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Impact of Human Immunodeficiency Virus on Hepatitis B-specific Immune Responses

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Declaration

I hereby confirm that the work presented in this thesis is my own work.

Dr. Roxana Monica LASCAR

Aknowledgements

Working on this project has been a great privilege as well as a steep and, at times, difficult learning curve. When I undertook this project I had almost no experience of working in an Immunology lab, and little previous research experience. It took me several years to complete the experiments of this study, writing and publishing of results. During this journey I accumulated fascinating knowledge of HIV and hepatitis immunology, worked with many fantastic people and towards the end of the project really enjoyed writing up my thesis!

I would like to thanks my supervisors, Dr. Richard Gilson for giving me the opportunity to work in such interesting field and Dr. Mala Maini for teaching me everything I know about Immunology and encouraging me along the way to see this work through. I would like to thank all my lab colleagues, especially Ross and Stephanie, for their practical advice and support with lab work. A big thank you to Prof. Rob Miller, who although was not directly involved with this project, provided me with his usual brief but very insightful advice and encouragement in the most difficult times.

Lastly, I would like to thank my family, which I built around me during the time of this project, especially my son Zach, who spent extra days in nursery for me to finish experiments, and to my second baby, who is waiting peacefully to come out into the world days after I have sent this work to the printers!

Abstract

Prior infection with hepatitis B virus (HBV) is a common occurrence in HIV infected subjects and an increasing cause of morbidity and mortality. The CD8 T cell response is crucial for the long-term control of the virus in patients resolving acute hepatitis B. I first examined the effect of HIV related immunodepletion on HBV-specific immune responses in patients who resolved HBV. A cross-sectional study showed a reduction in HBV-specific CD8 responses in HBV immune patients with HIV infection compared to those without. Longitudinal study of a subgroup of patients examined whether this attrition could be reversed by effective antiretroviral therapy. The introduction of highly active antiretroviral therapy (HAART) resulted in recovery of some HBV-specific CD8 and CD4 responses, in association with restoration of CD4 counts. These data provided a mechanism for the observed impairment of HBV control in the setting of HIV infection and support the ability of HAART to reconstitute functionally active responses.

I also studied the HBV-specific cellular immune responses in HIV negative patients who resolved acute hepatitis B without symptoms, a group which has never been studied immunologically, but represents a significant proportion of hepatitis cases acquired in adulthood. In the last chapter I focused on the chronic hepatitis B carriers co-infected with HIV and the impact of HIV and HBV treatment. I showed that reconstitution of some HBV-specific T cell responses can also occur in HIV-positive patients after a reduction in HBV load. This potential to recover T cell responses provides support for the addition of anti-HBV therapy in the treatment of co-infected patients.

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CHAPTER 1

1.Introduction

1.1 Study rationale

Human immunodeficiency virus (HIV) and hepatitis B virus (HBV) are major causes of morbidity and mortality worldwide and, due to shared routes of transmission, are often encountered together. The prevalence of chronic HBV infection among HIV infected people ranges from 6-13% in most cohorts and 64-84% of HIV patients have serological markers of natural immunity following previous HBV exposure (Beck, Mandalia et al. 1996) (Combe, La Ruche et al. 2001) (Hadler, Judson et al. 1991; Scharschmidt, Held et al. 1992). HBV is a non-cytopathic virus, in which the immune response to the virus is responsible for both control of virus replication and liver injury. In the context of HIV-related immunodepletion, studies have shown an increased HBV replication (Gilson, Hawkins et al. 1997) (Colin, Cazals-Hatem et al. 1999) and an increase in the rates of progression to chronicity (Sinicco, Raiteri et al. 1997). A decrease in necro-inflammatory activity was also noted (Gilson, Hawkins et al. 1997) (Colin, Cazals-Hatem et al. 1999). Hepatitis related morbidity and mortality has increased in the context of improved overall survival of HIV positive patients treated with highly active antiretroviral treatment (Puoti, Spinetti et al. 2000; Martin-Carbonero, Soriano et al. 2001; Soriano, Martin-Carbonero et al. 2001; Thio, Seaberg et al. 2002). Advances in the field of antiretroviral agents, some of them with additional anti-HBV activity, have also brought a renewed interest in treating HBV in this population.

The mechanisms by which HIV may affect HBV control at the level of cellular immune responses have been poorly studied, primarily due to the lack of sensitive immunological techniques. Use of lymphocyte proliferation assays to mitogens, Candida and tetanus antigens were insufficient to explain differences in the cellular responses between HIV negative and HIV positive patients (Rector, Govindarajan et al. 1988). The mechanisms behind the somewhat paradoxical decrease in necro-

inflammatory activity noted in the context of immunosuppression, the recently reported increase in liver related morbidity and mortality of HBV HIV co-infected patients in the context of HAART remain poorly defined. Our study aimed to dissect the impact of HIV related immunodepletion on the HBV-specific T cell responses across a wide range of HIV positive patients who have been exposed to hepatitis B. Secondly, we studied the impact of highly active antiretroviral therapy on the cellular responses to HBV.

Understanding the immune responses to hepatitis B in patients with HIV infection may have several benefits. From a scientific perspective, studying a clinically important co-infection could further extend our knowledge of HIV and its subsequent immune impairment. Secondly, studying hepatitis B infected patients who are immunosupressed may improve our understanding of the immuno-pathogenesis of hepatitis B. Additionally, this study was an opportunity to investigate whether HAART may lead to recovery of HBV-specific immune responses. On a clinical level, our study aimed that a better understanding of HBV-specific cellular immune responses in the context of HIV may provide a scientific basis for optimizing treatment in this group of patients. Given the wide choice of antiretrovirals available, it would be beneficial to assess the potential for recovery of HBV-specific immune in an immunodeficient population as well as whether the addition of anti-HBV agent was necessary.

Until recent years, studies of viral infections have been hampered by a lack of sensitive techniques able to identify antigen-specific cells directly ex vivo. Major technological advances in immunology such as tetramers and intracellular cytokine staining have now permitted for the first time a detailed examination of the dynamics of T cell responses to human infections.

The aim of this project was to analyse the impact of HIV related immunodeficiency on HBV-specific immune responses and whether such responses could be recovered after starting anti-retroviral therapy. Natural history studies of HIV-HBV co-infection have

suggested an impaired immune control of HBV during HIV, with increased HBV replication(Gilson, Hawkins et al. 1997) (Colin, Cazals-Hatem et al. 1999) and increased rates of progression to chronicity in the context of HIV infection. The mechanisms by which HIV may affect HBV control at the level of cellular immune responses have been poorly studied previously, primarily due to the lack of sensitive immunological techniques. In this project the immune responses to hepatitis B virus (HBV) during HIV infection were studied using such sensitive techniques.

We studied HBV-specific immune responses in HIV infected patients with different degrees of control of HBV replication, including patients with natural immunity to hepatitis B and chronic carriers of HBV. I first analysed HBV-specific immune responses in HIV negative and positive patients who controlled HBV. Patients who controlled acute hepatitis B and acquired natural immunity are the most likely group to have strong multispecific T cell responses (Bertoletti, Ferrari et al. 1991; Nayersina, Fowler et al. 1993; Rehermann, Fowler et al. 1995), which are preserved over time (Penna, Artini et al. 1996; Rehermann, Lau et al. 1996) and therefore represented an ideal group on which to study any impact that HIV related immunodepletion may have. I then analyzed the impact antiretroviral therapy may have on HBV-specific immune responses in HIV positive patients with natural immunity to HBV. Secondly, I studied HBV-specific T cell responses in patients who were HIV positive HBV chronic carriers and followed them up longitudinally after staring HAART.

In order to understand the impact HIV may have on HBV, I first outline the most recent available data on the immuno-pathogenesis of HIV and HBV infection as well as an overview of the relevant cellular immune responses that may play a role in viral control.

1.2 Overview of immune responses

Immunity is the state of protection from infectious diseases and can be innate (nonspecific) and acquired (specific) immunity. Both components cooperate in the process of defending the host from the invading pathogen, with the innate mechanisms providing a first line of defence, until an acquired immune response develops. Most infectious agents encountered by a healthy individual are cleared within days by the innate immune responses. When a microorganism eludes this barrier, a specific response is enlisted, which supplements the innate responses and is more effective.

1.2.1 Innate non-specific immunity

This comprises the basic mechanisms of resistance to disease that an individual is born with and includes a wide variety of mechanisms of protection against pathogens. There are the anatomic barriers, such as skin and mucous membranes that prevent pathogen entry. Physiologic barriers include temperature, pH, soluble factors such as interferons (produced by virus infected cells), lysozyme (hydrolytic enzyme found in mucous secretions) and complement, which lyses microorganisms or facilitates phagocytosis. The phagocytic/ endocytic barriers comprise cells, which are able to internalize (endocytose) foreign molecules and specialized cells (monocytes, neutrophils, tissue macrophages), which internalize (phagocytose), kill and digest microorganisms. There are also barriers created by the inflammatory response, where tissue damage is followed by a complex series of events, which facilitates the influx of phagocytes and serum proteins with antibacterial activity.

The past few years have seen an increased interest in the field of innate immune responses during viral infections and brought more insight into the strong reciprocal integration of innate and adaptive responses. During a viral infection, the presence of double-stranded viral RNA triggers production of type I interferons, IFN α/β (Ishikawa

and Biron 1993). IFN α/β directly interferes with viral protein synthesis (via dsRNA-dependent protein kinase) as well as by further stimulating the recruitment and activation of macrophages. Activated macrophages secrete cytokines and chemokines, which contribute to recruitment of NK cells, which are able to recognize infected cells prior to the upregulation of MHC molecules (Lanier 1998). NK and NK-T cells respond immediately after infection by producing IFN-γ and killing infected cells (Biron 1997; Biron and Brossay 2001). The dendritic cells (DCs) are a link between the innate and adaptive immune responses, maturing under the influence of signals received from the innate immune response and activating further antigen-specific T-cell responses.

The innate immune responses are characterised by a variety of mechanisms of recognition of foreign pathogens, which could be grouped into three strategies: 'pattern recognition' or recognition of microbial non-self, recognition of 'missing self' and recognition of 'induced or altered self'. The recognition of microbial non-self, or pattern recognition is the most commonly used one, where a family of Toll-like receptors play a central role (Beutler 2004).

NK cells and cytotoxic T cells use a combination of mechanisms to lyse target cells. These include calcium-dependant granule exocytosis and release of cytotoxic proteins (perforin and granzymes). Another mechanism used by NK cells to induce cell death is via FasL, constitutively expressed or induced upon interaction with target cells and inducing calcium-independent Fas-mediated apoptosis (Berke 1997). Another mechanism involves membrane bound or secreted cytokines belonging to the TNF family, including the TNF-related apoptosis-inducing ligand (TRAIL) (Wiley, Schooley et al. 1995). During differentiation, NK cells sequentially and differentially use distinct members of the TNF family or granule exocytosis to mediate cell death. Among the host responses enhancing NK cell function, there is a close interaction with other cell

types producing cytokines, such as IFN α/β, IL-12, IL-15 and IL-18, which promote NK cell cytotoxicity and IFN-γ production (Biron and Brossay 2001).

1.2.2 Specific acquired immunity

This is an adaptive immune response, which has four main attributes: antigen specificity, diversity, immunologic memory and self/non-self recognition. The cells involved in generating an effective immune response are the lymphocytes and antigen presenting cells.

1.2.2.1 Lymphocytes

Lymphocytes are responsible for the development and maintenance of specific immunity and are comprised of two main populations, T cells and B cells, which produce and express specific receptors for antigens.

B-lymphocytes are produced in the bone marrow and have the ability to transform into plasma cells following antigenic stimulation and to produce antigen-specific antibodies.

T lymphocyte responses are the subject of the present study. The importance of T cells has come into focus in the last two decades, with the emergence of AIDS and the search for a vaccine. This subset of cells plays a crucial role in controlling viral infections, as well as malaria and TB. A better understanding of cellular immune responses could provide strategies for the development of vaccines against other persistent viral infections, such as HIV or HCV, as well as for cancer therapy, autoimmune diseases or transplant rejection.

T cells originate from bone marrow stem cells and develop into T precursor cells that migrate to the thymus where they multiply and differentiate. The rate at which the thymus produces T cells is very high in childhood and declines thereafter. Mature T cells are long lived and re-circulate, comprising about 70-80% of lymphocytes in blood

and lymph. The maturation of T cells is characterized by a sequential appearance of certain cell surface molecules. Among the first surface molecules to appear are CD3, T cell receptors (TcR), CD4 and CD8. Thus immature thymocytes are $CD3^+CD4^+CD8^+$. Cortical T cells lose either CD4 or CD8 molecules to become $CD3^+TcR^+CD4^+$ also known as T helper cells or $CD3^+TcR^+CD8^+$ referred to as T cytotoxic cells. Mature T cells migrate to the medulla of the thymus from where they exit into the systemic circulation.

The T-cell receptor (TcR) is a unique antigen-binding receptor on the membrane, which can only recognize antigen that is associated with cell membrane proteins known as major histocompatibility complex (MHC). One function of MHC molecules is to present antigens to T cells. Lymphocytes in the thymus are exposed to various endogenous (self) proteins, particularly the products of MHC. Some nascent T cells that have specificity towards self MHC molecules are eliminated (negative selection), while remaining T cells become "educated" to recognize foreign antigenic peptides that are associated with self MHC (positive selection). Thus, antigen recognition by T cells becomes "MHC restricted," that is, the mature T cell recognizes its specific antigen only if that antigen is presented by the correct MHC molecule. Two kinds of MHC genes, class I and class II, are involved in the development of T cells. Class I MHC genes encode glycoproteins expressed on the surface of all nucleated cells, where they present peptide antigens necessary for the activation of T cytotoxic cells. Class I MHC molecules contain a large α chain associated noncovalently with the much smaller β_2-microglobulin molecule. The α chain is a polymorphic transmembrane protein encoded by genes within the A, B and C regions of the human HLA complex. β_2-microglobulin is a non-variant protein encoded by genes on a different chromosome. Class II MHC genes encode glycoproteins (with a heterodimer structure- α and β chain), which are expressed primarily on antigen presenting cells (macrophages, dendritic cells, B cells),

where they present processed antigenic peptides to T helper cells. Several hundred different allelic variants of class I and II MHC molecules have been identified, MHC being the most polymorphic genetic complex in humans, although any individual can only express up to 6 different class I molecules and up to 12 different class II molecules. Such differences in MHC allele expression have been associated with susceptibility to infectious or autoimmune diseases.

In the course of selective adaptation, T cells learn to recognize foreign antigens in association with protein products of either MHC class I or II genes. It was in the 1970s, when the experiments of Doherty and Zinkernagel, reviewed in (Zinkernagel and Doherty 1997) demonstrated that antigen recognition by T cells is specific not only for viral antigens but also for a MHC molecule. T cells were shown to recognize antigen only when presented on the membrane of a cell by a self MHC molecule, and this attribute of self MHC restriction distinguishes recognition of antigen by T cells from that by B cells.

T helper's cells (CD4+ T-cells) interaction with antigen-MHC class II molecule complexes is followed by cell activation and secretion of cytokines. The secreted cytokines play a crucial role in activating B cells, cytotoxic T cells, macrophages and other cells participating in the immune response. Two types of helper responses have been described, designated Th1 and Th2, which are characterized by different cytokine environments. During a Th1-like response, the predominant cytokines are IL-2, IFN-γ and TNF-β, whereas a Th2 response is characterized by cytokines such as IL-4, IL-5, IL-6 and IL-10. Th1 response is responsible for cell-mediated functions, such as activation of CTLs or delayed hypersensitivity reactions. Th2 functions more effectively as a helper for B cell activation.

Under the influence of cytokines, a CD8+ T cell that recognizes antigen-MHC class I molecule complexes proliferates and differentiates into an effector cell- cytotoxic T cell

(CTL). CTLs are an important host defence against viruses and some tumour cells. They recognize viral peptides that are processed intracellularly and presented to the cell surface as a trimolecular complex with β2-microglobulin and HLA class I. There is experimental evidence showing that CTLs can lyse infected cells prior to further production of progeny virions (Yang, Kalams et al. 1996). In addition to lysis, recognition of infected cells through the epitope specific TCR also leads to release of soluble antiviral factors (Yang, Tran et al. 1997). An example of non-cytopathic killing is HBV infection, where TNF-α and IFN-γ production by CTL leads to clearance of virus from hepatocytes with minimal hepatocyte destruction. (Guidotti, Ishikawa et al. 1996). Further confirmation of the central role CTLs play in controlling viral infections comes from studies of LCMV, where more than 59% of splenic CTLs are LCMV-specific during the acute infection in mice. (Murali-Krishna, Altman et al. 1998). During human acute EBV, up to 44% of peripheral CD8 are EBV-specific (Callan, Tan et al. 1998). Following this massive expansion of antigen-specific cells, lysis of infected cells and production of cytokines and chemokines, long term control of viraemia usually ensues (Murray, Kurilla et al. 1992) (Khanna, Burrows et al. 1992). Viral control is however not always the outcome in several viral infections, including HIV, HCV and HBV, which are characterized by persistent infection. Accumulating data indicate numerous qualitative, in addition to quantitative defects in the T cell response mounted during chronic viral infections (Welsh 2001). A better understanding of such defects is required in order to develop immunotherapeutic strategies suitable for use in the setting of persistent viral infection.

In addition to their marked antigen specificity, T cells have the capacity to exhibit memory. It is this ability to mount a more rapid and stronger immune response which, in cooperation with antibody responses, leads to quick control of secondary infections and elimination of pathogens. Experimental evidence mostly from the LCMV model,

suggests that among the attributes of CD8 memory cells, conferring long-term immunity are:

- Higher precursor frequency of antigen-specific T cells in immune animals compared with naïve animals (Hou, Hyland et al. 1994; Murali-Krishna, Altman et al. 1998; Whitmire, Asano et al. 1998).
- Reprogrammed gene expression profile, including genes that encode IFN-γ and cytotoxic molecules, allowing memory CD8 T cells to express larger quantities of such proteins more rapidly. (Bachmann, Speiser et al. 1999; Grayson, Murali-Krishna et al. 2001). Different pattern of surface proteins involved in cell adhesion and chemotaxis, allowing them a better surveillance of peripheral tissues, reviewed in (Moser and Loetscher 2001).
- Longevity over time, due to homeostatic cell proliferation (Murali-Krishna, Altman et al. 1998). Cytokines such as IL-2, IL-15 and IL-7 play important roles, but the fine mechanisms by which this process occurs has not been well characterized (Ku, Murakami et al. 2000; Schluns, Kieper et al. 2000).

It is based on these two attributes of antigen specificity and immunological memory (ability to recognize a previously encountered antigen and produce antiviral cytokines) that the methods to study T cell responses have been devised.

1.2.2.2 Flow cytometry, HLA-A2 staining, tetramer and intracellular cytokine staining

The techniques of tetramers and intracellular cytokine staining have permitted, for the first time, an accurate quantification of virus specific CD8 responses directly ex-vivo and with the aid of flow cytometry, permitted further functional characterization at an individual cell level.

In this project I studied HBV-specific CD8 responses in peripheral blood only. It would have been interesting to be able to study HBV-specific CD8 responses in the liver in HIV-HBV co-infected patients. This would have however been unethical in the context of patients who controlled HBV replication and acquired natural immunity. Previous studies of HBV-specific CD8 responses in HIV negative HBV chronic carriers showed still a low level of HBV-specific CD8 in the liver, although marginally enriched compared to peripheral blood (Maini, Boni et al. 2000).

Patients included in this study were selected for the expression of the HLA-A2 allele, due to the fact that the immunodominant epitopes for HBV were well characterized in this population. HLA-A2 staining is described in Chapter 2.

The techniques of tetramers and intracellular cytokine staining are based on the mechanisms of CD8 recognition of virally infected cells which express the MHC class I molecules. In humans, these are known as human leukocyte antigen- HLA class I, represented by HLA-A, B and C and are expressed on most nucleated cells. *Fig 1.1* schematically represents CTL recognition of MHC-class I/peptide complexes on the surface of an infected cell. During a viral infection, viral antigen is initially processed by the ubiquitous antigen-processing pathway, resulting in cleavage of viral proteins into short peptides (8-12 aminoacids)(Townsend, Elliott et al. 1990). Peptides are then transported into the endoplasmic reticulum, where they are loaded into the HLA molecules. This binding is very specific, only peptides of certain specificities, largely dictated by their anchor residues, can bind each MHC allele efficiently. The additional

binding of β2-microglobulin stabilizes the complex, which is then transported (via trans-golgi apparatus) to the cell surface and exposed to the cells of the immune system.

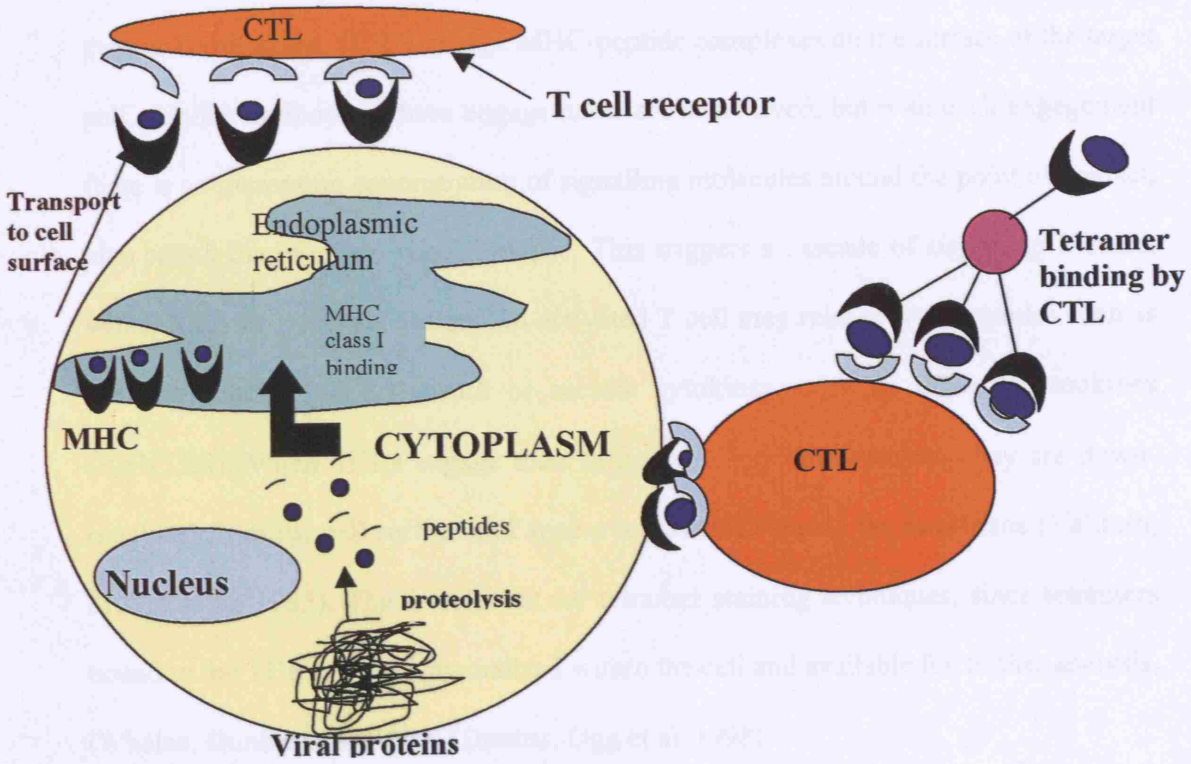


***Fig. 1.1** Presentation of viral antigens to CD8 cells and schematic tetramer binding.* Initial processing of viral antigen is initially processed results in cleavage of viral proteins into short peptides (8-12 aminoacids). Peptides are then transported into the endoplasmic reticulum and loaded into the HLA molecules. This binding is very specific, only peptides of certain specificities can bind each MHC allele efficiently. The additional binding of β2-microglobulin stabilizes the complex, which is then transported to the cell surface and exposed to the cells of the immune system. Only a minority of the host MHC molecules on the surface of an infected cell will express a particular MHC-viral peptide complex. Only a specific subgroup of the CD8 T cells will interact specifically with these low numbers of viral peptide-MHC complexes. The interaction occurs via the T cell receptor (TCR), which is unique for a certain T-cell clone. TCR's engage MHC-peptide complexes on the surface of the target cell. Schematically, the tetramer is represented as four HLA I-peptide complexes, where the peptide represents a defined CTL epitope. This permits binding only to the CTL specific for this epitope and not to CTLs of unrelated specificities.

Only a minority of the host MHC molecules on the surface of an infected cell will express a particular MHC-viral peptide complex. Only a specific subgroup of the CD8 T cells will interact specifically with these low numbers of viral peptide-MHC complexes. The interaction occurs via the T cell receptor (TCR), which is unique for a certain T-cell clone. TCR's engage MHC-peptide complexes on the surface of the target cell. Unlike antibodies, these engagements are short lived, but with each engagement there is an increasing concentration of signalling molecules around the point of contact, also called the immunological synapse. This triggers a cascade of signalling kinases, which leads to T cell activation. An activated T cell may release lytic granules such as perforin, leading to cell death or secrete cytokines, such as IFN-γ, chemokines (RANTES). When TCRs engage their target MHC-peptide complex they are down-regulated from the cell surface and appear in vesicles beneath the membrane (Valitutti, Muller et al. 1995). This is relevant for tetramer staining techniques, since tetramers bound to the TCR are then internalized within the cell and available for further analysis. (Whelan, Dunbar et al. 1999) (Dunbar, Ogg et al. 1998)

Previous experiments used mono or di-mers in an attempt to study CD8-Tcell responses. (Madden, Garboczi et al. 1993), but the MHC class I-peptide and TCR interaction although of high affinity, has a fast off-rate and complexes were not stable enough to permit staining. Tetrameric complexes of class I-peptides were only produced by Altman few years later (Altman, Moss et al. 1996), by the addition of a specific site for enzymatic biotinylation at the tail of the MHC class-I molecule (HLA-A201 was used). The appropriate enzyme, BirA, was used to create molecules, which could be bound in tetrads by streptavidin, to which fluorochromes are attached. This multimerisation allowed stable binding and visualization of antigen-specific CD8 cells. *Fig 1.2* illustrates schematically the specific binding of tetramer to an HBV-specific

CTL, where the A2 HB core 18-27 tetramers bind specifically to HB core 18-27 CTLs and not to CTLs of unrelated specificities

***Fig. 1.2** Schematic tetramer binding*

This figure illustrates a tetramer consisting of 4 HLA A2 molecules bound with the HBV peptide representing common CTL epitope, which is labelled with a fluorochrome. This allows binding to the T cell receptor of CTL's specific for this epitope and not to CTL of unrelated specificities.

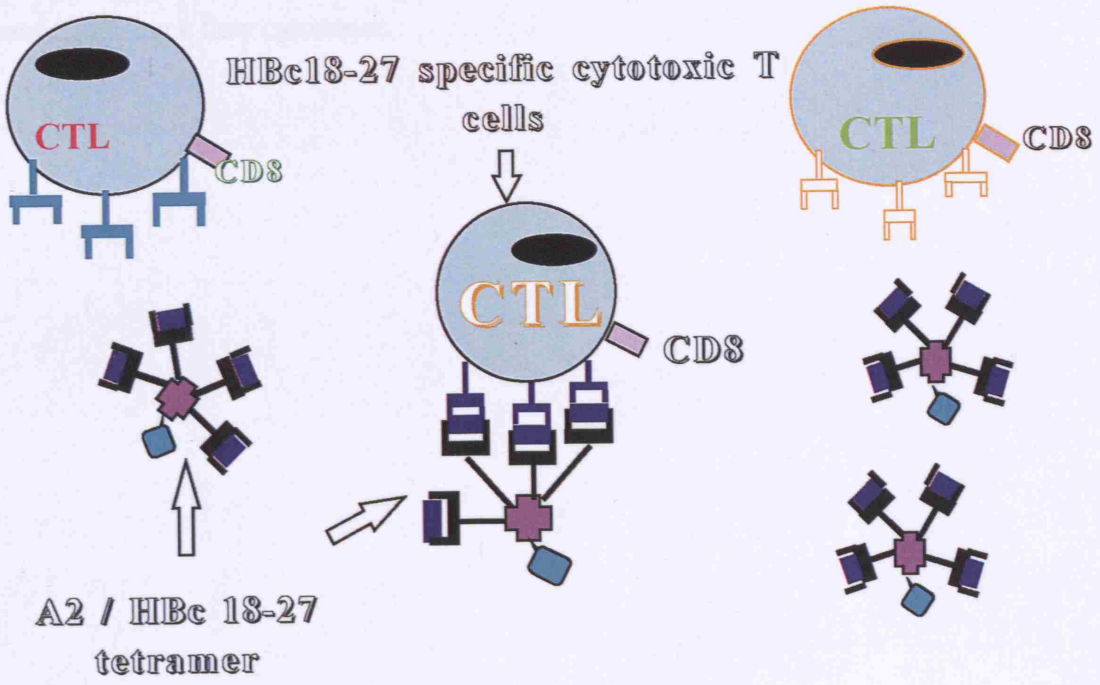
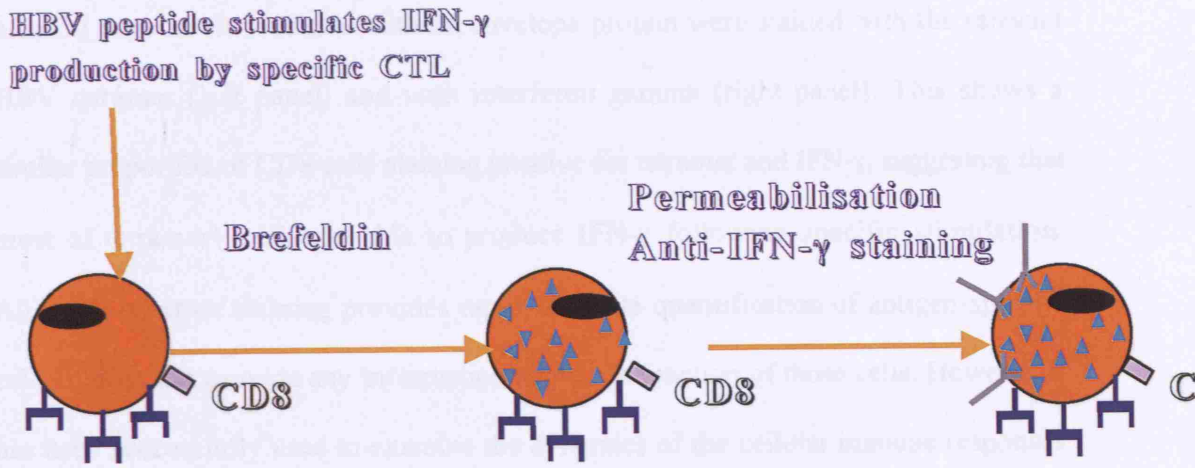


Fig 1.3 schematically represents the principle of the method for intracellular cytokine staining. This is based on the fact that antigen-specific T cells will produce cytokines, (such as IFN-γ) following stimulation with a previously encountered antigen. The addition of Brefeldin A permits the cytokines to be retained within the cell, and after permeabilisation, allows staining with fluorescently labelled anti-cytokine antibody, as well as surface antibodies for CD4 or CD8. Cells can then be analyzed and accurately quantified using a flow cytometer.

Fig. 1.3 Principle of the method for intracellular cytokine staining.

