mother to child we investigated the role of placenta. The placenta provides a barrier between the maternal and fetal circulation, but limited attentions has been given to its role in the transmission of HIV-1. The protective role of the placenta during gestation was suggested by the finding that caesarian sections reduce the risk of transmission of human HIV-1virus from mother to child (Kind *et al.,* 1998). Characteristics of virus itself may also influence maternal transmission of HIV-1. Recent evidence points to the importance of chemokine receptors on the surface of lymphocytes (Carrington *et al.,* 1999; Kostrikis *et al.,* 1999). In addition, selective pressure in the placenta during HIV-1 infection could be potentially involved in virus selection from mother to child. The CCR5 chemokine receptor is required for R5-using HIV-1 strains to infect target cells and these are supposed to be predominantly transmitted from mother to infant (van't Wout *et al.,* 1994). Previously, Patterson BK *et al* have shown that chemokine receptor expression on immune cells is regulated by Th1 and Th2 cytokines (Patterson *et al.,* 1998; Patterson *et al.,* 1999). Several studies support the hypothesis that production of IL-4 and IL-10 may permit allograph tolerance (Piccinni *et al.,* 1998) and maintenance of pregnancy (Chaouat *et al.,* 1996; Deneys and De Bruyere, 1997). However, HIV-1 infection may create a milieu that supports increased risk of spontaneous abortion in HIV-1 infected women (Langston *et al.,* 1995). Our data showed that placentae from TNT women maintained a normal Th2 cytokine milieu whereas TT women expressed Th1 cytokines. The mechanism underlying the shift from a Th2 cytokine dominance to a Th1 in TT placentae is not clear. However, the TT placental tissues studied in our cohorts were associated with *chorioamnionitis* or villitis, which is regularly found in placentae of HIV-1 infected women. Because the Th2 cytokine in TT placentae did not result in spontaneous abortion, a shift in cytokine expression from Th2 to Th1 may likely to be a late event in the transmitting women.

Quantification by *in situ* imaging in placenta tissues indicated that Th2 expressing cytokines milieu in TNT women drove the expression of CXCR4 and resulted in a normally low CCR5: CXCR4 ratio in placental tissue (paper V). Moreover, the CCR5: CXCR4 ratio was significantly increased in the TT placenta along with increased number of IL-2 mRNA and protein expression. For the first time we described the role of CCR5 expression levels and its linkage to cytokine expression in placenta sections which could be involved in selection for vertical transmission. It is noteworthy that observed up-regulation of CXCR4 could also be mediated by progesterone

(Patterson *et al.,* 1998). Morphological examination revealed that CCR5 and CXCR4 were expressed on placental macrophages and lymphocytes but not in trophoblasts.

To confirm selective utilization of chemokine receptors by HIV-1 in infected tissues we performed a sensitive *in situ* hybridization along with immunophenotyping of these receptors. Simultaneous immunoflurescence/ultrasensitive *in situ* hybridization for HIV-1 *gag-pol* mRNA revealed that HIV-1 almost exclusively infects HIV-1 CCR5-expressing cells in placentae from TT, whereas HIV-1 infects predominantly CXCR4-expressing cells in TNT placentae. A statistically significant increase in the total number of productively infected cells and in the number of viral particles produced per cell was demonstrated in TT (214 copies/cell) placentae compared to TNT (78 copies/cell). These data suggested that the Th2 cytokine milieu and concomitant low CCR5:CXCR4 ratio may prevent HIV-1 replication in the placenta.

Characterization of a novel HIV-1 inhibitory factor

A second hypothesis we favored was based on findings from previous work; up-regulation of IL-4 in low viral load TNT that concerns the possibility of prevention of HIV-1 replication in the placenta. Previous reports have shown that progesterone promotes the development of T-helper cells that produce IL-4 and IL-10 cytokines (Piccinni *et al.,* 1995). Studies in HIV-1 transgenic mice have demonstrated that pregnancy-related hormone human chorionic gonadotropin (β-hCG) exert a HIV-1 inhibitory effect (De *et al.,* 1997). However, subsequent studies proposed that an unidentified factor associated with β-hCG inhibited HIV-1 replication (Lunardi-Iskandar *et al.,* 1998). LIF is well characterized for its role in blastocyst implantation (Stewart *et al.,* 1992) and maintenance of pregnancy (Piccinni *et al.,* 1998). However, LIF is constitutively expressed throughout normal pregnancy in the placenta, but not in the peripheral blood of pregnant women. LIF knockout mice were unable to implant blastocysts resulting in infertility (Stewart *et al.,* 1992). The receptor for LIF (LIF-βR) is thought to play a role in trophoblast growth and differentiation in the human placenta. In human placenta, cells of the trophoblast lineage express LIF-βR and the major site of LIF mRNA expression in leukocytes is the maternal decidua (Sharkey *et al.,* 1999). Moreover, IL-4 production up-regulates the production of LIF by T cells.

We demonstrated a significant elevation of IL-4 and IL-10 mRNA and protein relative to IL-2 mRNA and protein expression ($p<0.02$) in TNT placentas. These data were further supported in paper VI with findings of upregulation of Th2 cytokines, particularly IL-4 (mRNA and protein) and its linkage to LIF production (mRNA and protein) in TNT placentae as quantified by *in situ* image analysis. We found upregulation

of LIF mRNA and protein expression which were significantly elevated in TNT placenta compared with TT placenta. In humans, LIF is secreted from the endometrial epithelium (Senturk and Arici, 1998), $CD16^{-}CD56^{+}$NK cells (Saito *et al.,* 1993) and Th2 T cells (Piccinni *et al.,* 1998). However, microscopic examinations in our study demonstrated that LIF was expressed in placental lymphocytes, decidua and endothelial cells. Further investigation is needed to identify the phenotypes of LIF-expressing cells in placental tissue.

