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Characterisation of CD8 T Cells in Mucosa Associated Lymphoid Tissue: Implications for Immune Control of HIV-1 Infection

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To my Family

Abstract

The role of CD8 T cells has been implicated in the partial immune control of HIV-1 infection. Ultimately these cells fail to control the infection and immune deficiency ensues in the absence of antiretroviral therapy. A primary focus of this thesis is the function of CD8 T cells in mucosa associated lymphoid tissue (MALT), a major site of HIV-1 replication, in particular in acute infection. In papers I and II, the expression of perforin in gut associated lymphoid tissue (GALT) was examined as an indicator of the cytotoxic CD8 T cell response during chronic HIV-1 infection and acute SIV infection. Considering the large size of the GALT, and the great number of potential CD4+ target cells for HIV-1, the contribution of CD8 T cells to control of viral replication at this site may be especially important. In paper I, it was found that the vast majority of CD8 T cells in rectal tissue, including HIV-1-specific cells, fail to express perforin at the protein level while perforin mRNA can be detected. However, rectal CD8 T cells express granzyme A, and are also capable of releasing IFN-γ upon stimulation with cognate peptide. The relative absence of lytic effector CD8 T cells in GALT may serve to protect the integrity of rectal mucosa under normal conditions, but might also provide an advantage to HIV-1 and other sexually transmitted viruses. For the examination of perforin expression at well-defined early time points post-infection, experimental SIV infection in rhesus macaques was studied (paper II). Significant increases in perforin protein and mRNA expression in the colon of macaques in acute SIV infection were observed. However, during chronic infection, despite ongoing viral replication, perforin expression returned to levels similar to those detected in naïve animals. These findings demonstrate the presence of a robust perforin-positive response in GALT CD8 T cells during acute, but not chronic, SIV infection. In paper III, a subset of human CD8 T cells was described that expresses the chemokine receptor CXCR5, which enables chemotaxis in response to CXCL13, produced by stromal cells within B cell follicles. CXCR5+ CD8 T cells are enriched in tonsil and found scattered in the follicular mantle and dark zone of B cell follicles. The early effector memory phenotype of these cells suggests that this subset is well equipped to influence B cell differentiation through direct cell-cell contact and through the production of soluble cytokines. In line with this, CXCR5+ CD8 T cells support the survival of tonsil B cells in vitro. This suggests a novel role for MHC class I-restricted CD8 T cells and extends their repertoire of functions. In paper IV, the expression of CD7 on CD8 T cells in HIV-1 infected adults and children was investigated to determine the disease relevance of effector and memory cells distinguished by the level of CD7 expression. It was found that CD8 T cells from HIV-1 infected patients display profound down-modulation of CD7 expression as compared to healthy subjects, and there was expansion of both CD7low and CD7negative effector subsets, in particular in patients with rapid disease progression. Down-modulation of CD7 on CD8 T cells correlates directly with HIV-1 load, and cells specific for HIV-1, EBV and CMV are predominantly CD7low effector cells. Furthermore, recovery of CD4 counts on antiretroviral treatment is associated with reversion of the skewed CD7 profile in CD8 T cells. Thus, effector CD8 T cell subsets distinguished by lowered CD7 expression expand in a manner that correlates with the magnitude of antigenic challenge, and contract in response to successful antiretroviral treatment. In summary, the work presented in this thesis contributes to the understanding of CD8 T cells in MALT and peripheral blood in health, as well as in HIV-1 disease.

List of Publications

This thesis is based on the following papers, which will be referred to by their roman numerals:

I.   Shacklett BL, Cox CA, **Quigley MF**, Kreis C, Stollman NH, Jacobson MA, Andersson J, Sandberg JK, Nixon DF.
     Abundant expression of granzyme A, but not perforin, in granules of CD8+ T cells in GALT: implications for immune control of HIV-1 infection.
     Journal of Immunology 2004 Jul 1; 173(1): 641-8.

II. **Quigley MF**, Abel K, Zuber B, Miller CJ, Sandberg JK, Shacklett BL.
     Perforin Expression in the Gastrointestinal Mucosa is Limited to Acute Simian Immunodeficiency Virus Infection.
     Journal of Virology 2006 Mar 80(6) 3083-3087.

III. **Quigley MF,** Granath A, Andersson J, Sandberg JK.
     CXCR5+ CCR7- CD8 T cells are Early Effector Memory Cells that Infiltrate Tonsil B cell Follicles.
     Manuscript submitted.

IV. Aandahl EM, **Quigley MF**, Moretto WJ, Moll M, Gonzalez VD, Sonnerborg A, Lindback S, Hecht FM, Deeks SG, Rosenberg MG, Nixon DF, Sandberg JK.
     Expansion of CD7low and CD7negative CD8 T cell effector subsets in HIV-1 infection: correlation with antigenic load and reversion by antiretroviral treatment.
     Blood 2004 Dec 1, Vol. 104, No. 12, pp. 3672-3678.

Contents

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
ART	Anti-retroviral therapy
AZT	Azidothymidine
CCR	CC chemokine receptor
CMV	Cytomegalovirus
CXCR	CXC chemokine receptor
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
EBV	Epstein Barr Virus
ELISA	Enzyme linked immunosorbant assay
ELISPOT	Enzyme linked immunospot assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
GALT	Gut Associated Lymphoid Tissue
HAART	Highly active anti-retroviral therapy
HLA	Human Leukocyte Antigen
HIV-1	Human Immunodeficiency Virus-1
HIV-2	Human Immunodeficiency Virus-2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Langerhan cell
LN	Lymph node
LP	Lamina propria
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MALT	Mucosa associated lymphoid tissue
MHC	Major Histocompatibility Complex
MIP	Macrophage inflammatory protein
NK	Natural Killer Cell
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositol 3-kinase
RANTES	Regulated on T cell activation, normal T cell expressed and secreted
SDF-1	Stromal cell derived factor-1
SEA	Staphylococcal Enterotoxin A
SEB	Staphylococcal Enterotoxin B
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SRBC	Sheep red blood cells
STAT	Signal transducers and activators of transcription
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TNF	Tumor necrosis factor
7'AAD	7' Amino actinomycin D
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation

Introduction

Human Immunodeficiency Virus-1 (HIV-1) has caused one of the most devastating epidemics in recorded history. Despite our substantial progress in understanding its transmission and pathogenesis, we are challenged by the harsh reality that now in 2006 the epidemic shows little sign of slowing in a global context. HIV/AIDS is the leading cause of death and loss of productive life years in adults aged 15-59 across the world (1). The virus debilitates the host's immune defense primarily by infecting and depleting a crucial cell in the adaptive immune system, the CD4 helper T cell. The diminishing capacity to replace such cells, combined with a plethora of other insults to the immune system, result in a progressive disintegration of immune defense. The host loses hold both of its immunological history with destruction of memory cells and of its capacity to meet new challenges, as the essential infrastructure of the immune system becomes compromised. Finally, otherwise manageable infections can kill the host. Any intervention that can reduce the burden of HIV-1 infection will have great benefits for health and hence social, economic and cultural stability in many parts of the world. An intervention such as a vaccine or immunotherapy will be more likely to succeed if based on a thorough understanding of disease pathogenesis (2). This thesis has focused on the role of an important effector cell in the partial immune control of HIV-1 infection, the CD8 T cell. Particular emphasis has been placed on the characteristics of CD8 T cells in mucosa associated lymphoid tissue (MALT), the primary site for HIV-1 replication and a common route of infection. By adding pieces to the jigsaw of HIV-1 pathogenesis, together we may soon see a chart that points towards a turning point in this epidemic.

Development of the HIV epidemic

The first medical reports that led to the definition of acquired immunodeficiency syndrome (AIDS) emerged in 1981 in California and New York City when a number of young homosexual men displayed symptoms of rare diseases normally associated with severely immuno-compromised patients (3, 4). The causative pathogen, HIV-1, was identified in the laboratories of Dr. Luc Montagnier at Pasteur Institute in Paris (5) and Dr. Robert Gallo at the National Institutes of Health, USA (6). It was hoped that a vaccine would be available within a short time but despite intensive efforts this remains one of the greatest challenges to the scientific community today. Tragically the virus has gone on to cause a global epidemic with an estimated 40.3 million people living with HIV-1 today and it has been responsible for the deaths of over 25 million people (1). In the advent of an effective vaccine, efforts are aimed at preventing further spread of the disease and to increase access to anti-retroviral therapy (ART). Current estimates show that only a fraction of those who would benefit from ART actually receive it, and this is mainly due to lack of healthcare infrastructure, poverty, and unawareness of their infection. The World Health Organisation (WHO) has recommended that

integration of prevention and care services will be key in reducing stigma, encouraging voluntary testing and raising awareness of prevention in all parts of the world (7).

The source of HIV-1, HIV-2 and SIV

There is persuasive evidence that HIV-1 and HIV-2 entered humans numerous times this century from monkeys, which harbor Simian Immunodeficiency Virus (SIV), as a result of persistent exposure to their blood while butchering them for meat (8). HIV-1 arose from SIVcpz of chimpanzees, while HIV-2, which has approximately 40-60% DNA sequence homology with HIV-1, developed from SIVsm endemic in sooty mangabeys of west Africa (9-12). Zoonoses of retroviruses from non-human primates to humans may be a relatively common occurrence, although a subsequent epidemic of the transmitted virus is fortunately rare (13). The virus that is responsible for the current HIV-1 epidemic has been estimated to have entered the human population between 1915 and 1940 in central Africa (14). A key feature of SIV infections in natural host monkeys is little obvious sign of illness, despite high virus titers and low immune activation (15). This is in sharp contrast to HIV-1 and HIV-2 infection in humans and to SIV infection in non-natural host monkeys (16). The balance of viral replication and immune activation achieved by the chimpanzees and sooty mangabeys is a result of thousands of years of evolving host pathogen interactions (17). However, there have been some recent intriguing descriptions of captive SIVsm+ sooty mangabeys with reduced CD4 T cell counts but with little, or much delayed, sign of illness (18, 19). A comparative analysis of the immune profiles of sooty mangabeys with normal or with reduced CD4 T cell counts should yield important insights into host control of SIV and may teach us valuable lessons about the optimal vaccine strategy for HIV.

Non-human primate models of HIV-1 infection

In 1985, lentiviruses were described in captive macaques that caused a comparable immunodeficiency to HIV-infected individuals (20-22). These animals had likely contracted the virus from natural host monkeys as a result of an experimental procedure or biting each other. It became clear that the infection of non-natural host macaques would become invaluable in transmission, pathogenesis, treatment and vaccination studies (23). Subsequently, the viruses SIVmac251 and SIVmac239 were generated from SIVsm to give pathogenic infection in non-African macaques (24-26). Initially, virus inoculation was performed intravenously but subsequently, models have been developed that mimic the predominant infection routes in humans, such as mucosal infection and multiple low-dose challenge models (27). The development of chimeric simian and human immunodeficiency viruses (SHIVs) that result in virions with the HIV-1 Env protein on its surface, and that is infectious in macaques, has made it possible to better

analyze the pathogenicity of HIV-1 *in vivo* (28). Aside from non-human primate models of HIV-1 infection, Feline Immunodeficiency Virus (FIV) infection of cats has also been used for the screening of new anti-retroviral therapies and vaccine candidates (29). The disadvantages of this model include the fact that FIV is less similar to HIV than SIV and less is known about the immune system of cats. However, the virus can be studied in its natural host and cats are smaller and less expensive to work with than monkeys.