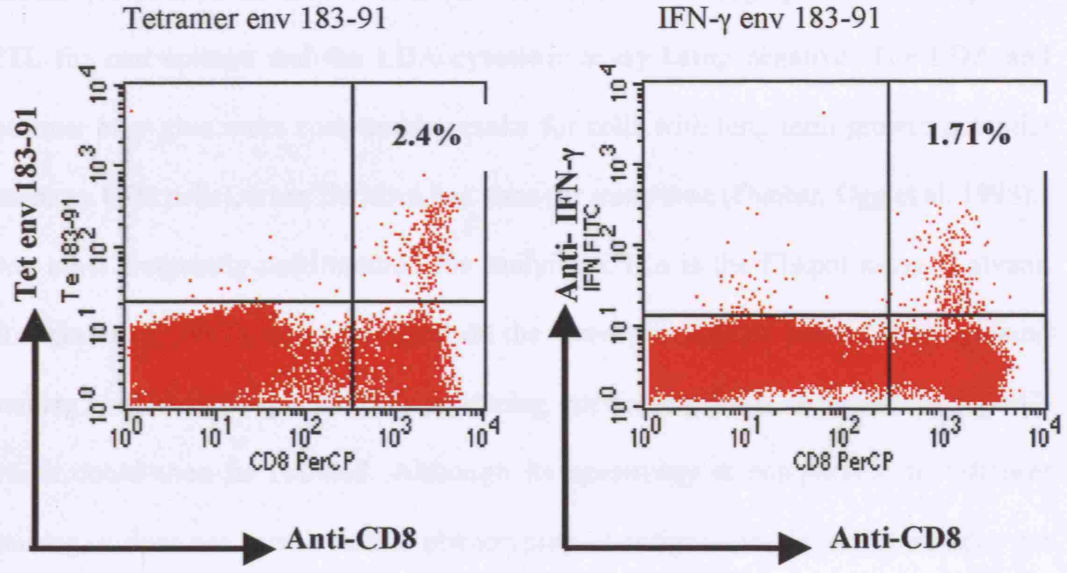
This method is based on the fact that antigen-specific T cells will produce cytokines, (such as IFN-γ) following stimulation with a previously encountered antigen. The addition of Brefeldin A permits the cytokines to be retained within the cell, and after permeabilisation, allows staining with fluorescently labeled anti-cytokine antibody, as well as surface antibodies for CD4 or CD8. Cells can then be analyzed and accurately quantified using a flow cytometer.



This method complements the tetramer staining and it is increasingly recognized that the two approaches should be used in conjunction, since some virus-specific CD8 cells may be tetramer negative (Reignat, Webster et al. 2002) and a proportion of tetramer positive CD8 cells may not produce IFN-γ. *Fig. 1.4* illustrates dot plots from a patient with natural immunity to HBV where peripheral blood mononuclear cells (PBMCs) after 10 days in vitro culture with an envelope protein were stained with the relevant HBV tetramer (left panel) and with interferon gamma (right panel). This shows a similar proportion of CD8 cells staining positive for tetramer and IFN-γ, suggesting that most of tetramer+ cells are able to produce IFN-γ following specific stimulation. Although tetramer staining provides rapid, accurate quantification of antigen-specific cells, it does not provide any information about the function of those cells. However it has been successfully used to examine the dynamics of the cellular immune responses ex vivo during an acute viral infection, revealing gross under-estimations of the virus-specific CTLs by older methods (Callan, Tan et al. 1998) (Tan, Gudgeon et al. 1999). Intracellular cytokine staining for IFN-γ, TNF-α, IL-2 and MIP-1β can be successfully combined with tetramer staining (Appay, Nixon et al. 2000) (Kostense, Raaphorst et al. 2001) (Sandberg, Fast et al. 2001). Since the production of tetramers bound with multiple different epitopes can be labour-intensive and costly, further detailed analysis across a broader range of epitopes lends itself to intracellular cytokine staining. A review of the combined use of tetramer and intracellular staining techniques has shown that most tetramer positive cells were able to produce cytokines, such as IFN-γ. (Appay and Rowland-Jones 2002)

***Fig.1.4** Comparison of tetramer and intracellular cytokine staining techniques.*

Data from the same patient after10 days *in vitro* stimulation with peptide from envelope group. Dot plot of staining for tetramer and anti-CD8 in the left panel, with HBV-specific CD8 cells that are tetramer positive and CD8+ (expressed as percentage of total CD8) being shown in the right upper quadrant. Dot plot of CD8 /IFN-γ staining in the right panel, with HBV-specific CD8 for the envelope epitope 183-91 seen in the right upper quadrant and expressed as percentage of IFN+ of total CD8.



Using the techniques of tetramer and intracellular cytokine staining has several proven advantages over the previously used methods, such as the limiting dilution analysis (LDA), which tests for CTL precursors and their killing potential in a chromium release assay. The numbers detected by LDA are often 50-500 times lower than those using tetramers. To detect a cytolitic activity, a considerable number of cell divisions is needed to take place in order to secure a detectable response, and antigen specific cells may be prone, in certain circumstances, to activation induced cell death. This is the case for instance during acute HBV, where there are discordant results between tetramer and LDA, (Maini, Boni et al. 1999), with the tetramer detecting up to 1% HBV-specific CTL for one epitope and the LDA cytotoxic assay being negative. The LDA and tetramer may give more comparable results for cells with long term growth potential (memory CD8 cells), when the virus has gone for some time (Dunbar, Ogg et al. 1998). One other frequently used method for studying CTLs is the Elispot assay (Lalvani, Brookes et al. 1997), which is based on the same principle as intracellular cytokine staining, with antigen-specific cells producing interferon gamma, detectable as "spots", which could then be counted. Although its sensitivity is comparable to tetramer staining, it does not permit further phenotyping of antigen-specific cells and does not exclude background interferon production from other cells, such as NK cells.

1.3 HIV epidemiology, structure, pathogenesis and treatment options

1.3.1 Global impact of HIV

The number of people living with HIV/AIDS in 2004 was 39.4 million (http://www.unaids.org/wad2004/EPIupdate2004_html_en/Epi04_02_en.htm#P16_3133), with an estimated 3.1 million deaths and 4.9 million new infections in 2004 alone. (AIDS Epidemic Update, Dec 2004 UNAIDS, Geneva) with the worst of the epidemic being centred on sub-Saharan Africa. The extent of the epidemic has been much greater than predicted ten years ago and the impact witnessed so far is only a fraction of the impact to come, unless concerted and effective measures are taken without delay. Since the identification of the retrovirus in 1983 by Luc Montagnier from a lymph node biopsy of a patient with AIDS, there has been an explosion of information about HIV. Never has so much been learned about a disease and its causative agent in such a short time. Although this has greatly expanded our understanding about the human immune system and allowed huge progress in the field of antiretroviral treatments, we are still short of an effective cure or preventative vaccine.

1.3.2 HIV Structure and Pathogenesis

HIV is a retrovirus. Like all viruses, HIV is a parasite that replicates within living cells of the host. It belongs to the lentivirus genus of retroviruses, which are cytopathic. Cytopathic retroviruses induce damage or death to the cells. The same group includes the simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV-1, HIV-2, HIV-0). HIV-1 infects humans, chimpanzees, pigtailed macaques and SCID-human mice, but causes immune suppression with development of AIDS only in humans. HIV-1 accounts for the majority of HIV infections worldwide, with HIV-2

being endemic in many countries of West Africa. HIV-2 strains are less pathogenic and appear to spread more slowly than HIV-1.

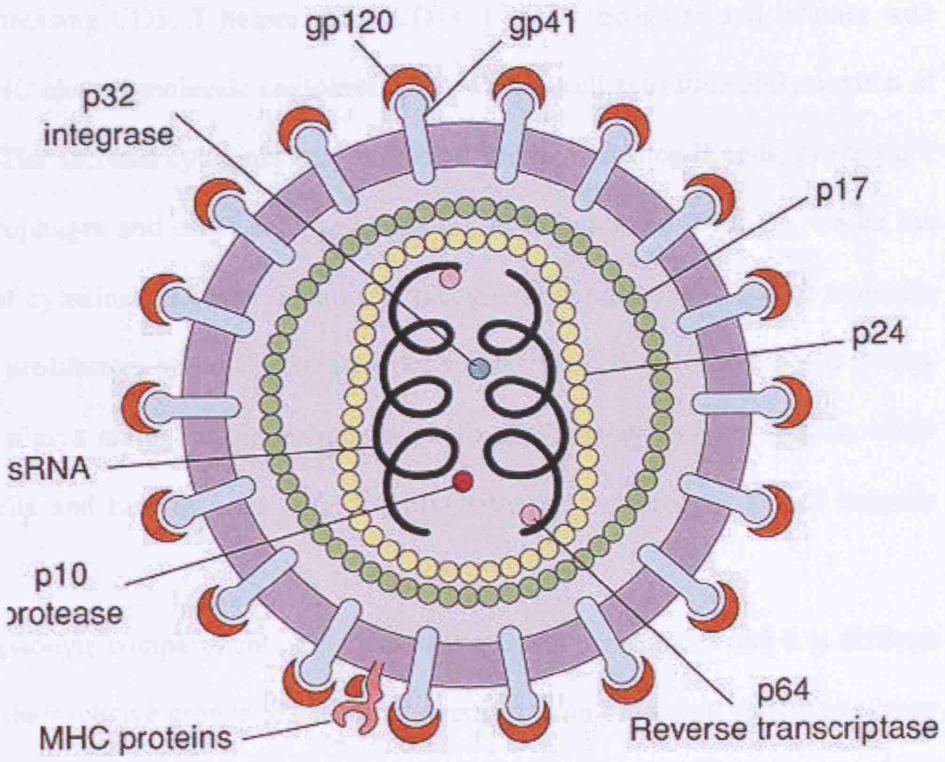
1.3.2.1 Structure of HIV

Human immunodeficiency viruses have an RNA genome and two associated molecules of reverse transcriptase, which catalyzes the "reverse transcription" of viral RNA into DNA. This enables HIV to be integrated into the host DNA and to use the cell's genetic machinery to make new virus. Other nucleoid proteins include the p10 protease and p32 integrase. Surrounding the viral genome and nucleoid proteins are two layers of core proteins, designated p17 and p24. The viral core, or nucleocapsid, is surrounded by an envelope derived from the host cell membrane, which is modified by the insertion of two HIV glycoproteins, gp120 and gp41, both proteins playing important roles in binding of HIV to cells. The HIV envelope is also studded with human proteins, including class I and II MHC molecules. *Fig 1.5* illustrates a diagram of HIV structure.

1.3.2.2 Entry of HIV into cells

The first step in HIV infection is binding of viral gp120 to receptors on target cells. The major cellular receptor for HIV is CD4, a glycoprotein expressed on the membrane of the T lymphocyte helper cell (Th). Chemokine receptors serve as co-receptors that guide the viral envelope glycoproteins into a conformation permitting fusion and entry into the cell. The main co-receptors used are CCR5 and CXCR4, HIV strains having an "R5" or "R4"

***Fig. 1.5** Schematic structure of HIV* (adapted from Kuby, Immunology 4th edition)
HIV has an RNA genome and two associated molecules of reverse transcriptase, which catalyzes the "reverse transcription" of viral RNA into DNA. Other nucleoid proteins include the p10 protease and p32 integrase. Surrounding the viral genome and nucleoid proteins are two layers of core proteins, designated p17 and p24. The viral core, or nucleocapsid, is surrounded by an envelope derived from the host cell membrane, which is modified by the insertion of two HIV glycoproteins, gp120 and gp41, both proteins playing important roles in binding of HIV to cells. The HIV envelope is also studded with human proteins, including class I and II MHC molecules.



tropism. (O'Brien and Moore 2000). Other cells that bind HIV include macrophages and dendritic cells. Macrophages are an important HIV reservoir, including microglia in the brain(Kaul, Garden et al. 2001). Dendritic cells bind HIV through the DC-SIGN receptors and carries it from the mucosal ports of entry to the lymph nodes. (Geijtenbeek, Kwon et al. 2000)

1.3.2.3 Destruction of CD4+ T-cells

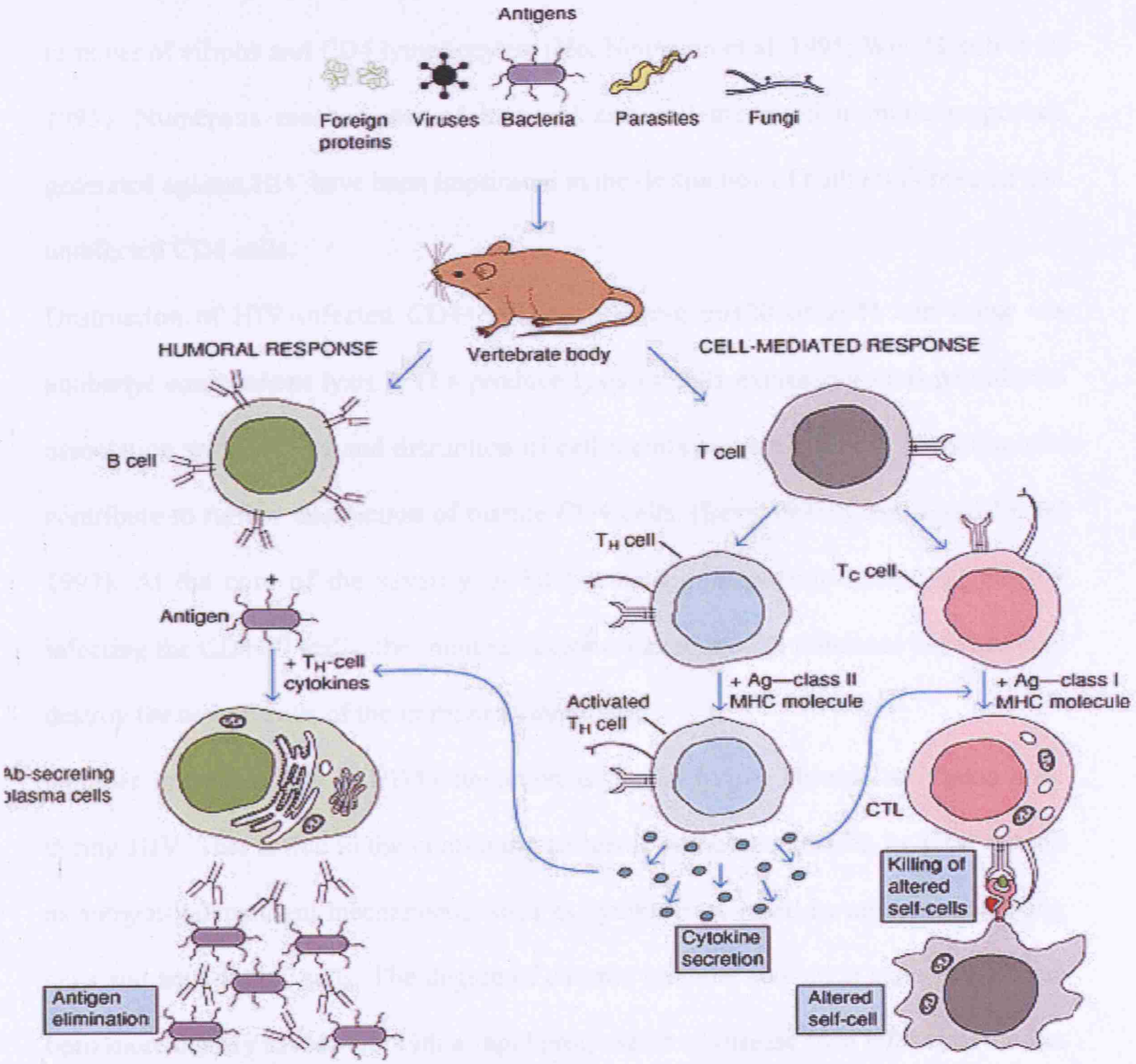
In order to understand the relevance of HIV affinity for CD4+ T cells, as well as the subsequent immune responses during HIV infection, it is essential to briefly review the particularities of T lymphocytes, which play a crucial role in the acquired (specific) immune responses. CD4 is expressed on the membrane of the T helper cells, cytotoxic T cells expressing CD8. T helper cells (CD4+ T-cells) recognize and interact with antigen-MHC class II molecule complexes, followed by cell activation and secretion of cytokines. The secreted cytokines play a crucial role in activating B cells, cytotoxic T cells, macrophages and other cells participating in the immune response. Under the influence of cytokines, a CD8+ T cell that recognizes antigen-MHC class I molecule complexes proliferates and differentiates into an effector cell- cytotoxic T cell (CTL). The CTLs play a major role in eliminating cells that display antigen, such as viral-infected cells and tumour cells. *Fig 1.6* illustrates an overview of T cell immune responses.

The T-lymphocyte compartment comprises multiple sub-populations and it is difficult to monitor their relative growth, death and movement in an individual patient over time (Haase 1999). HIV infection results in a progressive loss of CD4+ T-cells from the circulation as well as total body stores, inducing both quantitative as well as qualitative defects.

Fig.1.6 Diagram of specific immune responses

(adapted from Kuby, Immunology 4th edition, 1997.)

The adaptive immune response comprises the humoral response (via B cells) and the cell mediated response, where the T helper cells play an essential role. CD4 is expressed on the membrane of the T helper cells, cytotoxic T cells expressing CD8. T helper cells (CD4+ T-cells) recognize and interact with antigen-MHC class II molecule complexes, followed by cell activation and secretion of cytokines. The secreted cytokines play a crucial role in activating B cells, cytotoxic T cells, macrophages and other cells participating in the immune response.



Several mechanisms are implicated in the HIV-mediated depletion of CD4+ cells, such as accelerated destruction of mature CD4+ cells, chronic immune activation and T-cell death, impaired production of new T cells, all occurring simultaneously.

Destruction of mature CD4+ cells can occur as a direct effect of HIV. Once the HIV virions begin to assemble and bud from the infected cell, extensive damage to the cell membrane occurs, leading to cell death. This was suggested in the early 1980s by experiments showing the cytopathic effect of HIV in tissue cultures and further validated in 1995 by experiments confirming the HIV tropism for CD4 and rapid turnover of virions and CD4 lymphocytes. (Ho, Neumann et al. 1995; Wei, Ghosh et al. 1995). Numerous mechanisms of humoral and cell mediated immune responses generated against HIV have been implicated in the destruction of both HIV-infected and uninfected CD4 cells.

Destruction of HIV-infected CD4+ cells expressing gp120 or gp41 can occur via antibody/ complement lysis. CTLs produce lysis of cells expressing viral peptides in association with MHC-I and disruption of cell membrane and syncytia formation can contribute to further destruction of mature CD4 cells. (Levy 1993; Casella and Finkel 1997). At the core of the severity of HIV-related immune impairment is that, by infecting the CD4+ T-cells, the immune responses generated to eliminate the virus will destroy the central cells of the immune system itself.

Another important role in CD4+ depletion is played by the chronic activation state during HIV. This is due to the continuing antigenic stimulation driven by HIV as well as antigen-independent mechanisms, such as cytokines released by antigen presenting cells and activated T cells. The degree of chronic immune activation state in HIV has been more closely associated with a rapid progression of disease than HIV viral load in some studies (Giorgi, Hultin et al. 1999) (Simmonds, Beatson et al. 1991). Different

techniques were used to investigate the chronic immune activation state associated with HIV (studies of T-cell telomere length, maturation phenotype and T-cell activation markers, Ki67 marker of cell proliferation). Although there were differences in the T-cell subsets found to be affected by HIV, all studies described a state of high T cell turnover with a high degree of cell proliferation and increased death affecting both CD4+ and CD8+ populations, with memory cells being affected more than the naive cells (McCune, Hanley et al. 2000) (Hellerstein, Hanley et al. 1999). This chronic activation state becomes particularly relevant in HIV infected patient affected by other chronic infections, such as malaria or TB, which drive the activation of the immune system and CD4 count decline can occur much faster. Such accelerated destruction of CD4 cells may lead to a very rapid depletion of helper cells, and this is indeed the case in vertically infected children or adults acquiring HIV later in life who have limited ability to regenerate new T cells The gradual decline in immune function seen in most individuals (progressing to AIDS over 5-10 years) is due to compensatory mechanisms of T-cell production, such as the bone marrow, functioning thymus or both, until regenerative failure of theses organs also occurs. The thymus is an important target for HIV (McCune 1997) (Douek, McFarland et al. 1998). Studies in mice have shown that thymocyte destruction is more rapidly effected by the CXCR4-utilising isolates of HIV, which affects progenitor T cells, whereas the CCR5-utilising isolates affect the more mature thymocytes (Su, Kaneshima et al. 1995; Berkowitz, Alexander et al. 1998). Studies in humans showed that there is a decrease in the number of cells bearing TCR excision circles (TRECs), which marks recent intrathymic TCR rearrangement, associated with HIV disease progression (Douek, McFarland et al. 1998; Zhang, Lewin et al. 1999). After use of HAART, there is evidence of regenerative changes in the thymus (Autran, Carcelain et al. 1997; Zhang, Lewin et al. 1999). T-cell production is also impaired via infection-mediated death of progenitor cells, cytokine dysfunction,

opportunistic infections of the bone marrow (CMV, MAI), or malignancies. Impaired production of new cells eventually results in the collapse of the immune system.

1.3.2.4 Cellular immune responses to HIV

Similar to other viral infections, such as CMV and EBV, HIV infection is characterized by the presence in large numbers of HIV-specific cytotoxic T cells (CD8+ T cells), which appear early in the infection and their presence correlates with a rapid decline in viral burden. CTLs recognize virus peptides presented by HLA class-I molecules. Different HLA types present different peptides and may affect the quality of the immune response (exemplified by HLA-B57 and HLA-B27 patients who are slow progressors). CD8 responses during acute infection follow the rise in HIV levels and when that response reaches a peak, virus levels fall, suggesting that CTLs are responsible. (Wilson, Ogg et al. 2000). The HIV-specific CTL response during acute infection is large, with 10% of total CD8 being HIV-specific (Kuroda, Schmitz et al. 1999; Wilson, Ogg et al. 2000). After the acute infection, an inverse relationship has been demonstrated between HIV levels and CTL responses (Ogg, Jin et al. 1998), although examination of a broader spectrum of epitopes did not support this correlation (Betts, Ambrozak et al. 2001). HIV-specific CTLs were shown to secrete cytokines (IFN-γ, TNF-α) (Meylan, Guatelli et al. 1993) (Emilie, Maillot et al. 1992), which affect virus replication in vitro and chemokines (MIP 1α, MIP1β, RANTES), which suppress HIV replication by downregulation of CCR5 (Cocchi, DeVico et al. 1995; Wagner, Yang et al. 1998). Although this strong CD8 response continues to be present throughout during the chronic stages of HIV at high frequencies (1-2% of total CD8 being specific for a dominant HIV epitope), it ultimately fails to control viral replication and leads to viral escape and a subsequent collapse of immune functions.

Recent studies combining the techniques of tetramer with intracellular cytokine staining have highlighted functional defects of HIV-specific CD8 cells. Although present in

large numbers during acute and chronic infection, HIV-CTLs have been shown to produce decreased levels of perforin (a protein important in triggering target-cell death) when compared with other CTLs such as CMV (Appay, Nixon et al. 2000), associated with expression of an immature phenotype (Champagne, Ogg et al. 2001). This could result in less efficient killing of HIV by CTLs. Defects in CTL function can play a major role in selection of virus escape mutants that can make it harder for the immune system to maintain control of the virus. Inefficient killing by CTL favours cells infected with mutant virus, which then escapes lysis. Insufficient breadth of the CD8 response may be a major factor contributing to the frequent failure of CTL control through development of viral escape mutations. Recent data indicate that the polyclonality of individual CD8 responses in addition to their multispecificity may be factors for consideration in the design of future CD8-inducing HIV vaccines (Lopes, Jaye et al. 2003). At the core of this failure of CD8 responses to successfully control HIV may be the lack of adequate CD4 help, leading to impaired functioning of dendritic cells as well as CD8+ T cells. Given the susceptibility of CD4+cells to destruction by HIV virus, it is not surprising that HIV-specific CD4 responses and recall antigen responses are poor in HIV infected patients (Clerici, Stocks et al. 1989). Only a minority of patients (referred to as long term non-progressors) are able to preserve good CD4 counts and very low HIV viral loads over time. There is a strong link between the weak CD4+ T cell responses and the suboptimal CD8 response to HIV. As the infection progresses, both T cell responses decline further, leading to the collapse of the immune system.

1.3.3 Highly Active Antiretroviral Therapy (HAART)

HAART was introduced in 1995 and consists of a combination of antiretroviral drugs, including

inhibitors of HIV transcriptase and protease. Currently, the main classes in use are:

- nucleoside reverse transcriptase inhibitors (eg.: zidovudine, lamivudine, didanosine, stavudine, abacavir)
- non-nucleoside reverse transcriptase inhibitors (eg.: nevirapine, efavirenz)
- protease inhibitors (eg.: nelfinavir, indinavir, ritonavir, lopinavir)
- fusion inhibitors (T20)
- nucleotide analogues (eg: tenofovir).

The consistent use of HAART leads to recovery of CD4 counts and reduction in HIV VL for considerable periods of time, usually years, until drug resistance develops. Adherence to therapy is crucial to achieving a good response, which is maintained over time.

The main benefit of HAART is the possibility of immune reconstitution. Studies have shown that CD4+ T cell reconstitution occurs in two phases. Firstly, the rapid increase in CD4 counts is attributed to a redistribution of T cells from lymphoid tissues (memory CD4 T cells) into peripheral blood (Cavert, Notermans et al. 1997) (Tenner-Racz, Stellbrink et al. 1998; Bucy, Hockett et al. 1999). This is followed by a second phase of increase of CD4 naïve and memory T cells in the periphery (Autran, Carcelain et al. 1997) (Pakker, Notermans et al. 1998), as well as continued thymic production (Sempowski and Haynes 2002). HAART also reduces the HIV-related chronic immune activation, as shown by a reduction in cell activation markers on CD4 T cells in peripheral blood (Autran, Carcelain et al. 1997) (Lederman, Connick et al. 1998) (Rizzardi, Tambussi et al. 2000) and tissues (Andersson, Fehniger et al. 1998). This contributes to a reduction in the high rate of cell death and promotes restoration of memory cell numbers and functional capacity.

CD4+ T cell responses against previously encountered pathogens, such as CMV (Komanduri, Viswanathan et al. 1998; Komanduri, Donahoe et al. 2001; Jansen, Piriou et al. 2006), EBV (Kostense, Otto et al. 2002) or Mycobacterium tuberculosis can be

restored by HAART (Hsieh, Hung et al. 2000; Lawn, Badri et al. 2005; Lawn, Bekker et al. 2005) and reviewed by (Carcelain, Debre et al. 2001). The clinical benefit of such immunological restoration has been confirmed by studies showing the safety of discontinuation of prophylactic treatments, such as PCP (Furrer, Egger et al. 1999) and CMV (Jouan, Saves et al. 2001) and reviewed in (Kovacs and Masur 2000). Other opportunistic infections such as cryptosporidiosis (Carr, Marriott et al. 1998) (Miao, Awad-El-Kariem et al. 2000) and Kaposi sarcoma respond directly to therapy with HAART alone (Gill, Bourboulia et al. 2002).

There have also been descriptions of syndromes of immune reconstitution due to HAART, such as CMV vitritis (Karavellas, Azen et al. 2001; Jevtovic, Salemovic et al. 2005), Mycobacterium Avium Complex lymphadenitis (Phillips, Bonner et al. 2005) or worsening of TB, occurring soon after starting HAART (Michailidis, Pozniak et al. 2005; Manosuthi, Kiertiburanakul et al. 2006) and attributed to restoration of pathogen specific immunity.

In this study we addressed the question of restoration of HBV-specific CD4 and CD8 responses with HAART in chapter 3 and chapter 5.

Despite these achievements of immune restoration against different pathogens, the HIV-specific CD4 responses have been shown to remain impaired in chronic HIV-1 infection, which may play a role on the viral rebound and worsening immune function seen after discontinuation of HAART. (Davey, Bhat et al. 1999; Garcia, Plana et al. 1999) (Neumann, Tubiana et al. 1999) (Hatano, Vogel et al. 2000). Some studies (Pitcher, Quittner et al. 1999) (Betts, Ambrozak et al. 2001) using the technique of IFN-γ expression (previous studies used lymphoproliferative assays after stimulation with HIV p24 antigen) showed that some HIV-specific CD4 responses were maintained during the chronic infection. More studies are needed to further characterize the functional capacity of these cells. One study suggested that the HIV-specific IFN-γ

producing CD4+ cells have impaired proliferative potential, which is further worsened by active virus replication (McNeil, Shupert et al. 2001). Studies of primary HIV infection- (Rosenberg, Billingsley et al. 1997 {Oxenius, 2002 #344; Oxenius, Sewell et al. 2002) showed that patients treated early during the acute infection were able to preserve some HIV-specific CD4 responses, albeit transiently. HIV-specific CD8 responses also decline after the introduction of HAART.

1.3.4 HIV Vaccines

Boosting HIV-specific immunity remains an outstanding question, with several vaccine trials underway. Although HIV has been fully sequenced and there is a good primate model, the search for a vaccine continues. The vaccines promoting neutralising antibodies and containing viral envelope did not work due the high virus variability and different susceptibility to neutralisation in cell culture compared to in vivo. The problem lied in the structure of the HIV envelope and the conformational changes in the envelope structure on binding to the target cells (Kwong, Wyatt et al. 1998; Wyatt, Kwong et al. 1998) (Kwong 2005).

Most current trials are T-cell vaccine trials looking at similar ways to stimulate an HIV-specific CD8 response, which will protect against infection or at least limit the immunosuppressant effect of HIV. Although the wealth of current T-cell HIV vaccine trials (reviewed (McMichael 2006)) differs in the immunogens, delivery systems and vectors used, most are testing the same hypothesis that the T cell immune response induced will protect against challenge doses of HIV in a similar way in which they protect against challenge doses of SHIV in macaques (Shiver, Fu et al. 2002). T cell vaccine clinical trial teams are using standardised assays (Maecker, Rinfret et al. 2005) looking at peptide antigen stimulated IFN-γ production as their main measure of vaccine immunogenicity, with either intracellular cytokine staining or elispot. So far, it has been shown that DNA stimulates a weak T cell response in humans, which is predominantly

CD4 (MacGregor, Ginsberg et al. 2002), but DNA prime with MVA (modified vaccinia virus Ankara) boost gives stronger responses than either alone, which peak at day 7 but are short lived (Vuola, Keating et al. 2005).

Although there is hope that such T cell vaccines will elicit an effective CD8 response, there is still uncertainty regarding the most effective antiviral functions of CD8 T cells during HIV infection. HIV CTLs of different specificities are implicated in acute versus chronic HIV infection (Goulder, Altfeld et al. 2001) and some T cell responses have been shown to have a suppressive effect leading to virus escape mutants, whereas others offer little protection (Altfeld, Allen et al. 2002). A more in depth understanding of the protective effects of CTLs and role of IFN-γ in HIV may be required prior to designing an efficient vaccine.

1.4 HBV epidemiology, structure, pathogenesis and treatment options

1.4.1 HBV global impact

Over 350 million people are infected with HBV worldwide, according to the Centers for Disease Control and Prevention (CDCP) and HBV alone is responsible for 1 million deaths each year. The highest prevalence of HBV is found in Southeast Asia, Africa and China, where vertical transmission and infected blood products continue to drive the epidemic ((Kane 1995);(Margolis, Alter et al. 1991). In Western Europe and Australia carrier rates are low to under 0.1% (Margolis, Alter et al. 1991; Kane 1995). Coinfection with HIV and HBV is common because of shared routes of transmission, but HBV is transmitted more efficiently both in men who have sex with men and intravenous drug users (Kingsley, Rinaldo et al. 1990). Up to 10% of HIV positive patients have chronic hepatitis B (Rustgi, Hoofnagle et al. 1984; Homann, Krogsgaard et al. 1991; Konopnicki, Mocroft et al. 2005).

1.4.2 Natural history of HBV infection

Hepatitis B has a wide spectrum of clinical manifestations. During the acute phase in adults, it can be entirely asymptomatic. In 30-50% of cases patients may present with jaundice (McMahon, Alward et al. 1985), but only rarely does it cause fulminant hepatitis (0.1-0.5% of adults). Children and neonates are usually asymptomatic. The outcome of acute HBV infection largely depends on the age at acquisition. Infections acquired in adulthood result in viral clearance in up to 90-95% of cases, whereas up to 90% of infants infected perinatally from highly infectious mothers (HBeAg+) will become chronic HBV carriers(McMahon, Heyward et al. 1980). The maternal HBV DNA load is an important determinant of chronicity, with only 10% of babies born to HB eAg negative/ anti-HB e+ (low infectivity carrier) mothers becoming persistently infected (Chang 2000). The importance of age and a fully developed immune system in resolving the acute infection suggests that HBV infection is a dynamic process, where

the host-virus interaction plays an important role. This will be further discussed in the pathogenesis section. Patients who fail to control the initial viral infection and acquire chronic carrier status (defined by the presence of HB s Ag at 6 months after the initial infection) are at risk of developing cirrhosis and end-stage liver disease in 15-40% of cases (Maddrey 2000).