The following experiments were performed to address the question of whether LIF production in TNT placenta could inhibit HIV-1 DNA or mRNA expression. Quantitative real time RT-PCR analyses showed a decrease in HIV-1 mRNA and DNA in placenta tissue from TNT women compared with placenta tissue from TT women. These findings supported the possibility of LIF of HIV-1 suppressive activity within the placenta. Using quantitative real-time RT-PCR to detect early (LTR-U5/R) and late (LTR-U3/*gag*) reverse transcripts for unspliced *gag–pol*, we determined the possible site of inhibitory activity of LIF *in vitro*. Based on this analysis of specific HIV-1 gene products, LIF-induced HIV-1 suppression was shown to take place before reverse transcription. This inhibitory site was distinct from CAFs anti-viral activity produced from $CD8^{+}$ T cells. As has been demonstrated (Mackewicz *et al.,* 1995), CAF inhibits HIV-1 replication at the level of transcriptions whereas viral entry and reverse transcription are unaffected. After binding to gp130-LIFRβ receptor heterodimer (Davis *et al.,* 1993), LIF upregulates Janus kinase (JAK), which ultimately phosphorylates tyrosine residues on the STAT family of transcription factors (Fujio *et al.,* 1997). We could also demonstrate that inhibition of HIV-1 replication was dependent on expression of gp130-LIFRβ on the surface of HIV-1 susceptible cells without involving co-receptors. Further studies are necessary to elucidate the exact mechanism of the LIF-mediated inhibition in HIV-1 infected cells. Moreover, inhibitory experiments demonstrated that LIF could inhibit HIV-1 replication with at least 1000-fold less concentration than β-chemokines in PBMCs and explant cultures (RANTES and MIP1α/β) (Cocchi *et al.,* 1995). The identification of LIF as an inhibitor of HIV-1 replication may lead to the development of new anti-HIV-1 treatment.

CONCLUDING REMARKS

Lymphoid compartments are major sites of HIV-1 replication. However, despite potent activation of both cellular and humoral immune responses, viral replication persists. Our data demonstrated an extensive proinflammatory activation and vigorous Th1 type cytokine but low Th2 cytokine expression in chronically HIV-1 infected tonsil and lymph nodes. In addition, we studied the effects of HAART on immune activation within LTs. HAART treatment resulted in a significant reduction of proinflammatory as well as Th1 cytokine expression in the LT. However, the pool of HIV-1 DNA containing cells remained virtually unchanged more than one year of HAART therapy. Moreover, the initial increased numbers of $CD8^+$ T cells were normalized by HAART treatment. The normalized immune activation in LT after HAART may be beneficial for the restoration of functional immune response and may also facilitate immune reconstitution by reducing the virus-driven immune activation. The combination of anti-retroviral therapy and immunological interventions such as performing immunizations with HIV-1 candidate vaccines may be feasible to augment HIV-1 specific immune responses strong enough to control viral replication even in the absence of HAART (Autran and Carcelain, 2000). Based on these findings, we hypothesized that the lack of elimination of HIV-1 infected cells was due to impaired cytolytic effector function in activated $CD8^+$ T cells. Killing of infected target cells can be mediated by either Fas-L/Fas interaction or perforin/grA mediated events. $CD8^+$ T cells in HIV-1 infected LT were characterized by significant upregulation of Fas-L (unpublished observation) and grA expression, while perforin expression was not concomitantly induced. This was in contrast to $CD8^+$ T cells obtained from acutely EBV infected patients, which revealed upregulation of both perforin and grA. Our data indicated a defective cytolytic activity in $CD8^+$ T cells at local sites of HIV-1 replication within LT. To elucidate the molecular mechanism involved in inhibition of perforin expression in HIV-1 infection, we set up short term *in vitro* cultures using freshly isolated peripheral blood cells from HIV-1 seronegative donors. Exogenous addition of Nef protein was found to mediate downregulation of perforin in $CD8^+$ T cells. Sequence alignments and molecular modeling of different Nef proteins suggest that the so-called disordered loop corresponding to residues 148-178 is a likely contributor to the observed Nef-mediated downregulation of perforin expression. However, this study did not provide a mechanism for the inhibition of perforin expression by Nef. The effect of Nef may be indirect. One intriguing possibility is that Nef induces secretion of soluble factors involved in regulation of perforin expression or at least is dependent upon the presence of antigen presenting cells. Future studies will be aimed to elucidate the molecular mechanism behind Nef-mediated downregulation of perforin.

Furthermore, the immunoregulatory role of cytokines on chemokine receptors involved in vertical transmission of HIV-1 was investigated in placental tissues. Our study indicated that expression of CCR5 and CXCR4 in placental tissues was associated with differing cytokine milieu in TNT and TT women. The chemokine receptor repertoire was consistent with an upregulation of IL-4 and IL-10 expression on TNT placenta compared to TT. We concluded that upregulation of CCR5 and Th1 cytokine in TT placenta was associated with HIV-1 vertical transmission. Furthermore, we found an association between Th2 type of cytokine expression and LIF production in placenta from TNT tissue. LIF is an important factor for blastocyst implantation and maintenance of pregnancy. LIF was shown to inhibit HIV-1 replication in a tropic independent manner. This inhibition was dependent upon the expression of LIF-Rβ (gp130) on the surface of HIV-1 susceptible cells. LIF upregulation in placenta from TNT HIV-1 infected women support our hypothesis that LIF might be a potent HIV-1 suppressive molecule and may lead to the development of new anti-HIV-1 treatment.

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