HIV viral life cycle and tropism
HIV-1 is a lentivirus and as such is a member of the family Retroviridae. HIV-1 and HIV-2 are the only known lentiviruses to infect humans. A schematic structure of HIV-1 can be seen in figure 1. This double-stranded RNA virus has a 9.2kb long genome containing 9 genes, encoding for 15 proteins (30). These include three major structural proteins; gag, pol and env, regulatory proteins; rev and tat, and accessory proteins; vif, vpu, nef, and vpr. The greatest variation between isolates occurs in the env region of the viral genome, which encodes for a glycoprotein in the virion membrane. HIV-1 reverse transcriptase lacks proof-reading activity and it has been estimated that a single mutation arises in each new virion (31). This genetic variation has been used as the basis for classification of subgroups of HIV-1 and HIV-2. To date there have been nine HIV-1 subtypes described (A-I) and five subtypes of HIV-2 (A-E). Even within each subclass there is enormous sequence variation as a result of viral mutation, and possibly also due to inter-subclass viral recombination (32). This variation presents a formidable challenge both to the immune system and to molecularly targeted anti-viral therapies and vaccines.
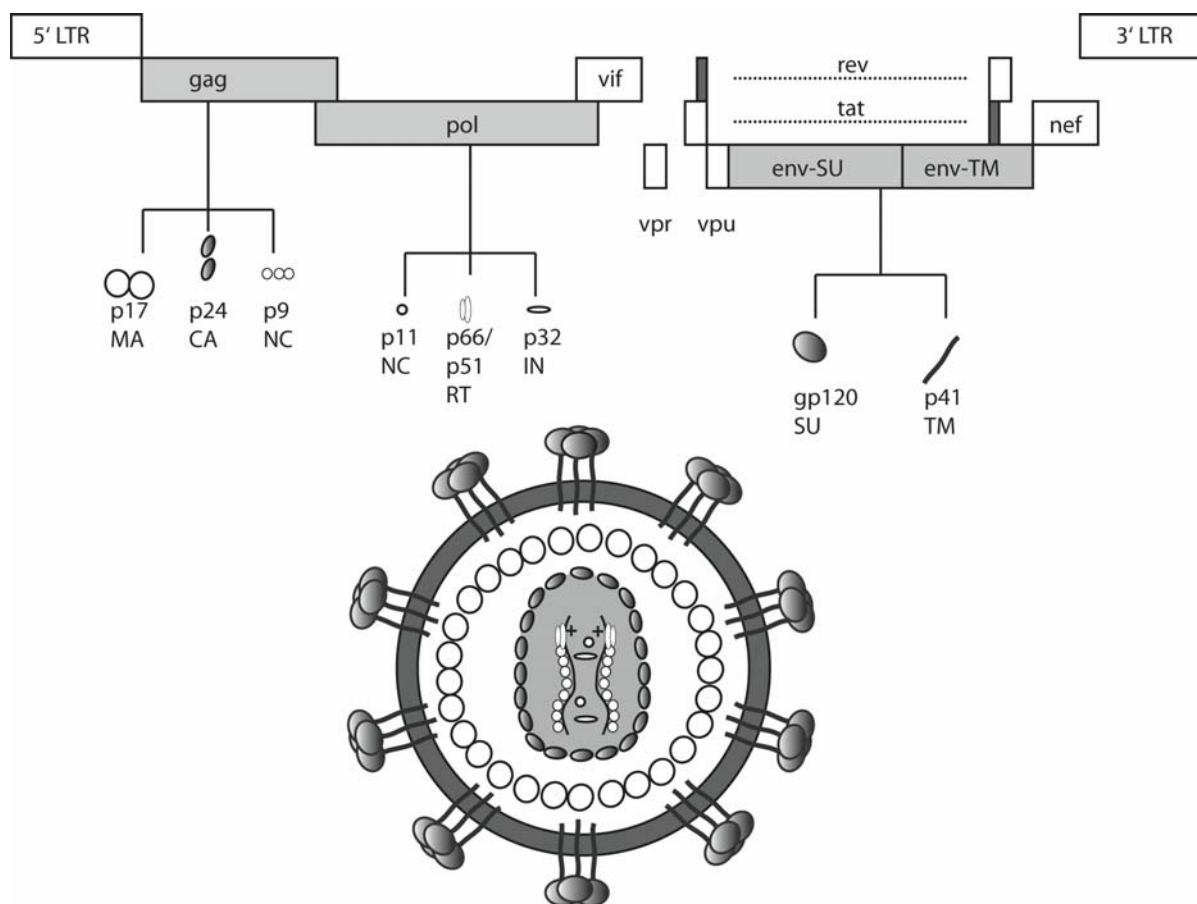
Figure 1. Schematic structure of the genome of HIV-1 and main structural features of the virion. The virus particle is 11nm in width with a lipid bilayer membrane surrounding the nucleocapsid. Gag encodes for the main structural proteins; capsid (CA), matrix (MA) and nucleocapsid (NC). Pol encodes for reverse transcriptase (RT), integrase (IN) and polyprotein processed (PR) proteins. The env gene encodes for the subunit (gp120) and transmembrane region of the virion (gp41). The accessory proteins include viral infectivity factor (vif, p32), negative effector (nef, p24), viral protein U (vpu) and viral protein R (vpr, p15). Regulatory proteins include Rev which encodes for p19, involved in regulation of viral gene expression and Tat which encodes for p14, involved in activating transcription. Neither the regulatory or accessory proteins have been detected in virion particles. Figure adapted from (33, 34).

HIV-1 enters a host cell when gp120 on its envelope binds to the cell surface glycoprotein CD4 (35). A conformational change is induced in gp120 and it subsequently binds to a chemokine receptor (36). This aids fusion as chemokine receptors are contained within lipid rafts that are of similar composition to the lipid-bilayer of the virion envelope (37). CXCR4 and CCR5 are the principle co-receptors for HIV-1. The usual function of chemokine receptors on the surface of cells is to guide their migration through blood and tissues along a chemokine gradient. *In vitro* experiments showed that the natural chemokine ligands for these co-receptors, RANTES, MIP-1α and MIP-1β for CCR5 and SDF-1 for CXCR4, could block the entry of HIV-1

virions into cells. This was achieved by a combination of out-competing the virus and by causing chemokine receptor down-regulation from the cell surface (38). It is not clear if higher levels of β-chemokines afford any protective effect *in vivo*. They may exert similar effects to the *in vitro* experiments. However, beta-chemokines may also mobilize HIV-susceptible cells leading to increased infection rates, in a 'double-edged sword' fashion (39). Interestingly, HIV+ individuals who have a 32 base pair deletion mutation in their CCR5 gene have slower disease progression (40). It has been speculated that the *Yersinia pestis* plague during the 14th and 15th century may have contributed to the increased frequency of this allele in European populations as CCR5 is also a principle receptor for this bacteria (41).

Upon successful fusion, the virus sheds its nucleo-capsid, and the viral enzyme reverse transcriptase transcribes viral RNA into DNA. Viral DNA then enters the nucleus and is integrated into the host genome by viral integrase. Some copies of viral DNA may remain integrated in the host genome for extended periods of time without being transcribed. In this manner the virus can remain hidden from the immune system and anti-retroviral therapies. When transcription does occur, the resulting viral structural and enzymatic proteins traffic to the plasma cell membrane, where viral protease cleaves and alters their structure. Finally the virus assembles within cholesterol-rich lipid rafts at the surface of the host cell and the new virion buds from the cell membrane (42).

HIV transmission between hosts
HIV-1 can be transmitted between hosts in a limited number of ways and, with awareness, transmission is highly preventable. In a majority of cases, the virus is passed across mucosal surfaces during sexual contact. Other modes of transmission include transfusion of infected blood and blood products, sharing of infected needles during intravenous drug use, and mother to child transmission (vertical transmission) during pregnancy, birth or breast-feeding. During sexual transmission, susceptibility of the host to infection varies with respect to the viral load of the infecting bodily fluid, availability of HIV-target cells, the presence of genital lesions or sores, the replication capacity of the infecting virus and the host's genetic makeup (27, 43). In women, the risk of transmission depends additionally on the hormonal cycle's influence of the vaginal mucosa thickness. Notable is the growing trend of increased infection of women in sub-Saharan Africa, mostly due to heterosexual contact, reflecting the greater natural susceptibility that women have to the infection (44). So it is clear that an understanding of immune responses in the mucosa is highly relevant to understanding HIV-1 transmission and consequent disease pathogenesis as a whole.

HIV dissemination within the host

Much of our insight into HIV dissemination has been garnered from the SIV model of mucosal transmission (45-47) and ex-plant tissue studies of HIV transmission across human mucosal tissue (48-52). HIV gains entry to the host in a variety of ways depending on the integrity of the mucosal surface and the frequency and activation state of locally susceptible cells. When the host epithelial surface is disturbed due to abrasions or sores, HIV-1 may have direct access to the primary target cell. In addition, a majority of CD4 T cells in the mucosa is of a memory phenotype that supports HIV-1 replication to a far greater extent than naïve cells. In the case of an intact mucosal epithelial surface, antigen presenting cells (APC) in the mucosa, primarily myeloid dendritic cells (DC) and langerhans cells (LC), trap HIV-1 and initiate infection (53). The principle mechanism involves high affinity binding of the gp120 subunit of the virion's envelope to a C-type lectin on the surface of immature DCs. The best characterized such C-type lectin is DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) (54). This binding however is not sufficient to trigger fusion of the virus. Rather it becomes internalized into endosomes in the DC, and can appear on the surface when the mature DC arrives in a draining lymph node and presents antigen to T cells (55). In this manner, the DC acts as a Trojan horse, ferrying infective virus to a site rich in HIV-1 target T cells. DCs themselves can also become infected, as many express CD4 and CCR5. However, the rate of viral replication is relatively low and it is thought that they contribute to establishment of the infection mainly by delivering virus to more susceptible T cells. Plasmacytoid DCs have also been shown to be susceptible to HIV-1 infection and may be a potential amplifier of virus in early infection (56). Binding of gp120 to DC-SIGN however does not appear to be critical for infection, as Rhesus macaques become infected with SIV irrespective of DC-SIGN expression (57) and a number of other C-type lectins, such as langerin on LCs, have been shown to also have an important role in HIV infection (58).

Recent studies in rhesus macaques have broadened our understanding of the very early events following SIV intravaginal infection (47). Within hours, the virus sets up a focus of local replication in CD4 T cells, macrophages and DCs in the subepithelial mucosa. Should sufficient replication-competent virus enter local draining lymph nodes by days 1-4, the virus replicates further in the numerous local target cells. Subsequent trafficking of free virus and SIV+ CD4 T cells to the bloodstream and gastrointestinal mucosa cements establishment of the infection, already by day 6-7. The sheer concentration of SIV target cells makes it virtually impossible for the host immune system to eliminate all infected cells. By this stage, the virus has managed to replicate sufficiently to mutate common neutralizing antibody and T lymphocyte epitopes (59, 60). The virus operates at a "too much, too fast" mode while the immune system apparently struggles to manage a "too little, too late" effort.

The virus can also infect a wide range of other cell-types, including kidney cells, microglial cells in the brain, and retina cells, but the incidence has not been fully characterized and is likely to be very low (61). Retention of HIV virions within follicular dendritic cell networks ensures that the adaptive immune system cannot detect the presence of the virus (62). In this manner, HIV hides from the immune system, a central mechanism in pathogen survival (63-66).

Apoptosis of lymphocytes in HIV-1 infection
One of the central and most complex questions in HIV pathogenesis research is what causes the depletion of CD4 T cells. There are three principle ways in which loss of T cells is evoked; 1) direct infection and over-expression of viral genes induce lysis of the host cell, 2) bystander killing of uninfected cells by viral pro-apototic proteins, and 3) killing of infected cells by HIV-specific CD8 T cells or antibody-dependant cell-mediated cytoxicity (ADCC). It is thought that during acute infection the majority of CD4 T cells that are depleted have been infected (67) and HIV-specific CD4 T helper cells have been reported to be more susceptible to apoptosis, likely as a result of becoming infected when recognizing infected DCs presenting HIV antigens (68). Nevertheless, in later stages of infection the number of productively infected cells appears to be relatively low, yet there is ongoing destruction of CD4 T cells and bystander killing is thought to be a significant contributor to the depletion of these lymphocytes (69). Mechanisms include increased expression of the death receptors CD95L and tumor necrosis factor-α (TNF-α)-receptor, thus enhancing susceptibility to FAS and TRAIL-mediated apoptosis respectively (70, 71). The HIV proteins env, tat and nef have been shown to cause the upregulation of CD95 on the surface of uninfected cells (72-74). However, considering that the virus' usage of a particular chemokine is a critical factor in disease progression (75), it is likely that direct viral lysis plays a key role in CD4 T cell depletion at all stages of disease.

Treatment of HIV-1 infection

When HIV-1 was identified as the cause of AIDS in the early 1980s, it was hoped that a vaccine would be developed within a short time and the virus would no longer be such a serious public health threat. Unfortunately, attempts to produce an effective HIV-1 vaccine have failed to date. While treatment of HIV-1 infection will not eliminate the virus, it brings significant improvements in the quality of life for the patient, such as a better-preserved immune system and prolonged survival. In addition, it may reduce HIV-1 transmission rates within a population as low viral load reduces the infectivity of the patient's body fluids.

The first antiretroviral drug, a nucleoside analogue called azidothymidine (AZT), was approved for use in 1987. However within one year AZT resistant HIV strains were reported. A study in 1991-1992 showed that the treatment of HIV-1 infected pregnant mothers with AZT for 4 weeks prior to birth reduced mother to child transmission from 30% to 8% and this represented a major clinical break-through. In 1995 and 1996, two new classes of antiretroviral drugs were launched, protease inhibitors and non-nucleoside analogues. By combining each of the three classes of available drugs, patients had a therapy that gave a high degree of viral suppression and reduced appearance of resistance mutations. This combination therapy was termed HAART, for highly active anti-retroviral therapy. It brought the hope that the virus could be eliminated from the infected individual with prolonged therapy. However, while this did not transpire, HAART enabled HIV-1 to be managed clinically as a chronic infection. From an immunological perspective, it gave important insights into the immune response to HIV-1 in the light of reduced antigen stimulation in the host (76, 77). In the late 1990s came reports of side-effects such as lipodystrophy, hypoglycemia and increased incidence of coronary infarctions in individuals on long-term ART (78). Current treatment regimens aim to suppress viral replication in a balance between avoiding development of resistance mutations in the virus and minimizing undesirable side-effects in patients. The arrival to the market of the fusion inhibitor T20, licensed in 2004, heralded a new drug target. The promising pipeline for integrase and novel reverse transcriptase inhibitors may soon make treatment optimisation more efficient (79).

Recommendations of when to initiate ART vary from country to country. Generally when infected subjects reach a level of 200-350 CD4+ cells per microliter of blood therapy is started. If viral load is high, treatment may commence at higher CD4 counts. Recent papers have made it clear that there is a continual ongoing destruction of CD4 T cells in mucosal tissues, even when this is not obvious in blood (80). Perhaps initiation of ART based on CD4 T cell depletion in the mucosa rather than blood would show greater clinical benefit, although this is a more invasive and costly measure. It is

estimated that only a small proportion of infected individuals who would benefit from ART have access to it today (1). The WHO initiative to bring ART to 3 million people by the end of 2005 showed much success, despite coming short of this goal. With the production of patented drugs at reduced cost, many of the world's poorer countries have been able to offer ART to their HIV-infected citizens. This is a clear investment in the future health of a country as Brazil illustrated in the early 1990s.

Background biology of T cells

It could indeed be relevant to here give an overview of the entire human immune system. Alas, due to space and time constraints, I here focus my attention on the biology of T cells. This is important background for the understanding of CD8 T cell effector function and differentiation, and the scope of HIV pathogenesis can be better understood when one appreciates the central role of CD4 T cells in the immune system.