1.4.3 HBV structure and pathogenesis

1.4.3.1 HBV structure

The hepatitis B virus is an enveloped DNA virus from the Hepadnaviridae family. It has a partially double stranded DNA genome that encodes four overlapping open reading frames (ORFs): the envelope gene (preS/S), core gene (preC/C), the X gene and the polymerase gene (P). (Wei Y, Clin Liver Dis 2001). HBV enters the hepatocyte via an unknown receptor and after translocation to the nucleus, the relaxed circular DNA is converted into fully double stranded HBV cccDNA (closed circular DNA). The cccDNA serves as template for the production of pregenomic RNA and messenger RNA (mRNA). The mRNA is translated into envelope and X proteins. The pregenomic RNA is capsidated into core particles and reverse transcribed into an open circular DNA molecule to become progeny core molecules. These progeny cores can either enter the nucleus to repeat the cycle and increase the pool of cccDNA or can acquire envelope protein and perpetuate the infection of other hepatocytes.

There are eight HBV genotypes (A-H), with A and D being the most common ones in the Western countries and India and B and C more common in Asia, E in Africa and F in central and South America, G and H in North America. The geographical distribution of the various genotypes relates to the different clinical pictures and degrees of severity of disease around the world. (Kao 2002).

1.4.3.2 Overview of HBV pathogenesis

In order to understand how HIV-related immunodeficiency may impact on control of HBV replication, the essential research done in the field of HBV immunology is reviewed.

HBV infection in immunocompetent adults results in over 90% of cases in a self-limited transient hepatitis followed by viral clearance and only 5-10% of patients become chronic HBV carriers (Hoofnagle 1981; Wright and Lau 1993). When infection occurs in neonates, over 90% of them will become persistently infected, suffering variable degrees of liver disease. HBV carriers are at risk of developing cirrhosis and hepatocellular carcinoma (Tiollais, Pourcel et al. 1985)

In dealing with the acute infection, the innate and adaptive immune responses, both humoral and cellular components play important roles. Clearance of acute infection is indicated by disappearance of HBV antigens, development of neutralizing antibodies together with strong CD4 and CD8 responses that persist over time. Good progress on HBV immunology occurred in recent years. The development of novel techniques such as HLA class I tetramers have permitted for the first time a detailed analysis of the cellular immune responses involved in acute and chronic HBV infection.

1.4.3.3 Host range and animal models

The narrow host range of HBV (man and chimpanzees) has limited studies of HBV pathogenesis. Other hosts of hepadnaviruses, the woodchuck, ground squirrel and Pekin duck have poorly characterized immune systems. An animal model for HBV, the HBV transgenic mouse, was created (Guidotti, Matzke et al. 1995), which was able to express all HBV gene products and replicate virus at high levels in the primary hepatocyte in vivo. This permitted further characterization of the mechanisms involved in HBV pathogenesis.

1.4.3.4 Antibody responses

Fig. 1.7 illustrates the time course of detectability of different antibodies. Detection of HB surface antigen alone may be the first serological marker of acute HBV. The first antibody to appear is anti-HBcore, followed by anti-HB e antibodies, which is an early sign of recovery. Antibodies against the S, pre S1 and preS2 regions are neutralizing and their appearance, which is T cell dependent (Milich and McLachlan 1986), coincides with clearance of HBs Ag and development of immunity. Anti-HBs titres are associated with protective immunity both after natural infection as well as vaccination. Patients who do not clear HB surface antigen, but acquire anti-HB-e Ab have lower HBV DNA (as a measure of HBV replication) and a better prognosis (low infectivity carriers) when compared with HBeAg positive patients (high infectivity carriers). There is however the exception of patients infected with a mutant HBV virus (mutation in the pre-core region), who display high viral load and tend to progress to severe liver disease inspite of undetectable HB e Ag .

Fig. 1.7

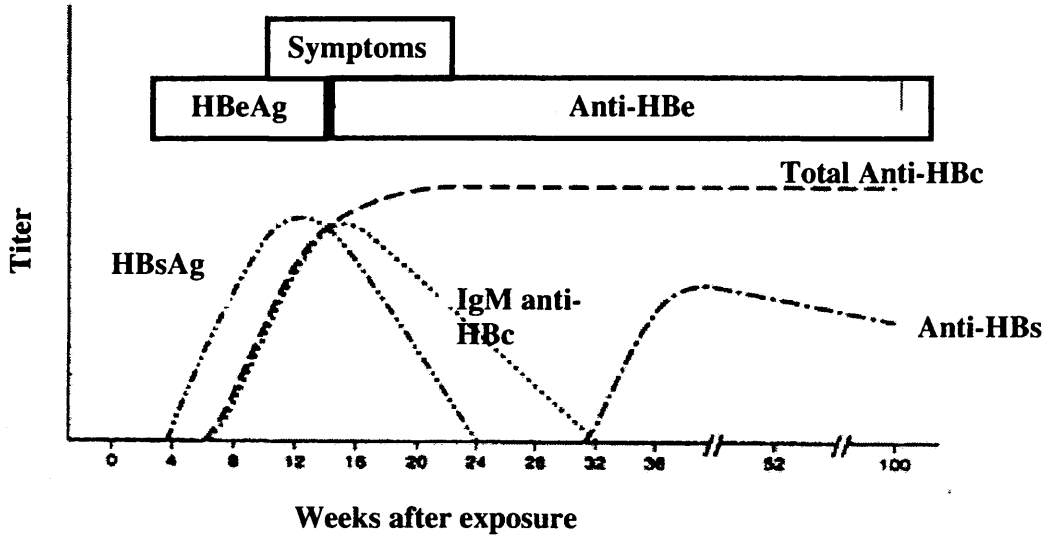


Fig.1.7 *Time course of detectability of different antibodies in a patient recovering from acute hepatitis B.*

Detection of HB surface antigen alone may be the first serological marker of acute HBV. The first antibody to appear is anti-HBcore, followed by anti-HB e antibodies, which is an early sign of recovery. Antibodies against the S, pre S1 and preS2 regions are neutralizing and their appearance, which is T cell dependent , coincides with clearance of HBs Ag and development of immunity.

1.4.3.5 Cellular immune responses in acute self-limited HBV

HBV is a non-cytopathic virus and the kinetics of virus-host interaction are crucial to the outcome of infection.

Similar with many viral infections, most cells recognise the presence of double stranded viral RNA (Ishikawa and Biron 1993) and respond with production of INF α/β, which interferes with viral replication by inducing 2'5'-oligoadenylate synthetase and dsRNA-dependent protein kinase (PKR), leading to inhibition of protein synthesis. (Vilcek 1996). INF α/β leads to recruitment and activation of macrophages. Activated macrophages secrete chemokines, such as MIP-1α (macrophage inflammatory protein 1α), which favours recruitment of NK cells to the liver (Salazar-Mather, Lewis et al. 2002). During acute HBV infection, NK cells may directly lyse infected cells or downregulate HBV replication by producing IFN-γ and TNF-α. In addition, NKT cells have been shown in the transgenic mouse model to produce INF-γ and downregulate HBV replication (Kakimi, Guidotti et al. 2000; Kakimi, Lane et al. 2001), and this process is amplified by IFN-γ dependent recruitment and activation of NK cells (Carnaud, Lee et al. 1999; Hayakawa, Takeda et al. 2001).

Patients who recover from acute infection with HBV have the ability to mount efficient HBV-specific helper and cytotoxic T cell responses that persist long term after resolution of infection (Penna, Artini et al. 1996) (Rehermann, Ferrari et al. 1996) . Such patients have strong and multispecific HBV-specific CD8 responses to a range of different epitopes within the core, envelope and polymerase regions (Bertoletti, Ferrari et al. 1991; Nayersina, Fowler et al. 1993; Rehermann, Fowler et al. 1995; Maini, Boni et al. 1999), which seem crucial in controlling viral replication. Chronically infected patients are characterized by weak or barely detectable HBV-specific cytotoxic CD8 responses (Bertoletti, Costanzo et al. 1994) (Chisari 1997; Maini, Boni et al. 2000). This association between strong antiviral CTL responses and outcome of infection was

further confirmed by extensive studies in the transgenic mouse model, and most convincingly by the recent studies in chimpanzees, showing loss of viral control, in association with CD8 depletion (Thimme, Oldach et al. 2001). These studies confirmed the role of CTL and stressed the non-cytopathic nature of HBV in the acute infection. HBV replication is abolished in the hepatocytes of the transgenic mouse by cytokine-dependent pathways, which do not require the destruction of hepatocytes (Chisari 1997) (Guidotti, Rochford et al. 1999). Cytokines such as IFN-γ and TNF-α are able to selectively abolish intracellular HBV replication without leading to cell death. HBV gene expression and replication was abolished in all the hepatocytes, the viral nucleocapsids disappeared from cytoplasm and viral RNA was degraded from nucleus under conditions of less than 1% hepatocyte destruction (Guidotti, Ishikawa et al. 1996) (Guidotti and Chisari 2000) (McClary, Koch et al. 2000)

Furthermore, patients recovering from acute infection have low but stable frequencies of HBV-specific CD8, which are maintained over time (Rehermann, Ferrari et al. 1996) (Webster, Reignat et al. 2004). This long term maintenance of memory CTLs may be associated with persistence of trace amounts of HBV DNA (antigen-dependent memory CD8) (Rehermann, Fowler et al. 1995) and could account for transmission seen in transplant recipients (Chazouilleres, Mamish et al. 1994) and blood transfusion recipients from HBV seronegative donors. Such "occult" HBV may be of relevance in the context of immunosuppression, either after chemotherapy or HIV-related, where reactivation from previous natural immunity to a high level HBV carrier state has been reported.

The maintenance of such HBV-specific memory CD8 responses is the focus of Chapter 3, where we analyzed the impact of HIV on HBV-specific CD8 memory responses in a group of patients with natural immunity to HBV. The crucial role CTLs play in controlling acute HBV infection has been reviewed here, however, like most viral

infections, it is likely that successful control depends on effective interactions between both the innate and adaptive immune responses. Although we did not specifically address the role of the innate immune responses to HBV in this study, it is increasingly recognized that they play an important role in controlling initial viral replication. This is reflected by the fact that, after initial infection, most HBV virions are cleared in the incubation phase, prior to the onset of clinical symptoms (Webster, Reignat et al. 2000). Chapter 4 describes two patients who recover from acute HBV inspite of very few detectable HBV-specific CD8 responses, but in the presence of strong CD4 responses and where the innate immune responses may have been sufficient for effective viral control.

1.4.3.6 Cellular immune responses in chronic HBV

Beside the central role played in controlling acute infection, CTLs are believed to be at the centre of initiating liver damage in chronically infected carriers. It was believed that in chronic HBV infection the degree of liver damage was related to the recognition of infected hepatocytes by HBV-specific CTL and thus the degree of liver damage was proportional to the number of HBV-specific CTLs. (Moriyama, Guilhot et al. 1990) (Chisari and Ferrari 1995)

The availability of HLA-peptide tetramers 5-6 years ago has made possible for the first time visualization of HBV-specific CD8 directly ex vivo in the liver and peripheral blood (Maini, Boni et al. 1999). These studies have shown that in circumstances of HBV viral persistence, the magnitude or breadth of HBV CTL does not seem to be directly proportional to the extent of liver damage. Chronic carriers without evidence of liver damage and with low HBV DNA have functionally active HBV-specific CTLs in the liver and peripheral blood (Maini, Boni et al. 2000). Carriers with significant liver damage have been found to have large infiltrates of CD8 T-cells on biopsy, which do

not seem to be HBV-specific, suggesting that antigen-nonspecific mononuclear cells rather than a cytolytic effect of HBV-specific CD8 cells may be responsible for the liver damage.

In chronic HBV carriers the HBV-specific CTL responses detectable in peripheral blood are usually narrow and focused against subdominant epitopes. Functional alterations, such as impaired tetramer binding have been described (Reignat, Webster et al. 2002). The hypo-responsiveness of T cells in chronic HBV has partly been attributed to the very high antigen loads, which may result in T cell exhaustion. Interestingly, treatment with the antiviral agent lamivudine is associated with an increase in both HBV-specific CD4 (Boni, Bertoletti et al. 1998) and CD8 cells (Boni, Penna et al. 2001) in HIV negative HBV chronic carriers. In chapter 5 I address the question of treatment of chronic HBV during HIV.

Many recent immunological studies have focused on a subpopulation of specialised T cells, which are able to actively regulate immune responses and are integral part of the T cell repertoire (Sakaguchi 2000; Maloy and Powrie 2001). These cells (Baecher-Allan, Brown et al. 2001) are mainly CD4+ and express the phenotypic marker CD25 and have been shown to suppress immunological responses against self (Sakaguchi, Sakaguchi et al. 1995) and foreign antigens (Franzese, Kennedy et al. 2005)

A recent study looked at CD4+CD25+ regulatory T cells in patients with chronic and resolved HBV infection and demonstrated that circulating regulatory cells modulate function and expansion of HBV-specific CD8 cells ex vivo, but failed to demonstrate a role in the pathogenesis chronic HBV infection (Franzese, Kennedy et al. 2005).

1.4.4 HIV-HBV coinfection

1.4.4.1 Natural History of co-infection

A recent large prospective study showed increased mortality attributable to liver disease in HIV-HBV co-infected patients (14.2/1000 person years) compared with patients infected with HBV alone (0.8/1000 person years). Patients with lower CD4 counts were at greater risk (Thio, Seaberg et al. 2002). Previous cross-sectional studies suggested an increased risk of cirrhosis in co-infected patients (Colin, Cazals-Hatem et al. 1999). The levels of HBV replication, measured by HBV DNA polymerase activity are increased in coinfected carriers (Perrillo, Regenstein et al. 1986; Gilson, Hawkins et al. 1997). Enhanced viral replication was confirmed in studies of HBeAg and BV DNA expression on hepatocytes (Goldin, Fish et al. 1990). This enhanced viral replication is associated with a lower rate of spontaneous HBeAg loss over time, from 20% in HIV negative to 4% in HIV positive patients (Mai, Yim et al. 1996; Colin, Cazals-Hatem et al. 1999). The rate of HB eAg loss was 12% in the HIV positive group, compared to 49% in the HIV negative arm over a median of 2.8 years of follow-up in a study done at the Mortimer Market Centre (Gilson, Hawkins et al. 1997) (where this project also took place). Research done in the field of natural history of HBV showed higher reactivation and reinfection rates in the context of HIV related immunosuppression. (Vento, S Lancet 1989, Lazizi Y JID 1998). Although HIV patients were reported to have lower ALT levels and necro-inflamatory activity (Colin, Cazals-Hatem et al. 1999) (Gilson, Hawkins et al. 1997), subsequent prospective studies showed an increase in morbidity and mortality in the HBV-HIV co-infected group, which was worse in patients with lower CD4 counts (Thio, Seaberg et al. 2002).

HIV natural history, on the other hand, does not appear to be influenced by HBV (Konopnicki, Mocroft et al. 2005), although there is an increased risk of antiretroviral related toxicity (Sulkowski 2003) (Saves, Vandentorren et al. 1999).

ALT flares in HBV/HIV co-infected patients can be drug related, usually within the first 6 months of starting a new HAART regimen. They may be related to immune restoration in sporadic cases of HBV clearance with HAART without anti-HBV agents (Carr and Cooper 1997) (Velasco, Moran et al. 1999) or may simply be due to spontaneous HBe Ag seroconversions. Flares have also been described in relation to discontinuation of lamivudine containing HAART (Altfeld, Rockstroh et al. 1998), or in association with the development of lamivudine resistance (Bessesen, Ives et al. 1999). Additionally, superinfection with other hepatotropic viruses must always be excluded.

1.4.4.2 Treatment of HBV in co-infected patients

Given the persistent nature of HBV and integration in the host genome, the aim of treatment is to improve virological control rather than viral eradication and to slow disease progression, by improving liver histology. Patients with high levels of ALT (>1.5 upper limit of normal) who are HbeAg positive and have HBV DNA> 10^4 copies/ml are at higher risk of HBV disease progression and may benefit from HBV treatment (http://www.bhiva.org/guidelines/2004/HBV/intromain.html) . Recent evidence suggests that HIV positive HBe Ag negative patients with HBV DNA> 10^4 copies/ml may also be considered for treatment (Keeffe, Dieterich et al. 2004).

The treatment options for HIV-HBV coinfected patients include:

- Interferon alpha;
- Nucleoside analogues (lamivudine, emtricitabine, entecavir);
- Nucleotide analogues (tenofovir and adefovir).

Current British HIV association (BHIVA) guidelines (Brook, Gilson et al. 2005) recommend treatment for patients who are HBeAg-positive and therefore have higher levels of viral replication. They are the principal candidates for treatment. Treatment should also be considered for HBeAg-negative patients with abnormal liver function

tests (LFTs) and in whom other possible causes of the abnormal LFTs have been excluded (such as super-infection with other hepatotropic viruses or drug toxicity). These patients are likely to be infected with a mutant virus, where a mutation in the pre-core region prevents the expression of HBeAg but the virus remains replication competent. Plasma HBV viral load provides a measure of viral replication, but levels are lower than in HBeAg-positive patients. HIV positive and HBeAg negative patients with HBV-DNA levels $>10^4$ copies/ml (or $>10^3$ copies/ml if they have decompensated cirrhosis) may also be considered for treatment of HBV (Keeffe, Dieterich et al. 2004). In some patients it may also be appropriate to treat in order to reduce infectivity at any HBV-DNA level.

Patients with normal liver transaminases who are HBeAg-negative with an HBV-DNA $\leq 10^4$ copies/ml are unlikely to have active liver disease or viral replication and should be treated conservatively.

Several issues must be taken into account when treating HBV-HIV co-infected patients. Firstly, HBV treatment can however be initiated on its own in patients with well preserved CD4 counts who are at high risk of HBV disease progression and the nucleotide analogue adefovir can be used (Delaugerre, Marcelin et al. 2002). Secondly, certain anti-HBV drugs (lamivudine, tenofovir) have dual HBV-HIV activity and must be used as part of HAART, to avoid developing HIV-drug resistance. Tenofovir, lamivudine or emtricitabine can be used as part of HAART. Thirdly, due to a reported increase in HBV lamivudine resistance in co-infected patients (Benhamou, Bochet et al. 1999) of up to 20-25% per year, lamivudine should no longer be used on its own for HBV treatment in co-infected patients. Discontinuing anti-HBV drugs when changing a HAART regimen can also cause significant morbidity (ALT flares) (Bessesen, Ives et al. 1999) and is therefore not recommended. Tenofovir (as well as adefovir) are active against resistant strains of lamivudine (Benhamou, Bochet et al. 2001).

In the case of HBV mono-infection, treatment is recommended in patients with HBe Ag+ hepatitis and abnormal liver function (ALT>2xULN) or necroinflamation at biopsy. Patients who are HBe Ag negative, but have detectable HBV DNA (>0.1 mil copies/ml) should also be considered for treatment (Lok 2000).

In chapter 5 I address the question of reconstitution of HBV-specific immune responses in co-infected patients.

CHAPTER 2

2. Methods

The aim of this project was to study the HBV-specific CD8 responses during HIV infection. As mentioned in introduction, this is novel work, which was made possible by the availability of new techniques enabling us to look at antigen-specific T cells directly ex-vivo. Such techniques- class I-peptide tetrameric complexes in conjunction with intracellular cytokine staining have only become available in recent years. (Altman J 1996 Science and Ogg G Science 1998) and are based on the cellular mechanisms of recognition by CTLs of virally infected cells. Quantification of virus-specific T cells was done with the aid of flow cytometry.

2.1 Basic principles of flow cytometry

We studied HBV-specific CD8 responses in peripheral blood only. Lymphocytes were isolated by density centrifugation over a step gradient consisting of a mixture of the carbohydrate polymer FicollTM. This yields a population of mononuclear cells at the interface that has been depleted of red blood cells and most polymorphonuclear leukocytes or granulocytes. This population is referred to as peripheral blood mononuclear cells (PBMC) and consists mainly of lymphocytes and monocytes. PBMCs were further analyzed and accurately quantified using a flow cytometer. The flow cytometer is equipped to detect and count individual cells passing through a laser beam and, when able to separate the identified cells, is also called a fluorescence activated cell sorter (FACS). Cells labelled with monoclonal antibodies to cell surface proteins (tagged with fluorescent dyes) and mixed with a larger volume of saline are pushed through a nozzle, creating a stream of singly spaced cells. As each cell passes through the laser beam, it scatters the laser light and the dye molecules are excited and fluoresce. Photomultiplier tubes detect the scattered light (giving information about the size and granularity of the cells), as well as the fluorescence emissions, quantifying the binding of the labelled monoclonal antibodies. This reflects the expression of cell

surface proteins by each cell. In the cell sorter, the signals are being converted into electrical signals by the optical and electronic system, providing a powerful tool for rapid analysis of thousands of cells. Data analysis can be done in the form of graphic representations by using either histograms (for a single acquired parameter) or bivariate dot plots. In my project, I used two-parameter data plots, which provided more information on the CD8 cells studied.

Fig 2.1 shows a dot plot of forward scatter (FSC- indicator of cell size) and side scattered (SSC- indicator of cell granularity or refractability). The dot plot of FSC/ SCC allows identification of the lymphocyte population alongside neutrophils and monocytes. Gating on the lymphocytes permits further specific analysis of this population and on the binding of tetramers or antibodies (such as CD4, CD8, anti-IFN), providing quantitative and qualitative information on the function of the cells. After gating on the lymphocyte population (R1) and further gating on the live cells (R2-not shown), cells stained with anti-CD8 and anti-IFN are shown in a dual parameter dot plot *(Fig 2.1)*. CD8+IFN+ cells are in the right upper quadrant.

Fig. 2.1

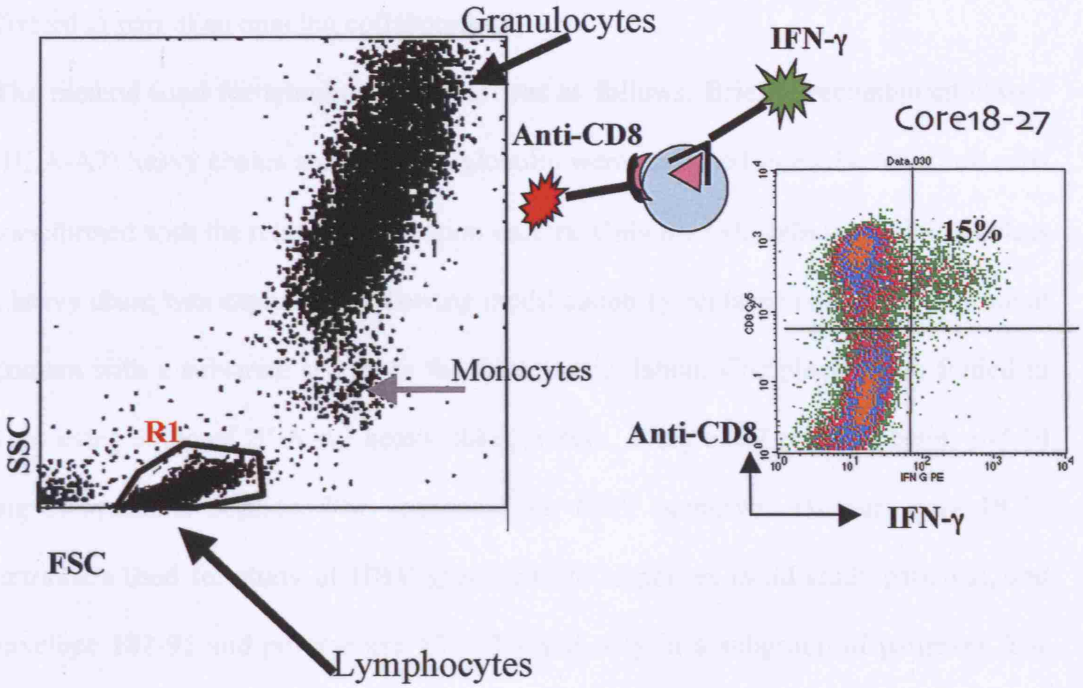


***Fig 2.1** Data analysis on FACS*

Dot plot of forward scatter (FSC- indicator of cell size) and side scattered (SSC- indicator of cell granularity or refractability). The dot plot of FSC/ SCC allows identification of the lymphocyte population alongside neutrophils and monocytes. After gating on the lymphocyte population (R1) and further gating on the live cells (R2-not shown), cells stained with anti-CD8 and anti-IFN are shown in a dual parameter dot plot. CD8+IFN+ cells are in the right upper quadrant and percentage is calculated using the CellQuest software.

2.2 Tetramers and Intracellular cytokine staining

2.2.1 Tetramers- Synthesis

In this project, all tetramers used were synthesized with HLA-A2, since most of the well described epitopes in HBV to date are HLA-A2 restricted. We mainly used an HLA-A2 tetramer bound with the core 18-27 peptide, an immunodominant HBV epitope, and occasionally used HLA-A2 tetramers bound with envelope or polymerase epitopes. These tetramers were kindly provided by Graham Ogg and colleagues from the IMM, Oxford as part of an ongoing collaboration.

The method used for tetramer synthesis was as follows. Briefly, recombinant class-I (HLA-A2) heavy chains and β2-microglobulin were produced in *Escherichia coli* cells transformed with the relevant expression vectors. Only the extracellular domain of class I heavy chain was expressed, following modification by replacement of the C-terminal domain with a substrate sequence for BirA biotinylation. Complexes were folded in vitro using 30mg of HLA-A2 heavy chain protein, 25mg of b2-microglobulin, and 10 mg of synthetic peptide. The sequences for HBV (genotype D) were core 18-27 (tetramers used for study of HBV-specific CD8 responses in all study patients), and envelope 183-91 and polymerase 575-83 (used only in a subgroup of patients). The HLA-A2/peptide complexes were biotinylated using purified BirA enzyme at a concentration of 5ug/ml, 0.5mmol/L biotin and 5-mmol/L adenosine triphosphate. The reaction was incubated at room temperature for 16 hr. Biotinylated HLA-A2/peptide complexes were recovered by fast protein liquid chromatography purification (using buffer containing 20mmol/L Tris, pH 8.0 and 50 mmol/L NaCl) and ion exchange chromatography (0-0.5 mol/L NaCl gradient). Tetramers were generated by mixing biotinylated protein complex with streptavidin-phycoerythrin at a molar ratio 4:1.

2.2.2 Isolation of PBMC

PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation.

2.2.3 Tissue typing

Screening for the HLA-A2 haplotype was performed by staining PBMCs with an anti-HLA-A2+ mAb (Incstar) followed by an FITC-conjugated sheep anti-mouse IgG second layer mAb and flow cytometric analysis.

2.2.4 Synthetic peptides

Peptides corresponding to the sequence of core 18-27, envelope 183-81, 335-43, 338-47, 348-57, polymerase 455-63, 502-10, 575-83, 655-63, 816-24 regions of HBV genotype D were synthesized. (Chiron Mimotopes Clayton, Victoria, Australia).

Table II.1 ***Peptides used for analysis of HBV-specific CD8+ T-cell responses***

Peptide	**Sequence**
Core 18-27	FLPSDFFPSV
Envelope 183-191	FLLTRILTI
Envelope 335-343	WLSLLVPFV
Envelope 338-347	LLVPFVQWFV
Envelope 348-357	GLSPTVWLSV
Polymerase 455-463	GLSRYVARL
Polymerase 502-510	KLHLYSHPI
Polymerase 575-583	FLLSLGIHL
Polymerase 655-663	ALMPLYACI
Polymerase 816-824	SLYADSPSV

2.2.5 HBV Antigens

Recombinant hepatitis B core Antigen (HBcAg; Sorin Biomedica, Saluggia, Italy) as obtained from bacterial extracts of Escherichia Coli K12 strain HB 101, harboring the plasmid carrying the HBcAg coding gene, as described previously (Pasek, Goto et al. 1979). Purity was 90% as previously reported (Ferrari, Bertoletti et al. 1991). Recombinant yeast derived preparation of HBsAg (Amgen, Thousand Oaks, CA) containing the S/p25 protein was used. Purity was higher than 90%.

2.2.6 HLA-A2 Tetramer and Antibody Staining

The soluble HBc18-27, HB envelope 183-91 and HB pol575-83 tetramers were produced as described previously (Maini, Soares et al. 1999). PBMC were isolated from heparinized blood samples by density gradient centrifugation on Ficoll-Hypaque. Cells ($0.5 \times 10^6 - 1 \times 10^6$) were incubated for 30 min at 37^0 with 1μg of PE-labeled tetrameric complex in RPMI 1640, 10% FCS in round bottom polystyrene tubes (Becton Dickinson). Cells were washed in PBS and then incubated at 4^0 for 30 min with saturating concentrations of directly conjugated anti-CD8-Cychrome (PE-Cy5) mAb (Sigma Chemical Co.). For one phenotyping experiment cells were also stained with a panel of FITC-conjugated antibodies consisting of anti-HLA DR, anti-CD45 RA and anti-CD38. Cells were washed twice and then analyzed immediately on a Becton Dickinson FACS using CellQuest^{TM} software. For analysis of circulating tetramer positive cells, approximately 5×10^5 cells were acquired within the live gate to ensure that at least 5×10^4 CD8 cells were available for analysis.

2.2.7 Production of T cell lines and IFN-γ staining

In brief, PBMC were seeded at a concentration of 0.3×10^6/ml in RPMI 1640, 10% FCS. Cells were stimulated with 1μM of the relevant peptide (the 10 peptides listed above) in a 96-well round-bottom plate. Recombinant Il-2 (10IU/ml)(Boehringer Mannheim, Germany) was added on day 4 of cell culture. Cells were re-stimulated on

day 10 with 1μM of the relevant peptide (5hrs), the last 4hrs with 10μg/ml Brefeldin A (Sigma-Aldrich, Missouri, USA). Cells were then stained with anti-CD8 (Pharmingen, San Diego, CA), followed by permeabilisation with 100 ul of a 1x solution Cytofix/Cytoperm ™ (Pharmingen). Anti-IFN-γ mAb (R&D, Minneapolis, MN) was then added (30 min at 4^0, dark) followed by two washes and immediate analysis on the FACScan (Beckton Dickinson, San Diego, CA) using CELLQuest ™ software.

For the intracellular cytokine staining experiments performed directly ex vivo, PBMC were incubated with 1μM of the relevant peptide for 5hrs in the presence of Brefeldin A followed by cell permeabilisation, staining and analysis as above.

For the *HBV-specific CD4+ cell enumeration*, PBMC were suspended at 1x 10^6/ml in RPMI 1640, 10% human serum and stimulated with 1μM HBV core antigen or surface antigen for 5hrs. Cells were washed, stained with anti-CD4 PE (Pharmingen) and subjected to intracellular cytokine staining as above. Some experiments were performed on short-term cell lines, in brief PBMC were seeded at a concentration of 0.3 x 10^6/ml in RPMI 1640, 10% human serum. Cells were stimulated with 1μM of the relevant antigen (HB core and surface, HB e Ag only available for one experiment) in a 96-well round-bottom plate. Recombinant Il-2 (10IU/ml)(Boehringer Mannheim, Germany) was added on day 4 of cell culture. Cells were re-stimulated on day 10 with 1μM of the relevant antigen (5hrs), the last 4hrs with 10μg/ml Brefeldin A (Sigma-Aldrich, Missouri,USA).

For all intracellular cytokine staining experiments responses were calculated by subtracting background IFN-γ production in a negative control well without peptide restimulation. Stimulation with anti-CD3 (Serotec, Oxford) and anti-CD28 mAb (Pharmigen, San Diego, CA), 1μM each, for 5hrs in the presence of Brefeldin A was used as a positive control. In relevant cases, the HIV gag 77-85 peptide was also used for intracellular cytokine staining.

For intracellular cytokine staining experiments ex vivo there were duplicate wells with un-stimulated cells for each experiment. A positive result was defined as any positive result after subtraction of background interferon production, which was greater than 0.02% of total CD8.

For intracellular cytokine staining after 10 days in vitro cell culture with 10 HLA-A2 restricted peptides, I performed a negative control for each condition. Cells were cultured in duplicate with 1uM of peptide and IL-2 was added to all cells at day 4, then analysed after 10-12 days of cell culture. For each well containing cells re-stimulated with the appropriate peptide there was a well of un-restimulated cells, which constituted a negative control. A positive result was obtained after subtraction of background interferon production and was greater than 0.02% of total CD8. All experiments with high background interferon production (defined as greater than 0.05% of total CD8) were excluded. Fig. 2.2 illustrates the use of negative controls and calculation of positive results.

Fig.2.2

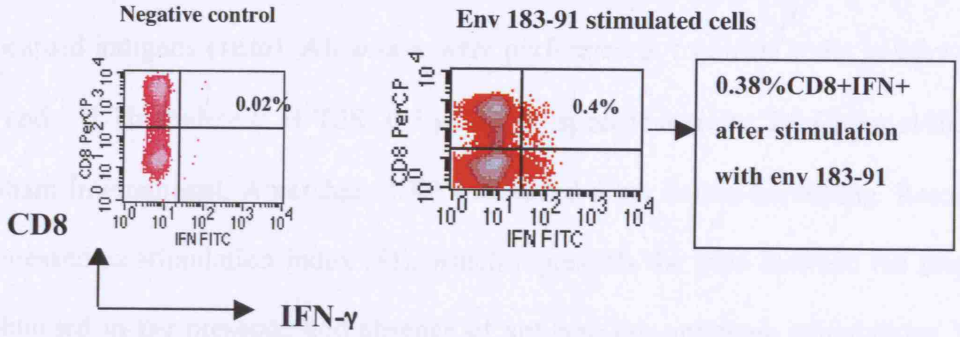


Fig. 2.2 *Intracellular cytokine staining dot plots after 10 days in vitro stimulation with peptide.*

CD8+ IFN+ can be seen in the right upper quadrant. In the case of this patient there was a positive response after stimulation with one of the envelope peptides. For each well containing cells re-stimulated with the appropriate peptide there was a well of un-restimulated cells, which constituted a negative control. A positive result was obtained after subtraction of background interferon production, in the case of this patient 0.38% of total CD8 produced IFN-γ after stimulation with env 183-91 peptide.

2.3 Proliferation Assay

PBMCs (2x 10^6 /ml) were incubated for 7 days at 37^0 C in the presence of HBV nucleocapsid antigens (1μm). All assays were performed in triplicate wells in 96-well plates and ^{3} H Thymidine (^{3} H-TdR; 0.5 μCi/well; specific activity, 2.0 Ci/mmol/liter; Amersham International, Amersham UK) was added 6 hrs before harvesting. Results are expressed as stimulation index (SI), which represents the ratio between the mean cpm obtained in the presence and absence of antigen. For antigenic stimulations, SI values above 4 were regarded as positive (> 2SD above the mean SI value obtained with each individual HBV protein or peptide in normal controls).

2.4 Virologic Assessment

HBsAg, antibody to the HBV surface (anti-HBs), total and immunoglobulin M anti-HBc, envelope HBV antigen (HBe Ag) and anti-HIV-1, anti-HCV were determined by commercially available enzyme immunoassay kits (Abbots Laboratories, Ortho Diagnostic System). The presence of HBV DNA level was quantified by commercial hybridization assay (Digene) with a detection limit of 1pg/ml (approx 3.5mil copies/ml).

2.5 Patients

Patients were recruited from the Genito-urinary medicine and HIV clinics at the Mortimer Market Centre with informed consent and local ethics committee approval. I have studied HBV-specific immune responses in HIV positive and HIV negative patients with different degrees of HBV control. Chapters 3 and 4 look at HIV negative and positive patients who controlled HBV and Chapter 5 on patients with chronic hepatitis B. Table II.1 summarises the groups of patients analysed and the methods used for studying HBV-specific CD4 and CD8 responses. Further characterisation of the patients included in the study is detailed in the corresponding chapters.

2.6. Statistical analysis

Statistical analysis was performed with SPSS for Windows.

Comparison of the number of patients with any HBV-specific CD8 responses detectable in the cross-sectional comparison of HIV negative and positive patients was done using the nonparametric Fisher's exact test. The non-parametric Mann-Whitney (Wilcoxon rank-sum) test was used to compare the groups taking into account the number and size of T cell responses for each patient.

Table II. 1 Patients with resolved HBV (natural immunity to HBV) HB surface Ag- /Anti-HB surface Ab + /Anti-HB core Ab+

HIV status	HBV status	Patient ID	Experiments for HBV-specific CD8 responses	Experiments for HBV-specific CD4 responses
HIV negative	HBV Asymptomatics	N1, N2, N3, N4, N5, N6, N7, N8, N9, N10, N11	Tet c18-27 (ex vivo and day 10) Day 10 and ex vivo [1] ICS for IFN-γ	Ex vivo and day 10 ICS for IL2/ IFN-γ
	HBV Symptomatic	N12, N13, N14	Tet c18-27 (ex vivo and day 10) Day 10 and ex vivo [1] ICS for IFN-γ	N/D
HIV positive HAART naïve	HBV Asymptomatic	P1, P3, P4, P5, P6, P7,P8, P9, P13, P14, P15, P16	Day 10 and ex vivo [1] ICS for IFN-γ	Ex vivo and day 10 ICS for IL2/ IFN-γ
	HBV Symptomatic	P10, P11	Day 10 and ex vivo [1] ICS for IFN-γ	N/D
HIV positive already on HAART	HBV Asymptomatics	P2, P12	Day 10 and ex vivo [1] ICS for IFN-γ	N/D
HIV positive Starting HAART	HBV Asymptomatics	P1, P5, P7, P8	Longitudinal follow-up of day 10 and ex vivo [1] ICS for IFN-γ	Longitudinal follow-up of ex vivo and day 10 ICS for IL2/ IFN-γ

Table II.2b Chronic HBV patients

HIV/HBV treatment history	Chronic HBV status and HBV DNA	Patient ID	Experiments for HBV-specific CD8 responses	Experiments for HBV-specific CD4 responses
HIV positive HAART naive HBV chronic carriers	High infectivity HBV (HBV DNA high)	Pc1, Pc2, Pc3, Pc4, Pc5, Pc6, Pc11	Tet c18-27	N/D
	Low infectivity HBV (HBV DNA low*)	Pc7, Pc8, Pc9, Pc10	Tet c18-27	N/D
HIV positive HBV chronic carriers Starting HAART +/- anti- HBV agent	High infectivity HBV (HBV DNA high)	Pc1, Pc2, Pc3, Pc4, Pc5	Tet c18-27 Longitudinal follow-up of day 10 ICS	Longitudinal follow-up of ex vivo and day 10 ICS IFNγ and proliferation as says

*low HBV DNA defined as undetectable HBV DNA with the Digene method (HBV DNA<3.5 million copies/ml)

Tet c18-27=tetramer core 18-27

Day 10 ICS= Intracellular cytokine staining for CD8/IFN-γ after 10 days of in vitro stimulation with panel of 10 HLA-A2 restricted peptides known HBV epitopes

[1]Ex vivo ICS= Intracellular cytokine staining directly ex vivo with same panel of 10 HLA A2 restricted peptides was performed in a subgroup of patients only

CHAPTER 3

3. Effect of HIV infection and antiretroviral therapy on hepatitis B virus (HBV)-specific T cell responses in patients who have resolved HBV infection.

3.1 Introduction

In this chapter we explore, for the first time, the effect of HIV related immunodepletion on HBV-specific CD8 responses in a group of patients who controlled HBV. The importance of CD8 T cell responses during acute HBV has been directly demonstrated by a recent study in chimpanzees (Thimme, Wieland et al. 2003), but has been previously suggested by the broad and multispecific CD8 responses found in patients who control the acute infection.(Bertoletti, Ferrari et al. 1991; Chisari 1997), (Nayersina, Fowler et al. 1993) (Rehermann, Fowler et al. 1995; Maini, Boni et al. 1999). Such responses were maintained up to 20 years following the clinical resolution of the acute infection. (Penna, Artini et al. 1996; Rehermann, Ferrari et al. 1996). In contrast, chronic HBV carriers are characterized by a hypo-responsiveness against HBV at a cellular level (Bertoletti, Costanzo et al. 1994; Maini, Boni et al. 2000). For this reason, in this chapter we will explore the effect of HIV on patients who controlled HBV infection (patients with natural immunity to HBV), rather than chronic HBV carriers, where the impact of any additional HIV-related immune depletion would be more difficult to examine. The natural history of acute HBV infection is heterogenous, with approximately 30% of adults displaying characteristic symptoms of jaundice and associated abnormal liver function tests (McMahon, Alward et al. 1985), but newborns and a significant number of adults can be asymptomatic during the acute infection. Immunological studies to date have only included patients with documented history of jaundice and raised liver transaminases. Patients without a history of jaundice, who were found to have natural immunity to HBV (anti-HBc+/antiHBs+) as part of routine screening prior to HBV vaccination represent a significant proportion of the HIV positive population (Hadler, Judson et al. 1991) (Scharschmidt, Held et al. 1992; Francisci, Baldelli et al. 1995). HBV-specific responses were compared in HIV positive and HIV negative subjects, focusing on those who have resolved HBV without

symptoms. Given that HBV infection is a dynamic process, where the very initial host-virus interactions (Webster, Reignat et al. 2000; Bertoletti, Maini et al. 2003) play a crucial role in the outcome of infection, it is possible that the initial degree of liver inflammation may be translated into different mechanisms of immunological viral control. Thus, by including patients who controlled HBV in the absence of any clinical symptoms, we sought to enhance our knowledge on HBV-specific immune responses.

We used the sensitive techniques of tetramers and intracellular cytokine staining for IFN-g to explore the breath of functionally active HBV-specific CD8 responses across a range of previously defined HLA-A2 restricted epitopes. (Penna, Chisari et al. 1991; Nayersina, Fowler et al. 1993; Rehermann, Fowler et al. 1995) In order to investigate to what extent any loss of HBV-specific immune responses could be reversed by antiretroviral therapy, a group of HBV immune patients starting HAART were studied prospectively. There is a growing body of evidence suggesting that HAART may lead to successful restoration of specific responses towards previously encountered pathogens (Dalod, Dupuis et al. 1999; Kostense, Otto et al. 2002; Wilkinson, Cope et al. 2002). Natural immunity to HBV provided a good model system to further explore the potential for functional restoration of virus specific CD4 and CD8 responses in the setting of controlled HBV replication.

3.2 Patients and controls

Patients were recruited from the genito-urinary medicine and HIV clinics at the Mortimer Market Centre with informed consent and local ethics committee approval. All patients had HBV serology confirming natural immunity to hepatitis B (HB surface Ag-, anti-HB core +, anti-HB surface +), and were further categorized according to whether they had a history consistent with symptomatic acute hepatitis. HBV-specific CD8 responses were compared in HIV positive and HIV negative patients. Patients with a past history of jaundice are referred to as "symptomatic resolved hepatitis" and those with serological evidence of past hepatitis B but in the absence of a history of symptoms are referred to as "HBV immune".

32 HIV positive gay men with natural immunity to hepatitis B were screened, of whom 16 were found to be HLA-A2+. Two patients had a history of symptomatic acute hepatitis B and 14 patients were simply HBV immune. Patients had a median CD4 count of 320 cells/mm ^{3} and a median HIV VL of 36,000 copies/ml (***Table III.1***). Fourteen patients were HAART naïve and two patients were already on antiretroviral therapy; four HLA-A2 + positive HAART naïve patients were followed up longitudinally after starting combination therapy.

Twenty five HIV negative patients (tested within the previous year) with natural immunity to HBV were screened to identify 14 who were HLA-A2+. Three patients had a history of symptomatic acute hepatitis and eleven had resolved HBV asymptomatically.

The HIV negative and HIV positive groups had similar demographic characteristics, with all but one asymptomatically resolved HBV patients being gay men, and all patients being Caucasian apart from one Asian in the HIV positive group. The HIV negative group had a mean age of 38 years (median 39 years), comparable to the HIV positive group with a mean age of 39 years (median 39 years).

Patient ID	CD4 cells/μl (nadir)	CD4%	HIV VL copies/ ml	HAART at recruitment	Commenced HAART
P1	320	19%	54,000	Naive	stavudine/ lamivudine/ nevirapine
P2	700 (160)	24.1	<50	stavudine/ lamivudine /nelfinavir	
P3	540	25.5	11,000	Naive	
P4	480	12.8	118,600	Naive	
P5	220	6.7	360,100	Naive	Zidovudine didanosine/ efavirenz
P6	290	22.3	2,200	Naive	
P7	260	20	296,700	Naive	zidovudine/ lamivudine/ efavirenz
P8	280	13.2	141,400	Naive	AZT/3TC/Efv
P9	510	25	8,800	Naive	
P10*	310	24.2	50,000	Naive	
P11*	320 (230)	13.7	13,000	Naive	
P12	350(240)	18	500	zidovudine/ lamivudine	
P13	190	15	426,000	Naive	
P14	150	21.7	173,400	Naive	
P15	640	22	800	Naive	
P16	710	19.5	21,600	Naive	
	CD4 nadir given in brackets where different from CD4 count at recruitment				

Table III.1 ***showing patients' CD4, HIV-VL and antiretroviral regimens used***

**Patients who had a history of acute resolved HBV*

3.3 Methods

3.3.1 Flow cytometry, HLA-A2 staining, tetramer and intracellular cytokine staining

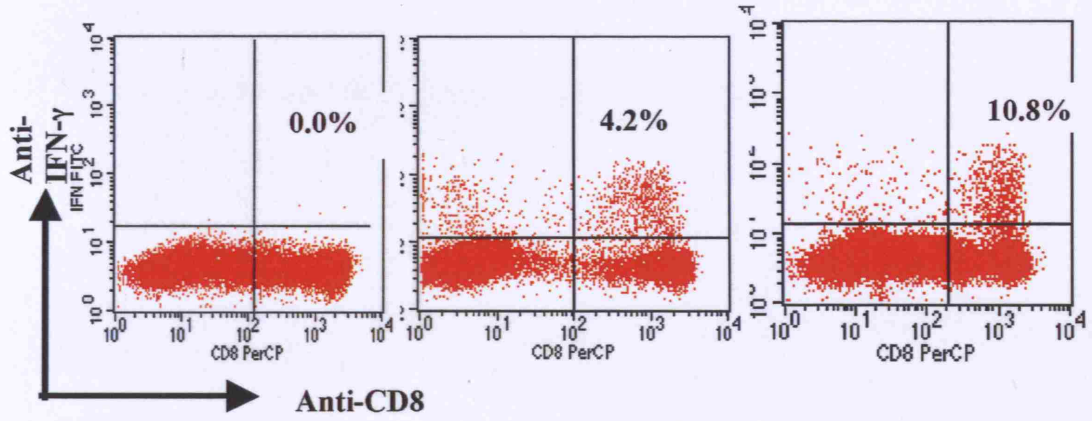
I reviewed in Chapter 1 the benefits of such novel techniques, which allowed for the first time visualization of antigen specific cells directly ex vivo, and with the aid of flow cytometry, permitted further functional characterization at an individual cell level.

In this study we analyzed HBV-specific responses in patients displaying the HLA-A2 allele, given that the HBV-specific epitopes have been previously well characterized in this population. The identification of HLA-A2+ patients by tissue typing and the staining techniques were described in the methods chapter.

In our experiments staining was done directly ex vivo as well as after short-term cell culture (with a panel of 10 HLA A2 restricted peptides, outlined in Chapter 2, table II.1). Given the relatively low frequencies of HBV-specific CD8 cells ex vivo, cells were expanded after 10 days in vitro culture with HBV proteins. This was previously shown not to alter the hierarchy of the immune response (Maini, Boni et al. 1999) (Maini, Boni et al. 2000). For each *ex vivo* or cell culture experiment, we performed negative controls of unstimulated PBMC, giving the background staining, which was subtracted from positive results. We also performed positive controls for each experiment. Fig. 1 illustrates dot plots of intracellular cytokine staining after 10 days in vitro stimulation in the same patient, where cells have been stimulated with a HBV peptide, a HIV peptide and CD3/CD28 as positive controls. In this case, the patient did not have a HBV-specific response when cells were stimulated with the HBV peptide core 18-27, but there was an expansion of HIV-specific CTLs against a gag epitope as well as when cells where stimulated with CD3/CD28.

Fig. 3.1 *Use of positive controls: stimulation with HBV peptide, HIV peptide and CD3/CD28.*

This HIV positive patient who had natural immunity to HBV did not have a HBV-specific response when cells were stimulated with the HBV peptide core 18-27, but there was an expansion of HIV-specific CTLs against a gag epitope as well as when cells where stimulated with CD3/CD28.



HBV peptide ***core 18-27***

HIV peptide ***Gag 77-85***

CD3/CD28 control

3.3.2 Statistical analysis

Comparison of the number of patients with any HBV-specific CD8 responses detectable in the cross-sectional comparison of HIV negative and positive patients was done using the nonparametric Fisher's exact test. The non-parametric Mann-Whitney (Wilcoxon rank-sum) test was used to compare the groups taking into account the number and size of T cell responses for each patient.

3.4 Results

3.4.1 Reduction of HBV-specific CD8 responses in HIV positive HBV immune patients

We initially conducted a cross-sectional study of HIV positive and HIV negative patients with natural immunity to HBV, either with or without a history of acute symptomatic infection. All patients were screened for CD8 cells producing IFN-γ in response to a panel of 10 peptides representing frequently recognized HLA-A2 restricted HBV epitopes. Results shown were calculated after subtraction of background staining in wells without peptide restimulation. In 3 HLA-A2+ HIV negative patients who had successfully resolved acute symptomatic hepatitis B 3-5 years previously, we demonstrated peptide-specific IFN-γ production for 6 or 7 of the 10 epitopes tested *(fig.3.2 and table III.1). Fig 3.2* compares the breadth and frequency of HBV-specific CD8 responses in 3 HIV negative and one HIV positive patient after 10 days of in vitro stimulation. P10 who had acute hepatitis B eight years previously showed recognition of 4 out of 10 epitopes tested with intracellular cytokine staining. Staining was also done ex-vivo (*fig.3.3*) in two HIV negative patients and two of the same HIV positive patients, where responses were only detected ex vivo in one of the HIV positive patients who had a history of acute symptomatic HBV infection eight years previously (P10), but not in P11 with acute infection more than 15 years previously *(fig 3.3).* The same patients were studied with the tetramer core 18-27 (*fig. 3.4*), where HBV-specific CD8 responses for this epitope showed slightly higher frequencies than those detected by intracellular cytokine staining, as previously reported. P11 had a low level response to tetramer core 18-27 ex-vivo and after 10 days in vitro stimulation, although this response was not detected by intracellular cytokine staining, possibly suggesting a functional impairment of this HBV-specific CD8 population in the context of HIV.

We also found that HIV negative patients who had resolved HBV infection without any prior symptoms of acute infection also had detectable CD8 responses to at least one HLA-A2 restricted epitope in 6 out of 11 cases (*Fig.3.5 and table III.2*). In some cases (e.g. patient N9, whose last risk of exposure was more than 10 years previously), responses had a similar level of mulspecificity and expansion potential as those seen in patients with symptomatic acute infection, who have formed the basis of all previous immunological studies of patients resolving HBV.

Table III.2

			Core 18-27	Env 183-91	Env 335-43	Env 338-47	Env 348-57	Pol 455-63	Pol 502-10	Pol 575-83	Pol 655-63	Pol 816-24
HIV neg	*HBV Asymptomatics*	N1	**0.7**	**0.3**	0	0	0	**0.1**	**0.1**	**0.1**	0	0
		N2	0	0	0	0	0	0	0	0	0	0
		N3	0	0	0	0	0	0	0	0	0	0
		N4	0	0	0	0	0	0	0	0	0	0
		N5	0	0	0	0	0	0	0	0	0	0
		N6	**0.8**	**0.6**	**2**	0	**0.1**	0	0	0	0	0
		N7	**0.4**	0	0	0	0	0	0	0	0	0
		N8	0	0	0	0	0	0	0	0	0	0
		N9	**14.8**	**1.5**	**1.3**	**0.8**	**6.3**	**6.6**	**0.2**	0	0	0
		N10	0	**0.9**	0	**0.1**	**0.1**	**0.1**	0	0	0	0
		N11	**2.3**	**1.4**	**0.8**	**0.9**	**1.6**	**0.4**	**1.1**	0	0	0
	HBV Symptomatics	N12	**5.0**	**1.0**	0	0	**1.4**	**2.4**	**0.3**	**0.7**	0	**0.8**
		N13	**20.4**	**0.9**	**0.4**	0	**6.9**	**12.3**	**1.3**	0	0	**0.4**
		N14	**1.1**	**1.2**	**0.5**	0	0	**7.9**	**0.6**	**0.5**	0	0
HIV pos	*HBV Asymptomatics*	P1	0	0	0	0	0	0	0	0	0	0
		P3	0	0	0	0	0	0	0	0	0	0
		P4	0	0	0	0	0	0	0	0	0	0
		P5	0	0	0	0	0	0	0	0	0	0
		P6	0	0	0	0	0	0	0	0	0	0
		P7	0	0	0	0	0	0	0	0	0	0
		P8	0	0	0	0	0	0	0	0	0	0
		P9	0	0	0	0	0	0	0	0	0	0
		P13	0	0	0	0	0	0	0	0	0	0
		P14	0	0	0	0	0	0	0	0	0	0
		P15	0	0	0	0	0	0	0	0	0	0
		P16	**0.1**	0	0	0	0	0	0	0	0	0
	HBV Symptomatics	P10	**0.6**	**1.1**	**0.1**	0	**0.4**	0	0	0	0	0

Table III.2 Cross-sectional study of CD8 response after 10 days in vitro stimulation

Table III.3

			Core 18-27	*Env 183-91*	*Env 335-43*	*Env 338-47*	*Env 348-57*	*Pol 455-63*	*Pol 502-10*	*Pol 575-83*	*Pol 655-63*	*Pol 816-24*
HIV neg	*HBV asymptomatics*	N1	**0.2**	0	0	**0.4**	**0.2**	**0.2**	0	0	0	0
		N7	**0.3**	0	0	0	0	0	0	0	0	0
	HBV symptomatics	N12	**0.1**	0	0	0	**1.2**	**2.1**	**0.05**	**0.5**	0	**0.7**
		N13	**4**	**1.2**	**0.2**	**0.05**	**0.3**	**1.8**	0	0	0	0
HIV pos	*HBV asymptomatics*	P1	0	0	0	0	0	0	0	0	0	0
		P4	0	0	0	0	0	0	0	0	0	0
	HBV symptomatic	P10	**0.8**	**0.8**	0	0	**0.7**	**0.6**	0	0	0	0
		P11	0	0	0	0	0	0	0	0	0	0

Table III.3 Cross-sectional study of CD8 responses ex vivo

Fig.3.2

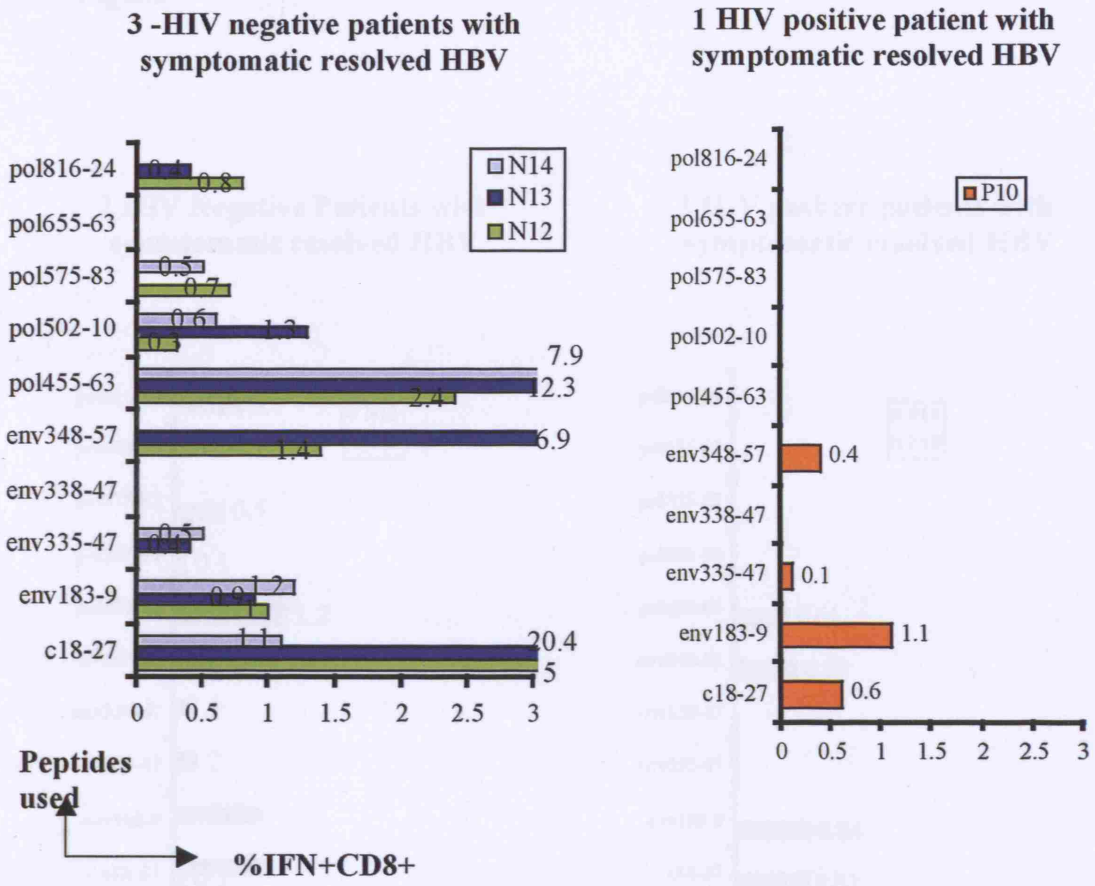


Fig.3.2 Intracellular cytokine staining data at 10 days in vitro stimulation with panel of 10 HLA-A2 restricted peptides in 3 HIV negative and one HIV positive patients who successfully resolved acute hepatitis B several years previously.

The HIV negative patients studied recognized 6-7 epitope and there were responses to 4 epitopes in the one HIV positive studied. The intensity of the HBV-specific responses detected are represented at the top of each bar as percentage of IFN+CD8+.

Fig.3.3

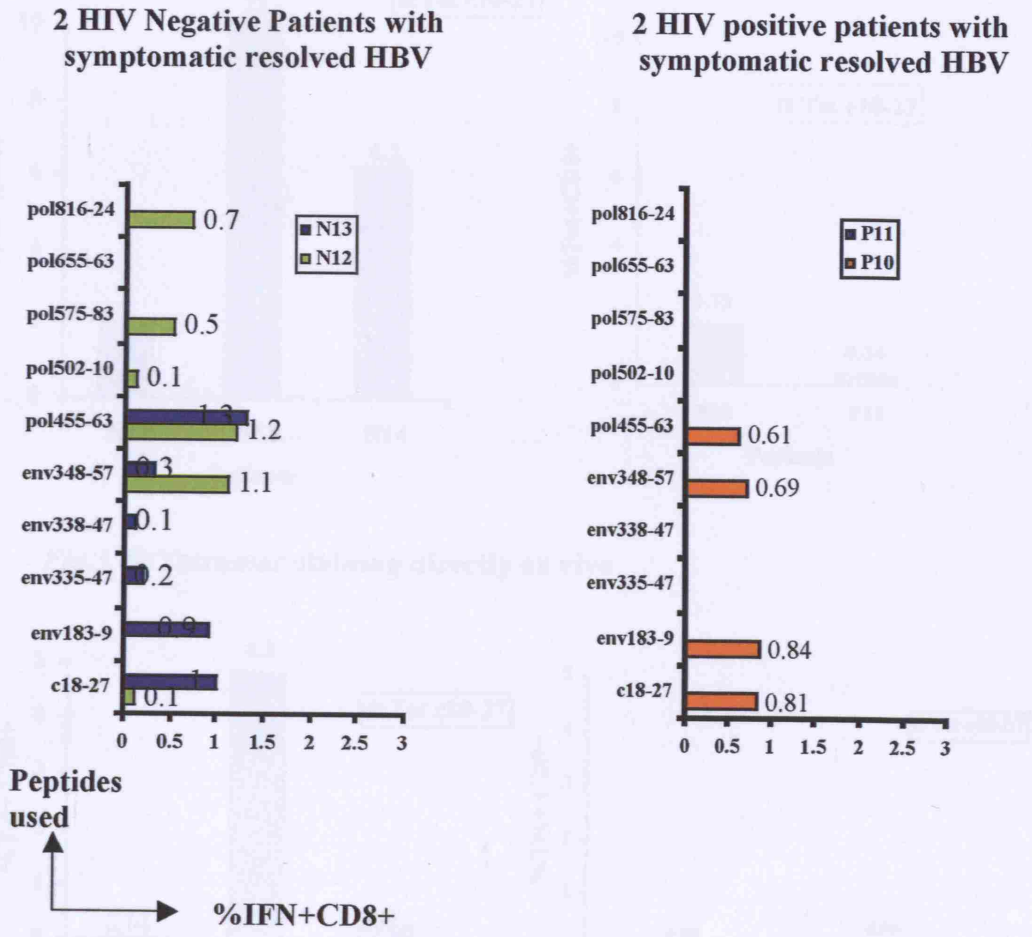


Fig.3.3 Intracellular Cytokine Staining directly ex vivo in two HIV negative and two H IV positive patients with a history of resolved symptomatic acute hepatitis B.

Cells were stimulated with panel of 10 HLA-A2 restricted peptides, represented on the vertical axis and the intensity of the responses detected is represented on the horizontal axis. HIV negative patients had preserved responses to 6 out of 10 HLA-A2 restricted epitopes. One HIV positive patient had detectable responses to 4 out of 10 HBV epitopes.

Fig 3.4a. **Tetramer staining at 10 days in vitro stimulation**

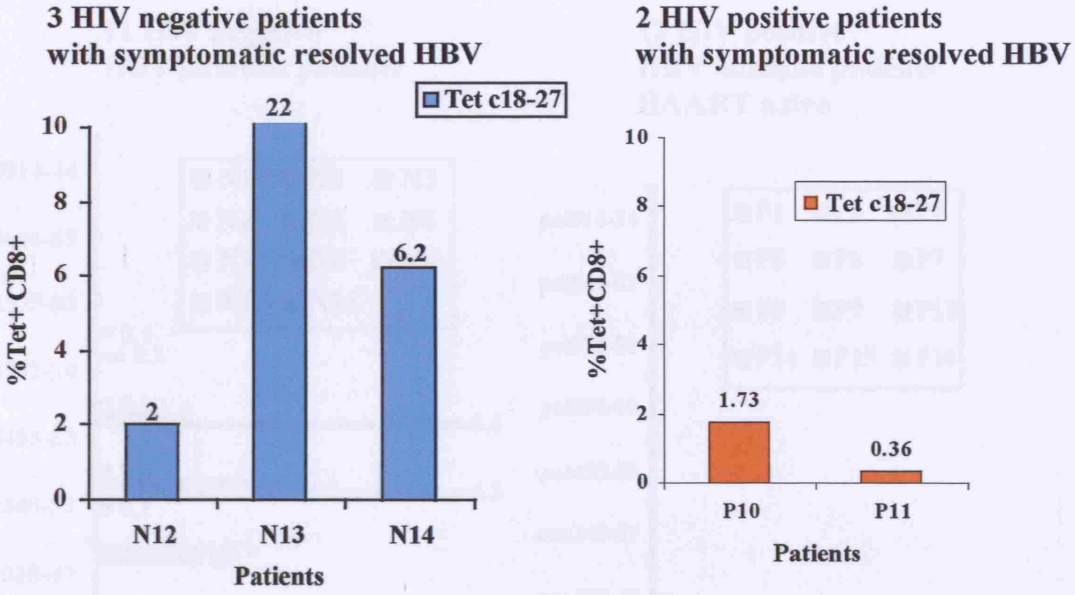


Fig.3.4b **Tetramer staining directly ex vivo**

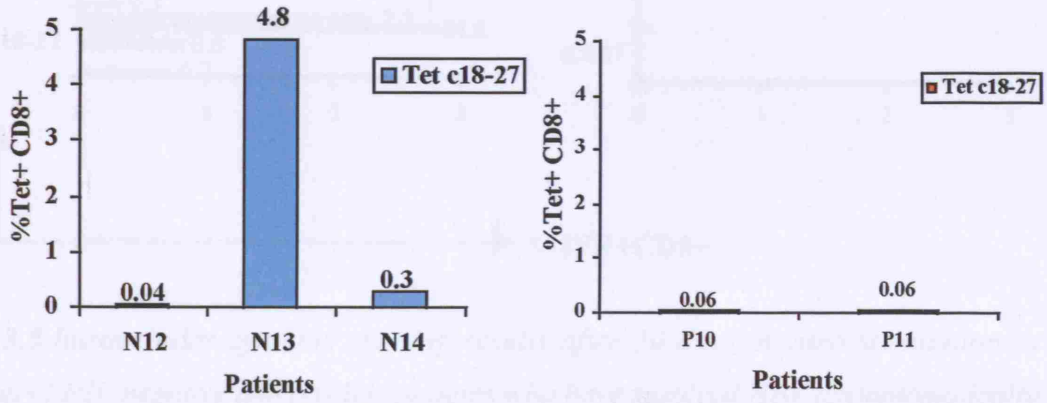


Fig 3.4a and b. *Tetramer staining with tetramer core 18-27 after 10 days in vitro stimulation and directly ex vivo in a group of HIV negative and HIV positive patients who have symptomatically resolved HBV*

The intensity of HBV-specific CD8 responses is indicated at the top of each bar. All patients tested had preserved responses to the HBV immunodominant epitope core18-27.