Many developmental and activation pathways are shared between CD8 T cells and CD4 T cells. However, their recognition of targets and execution of effector functions are different. Each T cell bears an estimated 30,000 identical antigen receptors on its surface, known as the T cell receptor (TCR). This receptor is composed of two heterodimeric polypeptide chains linked by a disulphide bond. The majority of T cells have a TCR composed of an alpha and a beta chain but a minority has a TCR made up of a gamma and a delta chain. The TCR features a variable region involved in antigen recognition, a constant region, a hinge region, a transmembrane region and a short cytoplasmic tail that requires other adaptor proteins to mediate intracellular signals when the T cell recognizes antigen. TCRs recognize antigenic peptides that are bound and presented by endogenous major histocompatibility complex (MHC) molecules (81, 82). The concept of 'MHC restriction' of T cells was pivotal for the understanding of how T cells are selected in the thymus and how they recognize antigen, and its discoverers, Rolf Zinkernagel and Peter Doherty, were awarded the Nobel Prize in Medicine in 1996. The genes that encode MHC molecules are the most polymorphic in the human genome and this allows scope for presentation and recognition of a very diverse set of peptides at the population level. Each individual also carries several different MHC class I and class II molecules which enables a diverse set of peptides to be targeted within a given host (83).

T cells undergo positive and negative selection in the thymus

T cells develop from lymphoid progenitor cells originating in the bone marrow but they mature in the thymus. There they rearrange their TCR genes in a reaction catalyzed by an endonuclease encoded by recombination-activating genes (RAG)-1 and 2 (84) and undergo the processes of positive and negative selection, reviewed in (85, 86). In brief, T cells which weakly recognize self-peptides presented by MHC class I and II molecules on epithelial cells are given survival signals. In this way, a T cell repertoire is developed that inherently recognizes a diverse set of endogenous antigen-presenting MHC molecules. T cells that strongly recognize self peptide-MHC (pMHC) complexes are eliminated, as subsequent effector function mediated against self-antigen is undesirable. The approximate 1-5% of thymocytes that survive and leave the thymus are termed naïve T cells and they circulate through the blood and lymphatic system in search of antigen that matches the specificity

of their TCR. As there are so few naïve T cells with the same TCR specificity, to increase their chances of meeting cognate antigen the adaptive immune system uses lymph nodes (LN) as meeting points for naïve T (and B) cells and APCs. T cells enter LN by traversing through thin-walled blood vessels called high endothelial venules. It is thought that most of the body's lymphocytes are recirculating and spend only about 30 minutes outside some lymphoid tissue before re-entering again (87).

Antigen presentation by MHC class I molecules
All nucleated cells express MHC class I molecules on their surface and their role is to present short peptide samples to their external environment. In this way, they can 'advertise' to other cells which proteins and peptides are currently in their cytosol, be they foreign or not. The peptides that bind MHC class I molecules are generated during the normal breakdown of all cellular proteins. This is mostly carried out by the proteasome, a large multicatalytic protease complex that degrades cellular proteins that have an ubiquitin sequence tag, and also mis-folded proteins that have been returned from the endoplasmic reticulum (ER). Peptides of 8-10 amino acids in length are optimal for binding to most MHC class I molecules, although longer peptides may also be presented (88). The final refolding of the MHC class I heavy chain, beta-2 microglobulin and peptide occurs in the ER with the carefully orchestrated help of a number of chaperone proteins. These include calnexin, tapasin, Erp57, and transporters associated with antigen processing (TAP) (89). Interestingly, expression of each of these proteins can be up-regulated in response to interferon (IFN) secreted by virally infected cells (90). The pMHC complex is stably expressed on the surface of the cell as long as the peptide remains bound and for this reason the vast majority of peptides presented by MHC class I are of intracellular origin.

Antigen presentation by MHC class II molecules
MHC class II molecules are expressed on the surface of APCs, and minimally on other cells. The peptides that bind MHC class II molecules are derived from proteins that have been phagocytosed or pinocytosed by the APC. These proteins are degraded by proteases such as cathepsin S and L within acidic endosomes. Newly synthesized MHC class II molecules are transported to the endoplasmic reticulum where they form a complex with the MHC class II invariant chain which binds to the peptide-binding groove and thus peptides from the ER are prevented from binding. The MHC class II invariant chain molecule then directs this complex to an endosomal compartment known as the MHC class II compartment (MIIC). Proteolytic degradation of the invariant chain in endosomes leaves the class II-associated invariant peptide (CLIP) in the peptide-binding groove. MHC class II–CLIP heterotrimers interact with a nonpolymorphic MHC-like dimer, human leucocyte antigen (HLA)-DM, which catalyses the dissociation of CLIP from the class II

molecule. Peptides from endocytosed or phagocytosed material are then loaded on to the now empty MHCclass II alpha/beta dimer. Peptide binding requirements for MHC class II molecules are less stringent as compared to MHC class I molecules, and peptides of 10-20 amino acids in length can be presented as they can lie within the rather open peptide-binding groove with the ends hanging out.

Cross-presentation of peptides can occur when antigen from the endocytic pathway becomes loaded onto MHC class I molecules and can be presented to CD8 T cells. DCs are particularly competent at this, and it is thought that the antigen arises from ingested apoptotic bodies and immune complexes (91). This is an important mechanism by which the immune system can direct CD8 T cell responses towards extracellular antigens. Production of type I interferons by a virally infected cell has been shown to increase the rate of cross-presentation (92) and there is evidence to show that DCs can cross-present HIV peptides on MHC class I molecules (93).

Recognition of antigen by T cells

When a T cell recognizes a pMHC complex, a signaling cascade is mediated by the TCR and its associated CD3 and co-receptor molecules, CD4 or CD8, that together initiates activation of the cell. Initial recognition of antigen or priming usually occurs in a lymph node to which professional APCs transport antigen from the site of infection. These APCs have high expression of costimulatory molecules required for activation of the T cell. The formation of the so-called immunological synapse is critical for sustained activation of the T cell and has been reviewed in detail elsewhere (94-96). Activation of a naïve T cell is referred to as 'priming', while activation of a memory T cell can be called 'boosting'. The result of this process can depend on a variety of factors, including what cell type has presented the antigen, and where and when the presentation is taking place (97). Co-stimulatory signals are particularly important as T cells that bind pMHC but fail to receive sufficient co-stimulation are liable to become 'anergic' or undergo apoptosis. Such co-stimulatory molecules include CD80 and CD86 on the APC, which bind CD28 on the T cell. Figure 2 shows the chief components involved in the formation of the immunological synapse in CD4 and CD8 T cells. Recent insights from intravital microscopy of mouse LNs have shown that T cells circulate rather rapidly through the paracortical region of the LN scanning a large number of DCs. When a pMHC complex is recognized, the T cells lose momentum and remain in a complex with the DC for a number of hours (98). Activation of a naïve T cell induces a program of intense proliferation within the LN that gives rise to many daughter T cells of identical TCR specificity in a process known as 'clonal expansion'. The differentiating T cell begins to express chemokine receptors and adhesion molecules appropriate for trafficking to the effector site and gene expression profiles appropriate to carry out effector functions. A certain number of the activated T cells will become memory

cells, and remain long-lived in the host providing more rapid response to antigen upon reencounter (99, 100). The understanding of T cell memory development is pivotal for the fine design of effective vaccines.
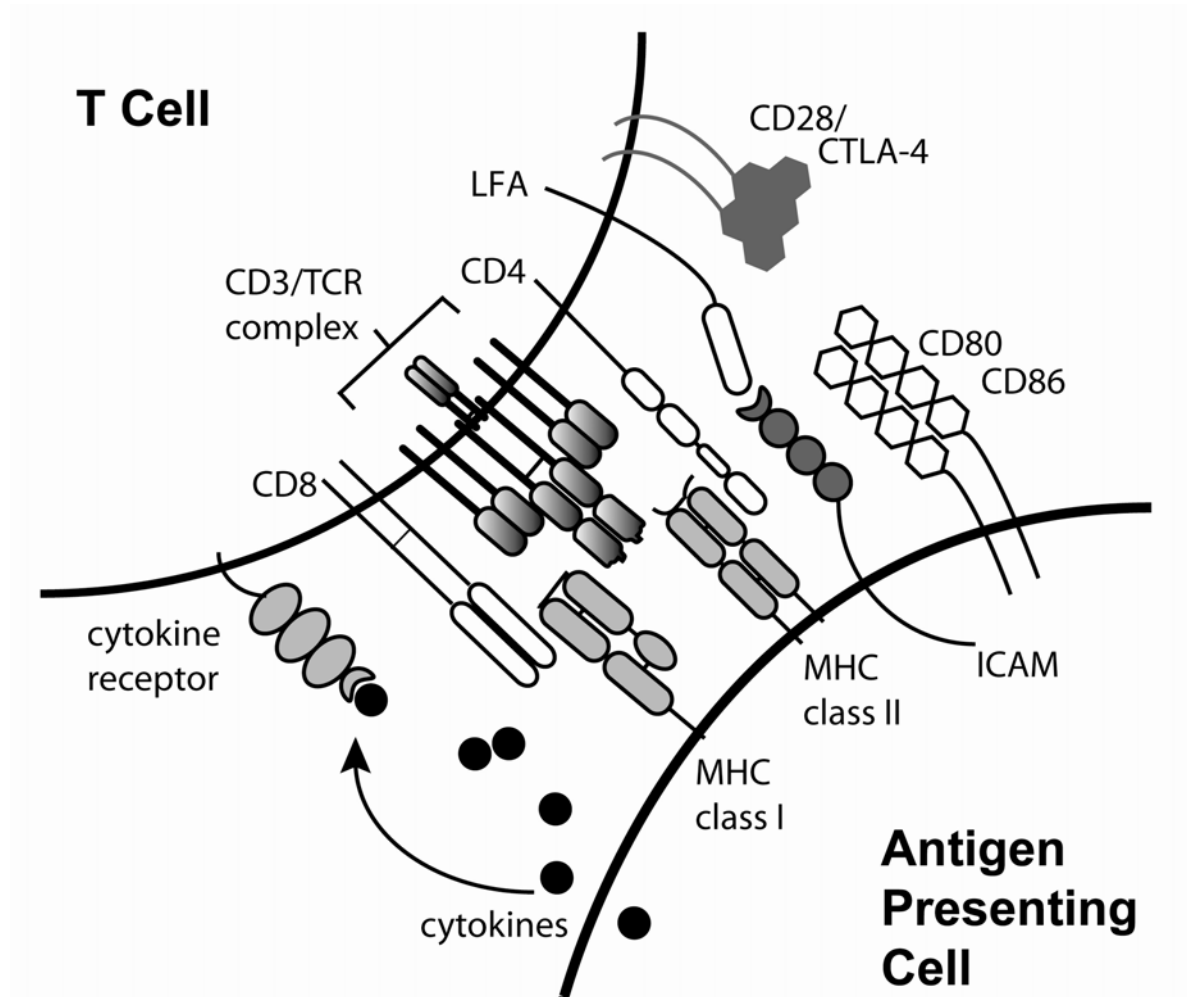

Figure 2. T cell recognition of antigen. In the case of a CD8+ T cell, the TCR binds to a MHC class I molecule and the α and β domains of the CD8 molecule bind to the α3 domain of the MHC class I molecule. In the case of a CD4+ T cell, the TCR binds to a MHC class II molecule and the D1 domain of the CD4 molecule binds to part of the β2 domain on the MHC class II molecule. This binding may be further stabilized by binding of lymphocyte function-associated antigen-1 (LFA-1) on the T cell and intercellular adhesion molecules (ICAMs) on the APC. In the presence of correct costimulatory molecules, signal transduction through the TCR is modulated by the CD3 molecule δ,ε,ζ, and γ subunits. The subsequent activation of a series of kinases, including Lck and protein kinase C, leads to activation of transcription factors, such as NF-κB, that govern expression of genes encoding for T cell effector proteins.
Diagram adapted from (94).

MHC class I tetramers

MHC class I tetramers are recombinant protein reagents designed to mimic the specific MHC-class I peptide complex that the T cell recognizes (101). By labeling T cells with a tetrameric pMHC it is possible to identify T cells of a defined specificity. The addition of a fluorescent label allows the cells to be detected by flow cytometry (102) and fluorescence microscopy (103). This technique revolutionized characterization of antigen-specific T cell populations following its development in the mid-nineties at Stanford University. Previously, these cells were identified using a read-out of their function, such as ELISPOT and chromium release assays. Such assays often underestimated the frequency of antigen-specific cells and made implicit assumptions about their functional capacity. MHC class II tetramers have also been developed (104), and used in a variety of immunological studies (105, 106). They are technically more challenging to produce, as the MHC class II complexes are less stable than those of class I. However, improvements are ongoing and MHC class II tetramers will continue to make a major contribution to the field of cellular immunology (107).

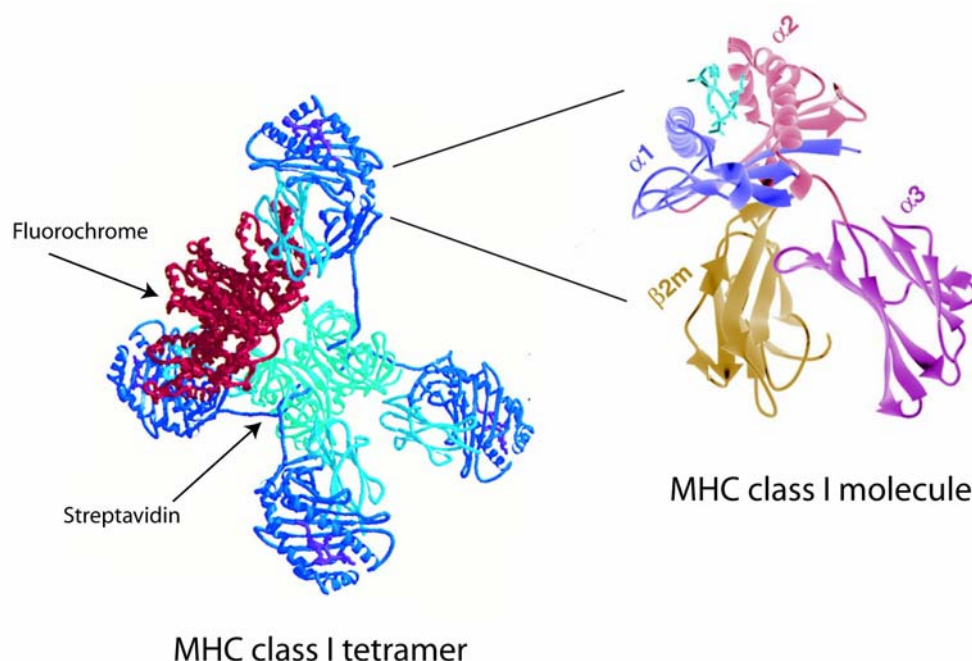Figure 4. Schematic outline of an MHC class I tetramer and an MHC class I molecule. The tetramer is composed of four pMHC complexes which each have been engineered to have a biotinylation site in the tail of the heavy chain and are linked by streptavidin. The crystal structure of MHC class I molecule was elucidated in 1987 by Bjorkman and colleagues and greatly improved our understanding of T cell recognition of target cells (82).