Fig. 3.5

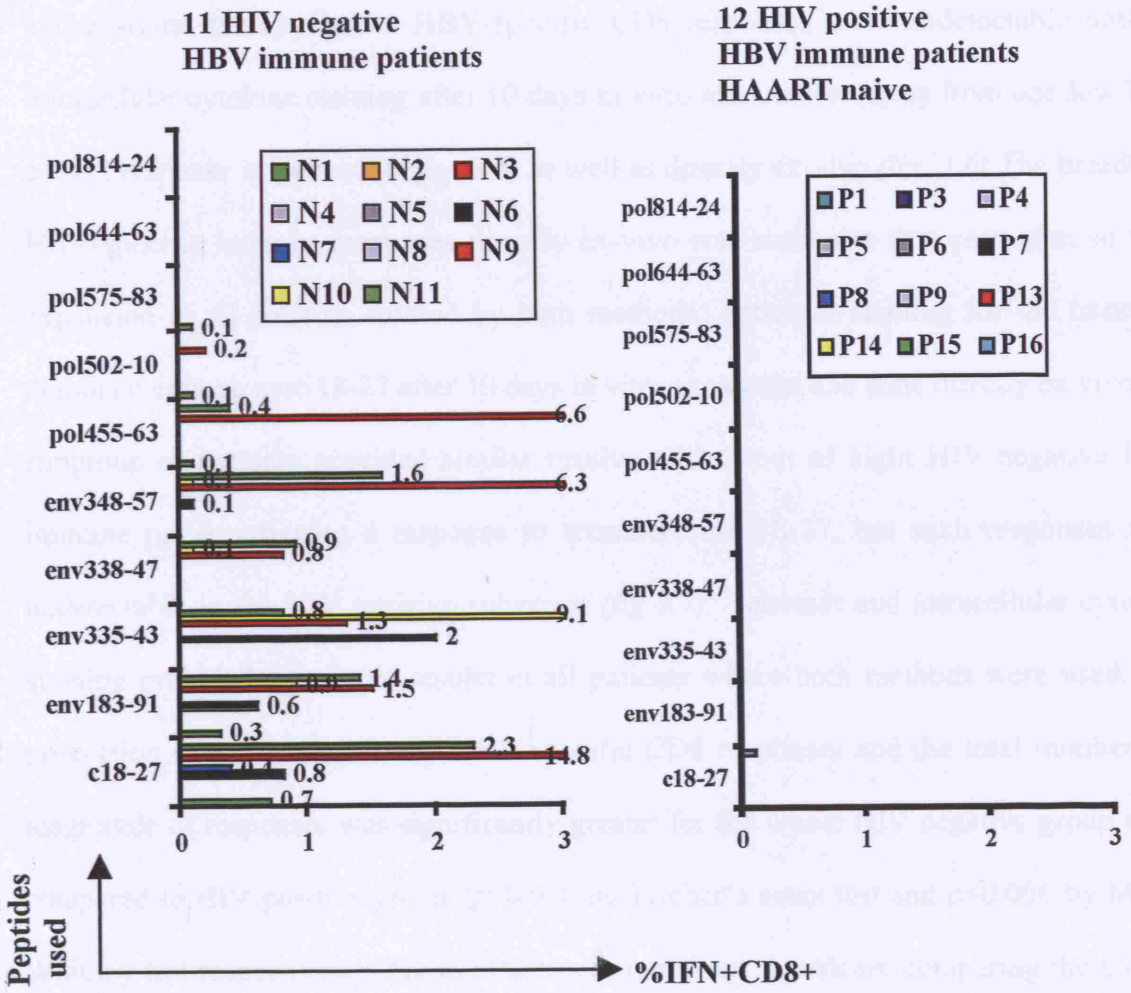


***Fig.3.5** Intracellular cytokine staining results after 10 days in vitro stimulation in a group of HIV negative and positive patients who have resolved HBV asymptomatically.* HIV negative patients who had resolved HBV infection without any prior symptoms of acute infection also had detectable CD8 responses to at least one HLA-A2 restricted epitope in 6 out of 11 cases. HBV-specific CD8 responses in the HIV positive group were markedly diminished.

In a similar group of 12 HAART naïve HIV positive patients who also had serological evidence of past HBV infection without a history of symptoms, HBV-specific CD8 responses were markedly diminished. This group was matched with the HIV negative HBV immune group for sex, age, ethnic origin and likely route of origin of HBV and were all antiretroviral therapy naïve. HBV-specific CD8 responses were undetectable both by intracellular cytokine staining after 10 days in vitro stimulation (apart from one low level c18-27 response in patient 16, *fig. 3.5)* as well as directly ex-vivo *(fig. 3.6)* The breadth of HBV-specific immune responses directly ex-vivo was similar to that seen after in vitro expansion in all patients studied by both methods. Tetramer staining for the immuno-dominant epitope core 18-27 after 10 days in vitro expansion and done directly ex vivo in a subgroup of patients provided similar results, with 3 out of eight HIV negative HBV immune patients having a response to tetramer core 18-27, but such responses were undetectable in the HIV positive subgroup *(fig 3.7)*. Tetramer and intracellular cytokine staining provided consistent results in all patients where both methods were used. The proportion of patients with any HBV-specific CD8 responses and the total number and magnitude of responses was significantly greater for the whole HIV negative group when compared to HIV positive group ($p=0.018$ by Fischer's exact test and $p=0.006$ by Mann-Whitney test respectively). These differences remained significant comparing the closely matched subsets in the HIV negative and positive groups who had resolved HBV infection without symptoms ($p=0.027$ for the proportion of patients with at least one response, Fisher's exact test and $p=0.01$ for the number and size of responses, Mann-Whitney test *(f i g . 3 . 8)*

Fig.3.6

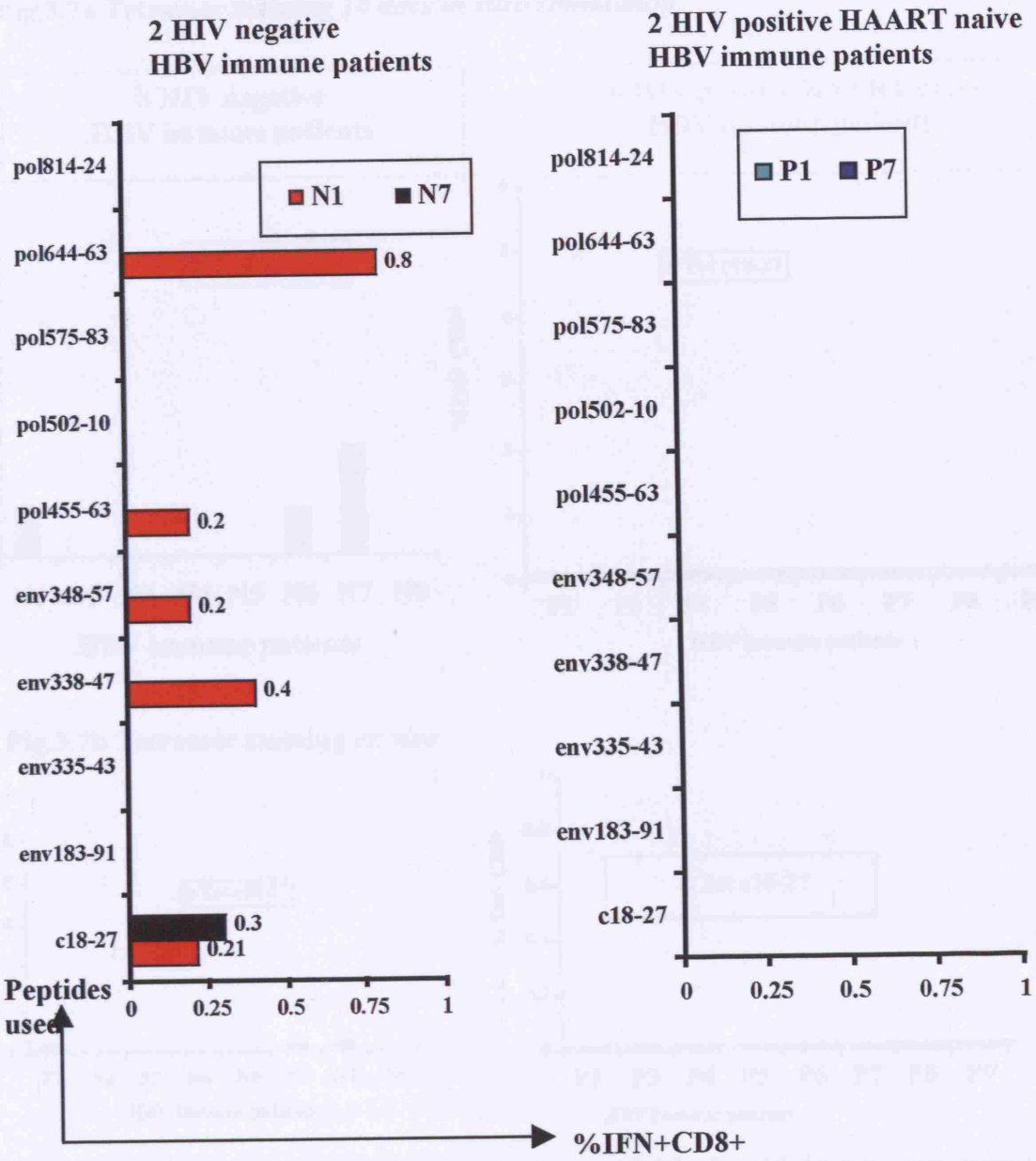


Fig.3.6 *Intracellular cytokine staining directly ex vivo in a subgroup of HIV negative and HIV positive patients who had resolved HBV asymptomatically.*

Two HIV negative HBV immune patients (right panel) had detectable CD8 responses ex vivo of similar breadth as detected after 10 days in vitro stimulation. HBV-specific CD8 responses in the HIV positive patients were undetectable ex vivo.

Fig.3.7a Tetramer staining *10 days in vitro stimulation*

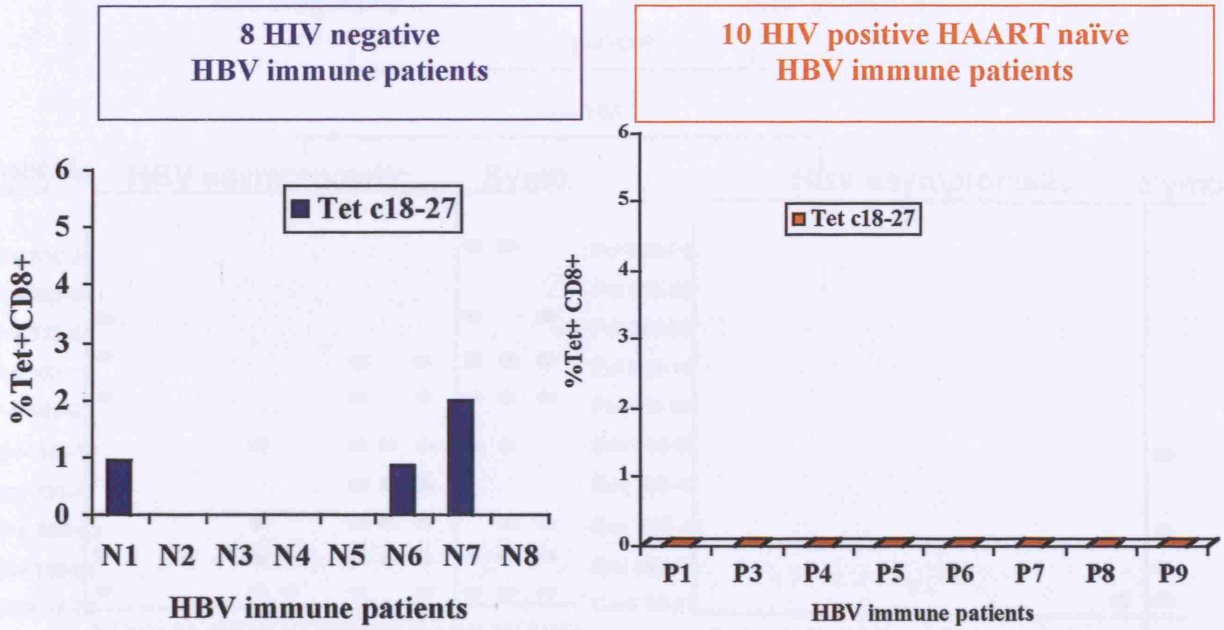


Fig.3.7b Tetramer staining *ex vivo*

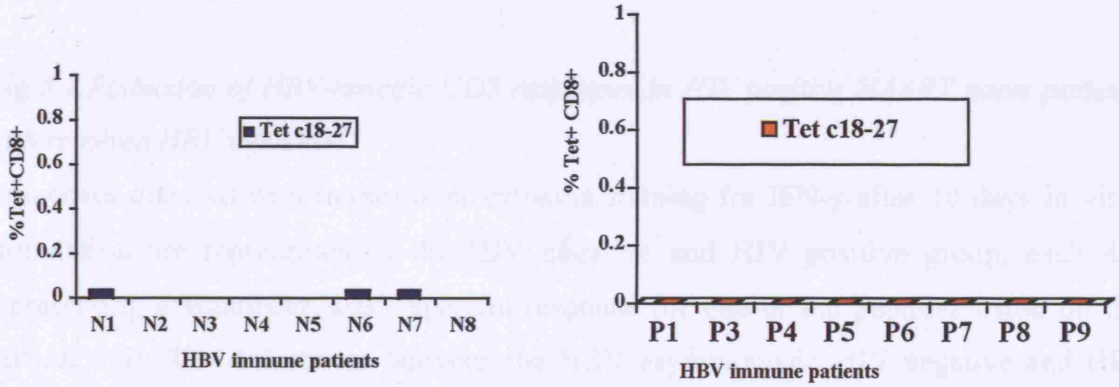


***Fig.3.7 a and b** Tetramer staining with tetramer core 18-27 after 10 days in vitro stimulation with c18-27 peptide (7a) and directly ex-vivo (7b) in HIV negative and HIV positive patients who had resolved HBV infection without any prior symptoms of acute infection.*

HIV negative patients (N1-N8, right panel) had detectable HBV-specific CD8 responses to at least one HLA-A2 restricted epitope in 3 out of 8 cases. HBV-specific CD8 responses in the HIV positive group (P1-P9) were undetectable ex vivo and after 10 days of in vitro stimulation.

Fig.3.8

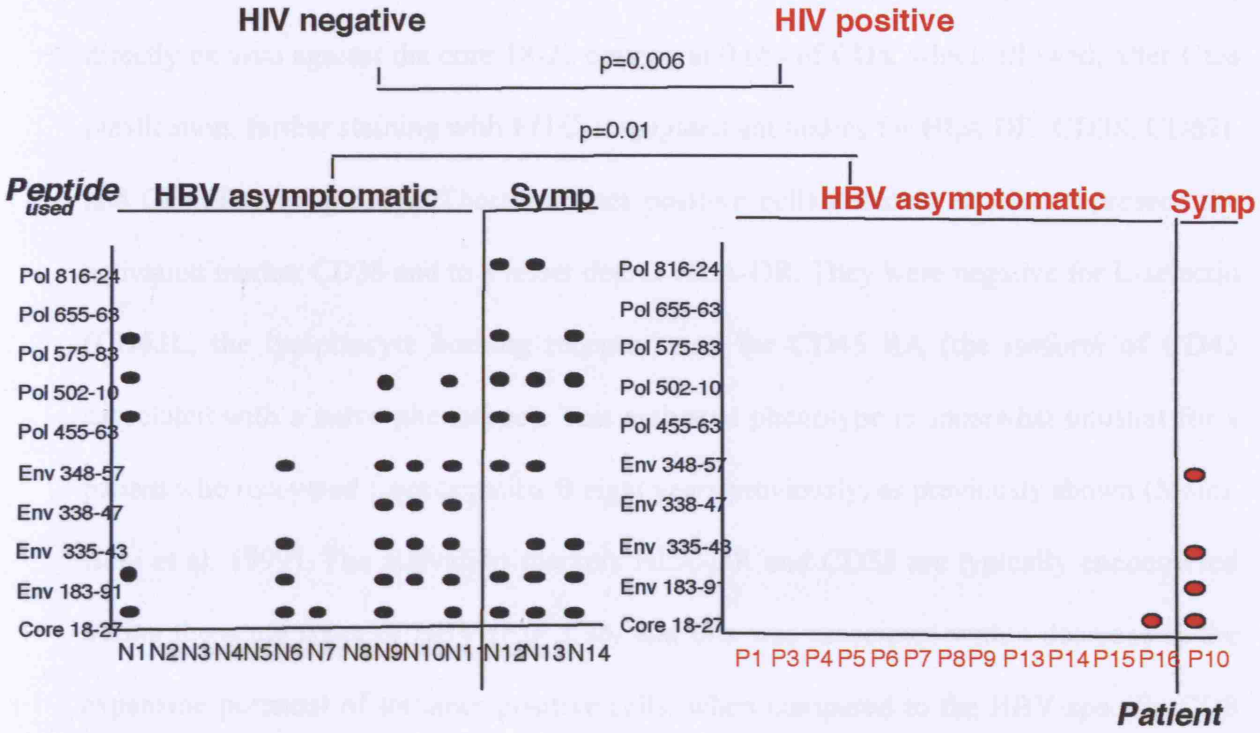


Fig 3.8*.Reduction of HBV-specific CD8 responses in HIV positive HAART naive patients with resolved HBV infection.*

Responses detected with intracellular cytokine staining for IFN-γ after 10 days in vitro stimulation are represented in the HIV negative and HIV positive group, each dot representing a significant HBV-specific response for one of the peptides listed on the vertical axis. The differences between the HBV asymptomatic HIV negative and HIV positive sub-groups were statistically significant and so were the difference between the HIV negative and HIV positive group.

3.4.2 Phenotype of HBV-specific CD8 responses in one HIV positive patient

We were interested in studying the phenotype of tetramer positive cells in the setting of HIV. Patient P10 had detectable HBV-specific CD8 responses detected by tetramer staining directly ex vivo against the core 18-27 epitope at 0.6% of CD8, which allowed, after CD8 purification, further staining with FITC-conjugated antibodies for HLA DR, CD38, CD62L and CD45RA. *(Fig.3.9a).* There tetramer positive cells detected ex vivo expressed the activation marker CD38 and to a lesser degree HLA-DR. They were negative for L-selectin (CD62L, the lymphocyte homing receptor) and for CD45 RA (the isoform of CD45 associated with a naïve phenotype). This activated phenotype is somewhat unusual for a patient who recovered from hepatitis B eight years previously, as previously shown (Maini, Boni et al. 1999). The activation markers HLA-DR and CD38 are typically encountered during the acute stage of HBV *(Fig 3.9b)* and this was associated with a decrease in the expansion potential of tetramer positive cells, when compared to the HBV-specific CD8 cells detected during the recovery phase *(Fig 3.9b).* The growth potential of HBV-specific CD8 cells is inversely related to the expression of cellular activation markers (Maini, Boni et al. 1999). The phenotype of the tetramer positive in the HIV negative chronic HBV carriers was also shown to express low levels of cellular activation markers CD38 and HLA DR (Maini, Boni et al. 2000). Although we could only study the phenotype of HBV-specific CD8 in one HIV positive patient, these results are consistent with the lower expansion potential of CD8 in the context of HIV. This could be accounted by the general immune activation state typical of HIV infected patients not taking HAART. HIV infection is accompanied by enhanced apoptosis, which affects mainly bystander cells (Finkel TH, Nat Med 1 1995 and Mueller YM Immunity 15, 2001). Interaction of FAS/ FAS ligand on infected cells may trigger apoptosis of virus-specific CTLs, although HIV-specific CTLs

were shown to be more prone to FAS-mediated apoptosis than HCMV CTLs in the same individuals (Mueller YM Immunity 15, 2001). There is no other data available on HBV-specific CTL's phenotype or apoptosis in the context of HIV.

Fig. 3.9a

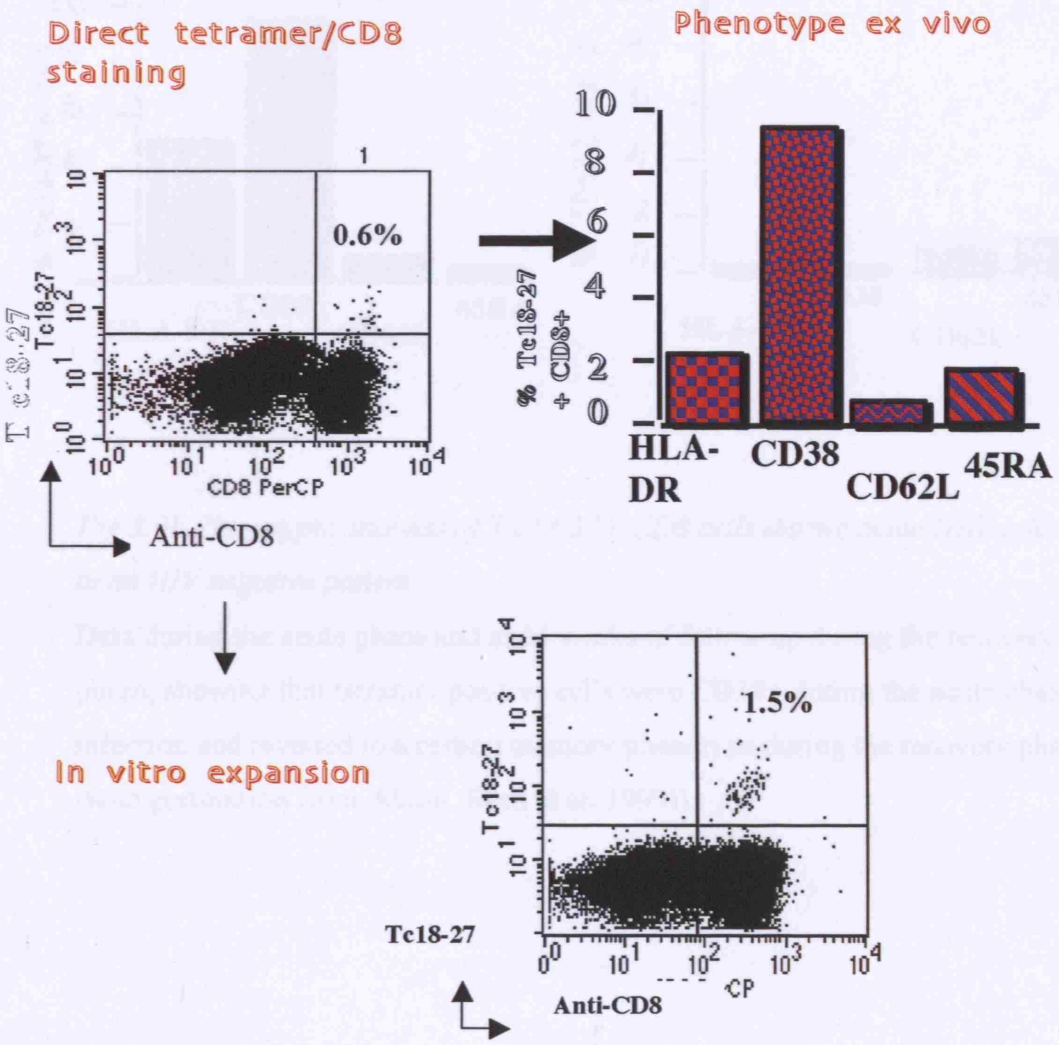


Fig. 3.9a *Direct tetramer staining with Tc18-27 and CD8 and phenotypic analysis of tet+ cells in a HIV positive patient with acute resolved HBV.*

Dot plot showing data after gating on CD8 cells, where there was a detectable population of tetramer positive CD8 cells after staining with tet c18-27 directly ex vivo. Further phenotypic analysis of tetramer positive CD8 cells with anti-HLA DR, anti-CD38, anti-CD62L and anti-CD45 RA shows that tetramer positive cells had an activated phenotype with CD38+. Percentage of CD38+, HLA-DR+, CD62L+, CD45 RA+ cells shown was calculated using CellQuest software.

Dot plot of tetramer staining with Tc18-27 at 10 days of in vitro expansion from the same patient.

Fig. 3.9b

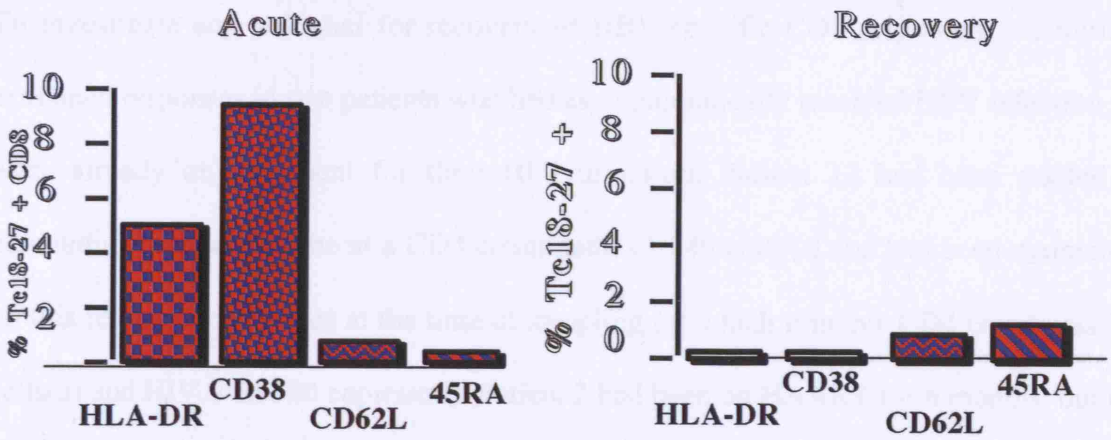


Fig 3.9b *Phenotypic analysis of T c18-27+ CD8 cells during acute HBV infection in an HIV negative patient.*

Data during the acute phase and at 14 weeks of follow up during the recovery phase, showing that tetramer positive cells were CD38+ during the acute phase of infection and reverted to a resting memory phenotype during the recovery phase (with permission from Maini, Boni et al. 1999)).

3.4.3 Some HIV-infected patients on HAART have detectable HBV-specific CD8 T cell responses

To investigate any potential for recovery of HBV-specific CD8 responses, we initially examined responses in two patients who had asymptomatically resolved HBV infection and were already on treatment for their HIV infection. Patient 12 had been started on zidovudine and lamivudine at a CD4 count nadir of 240 cells/μl and had been maintained on this regimen for 3 years at the time of sampling (at which time his CD4 count was 350 cells/μl and HIV load 500 copies/ml). Patient 2 had been on HAART for 6 months, but had substituted didanosine with lamivudine one month prior to sampling. His CD4 count had increased from a nadir of 160 to 700 cells/μl, with viral load suppressed below 50 copies/ml. Both these patients had CD8 responses to three out of 10 HBV-specific HLA restricted epitopes tested *(fig 3.10),* which after 10 days expansion were of a similar magnitude to those seen in the HIV negative HBV immune patients. This contrasted with the lack of responses detectable in 11 out of 12 untreated HIV negative HBV immune patients with an equivalent pattern of HBV immunity without a history of symptomatic acute infection *(fig 3.10).* HBV-specific CD8 responses were also detectable in the treated HIV patients but not in the untreated HIV positive HBV immune patients tested directly ex vivo *(Fig 3.10).*

Fig.3.10 *HBV-specific CD8 responses after in vitro expansion and directly ex vivo in HIV positive patients with natural immunity to HBV. HAART treated and antiretroviral naïve HIV positive patient were compared.*

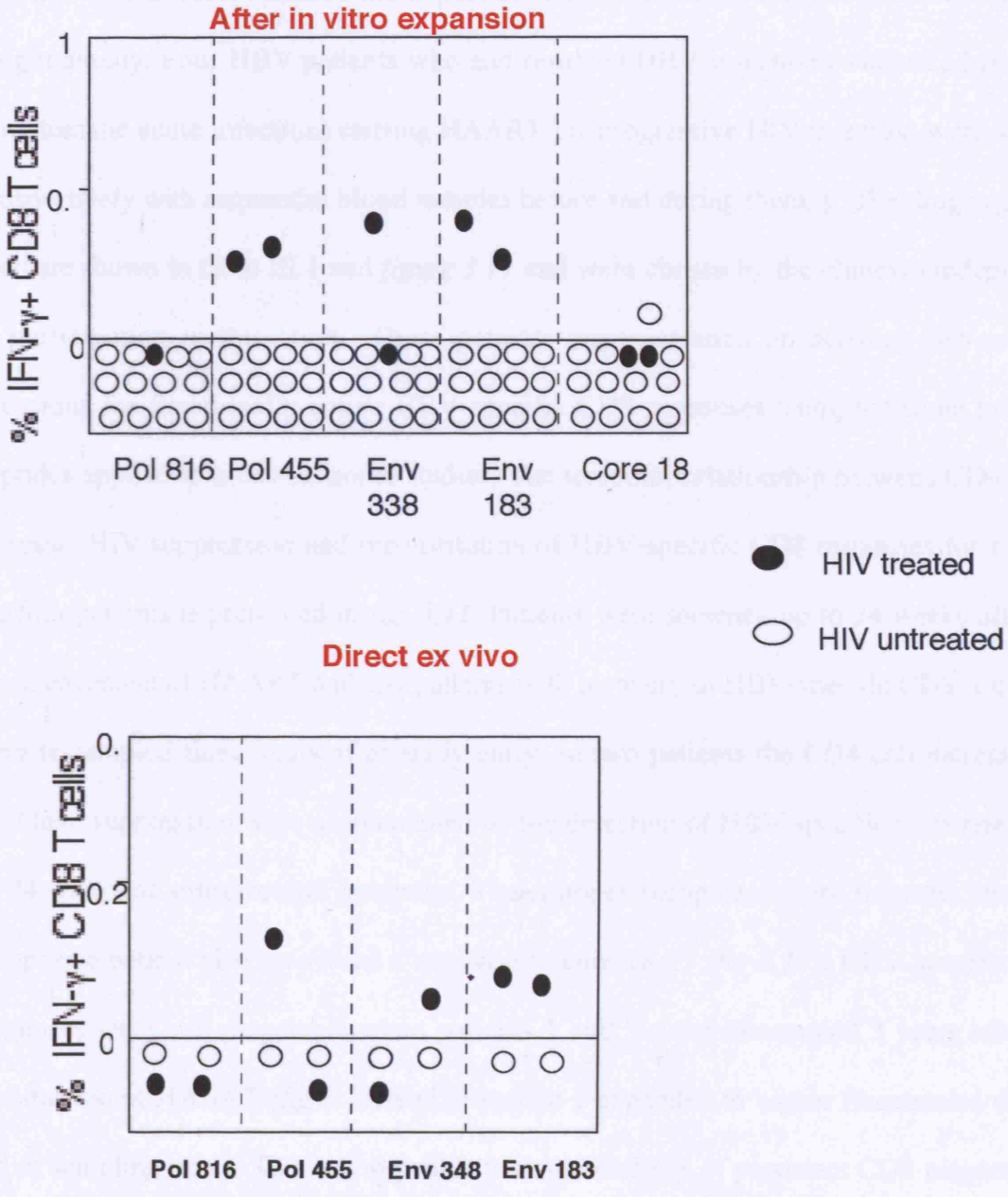


Fig. 3.10 Two HIV positive HBV immune patients on HAART had CD8 responses to three out of 10 HBV-specific HLA restricted epitopes tested with intracellular cytokine staining after 10 days in vitro stimulation and directly ex vivo.

3.4.4 Recovery of HBV-specific CD8 responses on longitudinal study of patients commencing HAART

The cross-sectional data suggested a decrease in HBV-specific CD8 responses in HIV positive HBV immune patients and a possible recovery of such responses in those taking HAART. We therefore studied the impact of HAART on HBV-specific immune responses longitudinally. Four HBV patients who had resolved HBV infection (without a history of symptomatic acute infection) starting HAART for progressive HIV infection were studied prospectively with sequential blood samples before and during therapy. The drug regimens used are shown in table III.1 and *figure 3.11* and were chosen by the clinician independent to participation in this study. These patients were screened on between two and six occasions for functionally active HBV-specific CD8 responses using the same panel of peptides applied in cross-sectional studies. The temporal relationship between CD4 count increase, HIV suppression and reconstitution of HBV-specific CD8 responses for each of the four patients is presented in *fig. 3.11.* Patients were screened up to 24 weeks after the commencement of HAART and any patients with recovery of HBV-specific CD8 responses were re-sampled three years after study entry. In two patients the CD4 cell increase and viral load suppression were accompanied by the detection of HBV-specific CD8 responses by 24 weeks of antiretroviral treatment. The epitopes recognized were from the envelope group; one patient also recovered a response to core 18-27 *(fig 3.11).* HBV-specific CD8 responses were still detectable when patients 1 and 7 were re-sampled 3 years after the introduction of HAART *(fig 3.11)* and in patient 1 expanded to higher frequencies than at earlier sampling times. This is compatible with the findings of persistent CD8 responses in patients studied cross-sectionally 6 months to 3 years after starting antiretroviral therapy.

Ex vivo analysis in P7 revealed a similar recovery of HBV-specific CD8 responses at week 24 (*Fig. 3.12a*), consistent in specificity and timing with the in vitro data *(fig 3.11).* This

would be more in keeping with a true increase in HBV-specific CD8 after HAART rather than a reduction of any inhibitory effect of HIV in cell culture after antiretroviral therapy. HIV gag 77-85 epitope are also shown, which declined following initiation of HAART, in accordance with previously reported data (Ogg, Jin et al. 1998; Dalod, Dupuis et al. 1999).

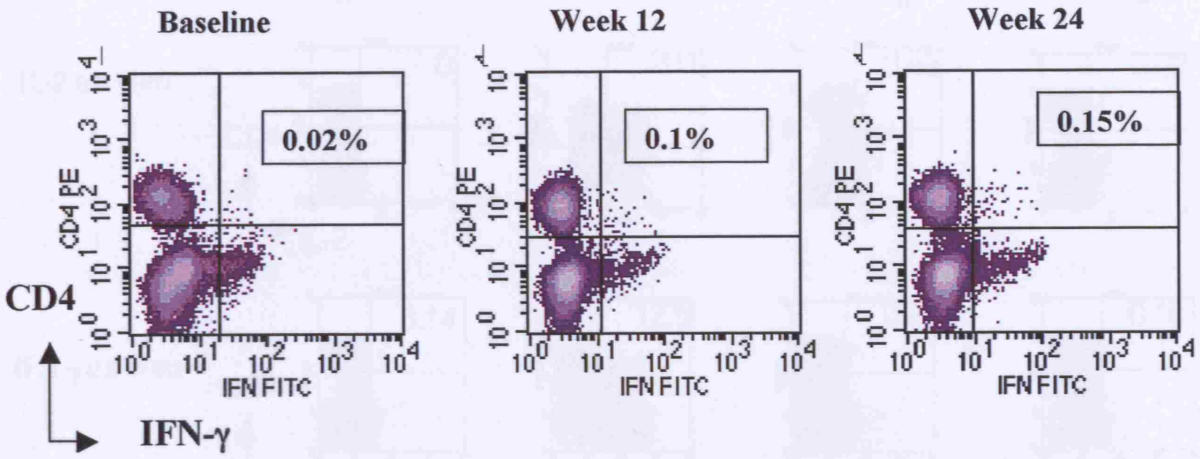
3.4.5 The increase in CD8 cells on HAART is accompanied by a reconstitution of HBV-specific CD4 responses

Patient (P7) had a substantial increase in the total CD4 count and rapidly achieved adequate viral suppression following antiretroviral therapy by week 12 *(Fig 3.11).* In order to dissect whether the CD4 count increase also included reconstitution of an HBV specific CD4 response, we performed HBV core antigen specific intracellular cytokine staining on CD4 cells directly ex vivo. We observed a detectable population of HBV-specific CD4 cells after 12 weeks, which reached 0.15% of total CD4 by 24 weeks *(Fig.3.12b),* and paralleled the increase in HBV-specific CD8 cells. *Fig 3.12c* illustrates a graphic representation of HBV-specific CD4 responses and HBV and HIV-specific CD8 responses after starting HAART.

In order to further investigate the potential role of enhanced CD4 help in reconstituting HBV-specific CD8 responses in these patients, we screened all the patients undergoing longitudinal study for HBV-specific CD4 responses at each time point. In light of recent data highlighting the potential importance of IL-2+ CD4 in maintaining adequate anti-viral CD8 (Day, Shea et al. 2001), patients in the longitudinal study were also tested at each time point for the presence CD4 cells able to produce IFN-γ and/or IL-2 on stimulation with HBV core and surface antigens. The assay to measure CD4 intracellular production of IFN-γ and/or IL-2 with or without stimulation with the hepatitis B core or surface antigens is shown in *fig.3.13.* As illustrated by the FACS plots in this treated patient, responses to overnight antigenic stimulation were easily detectable in some cases (IL-2+ CD4 responding to HBcAg). In other examples (IFN-γ+ CD4 with HBcAg and HBsAg), the responding populations were only slightly above background but could be confirmed by their ability to increase after antigen-specific in vitro expansion *(Fig. 3.13).*

The temporal relationship between CD4 count increase, HIV suppression and reconstitution of HBV-specific CD4 and CD8 responses for each of the four patients is presented in *figure 3.14*. CD4 responses to the core and surface proteins of HBV were not detectable in any of these HBV immune patients with HIV-related CD4 lymphopenia (CD4 < 350 cells/μl) prior to the start of antiretrovirals *(Fig. 3.14)*. All four patients developed detectable CD4 responses to both core and surface antigen from week six onwards, which persisted for the duration of follow-up (at least six months in two patients and at least three years in the other two, *fig 3.14*).

Fig 3.12b *HBV-specific CD4 responses after starting HAART in one patient (P7) as detected by intracellular cytokine staining directly ex vivo after 6 hrs stimulation with HBV core antigen.*
Dot plots of IFN/CD4 staining at 3 time points show a detectable population of HBV-specific CD4 cells after 12 weeks, which reached 0.15% of total CD4 by 24 weeks. CD4 counts and HIV-VL are represented under each time point.



CD4 abs *(x10⁶/l)*	**260**	**410**	**670**
HIV VL *(c/ml)*	**225,200**	**<50**	**<50**

3.12c

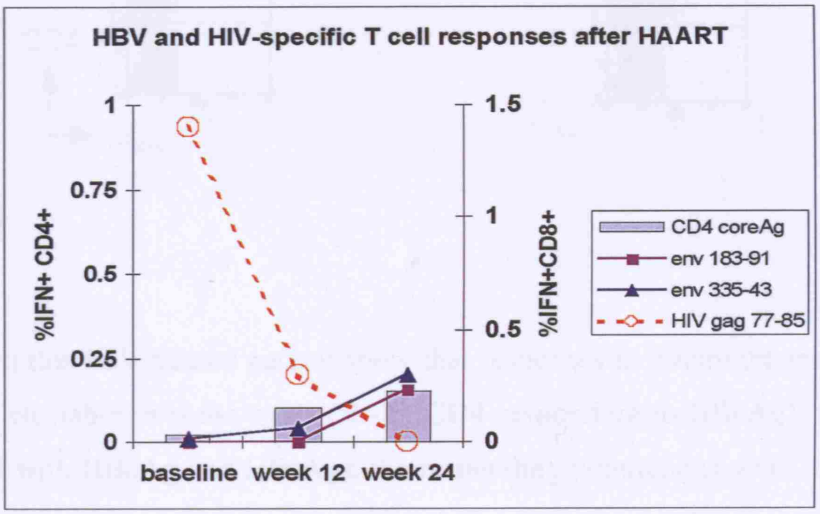


Fig. 3.12c *Graphic representation of HBV-specific CD4 responses and HBV and HIV-specific CD8 responses after starting HAART.*
There is an increase in HBV specific CD4 and CD8 responses accompanied by a decline in HIV-specific CD8 responses after starting HAART.

***Fig. 3.13* Screening for IL-2 and IFN-γ producing HBV-specific CD4.**

Intracellular cytokine staining of CD4 from HIV positive HBV resolved patient after 6 hrs in vitro stimulation

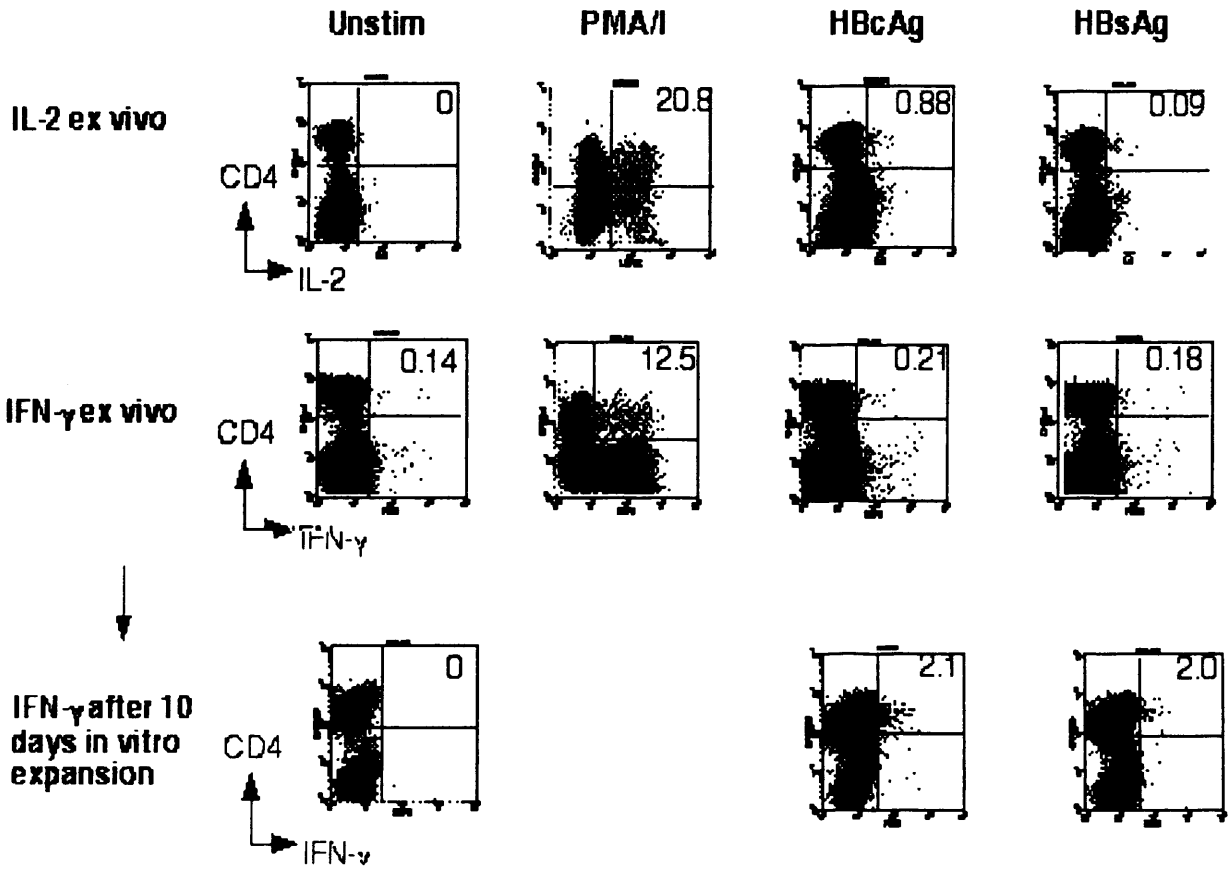


Fig 3.13.

FACS plots in this HIV treated patient show that responses to overnight antigenic stimulation were easily detectable in some cases (IL-2+ CD4 responding to HBcAg). In other examples (IFN-γ+ CD4 with HBcAg and HBsAg), the responding populations were only slightly above background but could be confirmed by their ability to increase after antigen-specific in vitro expansion. PMA/Ionomicin was used as a positive control.

Although some of these CD4 responses were of low frequency, many of them were able to produce IL-2 in addition to IFN-γ, and compatible with this, were able to expand in vitro. An exception was P8 who developed CD4 cells specific for HBcAg and HBsAg 24 months after starting HAART which were able to produce IFN-γbut not IL-2 *(Fig. 3.14);* consistent with this, proliferative responses to HBcAg and HBsAg remained undetectable in this patient (data not shown). Three of the four patients were given lamivudine-containing combinations; P5, who also developed strong HBV-specific CD4 responses, was not treated with any drugs with anti-HBV activity. All patients achieved adequate viral suppression and good CD4 recovery but the patient with the highest CD4 nadir, lowest starting viral load, and most rapid viral load suppression, had the most effective reconstitution of HBV-specific CD4 and CD8 responses (P1, fig. 3.14). *Fig.3.15* summarises the lack of CD4 responses to the core and surface proteins of HBV in five HBV immune patients with HIV-related CD4 lymphopenia (CD4<350 cells/μl) prior to start of antiretrovirals. This contrasts with HBV-specific CD4 responses in six HIV negative patients also with asymptomatic resolved HBV. The peak responses seen in HIV infected patients on HAART are comparable in frequency and magnitude to those seen in the HIV negative group *(Fig.3.15)*

3.5 Discussion

In this study we explored the effect of HIV immunodepletion and HAART related immune restoration in reshaping immune responses to HBV.

HIV infection represents a major insult to the immune system, directly impairing the CD4 cells and causing large polyclonal expansions of CD8 cells. The CD4 help is at the core of providing help in the development and especially for the maintenance of CD8 responses (Kalams and Walker 1998). This leads to failure of the immune system to control HIV infection, but how this affects the memory responses to clinically relevant pathogens has been the subject of several recent studies.

Our unique individual T cell repertoire is continuously shaped by exposure to the various antigens one comes in contact with during a lifetime. After the primary response developed following an encounter with a viral pathogen, a pool of antigen-specific cells forms, which need to be accommodated by our finite immune system. Memory CD8 T cells were initially believed to be a population of long-lived T cells persisting after viral clearance, although some evidence suggest a requirement for continued antigenic stimulation for their survival. (Gray and Skarvall 1988; Gray and Matzinger 1991; Gray 2000) (Oehen, Waldner et al. 1992). Most viruses are rarely completely expelled by the immune system (Ciurea, Klenerman et al. 1999), which would be in keeping with the fact that memory and protective immunity coexist with trace amounts of viral antigen. In our study we observed a decrease in the breadth of functionally active HBV-specific CD8 responses in association with HIV infection in HBV immune patients. Accumulating data suggest that residual virus is kept under tight control by an ongoing immune response in such patients (Penna, Artini et al. 1996; Maini, Boni et al. 1999) (Rehermann, Ferrari et al. 1996) Thus the reduction in HBV-specific CD8 responses observed here provides a mechanism that could contribute to the increased risk of reactivation of HB surface antigenemia seen in HIV infection and

other situations of clinical immunosuppression (Chazouilleres, Mamish et al. 1994).(Waite, Gilson et al. 1988)

In order to accommodate memory responses to new pathogens, some pre-existing memory cells are deleted through the process of attrition, some memory responses being more affected then others. Similarly, data from murine models show that heterologous viral infections can quantitatively and qualitatively alter the memory pool of existing antiviral CD8 (Selin, Vergilis et al. 1996; Selin, Lin et al. 1999; McNally, Zarozinski et al. 2001). These studies show that not all memory cells are affected equally. During a viral infection, "bystander" memory cells not specific for the virus also undergo apoptosis (McNally, Zarozinski et al. 2001) (McNally and Welsh 2002). These mechanisms would support the hypothesis that the lymphoid system has a finite number of niches for which there is a constant competition. When new memory cells are being generated, a similar number would be deleted as part of a homeostatic process. (for a review, (Gray 2000)). How an antigen gains a place in the memory pool is not completely understood, although in mice the clonal burst size and the T-cell differentiation at time of acute infection may play an important role (at least 5 cell divisions would be required for cells to survive in memory) (Hou, Hyland et al. 1994) (Opferman, Ober et al. 1999).

To date, immunological studies of HBV immunity following acute infection have only included patients presenting symptomatically. However a large proportion of patients are found to have immunity to HBV without any prior symptoms of acute infection (Redeker, Mosley et al. 1975; Parry, Brown et al. 1978). Although the duration since initial HBV exposure and the degree of initial liver inflammation were not always known, a number of HIV negative patients in this potentially heterogeneous group had preserved HBV-specific responses over time. Whilst we cannot exclude the possibility that HIV infected patients had a longer interval between resolution of HBV and sampling, the efficient long-term

preservation of HBV-specific CD8 responses makes this unlikely to account for their lack of responsiveness. The stronger multispecific CD8+ responses characteristic of patients with a clear history of acute symptomatic HBV infection may be better maintained in the context of HIV infection as suggested by one of the patients studied in this category. A second patient in this subgroup had maintained a low response to the core epitope as detected by tetramer staining, but these HBV-specific CD8 cells were functionally impaired and unable to produce IFN-γ.

Data describing the impact of HIV on responses to other viruses suggest that the persistence of detectable responses is related to the magnitude of antigen specific response generated by each virus. For example, EBV and CMV are both associated with stable high frequency memory CTL populations, which although reduced by HIV infection, remain easily detectable (Carmichael, Jin et al. 1993; Dalod, Dupuis et al. 1999; Komanduri, Donahoe et al. 2001). Since the responses generated in association with control of HBV are typically of lower frequency than those to viruses such as EBV and CMV, it is not surprising that few remain detectable in patients infected with HIV. Another study (van Baarle, Hovenkamp et al. 2001) revealed maintenance of numbers but functional impairment of EBV-specific CD8 associated with subtle increases in EBV load and progression to non-Hodgkins lymphoma on long-term follow-up.. Thus careful long-term assessment of HBV/HIV co-infected patients would be required to examine the virological impact of the partially impaired HBV-specific CD8 response in this group of patients. A reduction of HBV-specific CD8 responses following HIV infection would also be consistent with the demonstrated ability of HIV to induce apoptosis of CTL of unrelated specificities through FasL- (Xu, Screaton et al. 2001) and TNF-(Herbein, Mahlknecht et al. 1998) mediated counterattack. Similarly, studies using tetramers for LCMV infection in mice have highlighted persistence of CD8 cells in a nonfunctional state under conditions of deficient

CD4 help (Zajac, Blattman et al. 1998) and data from murine models show that heterologus viral infection can quantitatively and qualitatively alter the memory pool to existing CD8. (Selin, Lin et al. 1999; McNally, Zarozinski et al. 2001)

We examined whether the decreases of HBV-specific CD8 observed during HIV infection could be restored by antiretroviral therapy. There is evidence from studies of the herpes viridae (EBV, CMV, KSHV) that HAART can restore specific T cell frequencies (Dalod, Dupuis et al. 1999; Rinaldo, Huang et al. 2000; Casazza, Betts et al. 2001; Wilkinson, Cope et al. 2002); and this correlates with a decrease in end-organ disease (Komanduri, Viswanathan et al. 1998) (Palella, Delaney et al. 1998 ; Ledergerber, Telenti et al. 1999). The two patients studied cross-sectionally and two of the four patients studied longitudinally restored functionally active HBV-specific CD8+ responses, which had expansion potential after in vitro stimulation. The fact that CD8 responses were only reconstituted in a proportion of these HBV immune patients is in accordance with our findings in HIV negative patients without a history of symptomatic HBV infection, and in line with a study of the impact of HIV infection and antiretroviral therapy on responses to Mycobacterium avium complex (Havlir, Schrier et al. 2000).

An important contribution to this CD8 reconstitution is likely to be a restoration of CD4 help associated with the increasing CD4 cell numbers. T cell help is known to be important for maintaining functionally active CD8 (Kalams and Walker 1998) and recent data has suggested a key role for IL-2-producing CD4 cells in anti-viral immunity (Day and Walker 2003; Younes, Yassine-Diab et al. 2003). In both the patients with restoration of HBV-specific CD8, we observed a concomitant recovery of IFN-γ+ and IL-2+ CD4 responses to HBV core and surface antigen following the introduction of HAART. These HBV-specific CD4 T cell responses were similarly observed in a patient taking an antiretroviral

combination not including lamivudine or other drugs with anti-HBV activity. Importantly, we demonstrated long term maintenance of both CD4 and CD8 T cells on prolonged follow-up of two patients, in contrast to the rapid but short-lived effects of lamivudine on HBV-specific T cell function reported in patients with ongoing HBV infection (Boni, Penna et al. 2003; Lascar, Gilson et al. 2003). There is a temporal correlation between the recovery of T cell responses observed in this study and those seen in other studies of HAART-mediated reconstitution of T cell function (reviewed in (Carcelain, Debre et al. 2001), with an initial recovery at around three months, but subsequent reconstitution continuing gradually) for at least two years (Notermans, Pakker et al. 1999). These findings should be extended to a larger patient group, since this study was not powered to detect an effect of nadir CD4 count or magnitude of changes in CD4 count or viral load on the extent of HBV-specific T cell reconstitution. However it is worth noting that the patient with the most effective CD4 and CD8 recovery was the one starting HAART with the highest CD4 nadir, lowest viral load and achieving most efficient containment of HIV to undetectable levels.

The findings of this study could be extrapolated to suggest that in patients treated for HBV/HIV co-infection, re-constitution of HBV-specific T cell responses may involve two distinct components; the recovery of responses associated with reduction in HBV load (19) as seen in treatment of HBV mono-infection (41, 42) and the more gradual, sustained reconstitution associated with prolonged suppression of HIV viraemia and CD4 recovery demonstrated here. Thus, antiretroviral therapy can lead to an increase in functional CD4 and CD8 responses to HBV, supporting the potential of HAART to reconstitute immune responses to clinically important pathogens.

CHAPTER 4

4. Characterisation of HBV-specific CD4 and CD8 responses during and following asymptomatic acute hepatitis B

4.1 Introduction

Studies of the immune responses during acute hepatitis B have so far focused on patients who present acutely with jaundice and serology confirms an evolving acute infection. However, only 30-50% of patients (McMahon, Alward et al. 1985) who acquire HBV develop the classical symptoms of jaundice, dark urine or pale stools, which prompts them to seek medical care during the acute illness. In certain groups, such as intravenous drug users (IVDU), homosexual men and HIV positive patients there is a high prevalence of natural immunity to HBV (Scharschmidt, Held et al. 1992; Beck, Mandalia et al. 1996; Combe, La Ruche et al. 2001). This is usually diagnosed as part of routine screening prior to HBV vaccination, and most of these patients do not have a history of jaundice. This may represent a selection bias, as only the more severe cases present to a doctor, thus limiting our knowledge about the immune processes involved in the early stages of HBV infection. The outcome of HBV infection is influenced by host-virus interactions involving the innate and adaptive components of the immune response. When acquired in adulthood, HBV infection usually results in a self-limited hepatitis, with development of neutralizing antibodies and strong HBV-specific CD4 and CD8 responses, which persist long term.

Classically, the course of acute hepatitis B evolves over a period of months (Chisari and Ferrari 1995). The first serological marker to be detected is HB surface antigen (HBs Ag), followed by HBe Ag and a rise in HBV DNA. The ALT peak usually occurs at 10-15 weeks after infection and after the peak of HBV DNA. A large burden of viraemia is cleared in the early stages of infection (during the incubation phase of the clinical disease), via mechanisms involving antiviral cytokines produced by cells of the innate and early adaptive immune responses, as shown in the transgenic mouse model(Guidotti, Ishikawa et

al. 1996; Guidotti, Rochford et al. 1999) and in humans (Webster, Reignat et al. 2000). This was further confirmed by studies in chimpanzees where strong cellular immune responses against HBV (Thimme, Wieland et al. 2003) were shown to play the crucial role. Essential to HBV viral control in humans appear to be the adaptive immune responses, especially the HBV-specific CD4 and CD8 responses, which are strong and multispecific in patients recovering from acute infection, but relatively weak in chronic carriers. HB core antibodies (HBc Ab) of the IgM class appear early and high titres suggest the acute nature of the infection. HBcAb of the IgG class will persist for life, irrespective of the outcome of the infection.

I describe here the cellular immune responses in two adult cases of acute hepatitis B in patients who attended a STD clinic in Central London and were diagnosed as part of routine screening. Both patients were previously healthy males, were entirely asymptomatic and their only risk for HBV infection was sexual exposure.

4.2 Case definitions, materials and methods

4.2.1 Patients

We present two HLA-A2+ patients with asymptomatic acute hepatitis B, identified as part of routine sexual heath screening. Patient 1 was a homosexual man with a history of recent unprotected sex with casual partners and patient 2 was a heterosexual man in a relationship with a new partner who was a chronic hepatitis B carrier. The diagnosis of acute hepatitis B was made on the basis of HBV serology markers indicating an evolving HBV serological profile. Neither patient had previously been tested nor vaccinated against hepatitis B. HBsAg alone was detectable initially, followed by appearance of anti-HBc IgM on subsequent samples. Recovery from acute infection was defined by the detection of anti-HBe and subsequently anti-HBs Ab.

4.2.2 Analysis of HBV-specific CD4 and CD8 responses

The techniques of HLA-A2 staining, tetramer and intracellular cytokine staining for CD8 responses were described in the methods chapter (Chapter 2). CD4 responses were studied with proliferation assays and intracellular cytokine staining as described previously (Chapter 2), and further characterized by using pooled core peptides where responses to whole core antigen were found. Pooled peptides from the 16 peptides (10-20 residues long), corresponding to the entire sequence of the core region encoded polypeptides (synthesized by Multiple Peptide System, La Jolla, CA) were chosen taking into account existing experimental evidence on the immunodominant T cell epitopes within the core antigen during acute hepatitis B in symptomatic patients (Ferrari, Bertoletti et al. 1991). Amino acid sequences of the synthetic peptides used and indicated by the amino acid position of the NH_2-terminus of the core derived polypeptides are as follows: core 1 (1-20), core 2 (20-34), core 3 (28-47), core 4 (38-54), core 5 (50-59), core 6 (50-69), core 7 (61-80), core 8 (70-89), core 9 (82-101), core 10 (100-119), core 11 (117-131), core 12 (120-139), core 13

(131-145), core 14 (140-155), core 15 (155-169), core 16 (169-183). Responses to two further core peptides, previously described HBV epitopes (core 123-136 and core 112-125) were also tested separately.

4.2.3 Virological assessment

HBV DNA was measured using the Digene assay and later tested by Taqman PCR assay in one patient.

4.3 Results

4.3.1 HBV serological data and liver function tests

Both patients were initially identified by the presence of HBs Ag alone suggesting an incubating acute hepatitis B. For comparison with our data I included *fig 4.1a*, which illustrates the classical course of acute HBV (HBV serological markers, HBV DNA and liver function tests) as described in patients presenting with jaundice (Rehermann and Nascimbeni 2005). *Fig. 4.1b* illustrates the evolving serological markers in patient 1. At the initial presentation this patient was found to be positive for HBsAg only, and on subsequent testing two weeks later he became positive for HBe Ag and the HBV viral load was 3.5 million copies/ml. He promptly developed anti-HBe Ab and anti-HBs Ab within 10 weeks from initial presentation suggesting an accelerated course of resolution of HBV infection. Patient 2 was also identified through screening when he was initially HBsAg positive only and on subsequent testing two weeks later he had already developed anti-HBe antibodies, followed by clearance of HBs Ag and anti-HBs Ab after 6 weeks of infection. Both patients remained asymptomatic, without any clinical or laboratory signs of acute hepatitis from the time of presentation until complete virological recovery. Patients were followed up with sequential (2-4 weekly) blood samples for HBV markers, liver function tests and further detailed immunological analysis of HBV-specific CD4 and CD8 responses.

Fig.4.1 a

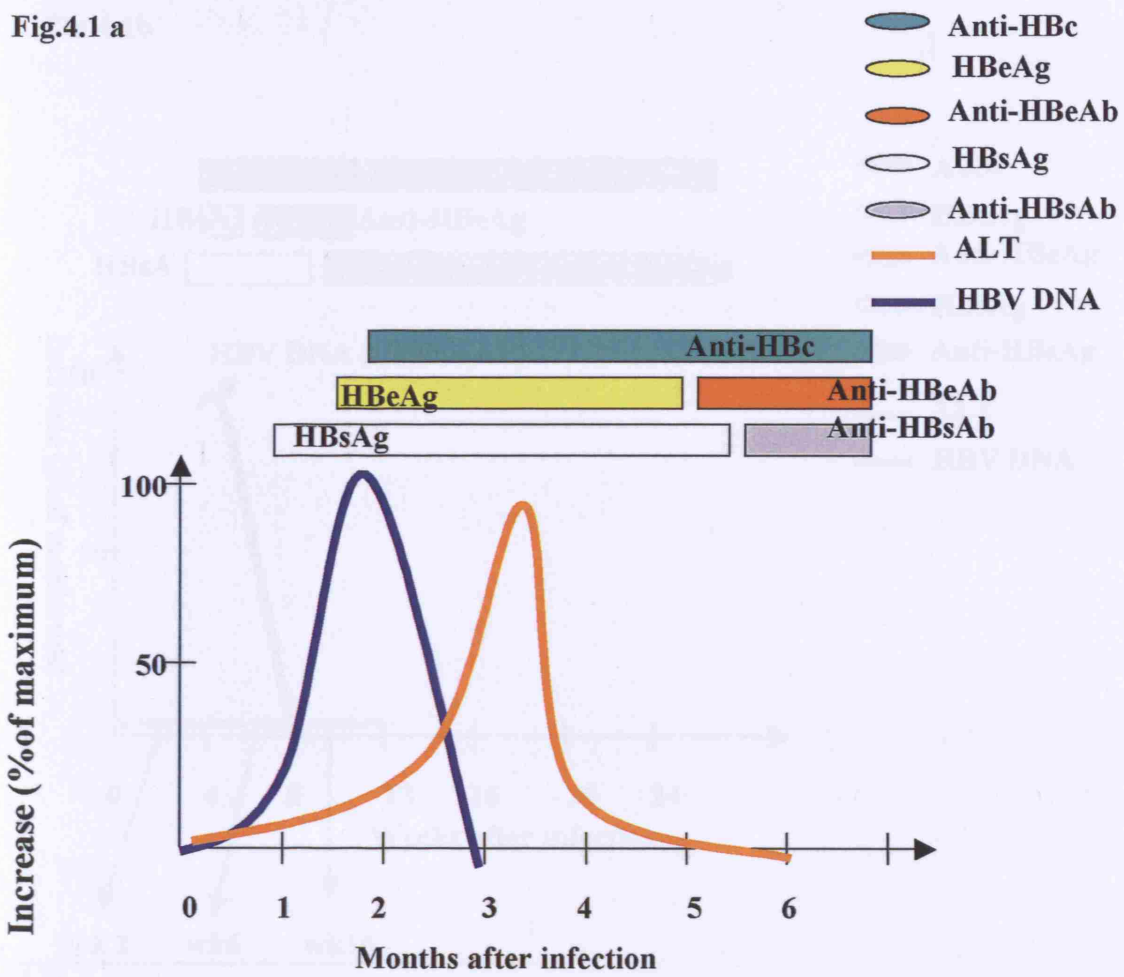


Fig. 4.1a *Representation of clinical, virological and immunological course of acute self-limited hepatitis B, adapted from (Rehermann and Nascimbeni 2005).*

The occurrence of various serological markers over time is represented as bars parallel to the horizontal axis. The ALT and HBV DNA are represented as percentages of the maximum elevation on the Y axis.

The first serological marker to be detected is HB surface antigen (HBs Ag), followed by HBe Ag and a rise in HBV DNA. The ALT peak usually occurs at 10-15 weeks after infection and after the peak of HBV DNA. A large burden of viraemia is cleared during the incubation time and prior to the onset of jaundice/ raised ALT. The occurrence of anti-HBe and later anti-HBs antibodies correlate with successful control of HBV infection.