Virus	Viral Protein	Protein	Amino acid Position	Amino acid Sequence
HIV	gag	Envelope	77-85	SLYNTVATL
HIV	pol	RT	476-484	ILKEPVHGV
EBV	BMLF-1	Lytic	280-288	GLCTLVAML
CMV	pp65	Envelope	495-503	NLVPMVATV
Influenza	M1	Matrix	58-66	GILGFVFTL

Table 2. The HLA-A0201 tetramers used in our studies detected T cells specific for these viral peptides. As HLA-A2 is the most common haplotype in Caucasians, we increased our chances of being able to identify antigen specific cells in our study subjects.

Effector functions of CD4 T cells

CD4 T cells play a central role in directing and regulating many processes in the adaptive immune response. They recognize peptide antigens presented by MHC class II molecules on antigen presenting cells and B cells. In the mid-1980s a new concept was developed using a mouse model that defined CD4 T helper populations based on their cytokine secreting profile. Upon recognition of specific antigen and activation of naïve CD4 T cells in the lymph nodes, a developmental decision is made to either become a helper T cell type 1 or type 2 (108). In this manner, the CD4 T helper cell focuses to favor the development of immune responses for intracellular or extra-cellular pathogens, respectively. The signals that instruct the CD4 T helper cell along its developmental path include duration of signal from the priming APC (109), IFNγ from the innate natural killer (NK) cell, choice of co-stimulatory molecule involved in activation and probably also other uncharacterized signals as of now. While these strict functional divisions are not as clear-cut in humans, the model has also been helpful in the study of several human immune responses. CD4 helper T cells secrete cytokines and express co-stimulatory molecules to promote proliferation and isotype switching in B cells, to support differentiation of effector and memory CD8 T cells, and to promote phagocytic activity in macrophages. Cytolytic CD4 T cells have also been described in humans (110) and appear to be expanded in chronic viral infections, such as HIV-1 and Epstein-Barr virus (EBV) (111-113). As CD4 T cells have such a diverse range of helper functions within the immune system, their loss in HIV disease impacts the development of many cellular immune responses.

Effector functions of CD8 T cells

CD8 T cells display considerable functional heterogeneity (114), and choice of effector function varies between response settings (115). Induction of apoptosis in target cells is one of their most important effector functions. CD8 T cells can degranulate to release pro-apoptotic enzymes, including granzymes and granulysin (116) and antiviral chemokines (117). Granule-mediated cytotoxicity is further described below. Cell surface bound members of the pro-apoptotic TNF receptor superfamily of proteins include CD95ligand and TNF-related apoptosis inducing ligand (TRAIL) and their ligation can induce death in target cells via activation of intracellular caspases. CD8 T cells can also secrete potent amounts of the antiviral cytokine IFN-γ, which can block viral replication and cause up-regulation of MHC molecules on the surface of an infected cell. IFN-γ can also activate macrophages, NK cells and promote isotype switching in B cells. CD8 T cells are an important source of interleukin-2, which promotes the proliferation of T cells and NK cells, and of TNF-β which is important in the activation of macrophages.

Granule-mediated cytotoxicity: the essential role of perforin

The coordinated expression and targeted release of perforin and granzymes by CD8 T cells is one of their most important mechanisms of eliciting cytotoxicity. This has been appreciated by evidence from perforin knock-out models of viral infection in mice (118) and the rare cases of defects of the human perforin gene, such as in familial haemophagocytic lymphohistiocytocis (FHL) (119). Granule-mediated induction of apoptosis involves irreparable damage to target cell DNA that brings about death of the cell. This process can be dependent on the activation of cellular caspases by granzyme B, or by caspase-independent interference with target cell DNA repair, mediated by granzyme A. This is a complex biochemical process that has recently been reviewed in detail (120). Upon recognition of the target cell, granules in the cytosol polarize towards the secretory domain of the immunological synapse. Granzymes constitute up to 90% of the granule, and together with perforin and carrier proteins, they are capable of inducing apoptosis in target cells. In humans, granzymes A and B are the most abundant and commonly expressed, but granzymes H, K, and M have also been described (121). These are all serine proteases capable of cleaving their substrates at highly specific sites. The granule-secreting cell is protected from self-destruction by cathepsin B on its surface (122). This targeted release of the contents of the granules ensures that damage to nearby cells is minimized. However, in the case of intense immune activation, such as HIV-1 infection and rheumatoid arthritis, some granule contents leak out and can be measured in plasma (123). If this has any pathological consequence has yet to be determined, but there have been some studies suggesting that free granzymes may have pro-inflammatory effects (120, 121).

The exact mechanism by which perforin mediates the action of granzymes is still under investigation. It had been thought that the main mechanism was via formation of pores in the target cell membrane to allow entry for granzymes, but this model has recently been subjected to revision (124, 125). Recent evidence suggests that the mannose 6-phosphate receptor can take up both granzymes and perforin from the surface of the target cell. Perforin then mediates release of the granzymes from target cell endosomes and initiation of apoptotic pathways. This mechanism is the subject of debate but it has been repeatedly shown that perforin is critical for granzyme-mediated apoptosis (120, 126). Granule mediated cytotoxicity is in particular a hallmark of natural killer cells (127), but can also be exhibited by other cells of the immune system such as neutrophils (128), T regulatory cells (129), CD4 T cells (110), and γδ T cells (130).

CD8 T cell differentiation pathways

From its exit from the thymus as a naïve CD8 T cell, to its targeted execution of effector function and formation of a memory compartment, there are many complex steps in the development of virus-specific CD8 T cell responses. CD8 memory T cells display diverse functional capabilities and it is not clear if these cells follow a linear pathway of development. The process is influenced by interactions with many other cells and products of the immune system, by the precise location of the CD8 T cell and the nature of the pathogen that the cells respond to. There are significant gaps in our understanding of this process, and in particular when it comes to the intricate scenario of chronic viral infection. Several models have been proposed to describe the development of effector and memory CD8 T cells in humans.

Sallusto and Lanzavecchia's model has placed emphasis on the lymphoid tissue homing profile of CD8 T cells (131). Expression patterns of the chemokine receptor CCR7 and the L-selectin CD62L can segregate CD8 T cells with 'effector memory' and peripheral tissue homing capacity from 'central memory' cells that circulate mainly through lymphoid tissue ready to respond to recall antigen. Appay et al have incorporated varying expression levels of the costimulatory molecules CD27 and CD28 to divide CD8 T cells into 'early', 'intermediate' and 'late' effector cells (132). In the setting of HIV-infection, Champagne and colleagues have used the expression profile of CCR7 and the T cell surface tyrosine kinase CD45 isoform to describe CD8 T cell subsets of antigen specific T cells as 'pre-terminally differentiated' or 'terminally differentiated' (133). Numerous other molecules have been identified and postulated to describe differentiation stages of T cells. These include cytokine receptors, adhesion molecules, enzymes and co-stimulatory molecules. Table 1 lists a number of these molecules, many of which were also used in studies in this thesis. To be able to phenotype CD8 T cells in a manner that would give reliable insight into their activation history and effector capacity is critical for a thorough evaluation of HIV vaccine-induced immune responses (2). Multi-color flow cytometry will be increasingly helpful in this regard and will raise the threshold of useful information available from a limited human tissue sample (134).

Molecule	Expressed on	Ligand	Function	Model
CD7	Hematopoietic cells, Thymocytes, T cells	Galectin-1 and PPI 3-kinase	Co-stimulation and induction of apoptosis	Aandahl (135)
CD27	T, NK cells, B cell subsets	CD70	Co-stimulation	Appay (132)
CD28	T cell subsets, Activated B cells	CD80, CD86	Co-stimulation	Appay (132)
CCR7	T, NK, B cell subsets, DCs	CCL19 CCL21	Chemokine receptor	Sallusto (131)
CD38	Activated T cells, plasma and germinal center B cells	-	NAD glycohydrolase	(136)
CD45RA /RO	T cells, B cell subsets, monocytes, macrophages	-	Tyrosine phosphatase isoforms	Champagne (133)
CD62L	T, B, NK cells, monocytes	GlyCAM (CD34)	Selectin	Sallusto (131)
CD127	T cells, pro-B cells, monocytes	IL-7	IL-7 receptor	(137, 138)

Table 1. Surface receptors used in CD8 T cell differentiation models.

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The CD7 based model of CD8 T effector cell differentiation
A new model of CD8 T cell effector differentiation was proposed by Aandahl et al in 2003 (135). It combined the varying expression levels of CD7, a cell surface molecule found on most T cells in the periphery, with other phenotypic and functional markers, to distinguish between up to four distinct CD8 T cell effector subsets as well as naïve and memory cells. An outline of their proposed model is shown in figure 3. This was one of the first models of CD8 T cell differentiation to give such a detailed description of multiple effector subsets. CD8 T cells with low CD7 expression could be segregated into cytokine producers or lytic effectors, as could CD7neg cells, although in addition CD7neg cells were less sensitive to apoptosis. CD8 T cells with high expression of CD7 were suggested to contain both naïve and memory cells, based respectively on their presence in cord blood and that CD8 T cells specific for a resolved viral infection expressed high levels of CD7.
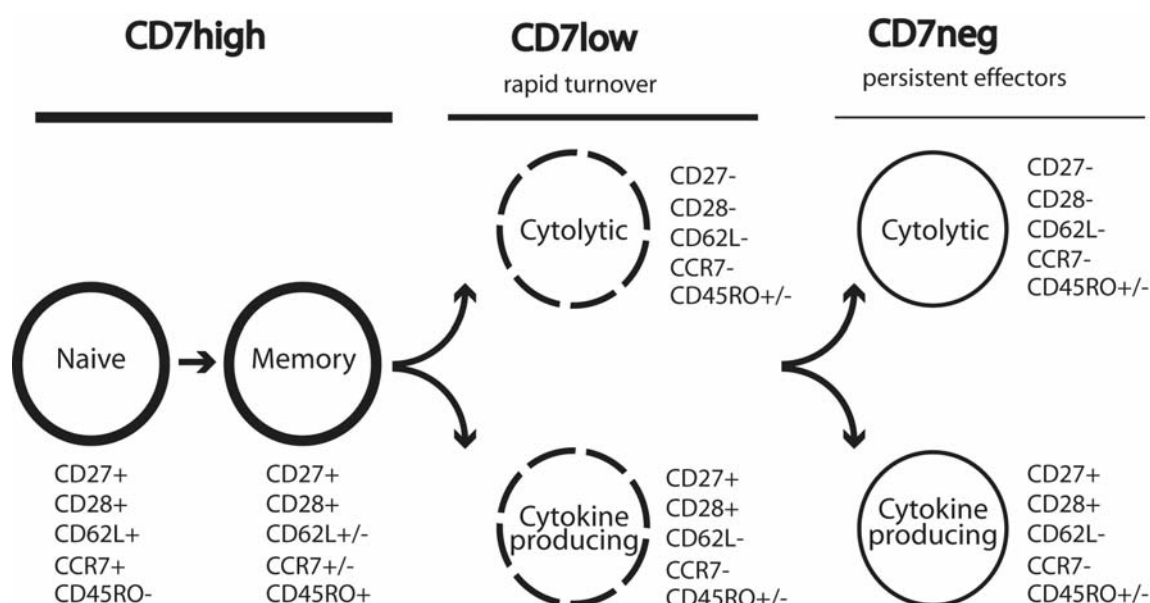

Figure 3. The CD7-based model of CD8 T effector cell differentiation.
Adapted from Aandahl et al (135).

The function of the CD7 molecule can give insight into its utility as a phenotypic marker. CD7 is a 40kDa transmembrane glycoprotein and is part of the immunoglobulin super-family. It is found on all T cells early in ontogeny and expression is retained on many T cells in the periphery. Dual roles have been described for CD7, in the induction of apoptosis and in co-stimulation. One ligand for CD7 is a beta-galactoside-binding lectin called

galectin-1. Galectins are a large family of endogenous glycoproteins widely expressed in various tissues (139). They induce apoptosis in activated lymphocytes by binding through cell surface oligosaccharides, including CD7. It has been reported that increased glycosylation of T cells in HIV-1 infection results in increased apoptosis mediated by binding to galectin-1 (140). The observation that CD7neg T cells are more resistant to apoptosis may may be explained by the fact that cell death cannot be initiated through the CD7 pathway (141). In its role as a co-stimulatory molecule, CD7 activates a lipid kinase called phosphatidylinositol 3-kinase (PI3K) (142). PI3K plays a role in many TCR signaling pathways and it is possible that low expression of CD7 is a consequence of prior signaling through CD7 and as such signaling through PI3K is part of effector cell differentiation.