Fig.4.1b

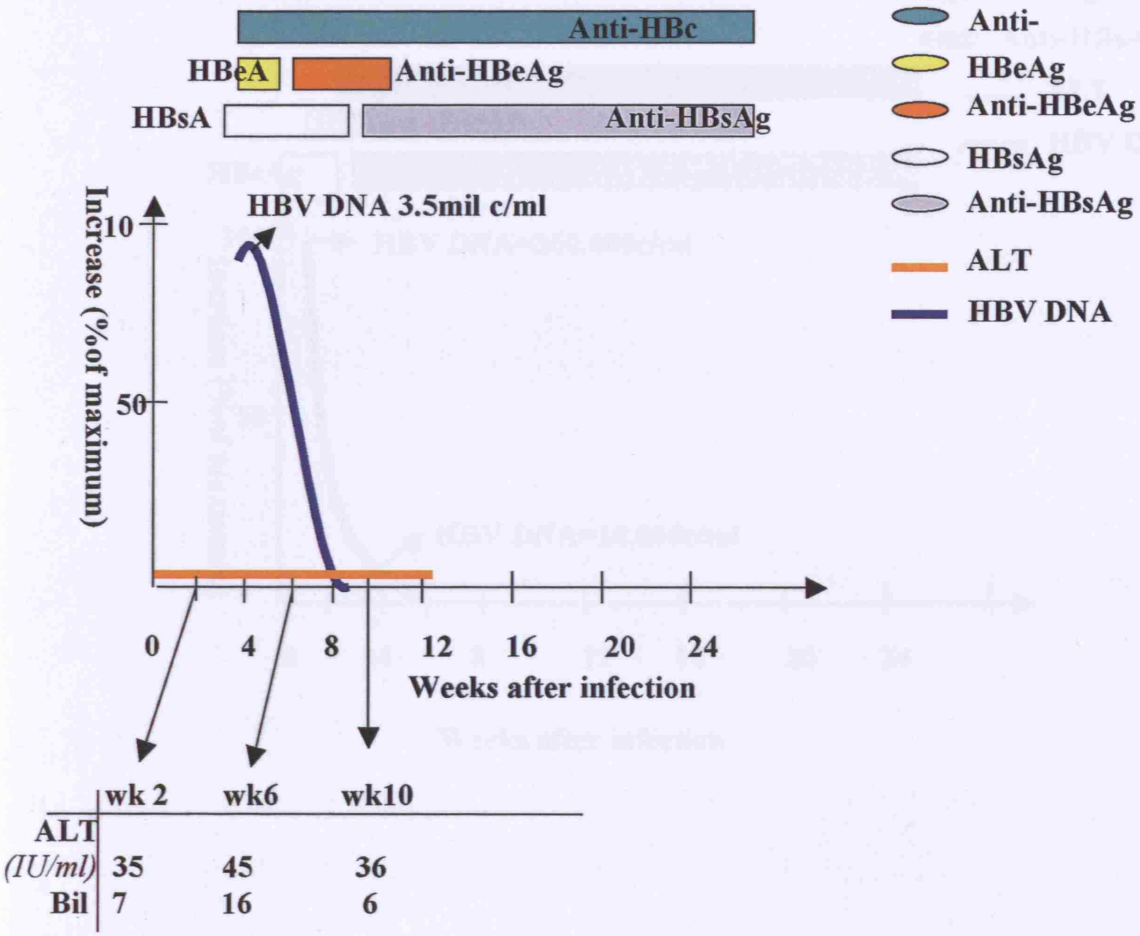


Fig. 4.1b *Representation of HBV serological markers and HBV DNA and ALT levels in patient 1.*

At the initial presentation this patient was found to be positive for HBsAg only, and on subsequent testing two weeks later he became positive for HBe Ag and the HBV viral load was 3.5 million copies/ml. He promptly developed anti-HBe Ab and anti-HBs Ab within 10 weeks from initial presentation suggesting an accelerated course of resolution of HBV infection. The peak of HBV DNA was 3.5mil copies/ml at week 2, and became undetectable at week 8. ALT levels remained normal throughout.

Fig.4.1c

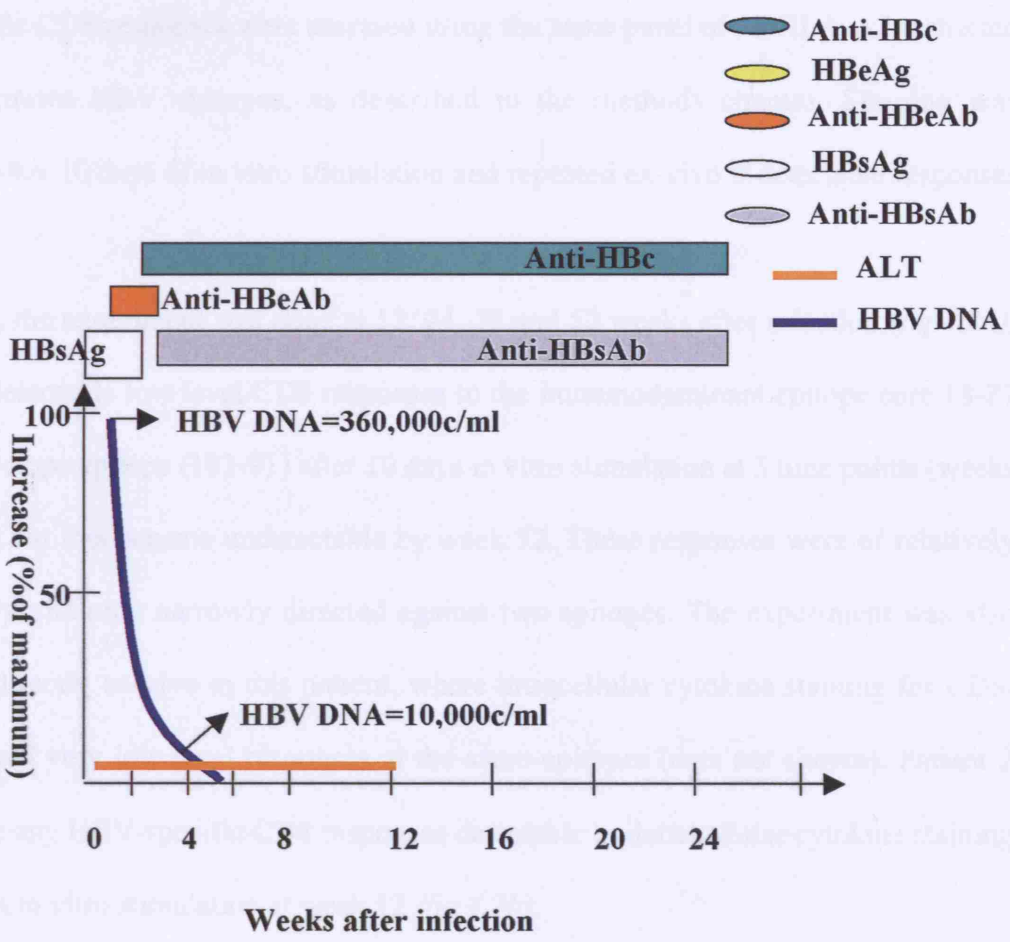


Fig. 4.1c *Representation of HBV serological markers and HBV DNA and ALT levels in patient 2.*

Patient 2 was initially HBsAg positive only and on subsequent testing two weeks later he had already developed anti-HBe antibodies, followed by clearance of HBs Ag and anti-HBs Ab after 6 weeks of infection. HBV DNA was 0.3 mil copies/ml at week 2 and decreased further to only 10,000c/ml at week 4. ALT levels remained normal.

4.3.2 HBV-specific CD8 responses

HBV-specific CD8 responses were assessed using the same panel of 10 HLA-A2 restricted peptides, known HBV epitopes, as described in the methods chapter. Staining was performed after 10 days of in vitro stimulation and repeated ex-vivo if detectable responses were found.

In patient 1, the experiment was done at 12, 24, 28 and 52 weeks after infection *(fig 4.2a)*. We found detectable low level CD8 responses to the immunodominant epitope core 18-27 and one envelope epitope (183-91) after 10 days in vitro stimulation at 3 time points (weeks 12, 24, 28), but this became undetectable by week 52. These responses were of relatively low intensity and only narrowly directed against two epitopes. The experiment was also performed directly ex-vivo in this patient, where intracellular cytokine staining for CD8/ IFN-γ showed very low level responses at the same epitopes (data not shown). Patient 2 did not have any HBV-specific CD8 responses detectable by intracellular cytokine staining after 10 days in vitro stimulation at week 12 *(fig 4.2b)*.

The CD8 responses in the two patients described were analyzed with the same panel of HLA-A2 restricted peptides used to characterize the cellular immune responses in patients with natural immunity to HBV described in Chapter 3. I described in both chapter 3 and in here patients who resolved HBV without symptoms or a history of jaundice, but only the two asymptomatic patients studied in this chapter were found to be in the acute phase of hepatitis B, allowing monitoring of liver function tests, viral load levels and evolution of hepatitis B serum markers.

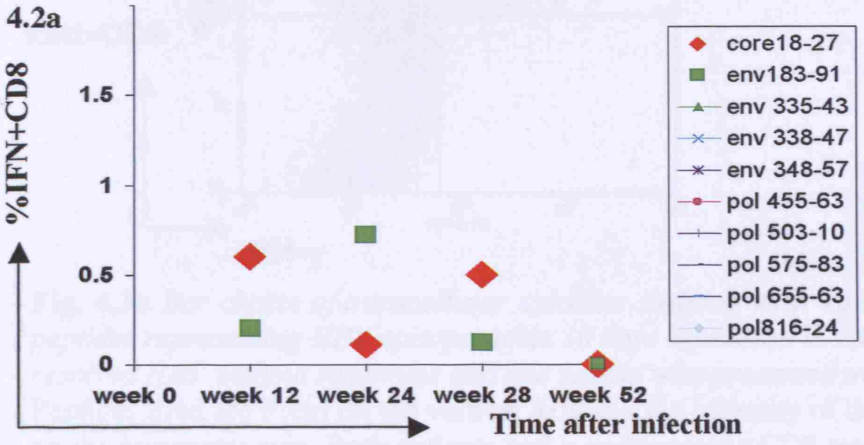
I have shown in Chapter 3 that patients who resolve hepatitis B asymptomatically can maintain HBV-specific responses, which can be expanded after 10 days in vitro stimulation with the appropriate peptide, and these responses are comparable with those previously

described in patients presenting with jaundice. *Fig 4.3a* shows a typical dot plot of intracellular cytokine staining for CD8/IFN after 10 day in vitro cell culture with the core 18-27 peptide from a patient who resolved acute symptomatic hepatitis B. *Fig 4.3b* compares charts from two patients who resolved acute hepatitis B with and without clinical signs of jaundice, showing that patients who are asymptomatic during the acute HBV illness can also maintain strong multispecific CD8 responses.

Fig.4.2a and b *Intracellular cytokine staining for CD8/IFN after 10 days in vitro stimulation with 10 HLA-A2 restricted peptides*

a.Patient 1 Data from 12, 24, 28 and 52 weeks after infection. HBV-specific CD8 responses are represented on the vertical axis as percentage of CD8+IFN-γ+ after subtraction of background. Detectable low level CD8 responses to immunodominant epitope core 18-27 and one envelope epitope (183-91) were found at 3 time points (weeks 12, 24, 28), but they became undetectable by week 52.

b. Patient 2 There were no detectable HBV-specific CD8 responses to any of the 10 peptides tested at week 12.



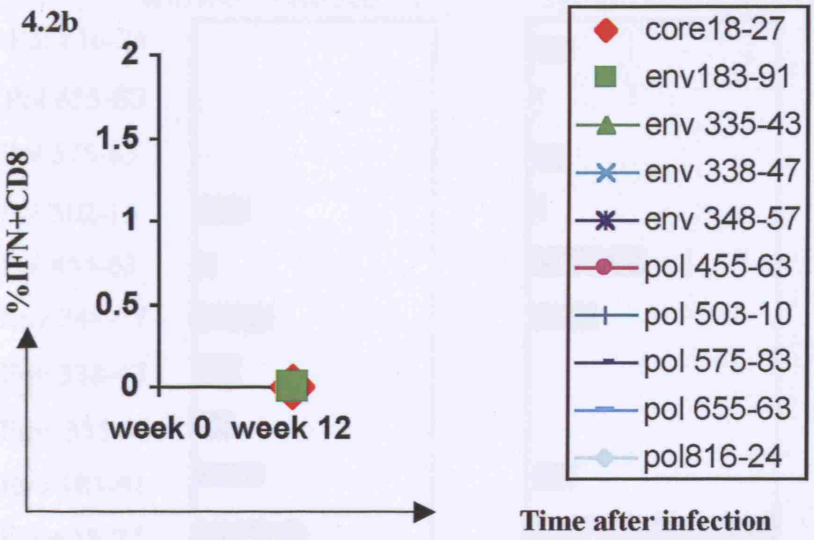


Fig 4.3a *Dot plot of intracellular cytokine staining after 10 days in vitro stimulation with the core 18-27 peptide in a patient with symptomatic resolved acute hepatitis.* After short term cell culture in vitro 15% of CD8 produce IFN-γ after stimulation with the core 18-27 peptide

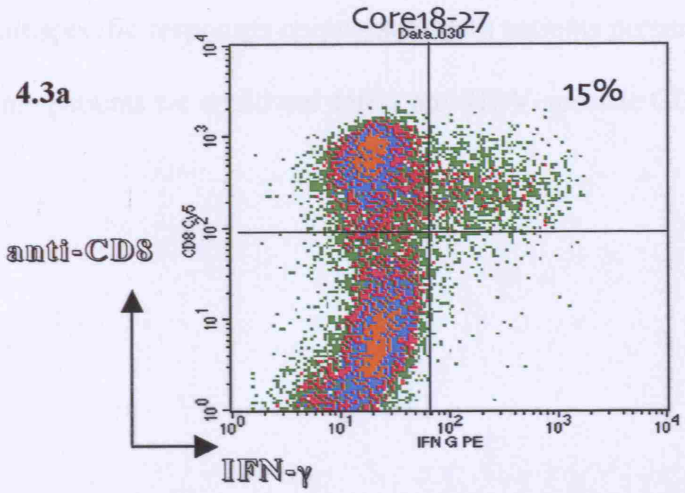


Fig. 4.3b *Bar charts of intracellular cytokine staining with 10 HLA-A2 restricted peptides representing HBV epitopes after 10 days expansion in vitro in a patient who resolved HBV without symptoms and one patient who presented with jaundice.* Peptides used are listed on the vertical axis and the intensity of detectable responses on the horizontal axis. Both patients had a multispecific CD8 response, recognizing 7 out of the 10 epitopes tested.

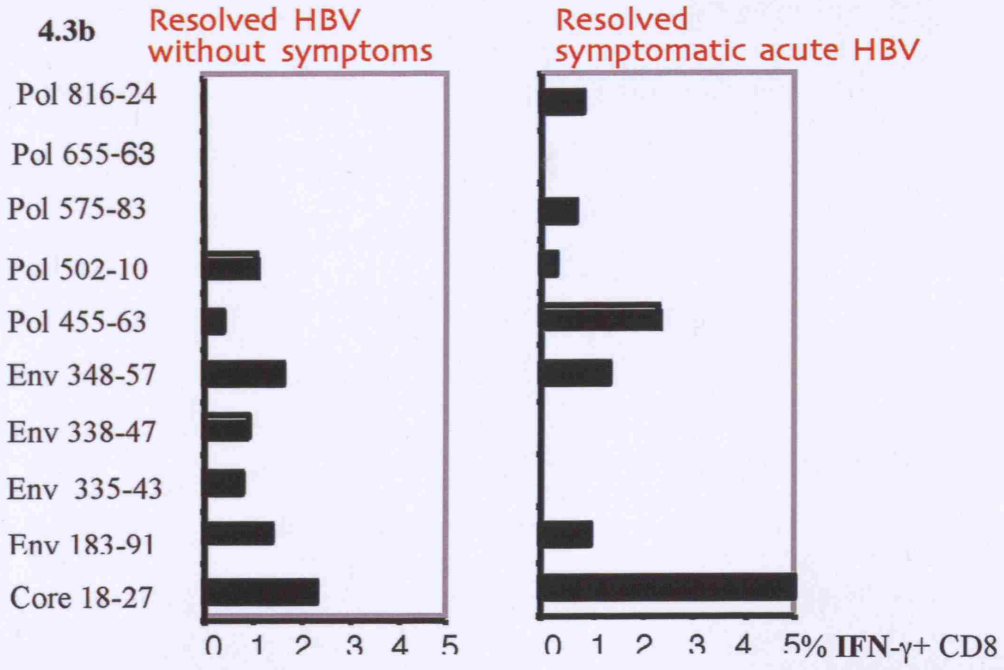


Fig 4.4 summarizes all responses detected with intracellular cytokine staining after 10 day in vitro stimulation in eleven patients who resolved HBV asymptomatically (but where the timing of the initial acute infection was not known) in comparison with 3 patients who had a history of jaundice during the acute HBV infection. This illustrates the wide spectrum of detectable HBV-specific CD8-responses, with some asymptomatic patients maintaining multispecific responses comparable with patients presenting acutely with jaundice, while in some patients we could not detect any HBV-specific CD8 responses.

4.3.3 HBV-specific CD4 responses

CD4+ T cells (helper cells) provide help for activation and differentiation of B cells and contribute to the induction and maintenance of HBV-specific CD8 cells. Together with CD8+T cells, CD4 cells may also secrete cytokines (IFN-γ, TNF-α), which inhibit viral replication. We assessed the presence and functionality of HBV-specific CD4 responses using the hepatitis B virus core, surface and e antigens, as well as a further selection of overlapping peptides from the core protein, in cases where detectable responses to the whole core protein were found. In order to dissect the nature of the T helper response (Th1 vs Th2) in the acute asymptomatic HBV infection, we performed staining for IFN-γ and IL-4. Proliferation assays were used for further confirmation of the results.

In patient 1 intracellular cytokine staining for CD4 and IFN-γ as well as IL-4 was performed after 8 days of in vitro stimulation with the same antigens. We show in *fig 4.5a* that there are HBV-specific CD4 responses to the core and e Ag, which could be expanded in vitro and produced preferentially IFN-γ rather than IL-4 for patient 1. Patient 2 showed detectable HBV-specific CD4 responses for the surface antigen at low level, which produced both IFN-γ and IL-4.

CD4 responses were also detected by proliferation assay *(Fig. 4.5b)* using the whole HB core Ag, HBs Ag and HBe Ag (only available in patient 1), showing significant reactivity to core and e Ag, with a stimulation index of 10.35 and 20 respectively in patient 1. The results of intracellular cytokine staining and proliferation assay were consistent, showing CD4 responses of similar specificities *(Fig 4.5a and 4.5b)*. Patient 1 was further tested for HBV-specific CD4 responses using a selection of overlapping peptides from the core region. There were detectable responses to peptides from core Ag, showing a multispecific CD4 response *(Fig 4.6)*. Proliferation assay results are also shown.

Fig 4.5a *Intracellular cytokine staining for CD4 and IFN-γ /IL-4 after 10 days in vitro stimulation with HBV proteins in two asymptomatic acute HBV patients.*
Significant HBV-specific CD4 responses to the core and e Ag were detected, which produced preferentially IFN-γ rather than IL-4 in patient 1 and a low level CD4 response to HBsAg in patient 2, which produced both IFN and IL-4.
Fig. 4.5b Proliferation assay results two asymptomatic acute HBV patients.
Patient 1 had a significant stimulation index for the HBV core and e antigen, consistent with the intracellular cytokine staining results. Patient 2 had a positive stimulation index for HB surface antigen, albeit at low level.

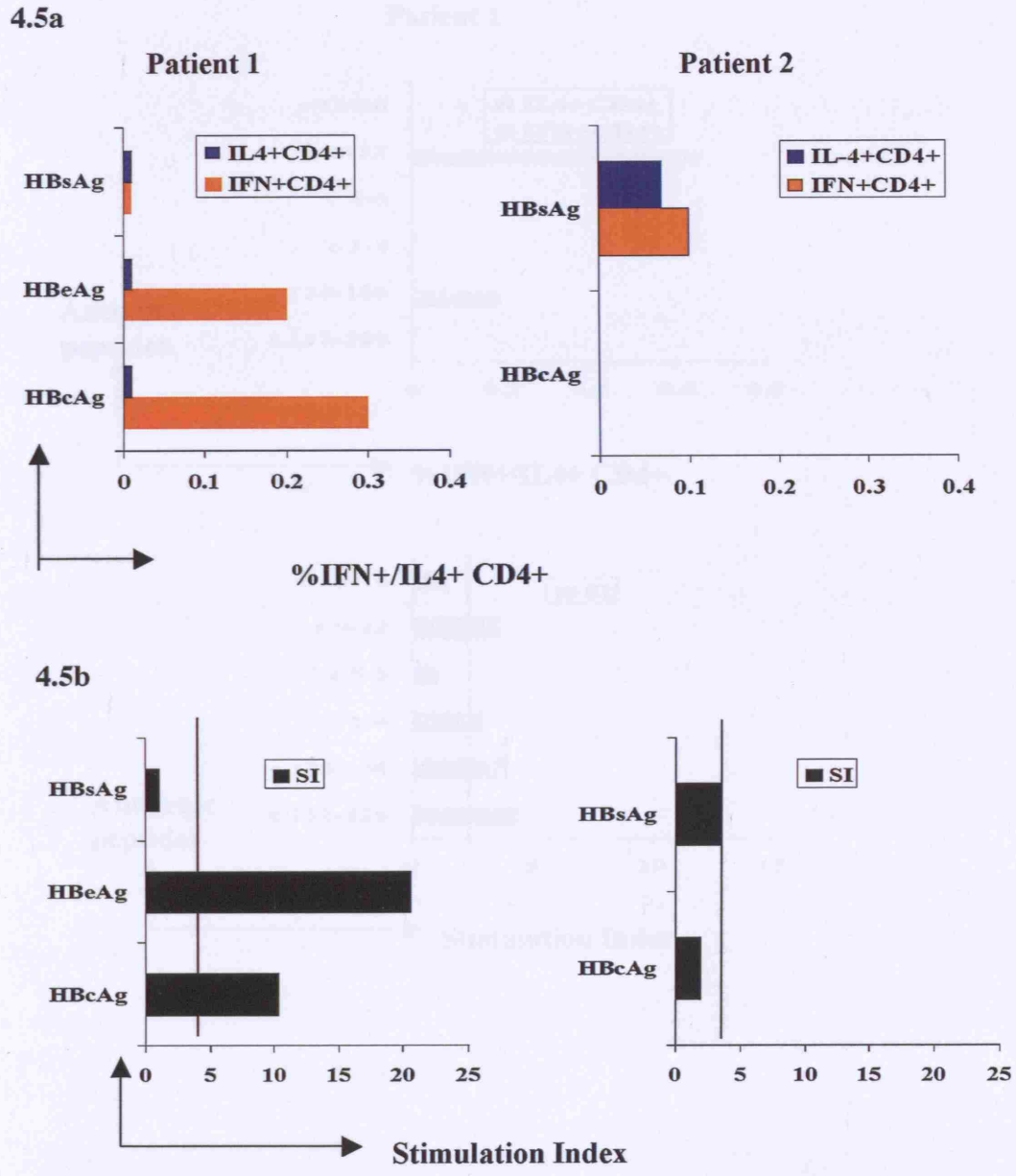
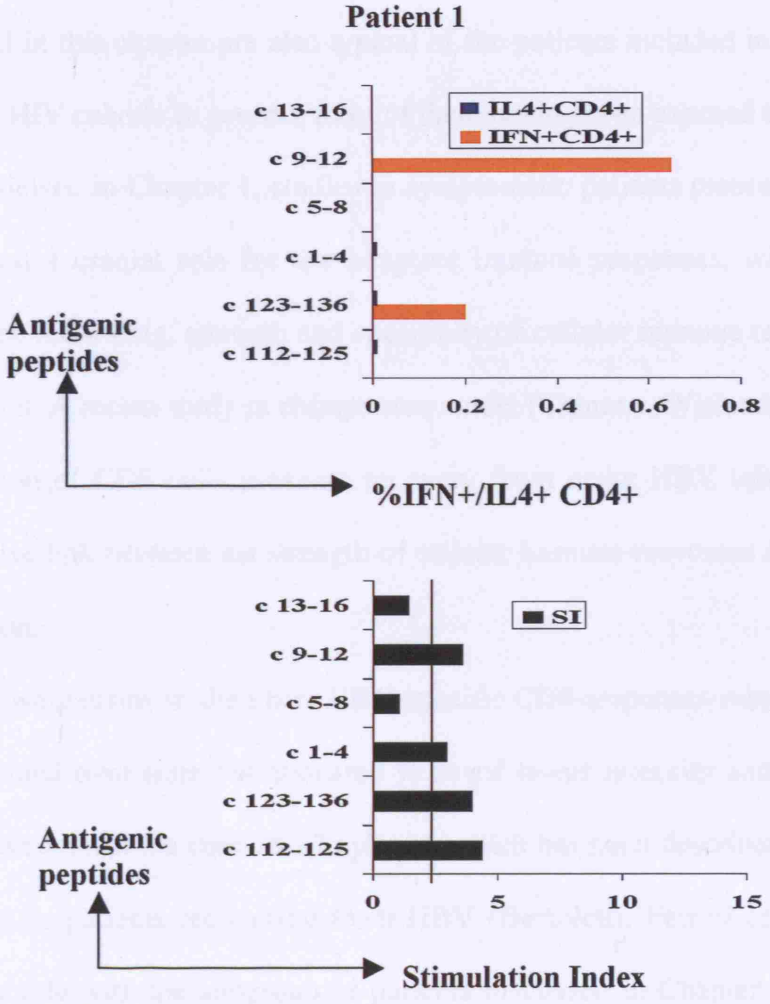


Fig. 4.6 *Intracellular cytokine staining for CD4 and IFN-γ /IL-4 after 10 days in vitro stimulation with a selection of overlapping peptides from the core region in one patient.*
Patient 1 was further tested for HBV-specific CD4 responses within the core region. There were detectable responses to peptides from core Ag, showing a multispecific CD4 response, which produced IFN-γ rather than IL-4. Proliferation assay showed consistent results



4.4 Discussion

The natural history of hepatitis B is complex and greatly depends on the age at time of infection, level of HBV replication and the host's immune status. Since acute hepatitis B acquired in adulthood may often follow a sub-clinical course (Redeker, Mosley et al. 1975; Parry, Brown et al. 1978); ((Roumeliotou and Papaevangelou 1992) the patients studied here are representative of this less well-studied subgroup. The two asymptomatic patients studied in this chapter are also typical of the patients included in this project (Chapter 3) and of HIV cohorts in general, most of them having been exposed to HBV in adulthood.

As reviewed in Chapter 1, studies in symptomatic patients presenting with acute hepatitis revealed a crucial role for the adaptive immune responses, with a strong association between the timing, strength and specificity of cellular immune responses and outcome of infection. A recent study in chimpanzee model (Thimme, Wieland et al. 2003) showed that depletion of CD8 cells prevents recovery from acute HBV infection, thus providing a causative link between the strength of cellular immune responses and outcome of the acute infection.

In the two patients studied here HBV-specific CD8-responses were detectable and could be maintained over time but appeared to be of lower intensity and only one patient had a response against the core 18-27 epitope, which has been described as the immunodominat epitope in patients recovering from HBV (Bertoletti, Ferrari et al. 1991). This data is comparable with the subgroup of patients discussed in Chapter 3, who controlled HBV asymptomatically, where six out of eleven patients studied had long-term persistence of CD8 responses. These asymptomatic patients also tended to have a narrower HBV-specific CD8 response of smaller intensity in most cases, although one asymptomatic patient did have a strong, multispecific CD8 response similar with that classically described in patients

presenting with jaundice (Penna, Chisari et al. 1991; Nayersina, Fowler et al. 1993; Bertoletti, Costanzo et al. 1994; Rehermann, Fowler et al. 1995). Since our experiments were done using the same techniques, such differences may be due to the wide spectrum of clinical (transaminase levels) and virological (HBV load) parameters of the individual infected.

We demonstrated that even in the absence of jaundice or any elevations in liver transaminases, HBV-specific CD4-responses producing IFN-γ were present, one patient showing a multispecific CD4 response, confirming a role for the cellular immune response even in the context of a sub-clinical infection. Similarly, the CD4 responses in patients with natural immunity to HBV studied in chapter 3 (data shown in chapter 3), where the duration from the acute infection was not known, were detectable in all patients studied. The role of strong CD4 responses with a predominantly T-helper 1 cytokine profile in patients recovering from acute hepatitis B was previously demonstrated (Ferrari, Penna et al. 1990; Jung, Spengler et al. 1991; Penna, Artini et al. 1996; Penna, Del Prete et al. 1997), all studies having focused on symptomatic patients. In addition, patient 1 showed strong CD4 responses to pooled overlapping peptides from core 9-12 (amino acid 82-139) and specifically to peptide core 123-136. Previous studies in acute symptomatic HBV patients have also identified sequences at the aminoterminal end of the core molecule, which were able to produce significant levels of T cell proliferation in a majority of patients (73% of patients showed responses to the core 117-131 peptide (Ferrari, Bertoletti et al. 1991).

The two patients studied here were not only asymptomatic, but also showed a rapidly evolving serological profile, with an accelerated course of resolution of HBV within a few weeks. HBV has been shown to have a long incubation period, being ignored by the immune system for 4-7 weeks (Chisari and Ferrari 1995) (Fong, Di Bisceglie et al. 1994) (Thimme, Wieland et al. 2003) (Berquist, Peterson et al. 1975). This is followed by

vigourous HBV replication for 1-2 weeks with high HBV loads of approximately 10^9 copies/ml (Whalley, Murray et al. 2001), which is accompanied by high levels of IFN and TNF production (Guidotti, Rochford et al. 1999), followed by the onset of adaptive immune responses and liver injury. Studies have also shown that most HBV DNA is cleared from circulation prior to the onset of symptoms and prior to liver injury and raised transaminases to occur (Thimme, Wieland et al. 2003) (Webster, Reignat et al. 2000). The patients studied here had relatively low HBV load of 3.5x 10^6 c/ml in patient 1 during the HBeAg positivity period (shown to be the time-point of maximal HBV replication in (Webster, Reignat et al. 2000) and only 360,000 c/ml in patient 2. It has been shown previously that patients progressing to chronicity tended to have higher levels of viral replication during acute HBV (Fong, Di Bisceglie et al. 1994), which would be in keeping with the rapid resolution observed in these two patients. One can also speculate that the innate immune responses during the early infection may have played a major role in the decline of viral replication and subsequent rapid clearance of virus from circulation in these two patients, but unfortunately I was not able to study this further in this work. The understanding of the immunological events during early acute hepatitis B has been markedly improved by studies from animal models, such as the transgenic mouse model (Guidotti, Matzke et al. 1995), the chimpanzee (Thimme, Wieland et al. 2003) (Guidotti, Rochford et al. 1999) and from HBV-related hepadnaviruses (reviewed by Ganem, D ; Fields Virology Vol2 p2923-2969) and only limited results are available from humans (Webster, Reignat et al. 2000) (Vento, Rondanelli et al. 1987).

It is as yet unclear which cells of the immune system during the natural HBV infection are able to mediate the early anti-viral effects, although a role for NK cells has been suggested in humans, where the NK cells numbers peak prior to the peak of HBV replication and is

followed 2-4 weeks later by HBV-specific CD8 responses (Webster, Reignat et al. 2000). The role of NK cells in early HBV has been further studied in the transgenic mouse, showing similar kinetics, although in the mouse model the NK cell response was not the consequence of direct HBV replication (Kakimi, Lane et al. 2001) but of galactoceramide exposure.

The first line of defense against HBV infection initially has been shown to be the type I interferons (Wieland, Guidotti et al. 2000) (McClary, Koch et al. 2000), although a recent study in chimpanzee using gene array studies found that HBV does not induce any genes during entry and expansion, when the immune system seem to be silenced early in the infection until the switching on of the IFN-γ production (Wieland, Thimme et al. 2004). In addition, the role of IFN-γ in controlling HBV replication has also been extensively studied in the transgenic mouse model, where NK T cells (Kakimi, Lane et al. 2001) and T cells (Guidotti, Ishikawa et al. 1996) producing IFN-γ may lead to downregulation of viral replication.