The role of CD8 T cells in immune control of HIV-1 infection

Do CD8 T cells play a role in the partial control of HIV-1 infection? As previously described, by the nature of their recognition of the intracellular contents of cells, CD8 T cells are uniquely equipped to respond to viral infections. It has been suggested that CD8 T cells play an important role in the clearance or control of a number of viral infections (143) including influenza (144), human cytomegalovirus (CMV) (145), measles (146), herpes simplex virus (147), hepatitis C virus (148) and EBV (149). A number of lines of evidence support the notion that CD8 T cells play an important role in HIV-1 immune control:

1. The virus tends to rapidly mutate genes encoding for amino acid residues important for CD8 T cell recognition, indicating a strong selection pressure exerted by these cells. This has been demonstrated for acute HIV-1 (150, 151) and SIV infection (152, 153), and for chronic HIV-1 (154). Viral escape from CD8 T cell epitopes resulted in the failure of vaccine-induced immune control in SIV (155) and mutations in the genes encoding for regions flanking common CD8 T cell epitopes have also been described to contribute to viral escape (156, 157).

2. The observed drop in plasma HIV-1 viral load during early infection coincides with the appearance of HIV-specific CD8 T cells in circulation. (158, 159).

3. Experimental depletion of CD8+ cells using monoclonal antibodies in SIV infected monkeys led to an increase in plasma virus load (160, 161).

4. Long term non-progressors have better CD8 T cell proliferative responses (162-164) and generally target broader CD8 T cell epitopes (165) than rapid progressors.

5. Ex vivo expansion of HIV-specific CD8 T cells clones and readministration to HIV-1 patients led to a temporary decrease in HIV-1 viral load (166).

However, as there have been no documented cases of eradication of HIV-1, it must be concluded that the CD8 T cell response is sub-optimal from the host's point of view. By teasing apart the various contributions of CD8 T cells to HIV-immune control, we get a more concise idea of what type of immune response an effective vaccine or treatment should aim to evoke. Most studies of T cell characteristics in HIV-1 infection are based on cells from blood, as it is plentiful and easy to sample. However, blood contains only an estimated 2-5% of the total lymphocytes (167), and may not reflect the overall state of the CD8 T cell compartment. This thesis examined the characteristics of CD8 T cells in mucosa associated lymphoid tissue (MALT), where the majority of HIV-1 replication occurs (168-172).

The structure and function of mucosa associated lymphoid tissue

Mucosa surfaces are found at all external parts of the body where skin is not present and the structure has arisen to provide a compromise between host needs and protection from the external environment. The MALT can be broadly considered as composed of two main compartments: inductive sites, where antigen is taken up and redirected, and effector sites, where the immune system responds to the mucosal antigen (173, 174). Antigen can be endocytosed by rare epithelial cells called M or micro-fold cells (175). These specialized cells have deep folds in their baso-lateral surface that can accommodate various APCs, T and B lymphocytes and macrophages. Thus the M cells enable other specialized lymphocytes which themselves do not sit in the epithelial surface to get exposure to pathogens. M cells can also be targeted by pathogens for invasion of the mucosal epithelium (176), including HIV-1 (177). Antigen can also be trapped directly by DCs that may be able to extend their dendrites out into the lumen of the mucosa (178, 179). Once antigen is captured, the APC transports it to the draining lymph nodes and T and B cell responses are initiated as appropriate. T cells primed in the mucosa are imprinted during differentiation with expression of trafficking molecules that allow homing to mucosal tissues to ensure that they carry out their effector functions there. For example, mucosal DCs induce expression of CCR9 or $\alpha 4\beta 7$ on T cells in order to redirect primed T cells back to mucosal sites (180). Their ligands are CCL25 (also known as thymic epithelial chemokine or TECK) and MADCAM-1 respectively, both of which are expressed by epithelial cells in the mucosa (181-183) and DCs in the mucosa have a special role in maintenance of tolerance (184). The barrier function of the mucosal surface is enhanced by the presence of highly viscous mucus that is rich in antibacterial peptides and enzymes and contains a high concentration of immunoglobulin A. There are numerous lymphocytes in the epithelial membranes e.g. B cells that secrete large amounts of IgA and intraepithelial T cells that can have immediate effector function.
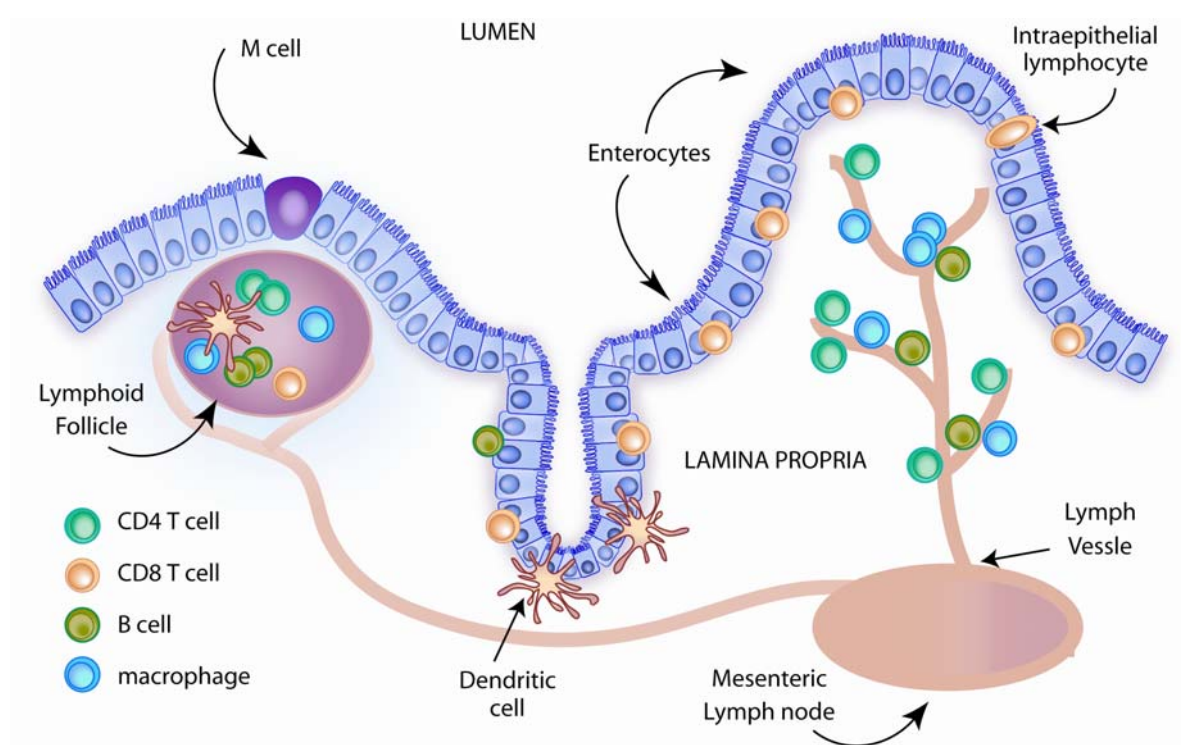
Figure 5. Schematic outline of the structure of gut associated lymphoid tissue.
Adapted from (174) with the kind assistance of Daria Donati.

Tonsil: Early point of contact with the mucosal immune system

Tonsil is an easily accessed mucosal lymphoid tissue that has been valuable in our studies of CD8 T cells. Its primary role is to take up antigen from air and food in the oral cavity, the entrance site to both the respiratory and the digestive system. The structure of a tonsil can be likened to a Peyers patch or to other mucosal lymphoid aggregates. It has a relatively large epithelial surface area (up to $300cm^2$) through the formation of many blind-ending crypts (185). Its surface contains specialized epithelial cells, similar to M cells of the intestine, which take up antigen and transpose it to the luminal side of the tissue (186). Beneath the epithelium lie numerous primary and secondary lymphoid follicles where antigen-specific T and B cells responses develop, similar to processes that occur in LNs. One important difference between a LN and a tonsil (and other mucosal lymphoid aggregates) is that antigen does not enter the tonsil or lymphoid aggregates through afferent lymphatic vessels. However, immune responses to systemic infections can also be initiated in mucosal inductive sites when antigen enters through blood (187). This likely happens in HIV infection, regardless of route of transmission of the virus.

The gastrointestinal mucosa

The gastrointestinal mucosa is extensive in size, with an estimated surface area 200 times the size of the skin (167). It has likely evolved in relation both to the need for optimal digestion of food and liquid and the constant threat of exposure to microbial pathogens. The mucosa surface is rich in a variety of

cell types including mucus-secreting goblet cells, hormone secreting endocrine cells, anti-microbial peptide secreting Paneth cells and absorptive enterocytes (188). In addition, the single layer thick epithelium maintains a barrier between the sterile interior and the pathogen-rich lumen of the gut. Most of the lymphocytes are found in the gastrointestinal mucosa (167). Inductive sites include Peyer's patches, found in the small intestine, and numerous other smaller lymphoid aggregates in the large intestine. The main effector sites include the lamina propria (LP) and lymphocytes in the epithelial lining. The LP contains a variety of cells of the immune system such as T cells, B cells, macrophages, and dendritic cells, as depicted in figure 5. The majority of T cells in the LP are of an effector/effector memory type and very few naïve cells are present. In addition, there are a number of unusual lymphocyte populations. T cells with a γδ TCR constitute up to 10% of gut T cells. These cells recognize antigen restricted by non-classical MHC molecules such as MICA and MICB (189), as well as some glycolipid antigens. They secrete cytokines and can exert granzyme-mediated apoptosis in target cells. It is not known if they are preferentially depleted in HIV-1 infection or how they behave under ART. A minority of lamina propria TCRαβ T cells has a CD8 molecule composed of two alpha chains rather than an alpha and a beta chain. These cells may be restricted by MHC class I, class II or non-classical MHC molecules. In humans, it is not known precisely what they recognize but their expression of CD8αα may be considered more as an indicator of an antigen-experienced phenotype (173).

Massive and early CD4 T cell depletion in gut in HIV-1 and SIV infection
As early as 1998, there were a number of reports on CD4 T cell depletion in the gastrointestinal mucosa in HIV-1 and SIV infection (190, 191). However it was not until examination of the early stages of SIV infection that it was appreciated that the most severe loss of CD4 T cells occurs in the gastrointestinal mucosa (190, 192). After both intravenous and mucosal inoculation of SIV, a dramatic drop in CD4 T cell numbers was seen in gastrointestinal mucosa, before such a loss could be seen in blood and other lymphoid tissues (190, 192). Recently, studies have shown similar findings in humans (67, 80, 193, 194).

There has been much speculation as to why CD4 T cell depletion is so severe in the gut. A major contributing factor is that a majority of T cells in the gut express high levels of the HIV co-receptors CCR5 and CXCR4 and they are present in large numbers (195, 196). In blood, CCR5+ CD4 T cells constitute a minor population of the total CD4 T cells. So while there is also a significant depletion of CCR5+ CD4 T cells in the blood and LNs during acute infection, the overall loss of CD4 T cells is not as striking as in the gastrointestinal mucosa (67). Mucosal lymphocytes are more sensitive to apoptosis and have a higher rate of turnover. As the mucosal immune system is constantly

challenged with antigen, it must be kept in a careful balance, ready to fight off pathogens but at the same time not targeting beneficial microorganisms. Consequently, mucosal CD4 T cells may be more sensitive to HIV-induced lysis.

Prior to the discovery of HIV-1, it had been observed that young adults in Africa died from a condition that was locally called 'slim disease' which gave symptoms of wasting, fever and stomach ache (197, 198). This reflects the extent to which HIV-1 infection manifests itself in the gut and it can harbor many opportunistic pathogens, such as amoeba, cryptosporidium, mycobacterium and CMV. In more advanced disease, morphological abnormalities in the gut are not uncommon, such as atrophy and fusion of the villi (199). While these symptoms may in part be due to the direct effects of HIV infection, reduced immunological protection against other gastrointestinal pathogens is also a contributing factor. Improvements in the sampling of human gastrointestinal mucosa and processing in the laboratory have helped to advance the study of HIV-1 pathogenesis. A rectosigmoid endoscopy, taking up to twenty five pinch biopsies, can yield 1-2 $\times 10^6$ mononuclear cells, sufficient for a number of cellular immune assays (200, 201).

Trafficking of lymphocytes is intimately related to their differentiation status

As the immune system has evolved to protect the integrity of the whole organism, the circulation of lymphocytes is pivotal to the functioning of immune defense. Movement of lymphocytes is governed by fine gradients of chemo-attractants and expression of chemo-attractant receptors on their surface (87). Extravasation from blood and lymph vessels into tissues involves a process of 'tethering' and 'rolling' where circulating lymphocytes lose velocity by loosely binding to selectins on vessel walls and then make more firm attachment to integrins and finally chemokine receptors (202). Once within the tissue, lymphocytes respond to directional migration and finite positioning in relation to each other. In general, chemokines are secreted molecules, and may be stored in cytosolic granules ready for fast release upon appropriate stimulation of the cell. They readily bind to sulphated glycoproteins on the surface of nearby cells and so form a local concentrated stable gradient. Chemokine receptors are G-coupled membrane proteins, and their ligation leads to a signaling process that can alter expression of various other effector proteins on their surface. The concept of ligand-induced desensitization is important, as use of a particular chemokine receptor can lead to its down-regulation and subsequent expression of a different chemokine receptor. Consequently, staining and detection of cell surface chemokine receptors can be challenging and requires careful consideration of staining conditions and inter-individual variations (203, 204).

Chemokine Receptor	Expressed on	Chemokine	Chemokine secreted by
CXCR5	B, T and some DC	CCL13	Follicular DCs and stromal cells
CCR7	T, NK, B cell subsets, DCs	CCL19, 21	DCs
CCR5	CD4 and CD8 T cells, NK cells, macrophages, DC	RANTES, MIP-1 α and β	CD8 T cells Inflammatory cells
CCR9	Gut homing T cells	TECK	Gut and thymus epithelial cells
CXCR4	Naïve T cells, NK cells, macrophages	SDF-1 Eotaxin	Stromal cells
Adhesion Molecule	**Expressed on**	**Ligand**	**Ligand expressed on**
CD62L	T cells	GlyCAM	High endothelial venules
$\alpha E \beta 7$ CD103	Gut homing T cells	E-cadherin	Epithelial cells of small intestine
$\alpha 4 \beta 7$ integrin	Gut homing T cells	MadCAM	Intraepithelial cells of small intestine

Table 2. Chemokine receptors and adhesion molecules, and their ligands of interest in this thesis.

The crux of HIV-1 pathogenesis

Even with the brief description outlined in this thesis, it should be clear that the pathogenesis of HIV-1 is a complex and heterogeneous issue. With the intense research efforts ongoing in the field, several mechanisms have been proposed to explain the basis of HIV-1 immunopathogenesis. The role of CD8 T cells in mucosa associated lymphoid tissue in HIV infection should be considered in the context of these settings. A number of the main proposed pathogenic mechanisms here are outlined here:

1. The propensity for HIV-1 to mutate at amino acid residues important for CD8 T cell recognition is a major challenge to the immune system. While there have been many documented cases of immune escape from immunodominant epitopes, there is also ongoing escape from subdominant epitopes (205).
2. Inefficiency of HIV-specific neutralizing antibodies (206). There is significant variability at common antibody targets and the high degree of gp120-associated glycosylation makes for poor neutralizing antibody epitopes (207). In addition, neutralizing antibodies only target free virus and have no effect on infected or latently infected cells.
3. 'Exhausted' HIV-specific CD8 T cells progressively fail to control viral replication (208). As CD8 T cells require help from CD4 T cells for programming to effector and memory development, their loss implicates a qualitative loss for the CD8 T cell. Eventually the host CD8 T cells cannot control virus replication.
4. Higher levels of immune activation are associated with poor disease progression. A number of different experimental approaches have supported this hypothesis in HIV-1 (209, 210) and SIV infection (211, 212). This is in contrast to the non-pathological infection of sooty mangabeys with SIVsm, where there is low level of immune activation despite high viral titers, as described earlier in this introduction.
5. The increased rate of T cell turnover in HIV-infection, combined with diminishing thymic output, is ultimately unsustainable for the host. There is evidence that both HIV-specific and non-specific T cells have a higher turnover rate in HIV-1 infected subjects (213). One study showed that there is an ongoing and progressive loss of naïve CD8 T cells (214). Other studies have pointed to the destruction of the thymus as a mechanism for reduced output of naïve T cells (215), as indeed an aging thymus has a lower naïve T cell output (216). The effects of chronic HIV-1 infection lead to 'premature aging' of the immune system (217).
6. The severe and early depletion of memory CD4 T cells impacts the development of B cell immunity, and CD8 T effector and memory cell development. In particular, the preferential infection and loss of HIV-specific CD4 T cells undermines immune responses to the virus (218).

7. Viral latency ensures that the host will have a perpetual source of virus even in the face of a strong immune response and ART (219-222). There are no known mechanisms to purge the body of such viral reservoirs. Diverse cell types are infected, including microglia of the central nervous system, kidney cells, macrophages, infected follicular DC networks (223-225).

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Aims of the Thesis

1. To investigate the use of the perforin mediated pathway of cytoxicity in the gastrointestinal mucosa during HIV-1 and SIV infection **(Papers I and II).**
2. To validate new monoclonal antibodies for the detection of perforin in rhesus macaques.
3. To characterize CD8 T cells that infiltrate B cell follicles of healthy individuals and investigate what unique properties these cells possess **(Paper III).**
4. To evaluate the utility of the CD7 model of CD8 T cell differentiation in the setting of HIV-1 infection. **(Paper IV).**

Results and discussion

Paper I: Abundant expression of granzyme A, but not perforin, in granules of CD8+ T cells in GALT: implications for immune control of HIV-1 infection

Paper II: Perforin expression in the gastrointestinal mucosa is limited to acute simian immunodeficiency virus infection

Considering that the gut associated lymphoid tissue (GALT) contains a majority of CD4+ HIV-1 target cells, the contribution of CD8 T cells to the control of viral replication may be especially important here. We set out to characterize the frequency of HIV-specific CD8 T cells in the gastrointestinal mucosa and the extent to which perforin and granzyme was expressed. As perforin and granzyme-mediated induction of apoptosis is an important pathway for elimination of HIV-infected cells (226), we examined the expression of these effector molecules as an indicator of cytotoxic function in CD8 T cells. In our study of HIV-1 infection (paper I), we assessed individuals not currently on ART, who had been infected for a mean of 10.2 years. For the examination of well-defined early time points post-infection, we used the SIV model of infection in rhesus macaques (paper II).

Validation of perforin detection reagents for studies in rhesus macaque SIV model
To date, the available anti-human perforin mAbs did not detect perforin in macaques. Due to the high degree of conservation of genes between humans and macaques, monoclonal antibodies (mAbs) to human proteins frequently cross-react with the corresponding macaque protein. However, the degree of cross-reactivity varies and needs to be properly evaluated for each mAb (227). Newly validated human perforin-specific mAbs (228) were evaluated for their cross-reactivity with macaque perforin and their ability to detect macaque perforin by intracellular flow cytometry and immunohistochemistry (IHC). As seen in figure 6, mAbs PF164 and PF344 both showed cross-reactivity with perforin from rhesus and cynomolgus macaque. By flow cytometry, these new antibodies detected macaque perforin while the δG9 mAb commonly used in staining of human cells did not stain at detectable levels (figure 6A). By IHC, PF164 and PF344 staining showed a specific granular and polarized expression of perforin (figure 6B).
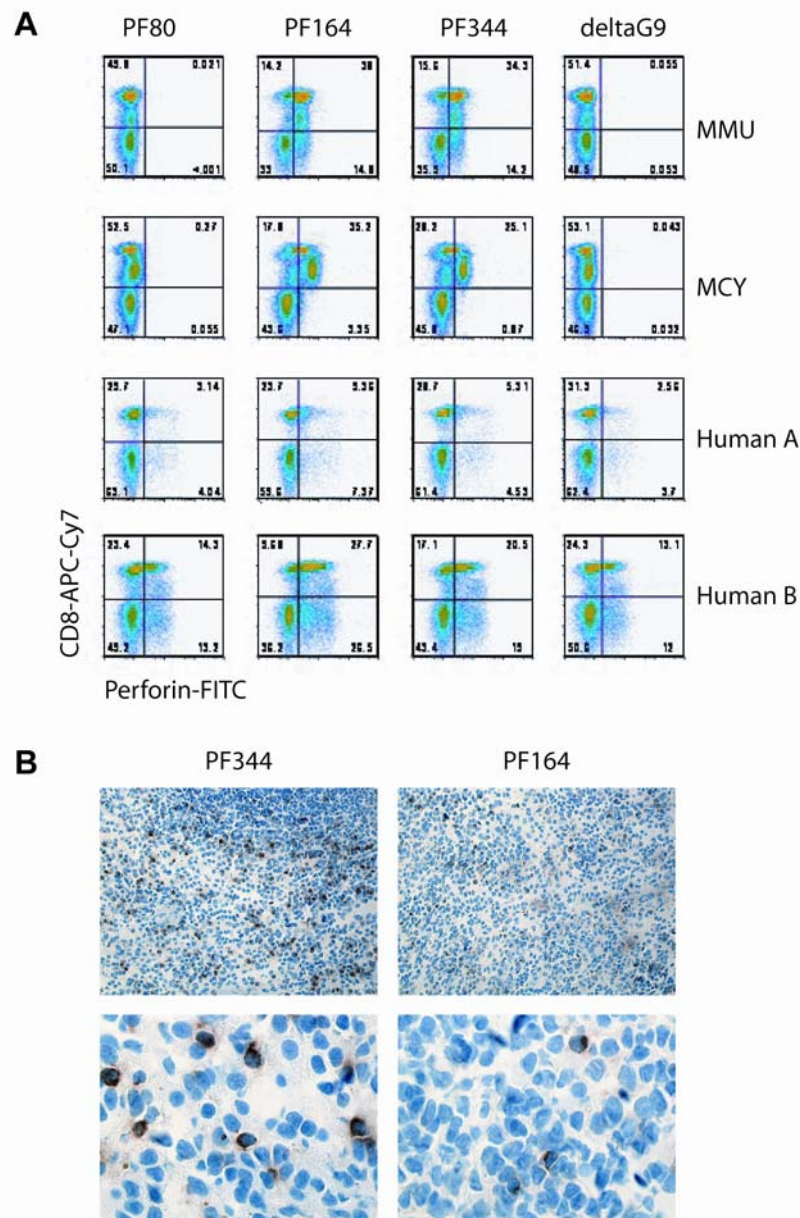
Figure 6. Detection of Intracellular perforin by flow cytometry and immunohistochemistry
(A) The anti-human perforin mAbs Pf-80, Pf-164, Pf-344 and mAb δG9 were analyzed for their reactivity with unstimulated human, cynomolgus macaque and rhesus macaque peripheral blood mononuclear cells (PBMC) by flow cytometry. The plots depict CD8+ and perforin+ expression in the CD3+ lymphocyte population. Positive staining of perforin was observed with mAbs Pf-344 and Pf-164 using human, cynomolgus macaque and rhesus macaque PBMCs, while Pf-80 and δG9 stained perforin in human but not macaque cells. Representative data are shown from at least four experiments with different donors. (B) The perforin-specific mAbs Pf-80, Pf-164 and Pf-344 were evaluated for staining of spleen sections in a healthy rhesus macaque by IHC. The optimal staining in terms of frequency of positive cells and intensity of staining was obtained using mAbs Pf-344 and Pf-164 whereas Pf-80 stained poorly (data not shown). The staining pattern showed the granular and polarized expression of perforin, in line with previously described observations in human tissue (228, 229). The brown color indicates perforin positive staining while the cell nuclei were stained blue by haemotoxylin. All stainings were performed under identical experimental conditions.

Chronic HIV-1 and SIV infection is characterized by low perforin protein expression in gastrointestinal mucosa CD8 T cells, similar to uninfected mucosa.

In papers I and II, the level of perforin protein expression in gastrointestinal mucosa T cells during chronic HIV-1 and SIV infection was investigated. In HIV-positive adults, perforin expression in CD8+ T cells in rectal biopsies was assessed by flow cytometry and immunohistochemistry. When compared to CD8 T cells from PBMC of the same individuals, perforin expression in rectal mucosa was significantly lower ($p<0.001$) (see figure 1B, Paper I). HIV-1 gag-specific CD8 T cells from the rectal mucosa, identified using HLA-A2 tetramers, did not express perforin, although >90% were positive for granzyme A (figure 3, paper I). Similarly, a low level of perforin protein expression was observed using IHC in the colon of macaques that had been SIV-infected for 180 days. There were no significant differences in perforin expression between tissues from naïve and SIV-infected animals (figure 2A, paper II). Perforin levels were consistently higher in the spleen than the rectal tissue of these animals (figure 2B, paper II), and also in PBMC (see figure 7, data not presented in paper II). Given the different methods used to measure perforin in tissue and PBMC, we refrained from direct comparisons, but this result was clearly in line with our findings in the chronic HIV-positive patients (paper I). In parallel, we did not observe a significant increase in mRNA for perforin in either the colon or spleen during chronic SIV infection in macaques, as quantified using real-time PCR (figure 2C+D, paper II). In gut CD8 cells from two HIV+ human subjects, we observed an increase in mRNA for perforin as compared to two HIV- subjects. If there are any significant differences in the dynamics of mRNA for perforin in humans versus macaques can only be determined by examination of a wider group of subjects.
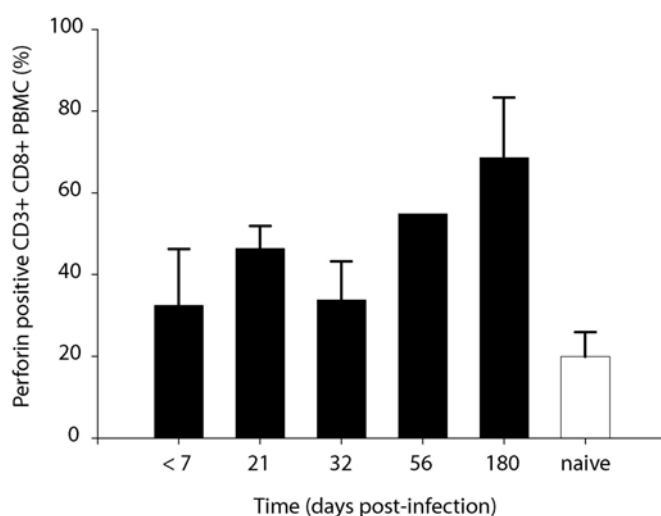

Figure 7.
Detection of intracellular perforin by flow cytometry in PBMC from SIV-infected rhesus macaques. No statistically significant differences were observed between the groups, using a multiple analysis of variance test.

Taken together, our results indicated that in both chronic HIV-1 and chronic SIV infection, the level of perforin expression in the gastrointestinal mucosa was similar to that of healthy controls, despite detectable viral load. Interestingly, pathological conditions in the gastrointestinal mucosa such as Crohn's disease and ulcerative colitis are associated with increase in perforin and granzyme expression (230-234) and over-expression of transcription factors associated with production of inflammatory cytokines (235). In the context of chronic HIV and SIV infection, one may speculate that the gastrointestinal mucosa may maintain a perforin-low state in order to reduce excessive damage to the integrity of the mucosa. It has been previously demonstrated that cytokines prevalent in the mucosal microenvironment tend to favor an anti-inflammatory state. This has been demonstrated for mucosal DCs (236), mast cells (237), macrophages (238) and T cells (239). We propose that the mucosal microenvironment similarly limits production of potentially damaging factors by CD8 T cells, notably perforin.

Increased expression of perforin in the gastrointestinal mucosa during acute SIV infection in rhesus macaques

Given the finding that the rectal mucosa of both healthy and chronically HIV-1 infected individuals expressed little to no perforin, we hypothesized that an absence of cytotoxic immune responses might contribute to the massive viral replication and subsequent depletion of CD4 T cells in the gastrointestinal mucosa observed in early infection (67, 80, 190, 240). It was thus important to undertake similar analysis at earlier timepoints post-infection. We examined colon and spleen tissue from rhesus macaques vaginally inoculated with SIVmac251 for the expression of perforin protein and mRNA at timepoints as early as one day post-infection. In this manner, we were able to track the kinetics of perforin expression in the gastrointestinal tract from the time of viral inoculation up until chronic infection. In parallel, we also analyzed perforin expression in PBMC by intracellular staining and flow cytometry. In addition, tissue SIV viral copy number was assessed to decipher when and to what extent infection had disseminated through the animal.

Already by day 4 post-inoculation, SIV RNA was detectable in the colon mucosa (see table 1, Paper II). In animals 21 days post-infection, SIV replication had reached high levels and this was concurrent with a significant increase in perforin protein and mRNA. It would have been interesting to examine tissues at a time-point between 7 and 21 days, as this is when the reported CD4 T cell depletion and influx of SIV-specific CD8 T cells begins (241, 242). Unfortunately, such material was not available. Perforin protein and mRNA levels were observed to decrease over time until 180 days post-infection, when levels were not significantly different from those observed in naïve animals, as discussed above. This differed from the spleen and the PBMC, where perforin protein levels did not vary significantly over the

timepoints examined, although there was a trend for increased perforin expression over time (figure 2B, paper II and figure 7). These results suggest that there is a robust but transient perforin positive response in the colon of acutely SIV-infected rhesus macaques that may lead to the cytolysis of SIV-infected cells. Both the spleen and the PBMC show a more stable expression of perforin, with no significant differences between early and chronic infection.

The appearance of SIV gag-specific CD8 T cells has been estimated to occur between 14 and 21 days post infection (241, 242). We observed a trend towards increased perforin in the colon of macaques already at day 7 post-inoculation (data not shown). Such early expression in the SIV+ colon raises the possibility that perforin was also expressed in CD8 T cells specific for antigens other than SIV. It may be that a change in the cytokine milieu of CD8 T cells in part led to the increased expression of perforin. The elevated levels of IL-2 and IL-15 observed in inflammatory bowel disease is thought to contribute to a higher frequency of activated perforin positive T cells (234).

No clear relationship between perforin expression and viral HIV/ SIV replication.

We speculated that the level of perforin expression observed in the gastrointestinal mucosa was related to the magnitude of viral replication. In our study of chronic HIV-1 infection, no significant relationship could be established between perforin protein levels in CD8 T cells of the rectal mucosa and plasma viral load (data not shown, paper I). It would have been worthwhile to investigate the possible relationship between rectal mucosa viral load and mucosa perforin levels, although unfortunately this tissue viral load data was not obtainable at the time. However in macaques with acute SIV infection, we were able to assess both tissue and plasma viral RNA load. Interestingly, we saw no significant difference in perforin protein expression in animals with high or low colon viral RNA loads (see table 1, paper II). This finding should be explored in a greater number of study subjects both in HIV-1 and SIV infection, but it does suggest that perforin protein expression is not directly proportional to viral replication. Prospective longitudinal studies of HIV-1 infection could help clarify the role of perforin in limiting viral replication.

Are CD8 T cells friends or foes during the severe CD4 T cell depletion observed in acute infection?

We originally hypothesized that mucosal CD8 T cells might fail to mount an adequate cytotoxic response during acute HIV infection, and hence the depletion of CD4 T cells is all the more severe during acute infection. However, the results presented in paper II, showing that CD8 T cells in the colon can mount a vigorous perforin positive immune response in early

infection, support two alternative scenarios. First, the cytotoxic response may be too late already from the beginning, making limited impact on viral replication. Second, this response may in fact contribute to a large part of the acute CD4 T cell depletion. As discussed in the background to this thesis, it is thought that a majority of CD4 T cells that get depleted in the SIV-infected gastrointestinal mucosa has been productively infected, and are susceptible to virus-induced lysis. Our results suggest that strong virus-specific cytotoxic immune responses may also contribute to the killing of many infected CD4 T cells. Other data show that induction of the FAS-FASligand pathway of apoptosis also contributes to the depletion of both SIV-infected and non-infected CD4 T cells in the gut in acute SIV infection (70). While these cytotoxic CD8 T cells may limit viral replication by eliminating virally-infected cells, they may also contribute to the pathology of HIV-1 infection during early infection by putting a strong selective pressure on the virus and promoting the appearance of escape mutants. To date, a relationship between magnitude of CD8 T cell responses and severity of CD4 T cell depletion in the gut during acute HIV-1/SIV infection, with disease progression has not been established.

What are the requirements for mucosal CD8 T cells to express perforin and become fully differentiated cytolytic cells?

While CD8 T cells in chronic HIV-1 and SIV infected gastrointestinal mucosa were largely perforin negative, those in early infection were clearly able to express this cytolytic molecule. Under what conditions could CD8 T cells in chronic infection produce perforin and become cytolytic? After virus-specific CD8 T cells have been primed in the Peyer's patch or draining mesenteric lymph node, it is thought that the final functional differentiation of mucosal effector T cells occurs at the effector site. This can be thought of as a two signal process: the first signal from priming in the LN to program proliferation and development of a mucosal tissue homing profile, and the second signal from cells at the site of infection that stimulate differentiation to full effector capacity (243). Signal II can be in the form of cytokines or co-stimulatory molecules. Cytokine-induced expression of perforin has been mapped to an enhancer region in the human perforin promoter that contains a STAT-binding site (244). STAT proteins are a family of transcription factors that are recruited to the cytoplasmic domain of engaged cytokine receptors. Subsequently they undergo a series of phosphorylations before going to the nucleus and influencing gene transcription. It is thought that STAT5 may regulate IL-2 induced perforin expression (244). An important source of these cytokines is the CD4 T cell. The depletion of virus-specific CD4 T cells from the gastrointestinal mucosa may leave local virus-specific CD8 T cells without this signal II. Hence we observe CD8 T cells in a perforin-negative state in chronic infection. Interestingly, in contrast to individuals with progressive disease, CD8 T cells from long term non-progressors demonstrate a better

proliferative capacity in response to autologous virus and can more readily upregulate expression of perforin (245). NK cells from lymphoid tissues also require IL-2 in order to be able to express perforin, whereas NK cells from blood to not require this cytokine for their lytic activity (246). Co-stimulation by CD70-expressing cells in the LP may influence the effector function of mucosal T cells by signaling via CD27 on the T cell surface (247). Concurrently, it has been shown that CD27 is important for maintenance of effector memory function in progressive HIV disease (248), while over-use of this stimulatory pathway can lead to T cell exhaustion (249). A recent study of chronic LCMV infection in mice demonstrated that exhausted mucosal CD8 T cells upregulated expression of the programmed death 1 (PD-1) receptor, an inhibitory receptor of the CD28 family. Blockade of this pathway using antibodies to its ligand restored effector function to the CD8 T cells, even in the absence of CD4 T cells (250) A therapeutic intervention to restore the proliferative and cytotoxic capacity of CD8 T cells in HIV-infected mucosal tissues may result in better immune control of the virus.

Quantifying virus-specific CD8 T cells in the gastrointestinal mucosa
A number of studies have shown that tetramer-positive SIV-specific CD8 T cells appear in the colon mucosa at 14 days post-infection (241, 242). It is technically difficult to measure the breadth of the CD8 T cell focus using MHC class I tetramers. However it is an important site to examine as the frequency of HIV gag-specific cells can be higher here than in the peripheral blood (251). A recent assessment has been made of the breadth of CD8 T cell epitopes targeted in the rectal mucosa of chronically infected individuals by ELISPOT using pools of HIV-1 peptides (252). This study showed a high level of similarity between responses to peptide pools in the rectal mucosa CD8 T cells as compared to PBMC. A finer analysis of the individual peptide-specific responses in the gut has revealed that there are important differences between blood and rectal mucosa in the breadth of responses (253). The study of these differences is important to our understanding of virus-host interactions.

It is challenging to make a comparative quantitative assessment of the influx or depletion of any particular cell type to a tissue. Such measurements are compounded by differing orientations of tissue sections, by influx of other cell types and by variations in baseline measurements, and are very laborious. However, an approximation may be possible by counting the absolute number of cells per unit area, or expressing the number of positive cells as a percentage of total cells within the field. We choose this latter method to estimate the change in the percentage of CD8+ cells in the macaque colon LP (data not shown).

When can low perforin expression be considered a defect in CD8 T cells?
There has been much speculation in the literature as to whether functional defects in HIV-specific CD8 T cells are a major contributing factor to HIV-1 disease progression (133, 254, 255). These suggested defects have ranged from defective signaling through the CD3 zeta chain (256), inability to express perforin (229, 257), and failure to mature and become fully differentiated effectors (133). A novel flow cytometry-based assay to investigate degranulation of T cells has recently been developed (258). There is evidence to suggest that, upon stimulation of TCR, CD8 T cells from chronically HIV-1 infected gastrointestinal mucosa do degranulate and secrete cytokines at equal or greater frequencies than PBMC, although they do not contain perforin (manuscript in preparation, BL Shacklett and JW Critchfield). Other studies have indicated that perforin expression may be limited in both tonsil and LNs in chronic HIV-1 infection (229). It can well be that this is an intentional limitation in the use of this cytotoxic pathway in order to limit tissue damage. Pathological conditions in the gastrointestinal mucosa are associated with increase in perforin and granzyme expression (230, 231). CD8 T cells may have become anergized by the presence of antagonist peptides presented on MHC class I molecules, likely in abundance given the high mutation rate in the virus at targeted CD8 T cell recognition sites. A number of studies have shown the expression of HLA-class I specific inhibitory NK cell receptors on HIV-specific CD8 T cells reduces their cytolytic capacity (259, 260), although to date mucosal CD8 T cells have not been examined extensively in this regard. Nevertheless, the study of many other chronic viral infections has shown that there is widespread functional heterogeneity within memory CD8 T cells (111, 114, 261-263). There is growing evidence that HIV-specific T cells that are 'polyfunctional' in nature and can exert a variety of effector functions in HIV-1 infection are associated with better disease prognosis (163, 264). It is our ongoing challenge to distinguish between what may be a defect and what may be an appropriate differentiation profile of a HIV-specific CD8 T cell response in MALT.

Future perspectives
The data presented in Paper I and II suggest a number of follow-up investigations of importance to the understanding of HIV-1 pathogenesis. Studies to investigate the CD8 T cell targeted epitopes in early SIV infection and compare to viral evolution would give insight into the initial interaction between host immune response and pathogen genetics. While the cytotoxic T cell response in early SIV infection may lead to the elimination of infected CD4 T cells, many of these cells would undergo virus-induced apoptosis anyway. Could this cytotoxic response simply promote the development of virus escape mutants without any major benefit for the overall reduction in viral load? Careful studies to map virus host interactions in the gastrointestinal mucosa may take us closer to understanding this issue. Are there any

therapeutic interventions that may serve to increase perforin expression in mucosal CD8 T cells? IL-2 treatment should promote the development of perforin-expressing CD8 T cells. However this has not been examined to date in the mucosa of IL-2 treated patients. It has been shown that treatment with polyethylene glycol interferon alpha can increase the proportion of T cells and NK cells that express perforin and this increase is associated with reduction in viral load (265, 266). If perforin expression also increased in the mucosal tissues of these treated patients has not been addressed. It is likely that an immunotherapy directed towards mucosal tissues will need to account for tolerogenic DCs in the gut.

Ultimately, prospective longitudinal studies of HIV-infection are necessary to clarify the role of perforin in limiting viral replication and rate of disease progression. It remains to be determined whether the lack of perforin expression by gut CD8+ cells during chronic HIV and SIV infection is a consequence of tight regulatory control of cytotoxicity within the mucosa, or if it reflects an aspect of viral-induced immunopathogenesis.

Paper III: CXCR5+ CCR7- CD8 T cells are Early Effector Memory Cells that Infiltrate Tonsil B cell Follicles

CD8 T cells in secondary lymphoid tissues typically position themselves within T cell zones to interact with APCs. This study described a subset of human CD8 T cells that express the chemokine receptor CXCR5, which enables chemotaxis in response to the chemokine CXCL13, produced by stromal cells within B cell follicles. By confocal microscopy, CXCR5+ CD8 T cells were observed scattered in the follicular mantle and dark zone of tonsil B cell follicles. Directly *ex vivo*, CXCR5+ CD8 T cells expressed high levels of the co-stimulatory molecules CD27 and CD28, the RO isoform of CD45, low expression of CD7 and high levels of granzyme A, a phenotype indicating that these are antigen-experienced cells. In addition, they were absent from cord blood, further supporting that CD8 T cells express CXCR5 post antigen recognition. Tonsil CXCR5+ CD8 T cells expressed CCR5, but interestingly, were negative for CCR7, thus distinguishing them from the frequently described CCR7+ central memory cells (131). Upon activation *in vitro*, CXCR5+ CD8 T cells upregulated expression of co-stimulatory ligands important for T cell–B cell interactions, CD70, OX40 and ICOS, could produce IFNγ and TNFα, but lacked perforin expression. These data demonstrate that CXCR5+ CD8 T cells have several characteristics of an early effector memory phenotype.

B cell requirements for survival in the germinal centre
Germinal centres are critical for production of high-efficiency neutralizing antibody (267, 268). B cell centroblasts in the dark zone proliferate intensively and selectively mutate the genes that encode their B cell receptors in a process known as somatic hypermutation. They then migrate to the light zone as B cell centrocytes and display antigen on their new B cell receptor. The centrocytes undergo a process of affinity maturation whereby B cells that produce high-affinity antibodies are given survival signals, and B cells with low affinity or auto-reactive antibodies go into apoptosis. The B cells that leave the germinal centre reaction may become antibody-producing plasma cells or long-lived memory B cells that reside in the bone marrow. The role of CD8 T cells in this process, if any, is unknown (269).

Our experiments investigated if co-culture with CXCR5+ CD8 T cells would confer any survival advantage on tonsil B cells. A significant increase in the survival rate of B cells was observed during co-culture with CXCR5+ CD8 T cells (see figure 4, paper III). We used two different methods to purify the CXCR5+ CD8 T cells from tonsil. Both methods were complicated by the fact that on average the target cell population was approximately 1% of total starting population of mononuclear tonsil cells. FACS based sorting was time-consuming and the yield was still relatively poor. Rosetting with sheep red

blood cells (SRBC) enabled excellent purification of B cells and consequently enriched for T cells (270). The SRBC ligand, CD2, is present on all T cells. However, the rosetted cells underwent a certain level of activation as measured by increased expression of cell surface CD69. Cells were kept on ice to minimize exacerbation of this activation. However, with both methods, the yield of T cells was too low to further optimize these experiments and efforts are ongoing to improve this. Other aspects of these T cells can be investigated including a more thorough analysis of cytokine production and acquisition of cytotoxic function.

There have been other reports in the literature of B cell processes supported by CD8 T cells. The regulatory effects of steroid hormones on antibody secretion by B cells in female macaques were shown to be CD8 T cell dependent (271). Also intriguing is the finding that CD8 T cells are required for the formation of ectopic germinal centres in rheumatoid synovitis (272). The mechanism behind these observations is unclear, but one can speculate that it involves T cell - B cell contact within a B cell follicle.

Clues to the antigen specificity of CXCR5+ CD8 T cells
We initially speculated that CXCR5+ CD8 T cells might be specific for EBV, as the tonsil B cell follicle has been recognized as a reservoir for this latent virus (273, 274). However, we failed to identify tetramer positive EBV-specific cells in tonsil and those that we did find in peripheral blood generally did not express CXCR5 (data not shown). Interestingly, recent studies have identified tonsil homing EBV-specific cells, by tetramer binding and expression of the integrin CD103/αEβ7 (275, 276) although expression of CXCR5 was not examined in these studies. As CD8 T cells are MHC class I restricted, they will recognize different antigens as compared to MHC class II-restricted CD4 T cells. So the CXCR5+ CD8 T cells infiltrating B cell follicles should recognize a different set of epitopes from CXCR5+ follicular helper CD4 T cells. Possibly, these CD8 T cells are specific for antigens that are cross-presented by B cells in the follicle. This has recently been shown to occur in a mouse model of CD8 T cell cross-priming against OVA-CpG (277).

Recent data from mouse models has suggested that CXCR5+ T cells may enter the B cell follicle to undergo a post-thymic rearrangement of their TCR genes and these cells had up-regulated expression of their RAG-1 and -2 genes (278). Could CXCR5+ CD8 T cells be undergoing TCR revision within the B cell follicle of healthy adults? While there is currently no evidence to support this, it is an interesting speculation that may have relevance for maintenance of tolerance and elimination of autoreactive T cells.

Potential role of CXCR5+ CD8 T cells in HIV-1 infection
The germinal center has been identified as a site for HIV replication (62, 225, 279-281). It contains several HIV target cells including CD4 T cells and follicular DCs. While we have seen that CXCR5+ CD4 T cells do not express high levels of CCR5, apparently they too can become infected. One group has reported that there are few CD8 T cells in HIV+ LN germinal centers and that they do not express perforin and beta-chemokines, and as such the follicle is an 'immunoprivileged site' (282). However, our data indicate that in healthy individuals CD8 T cells do infiltrate the germinal center using CXCR5. One might spectulate that in the case of CXCR5+ CD4 T cell depletion, CXCR5+ CD8 T cells may provide critical B cell help. Preliminary data indicate that CXCR5+ CD8 T cells are expanded in the blood of HIV-1 patients (figure 8), and no major phenotypic differences can be determined when compared to CXCR5+ CD8 T cells of healthy individuals. There has been a recent report suggesting that serum levels of CCL13, the ligand for CXCR5, are elevated in HIV-1 infection and return to normal levels on ART (283). Mice that lack CD4 T cells due to having their MHC class II genes knocked-out have normal B cell follicles and they are able to support plasma cell differentiation and isotype class switching (284). It has not been investigated if CD8 T cells have a role in supporting B cell processes in these animals.
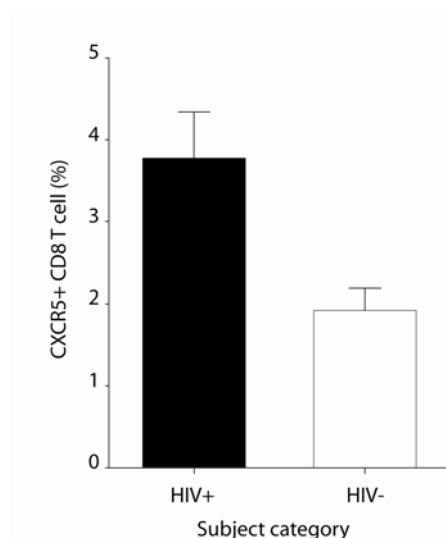

Figure 8. Percentage of CD8 T cells that express CXCR5 in the blood of HIV+ and HIV- individuals. PBMC were stained as described in paper III, and analyzed by flow cytometry.

Future Perspectives
The finding that CXCR5+ CD8 T cells locate to B cell follicles and may play a role in supporting B cell survival and function, suggests a novel role for MHC class I-restricted CD8 T cells. Future experiments will investigate if CXCR5+ CD8 T cells can support isotype switching or antibody production by B cells and more thoroughly define the cytokine-producing profile of these cells. Finally, the role of CXCR5+ CD8 T cells in HIV-1 infection will be further explored to investigate if they make any unique contributions to virus immune control.

Paper IV: Expansion of CD7low and CD7negative CD8 T cell effector subsets in HIV-1 infection: Correlation with antigenic load and reversion by antiretroviral treatment

Evidence that CD7 is a CD8 T cell differentiation marker with disease relevance

The antiviral response of CD8 T cells involves the differentiation of naïve T cells into distinct types of effector and memory cells. A previous report has indicated that effector and memory subsets may be distinguished by the level of CD7 expression (135). We have investigated the expression of CD7 on CD8 T cells in HIV-1 infected adults and children to determine the disease relevance of cell subsets defined by CD7. CD8 T cells from HIV-1 infected patients displayed profound down-modulation of CD7 expression as compared to CD8 T cells in healthy subjects, with expansion of both CD7low and CD7neg effector subsets. Loss of CD7high cells was particularly pronounced in patients with rapid disease progression. Down-modulation of CD7 on CD8 T cells correlated directly with HIV-1 load, and cells specific for HIV-1, EBV and CMV were predominantly found in the CD7low effector cell subset. Furthermore, recovery of CD4 T cell counts on ART was associated with reversion of the skewed CD7 profile in CD8 T cells. Thus, effector CD8 T cell subsets distinguished by lowered CD7 expression expand in a manner that correlates with the magnitude of antigenic challenge, and contract in response to successful antiretroviral treatment.

Potential mechanisms behind the phenomenon of CD8 T cell skewing towards a CD7low profile in HIV-1 infection

The skewing of the CD8 T cell compartment towards a predominantly CD7low profile was particularly striking in the group of patients with fast disease progression (p=<0.005, figure 5, paper IV). This is the patient group with the highest HIV-1 viral load burden, and lowest CD4 T cell count. This expansion of CD7low effector CD8 T cells was likely in response to HIV-1 and other infections. Conversely, in patients with reduced antigen load due to ART, this skewed pattern normalized and the patients regained a CD7 profile more similar to HIV negative individuals.

On the other hand, this skewed CD7 low profile may also be partly due to galectin-1 mediated apoptosis of CD7high CD8 T cells. While we did not examine levels of the CD7 ligand in any of the study subjects, other reports have suggested that galectin-1 levels are increased in HIV-1 infection and particularly in patients with faster disease progression (140, 285). It is not known what level of CD7 expression is required for induction of apoptosis by this pathway, if CD7high and CD7low cells are differentially susceptible or if CD7neg cells are protected from death in this manner. Susceptibility of CD4 T cells to galectin-1 mediated apoptosis may differ from that of CD8 T cells. However, the fact that we observed a similar skewing in the CD4 T cell

compartment only in patients with CD4 counts of less than 200 cells per μl questions whether it is galectin-1-mediated depletion of CD7high cells that contributes to the observed pattern. Further studies are needed to understand the contribution of galectin-1 mediated apoptosis to HIV-1 disease pathogenesis.

Differentiation of CMV and EBV-specific effector CD8 T cells in HIV-1 infection

The observation that EBV- and CMV-specific tetramer-defined CD8 T cells of HIV+ individuals displayed a more pronounced CD7low phenotype than CD8 T cells of healthy individuals, suggests that these virus specific cells may have been driven into effector phase (figure 4B, paper IV). There have been many reports of latent virus reactivation in HIV+ and other immuno-compromised patients (286-288). While there were no reported symptoms of EBV and CMV reactivation from a clinical point of view in the patients that we studied, it would have been interesting to have viral load data to verify this. Influenza infection is usually resolved with elimination of the virus and no known reservoir persists in the host. Influenza-specific CD8 T cells displayed a CD7high phenotype in healthy subjects, consistent with these cells forming part of a long-term memory subset. Unfortunately, data on the CD7 profile of influenza-specific CD8 T cells from HIV-1 infected patients was not available. In fact, we failed to detect influenza-specific CD8 T cells in our HIV+ study subjects using HLA-A2 tetramers (data not shown). While this can vary between donors, it may be that with the massive expansion of HIV-specific CD8 T cells, the frequency of influenza specific CD8 T cells falls below the limit of detection for tetramer staining. One could speculate that influenza specific CD8 T cells in HIV-infected patients too would express high levels of CD7, providing that the patient was not suffering from a current influenza infection. However, it could also be possible that influenza specific CD8 T cells would share a CD7 expression pattern similar to EBV and CMV, and that HIV-1 induced immune activation could lead to activation of influenza specific cells even in the apparent absence of their cognate antigen.

Can the CD7-based model of CD8 T cell differentiation be useful in the study of mucosal CD8 T cells?

Our study shows that the CD7 model of CD8 T cell differentiation has disease relevance in the setting of HIV-1 infection. Levels of galectin-1 are known to vary in inflamed tissues and consequently apoptosis could be more frequent (289). Considering that the majority of HIV-1 viral burden may be within the mucosal tissues, it is of interest to see if this model can also be applied to CD8 T cells from this site. We reported that a skewing of CD7 expression from high to low and negative occurs in relation to disease severity, and reduction of viral load by antiretroviral treatment normalizes this pattern. It could well be that the CD7 model of CD8 T cell differentiation gives an even more

sensitive description of immune activation in mucosal tissues. We subsequently used the CD7 marker for the phenotypic analysis of CXCR5+ CD8 T cells of healthy individuals in paper III of this thesis and found these cells to be of an effector profile, consistent with other phenotypic analyses.

Future perspectives

In conclusion, the CD7-based model of CD8 T cell differentiation could be broadly applicable to other viral infections, as well as in the assessment of vaccine-induced immunity, and will be further explored. The role of galectin-1 in inducing CD7-mediated apoptosis in mucosal tissues is interesting and its contribution to the high T cell turnover rate in HIV-1 infection will be investigated.

Concluding remarks

This thesis has dealt with the characterisation of CD8 T cells of the MALT and the blood in HIV-1 infection. Evidence has been presented to suggest that effector function of CD8 T cells varies significantly depending on the location of the cell, and the stage of disease (papers I and II). It has also been shown that tonsil mucosal tissue contains a subset of early effector memory CD8 T cells with a unique phenotype that may support B cell differentiation (paper III). Finally, it has been demonstrated that effector CD8 T cell subsets expand and contract in close relationship to HIV-1 viral load and that these subsets can be defined by their expression of varying levels of CD7 (paper IV).

The relatively low rate of transmission of HIV-1 per exposure by heterosexual contact (290, 291), suggests that the host has a certain barrier to HIV-1 infection, which can be physical and/or immune mediated. The observation that highly HIV-1 exposed sex workers, who persistently test HIV-1 seronegative, have detectable CD8 T cell responses and neutralizing antibodies specific for HIV antigens suggests that a mucosal vaccine may already be working in nature (292, 293). A more extensive analysis of mucosal immune responses in these study subjects may elucidate the type of immune response a HIV-1 vaccine should aim to elicit. By complimenting an individual's natural barrier to mucosal pathogens including HIV-1, with anti-viral microbicides, transmission rates could be reduced. Recent data showed that macaques were protected from vaginal challenge with pathogenic SHIV when treated with a microbicide containing HIV-fusion inhibitors (294). These promising results give hope that such preparations may become available for human use within the near future.

In individuals who do become infected with HIV-1, it appears that virus dissemination and establishment of viral latency occurs within a matter of days after infection. There is an early and severe depletion of CD4 T cells from mucosal tissues and this T cell loss is ongoing through-out the course of disease (295). Thus, it is of vital importance to target HIV-vaccines and therapies to MALT. Much of our understanding of CD8 T cell biology in HIV-1 infection has come from studies of peripheral blood, and these extensive and insightful investigations from the past twenty years have helped to refine our hypotheses on virus-host dynamics within mucosal tissues. Hopefully, this will soon lead to clearer definitions of immune correlates of protection in MALT, crucial for the evaluation of vaccine-induced immune responses, and immune reconstitution during ART.

Máire Quigley

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