Interestingly, even HBV-non-specific stimuli and unrelated pathogens can also stimulate IFN–γ mediated downregulation of HBV through macrophages, NK T cells and HBV non-specific cells (Guidotti, Borrow et al. 1996) (Kakimi, Guidotti et al. 2000). In the transgenic mouse model, there is also evidence for a role of NK T cells during the acute phase of HBV infection. NK T cells express both NK and T cell markers and have a limited T cell repertoire (Bendelac 1995; Bendelac, Lantz et al. 1995). NK T cells become activated after experimental stimulation with α-galactosylceramide, which after presentation by CD1 molecules can lead to activation of NK T cells, IFN-γ production and downregulation of HBV replication in the transgeni

Another contribution of the innate immune responses to HBV viral eradication may be

dendritic cell population, such as MIP1α (macrophage inflammatory protein 1α), MIP1β (macrophage inflammatory protein 1β), RANTES (regulated upon activation, normal T cell expressed and secreted), which enhance recruitment of antigen-specific and non-specific cells to the liver. (Salazar-Mather TP, JCI 2002, Salazar-Mather TP J Immunol 1996).

To conclude, it appears that even in the context of asymptomatic acute hepatitis B HBV-specific T cell responses may play a role, as suggested by a multispecific IFN-γ producing T-helper response in one patient. In all the patients who resolved HBV asymptomatically studied in Chapter 3 and this chapter, HBV-specific CD8 responses ranged from being multispecific, to being directed only to the immunodominant HBV epitope core18-27 or undetectable with any of the peptides used in this study. This may be a reflection of the complex mechanisms involved in viral control during HBV as well as of the different modes of acquisition of infection and the wide clinical spectrum of the acute illness. It would be interesting to study further the immunological events during the incubation time and prior to the onset of adaptive responses and clinical symptoms, such as the role of NK T cells and IFN-γ and their relation to the outcome of infection.

CHAPTER 5

5. A longitudinal study of HBV-specific T cell responses in HIV/HBV co-infected patients commencing HAART.

5.1 Introduction

Because of the shared transmission routes for HIV and HBV, a large majority of HIV infected patients have also been infected with HBV (Scharschmidt, Held et al. 1992) (Puoti, Spinetti et al. 2000). HIV infection has been shown to result in higher rates of chronic HBV carriage, resulting in 10%-15% of HIV infected patients remaining co-infected with HBV (Konopnicki, Mocroft et al. 2005) . Recent evidence suggests that HBV-HIV co-infected patients (Thio, Seaberg et al. 2002),especially those with low CD4 counts, are at significantly increased risk of mortality due to liver disease. The introduction of HAART has been associated with an increase in liver-related morbidity and mortality in HIV cohorts. (Martin-Carbonero, Soriano et al. 2001) (Puoti, Spinetti et al. 2000) This may relate not only to hepatotoxicities of these drugs (Bonfanti, Landonio et al. 2001; Reisler 2001; Sulkowski 2003) but also to the prolonged patient survival allowing time for slowly progressive viral hepatitis to become clinically relevant (Palella, Delaney et al. 1998). Furthermore, the introduction of HAART in such co-infected patients has raised a number of as yet unanswered therapeutic dilemmas. Will effective immune reconstitution associated with HAART result in recovery of HBV control as seen in the case of a number of other opportunistic infections or is it necessary to include a drug with specific activity against HBV? Conversely, can anti-HBV treatment be effective even in the setting of sub-optimal immune reconstitution?

An important consideration in addressing these questions is the impact of different HAART regimes on HBV-specific T cell responses in such co-infected patients. Strong HBV-specific $CD8^+$ T cell responses are associated with control of infection (Bertoletti, Ferrari et al. 1991; Nayersina, Fowler et al. 1993; Rehermann, Fowler et al. 1995), whereas these responses are barely detectable (Maini, Boni et al. 2000) or of altered function (Reignat, Webster et al. 2002) in chronic infection. Recent studies have revealed that the hypo-

responsiveness of both CD4 (Boni, Bertoletti et al. 1998) and CD8 (Boni, Penna et al. 2001) T cell responses characteristic of chronic HBV infection can be partially overcome by the reduction in HBV load induced by the reverse transcriptase inhibitor lamivudine. However, HBV-specific T cell responses have not been studied in HIV patients with chronic HBV infection and there are no available data on whether these responses can also be re-constituted by HAART or by effective anti-HBV therapy in the context of immunosuppression.

Here we present a study of the changes in HBV-specific T cell responses following the commencement of HAART in HIV/HBV co-infected patients. T cell responses are examined using MHC/peptide tetramers and intracellular cytokine staining in conjunction with changes in HIV and HBV load, CD4 count and ALT levels before and after the commencement of HAART.

5.2 Materials and Methods

5.2.1 Patients

20 HBV-HIV coinfected patients were recruited from the HIV clinic at the Mortimer Market Centre, London with informed consent and local ethics committee approval.

All patients were known HIV positive and had HBV serology confirming chronic HBV carriage of high infectivity (HBs Ag+, HBeAg+) or low infectivity (HBs Ag+, HBeAg-, anti-HBeAg+). Eleven HLA-A2+ patients were studied cross-sectionally; three patients who commenced HAART and two patients who started HBV treatment were also followed longitudinally with sequential blood samples for changes in the HBV-specific CD4 and CD8 responses.

Details of the patients' CD4, HIV viral load, HBV serology, ALT, HBV DNA and HAART regimen are presented in table V.1. Patients with low infectivity HBV had normal ALT levels (<50IU/ml) and low HBV DNA (undetectable with the Digene assay). Patients with hepatitis B of high infectivity had high ALT (>1.5 upper limit of normal) and high HBV DNA.

5.2.2 Methods

The techniques of tetramer and intracellular cytokine staining have been described in Chapter 2. In brief, peptides corresponding to the sequence of core18-27, envelope 183-91, 335-43, 338-47 and 348-57 and polymerase 455-463, 502-510, 575-583, 655-663 and 816-824 regions of HBV genotype D were synthesized by Chiron Mimotopes and were found to be >90% pure. PBMC were expanded in vitro with 1 μmol/L of the relevant HBV peptide for 10 days and then were subjected to peptide restimulation in the presence of Brefeldin A for 6h. Cells were stained with anti-CD8, were permeabilised with Cytofix/Cytoperm (BD Biosciences) and were stained with anti-IFN-γ MAb, to allow flow cytometric analysis of peptide-specific CD8 cells. HBV-specific CD8 responses were measured at baseline and at

4, 8, 12 and 24 weeks after starting HAART. HBV-specific CD4 cell responses were measured at baseline and at 24 weeks, by intracellular cytokine staining, after stimulation with either hepatitis B surface antigen (HBsAg) or hepatitis B core antigen (HBcAg) for 10 days followed by restimulation for 6 h in the presence of Brefeldin A. The frequencies of the HBV-specific responses shown represent the IFN-γ CD8+/ CD4+ responses detected after restimulation with the appropriate peptide after subtraction of background IFN-γ production (frequency of unstimulated IFN-γ CD8+/CD4+ T cells).

5.3 Results

5.3.1 Undetectable HBV-specific CD8 responses in HBV-HIV coinfected patients

Twenty patients with chronic hepatitis B and HIV were screened for the presence of the HLA-A2 allele and 11 were found to be HLA-A2+, of whom seven were HBe Ag positive and 4 HBeAg negative and had anti-HBe antibodies detectable *(Table V.1)*. All A2+ patients were screened with the HLA-A2 tetramer specific for the immunodominant core 18-27 epitope for the presence of HBV-specific CD8 responses. The staining was performed directly ex vivo (data not shown) as well as after 10 days stimulation in vitro in the presence of core 18-27 peptide *(fig 5.1)*. No patients had detectable responses to the immunodominant epitope core 18-27. The patients who were followed up longitudinally (P1-P5) were also screened with intracellular cytokine staining after 10 days of in vitro stimulation with 10 HLA-A2 restricted peptides (this baseline data is presented in *fig. 5.2-fig. 5.4* for each individual patient prior to starting HAART or anti-HBV treatment). Only one patient (P3) had an isolated low-level response to an envelope epitope. A longitudinal study was carried out to examine the impact of HAART (3 patients) or anti-HBV treatment (2 patients) on the HBV-specific CD4 and CD8 responses.

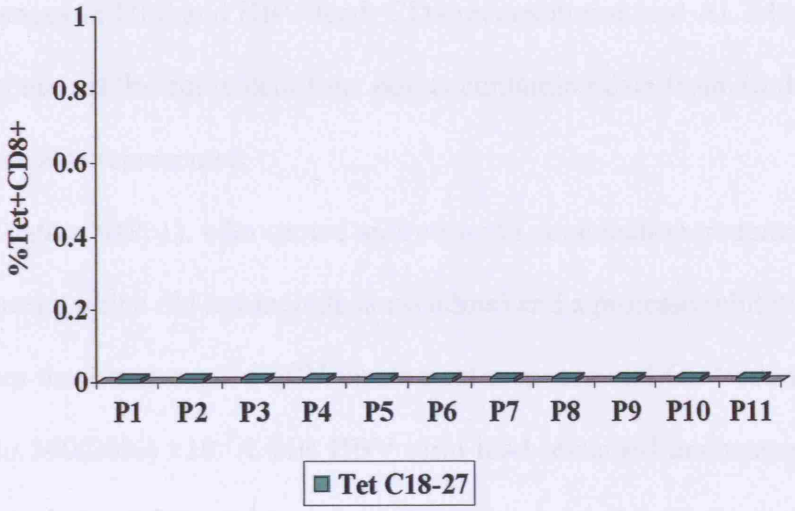
Table V.1

Patient ID	CD4 (%) X10 ^{9}/l	HIV VL c/ml	HBe Ag	Anti-Hbe Ab	HBV DNA (pg/ml)	ALT	HAART
Pc1	120 (12%)	604,000	+	-	>6,000	86	Commenced on DDI/abacavir/nelfinavir
Pc2	100 (7.7%)	250,000	+	-	>6,000	187	Commenced on AZT/lamivudine/ efavirenz
Pc3	70 (6%)	45,000	+	-	75	202	Commenced on AZT/lamivudine/ efavirenz
Pc4	500 (22%)	<50	+	-	>6,000	138	didanosine/stavudine/ efavirenz followed by stavudine switch to lamivudine
Pc5	350 (26%)	1,600	+	-	>6,000	202	AZT/DDI, and adefovir added for HBV treatment
Pc6	340 (9%)	6,700	+	-	>6,000	108	None
Pc7	420 (19%)	5,600	-	+		46	None
Pc8	820 (37.2)	<50	-	+	Not detected	24	None
Pc9	270 (18%)	16,100	-	+	Not detected	43	None
Pc10	400 (37%)	1,800	-	+	Not detected	30	None
Pc11	360 (11%)	800	+	-	3150	261	DDI/abacavir/efavirenz

Table V.1 showing details of the CD4/ HIV VL, HBV DNA and HBV serological markers and HAART used in the 11 HIV/HBV co-infected patients studied in this chapter.

Fig. 5.1 *Screening with tetramer core 18-27 after 10 days in vitro stimulation of HIV positive HBV chronic carrier patients.*

No HBV-specific CD8 responses were detected in the 11 HBV chronic patients tested



5.3.2 Impact of HAART on HBV-specific immune responses 3 HIV-HBV co-infected patients

All patients included were HBV chronic carriers of high infectivity (HBeAg+) with elevated ALT (>1.5x normal). The choice of antiretroviral drug regimen was left to the discretion of the clinician.

Fig 5.2-5.4 dissect the time course of responses in the 3 HAART treated patients in relationship to changes in HIV and HBV load, CD4 reconstitution and ALT levels. HBV-specific CD8 responses at the equivalent time points containing data from 10 days after in vitro stimulation are also represented.

Fig 5.2 illustrates patient 1(Pc1), who started antiretroviral combination treatment with two nucleoside analogues, (which did not include lamivudine) and a protease inhibitor. After 24 weeks of follow-up there was a good HIV virological response and CD4 count increased from 100 (12%) to 340(20%) x10 ^{9}/l. His HBV viral load remained unchanged and there were no fluctuations in his ALT, which remained mildly elevated throughout. There were no ALT flares during the follow-up period. HBV-specific CD8 responses remained undetectable at three time points during the six-month follow up. He had no HBV-specific responses detectable at any time points during the 24 week of follow-up.

Fig 5.3 illustrates data from patient 2, who had significant HBV disease, having been diagnosed with active hepatitis and significant fibrosis on liver biopsy and membranous glomerulonephritis with nephrotic syndrome and marked HBcore antigen staining on kidney biopsy. He was started on a lamivudine-containing regimen, which resulted in a good increase of his CD4 count and undetectable HIV viral load by 24 weeks of therapy. The HBV DNA dropped significantly, however, he remained a carrier of high infectivity,

albeit at decreased HB surface antigen titres. No detectable HBV-specific CD8 responses were found at the one time point available (24 weeks).

Fig 5.4 shows data from patient 3, who was a HBV carrier of high infectivity having been diagnosed HIV positive as part of screening pre-liver transplant. He had cirrhosis and decompensated liver disease. At the time of diagnosis, he had a low CD4 count at 70x 10^{9}/l (6%) and was commenced on lamivudine containing HAART. He had a good virological response to HAART. His ALT normalized after 12 weeks of therapy and HBV DNA was undetectable by week 24 and he became HBe Antigen negative without detectable anti-HBe Ab. He had low frequency CD8 responses against envelope 348-57 epitope at baseline, which disappeared on subsequent testing. At week 3 and week 24 there were detectable CD8 responses at higher frequency to other epitopes from the envelope protein. Cells were also tested with tetramer for the envelope 183-191 epitope ex vivo (data not shown) and after 10 days in vitro stimulation at all time points and at week 24 the reconstituted cells were shown to be able to bind the tetramer.

Fig.5.2 Longitudinal follow-up data from patient 1 after starting HAART. CD4 counts, HIV load, HBV DNA and ALT levels are represented together with the evolution of HBV markers and HBV-specific CD8 responses.

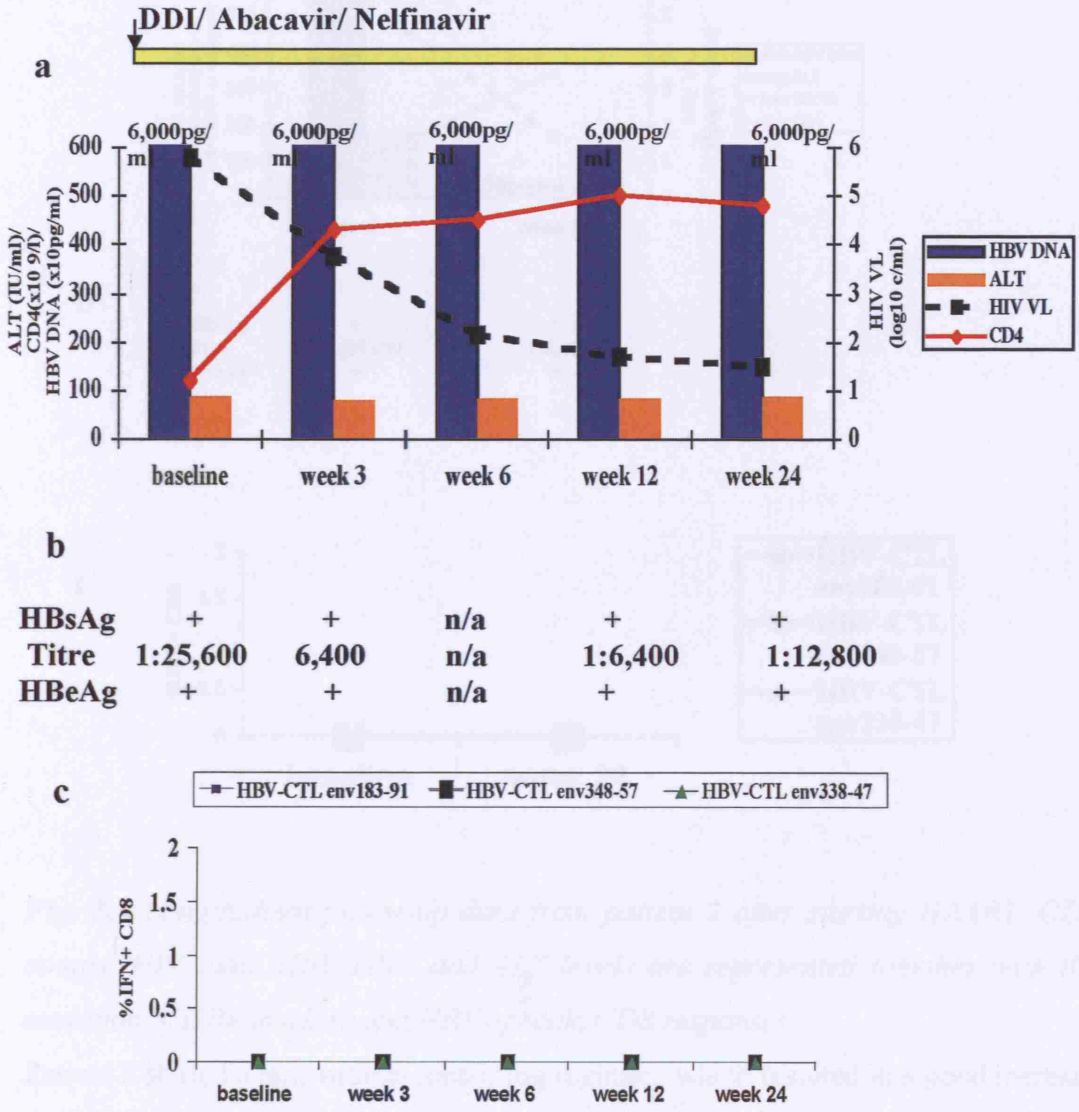


Fig 5.2 illustrates patient 1, who started antiretroviral combination treatment with a non-lamivudine containing HAART regimen. Fig2a shows the HIV VL, CD4 counts responses after starting HAART as well as ALT and HBV DNA levels during therapy. Fig 5.2b represents the evolution of HBV markers, including HBsAg titres. Fig 5.2c shows the HBV-specific CD8 responses studied with intracellular cytokine staining after 10 days in vitro stimulation with a panel of 10 HLA A2-restricted peptides. There were no HBV-specific responses detectable at any time points during the 24 week of follow-up.

Fig.5.3 *Patient 2*

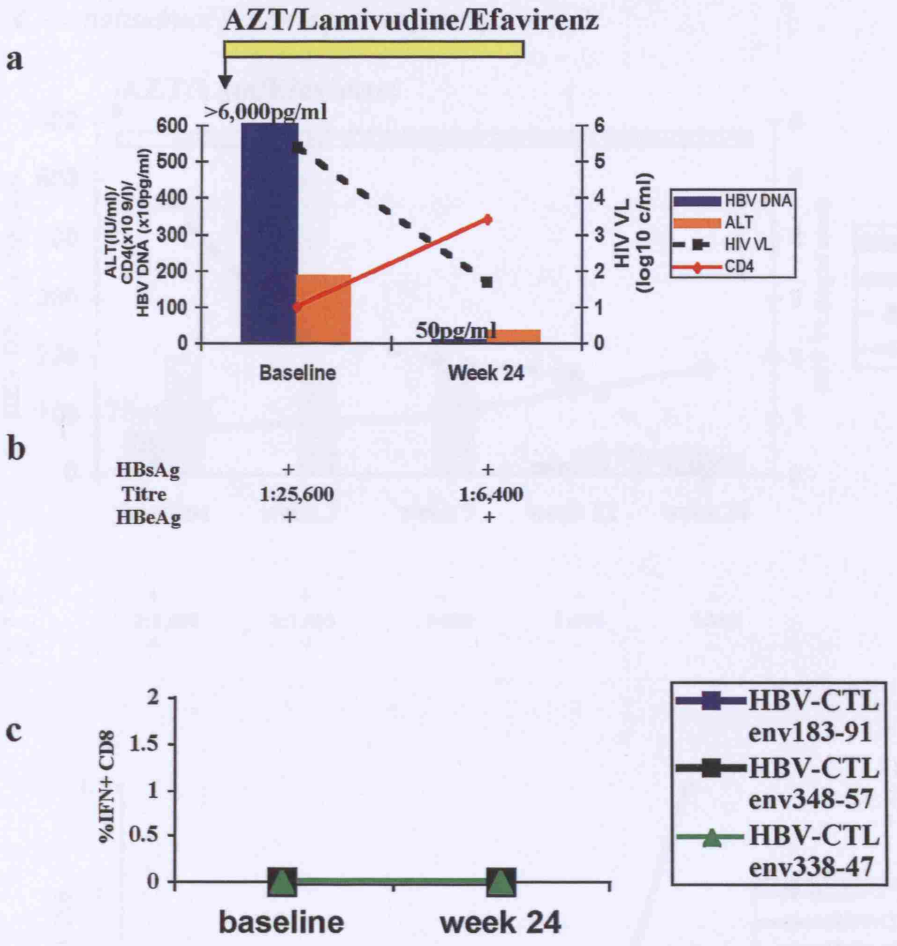


Fig. 5.3 *Longitudinal follow-up data from patient 2 after starting HAART. CD4 counts, HIV load, HBV DNA and ALT levels are represented together with the evolution of HBV markers and HBV-specific CD8 responses.*

Patient 2 started a lamivudine-containing regimen, which resulted in a good increase of his CD4 count and undetectable HIV viral load by 24 weeks of therapy-*fig 5.3a*. The HBV DNA dropped significantly, however, he remained a carrier of high infectivity, albeit at decreased HB surface antigen titres-*fig 5.3b*. No detectable HBV-specific CD8 responses were found at the one time point available (24 weeks)-*fig. 5.3c.*

Fig.5.4 –Longitudinal follow-up data from patient 3

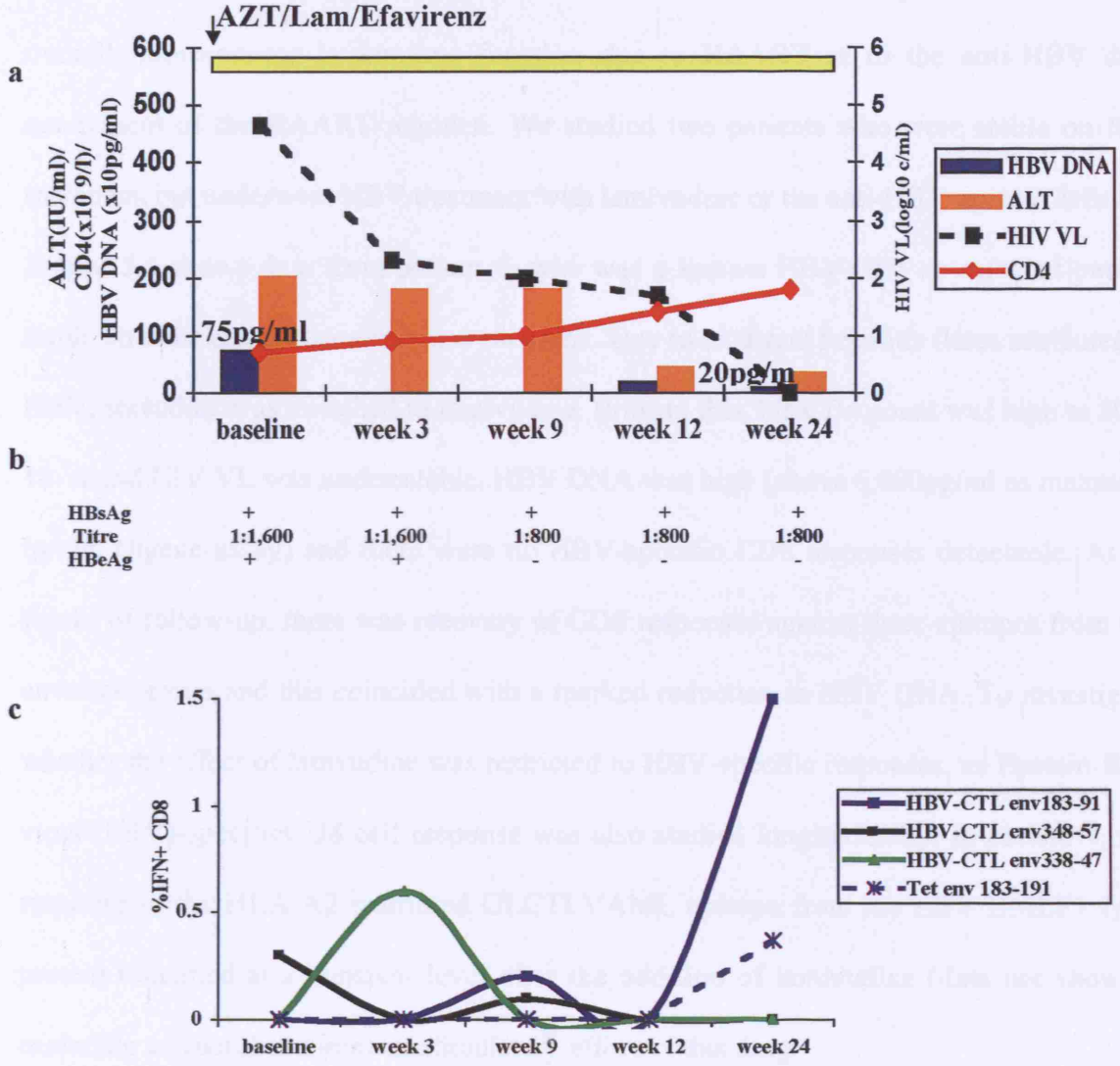


Fig 5.4 Patient 3, who started antiretroviral combination treatment with a lamivudine containing HAART regimen. Fig4a shows the HIV VL, CD4 counts responses after starting HAART as well as ALT and HBV DNA levels during therapy.Fig 4b represents the evolution of HBV markers, including HBsAg titres. This patient became HBeAg negative at week 9. Fig 4c shows the HBV-specific CD8 responses studied with intracellular cytokine staining after 10 days in vitro stimulation with a panel of 10 HLA A2-restricted peptides. At week 3 and week 24 there were detectable CD8 responses to other epitopes the envelope group.

5.3.3 Impact of HBV treatment on HBV-specific immune responses in HIV co-infected patients.

We attempted to further dissect whether the increase in HBV-CTL responses was due to an overall improvement in immune function due to HAART or to the anti-HBV drug component of the HAART regimen. We studied two patients who were stable on HIV treatment, but underwent HBV treatment with lamivudine or the anti-HBV agent Adefovir. *Figure 5.5* shows data from patient 4, who was a known HBV-HIV co-infected patient stable on didanosine, stavudine, and efavirenz. Due to recurrent hepatitis flares attributed to HBV, stavudine was switched to lamivudine. Prior to this, his CD4 count was high at 500x 10^{9}/l and HIV VL was undetectable. HBV DNA was high (above 6,000pg/ml as measured by the Digene assay) and there were no HBV-specific CD8 responses detectable. At 12 weeks of follow-up, there was recovery of CD8 responses against three epitopes from the envelope group and this coincided with a marked reduction in HBV DNA. To investigate whether the effect of lamvudine was restricted to HBV-specific responses, an Epstein-Barr virus (EBV)-specificCD8 cell response was also studied longitudinally. In patient 4, the response to the HLA-A2 restricted GLCTLVAML epitope from the EBV BMLF1 lytic protein remained at a constant level after the addition of lamivudine (data not shown), excluding a generalized immunostimulatory effect of this drug.

Figure 5.6 illustrates data from patient 5, who was an HIV-HBV chronic carrier patient who commenced Adefovir dipivoxil at a dose with therapeutic activity against HBV but not HIV (10mg OD). He had been stable on suboptimal HIV therapy (AZT/DDI) for the past three years and was reluctant to change this treatment. Figure 5.6a shows the normalization of ALT and decline in HBV DNA after starting Adefovir. There was a marked reduction in HBV DNA, however the patient remained HBe Ag positive throughout (fig 5.6b). The CD4 count remained stable and there was a non-sustained mild increase in HIV viral load by

week 12. After 3 weeks of Adefovir, we observed recovery of HBV-CD8 responses to envelope 183-191 epitope, which increased further in frequency by week 12 and disappeared by week 24 (graphic representation of HBV-specific CD8 responses in *fig 5.6c* and dot plots of responses to 183-191 epitope in figure *5.6d*). Responses to another envelope epitope were also briefly detectable at 6 weeks. Results derived from 10-day in vitro cultures do not necessarily represent circulating frequencies, but are a reflection of the proliferative potential of these cells. Intracellular cytokine staining was therefore performed directly ex vivo in patient 5 and confirmed that identical CD8 cell specificities became detectable with the same time course as after 10 days in vitro expansion (data not shown). Tetramer staining with the envelope 183-191 tetramer (10 day data represented on graph in *fig. 6c*) showed similar results.

Fig. 5.5 Impact of HBV treatment on HBV-specific immune responses in patient 4.

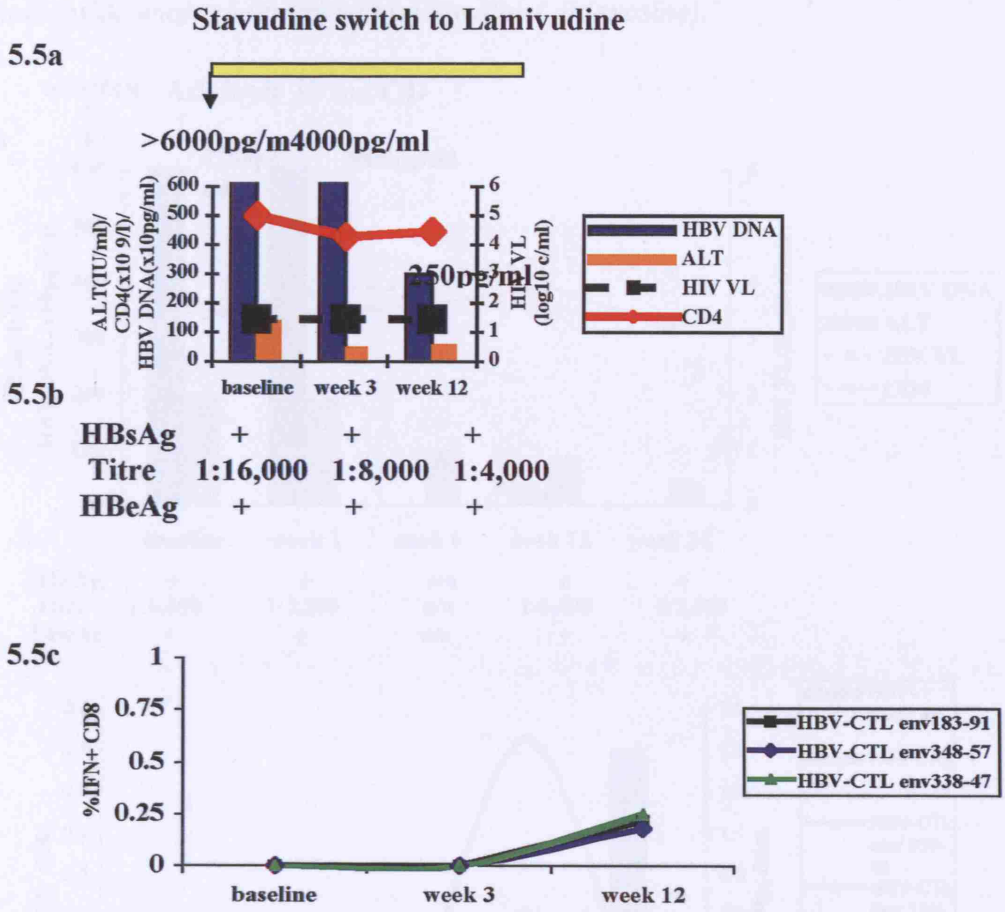


Fig. 5.5a shows decrease of ALT and decline of HBV DNA after stavudine was switched to lamivudine as part of HAART; CD4 and HIV VL remained unchanged. **Fig. 5.5b** shows a decline in HBsAg titres. **Fig 5.5c** represents detectable HBV-specific CD8 responses with intracellular cytokine staining after 10 days in vitro stimulation. At 12 weeks of follow-up, there was recovery of CD8 responses against three epitopes from the envelope group and this coincided with a marked reduction in HBV DNA.

Fig.5.6 Impact of HBV treatment with adefovir dipivoxil on HBV-specific immune responses in one HIV positive HBV chronic carrier patient (patient 5) on dual nucleoside analogue treatment (zidovudine/ didanosine).

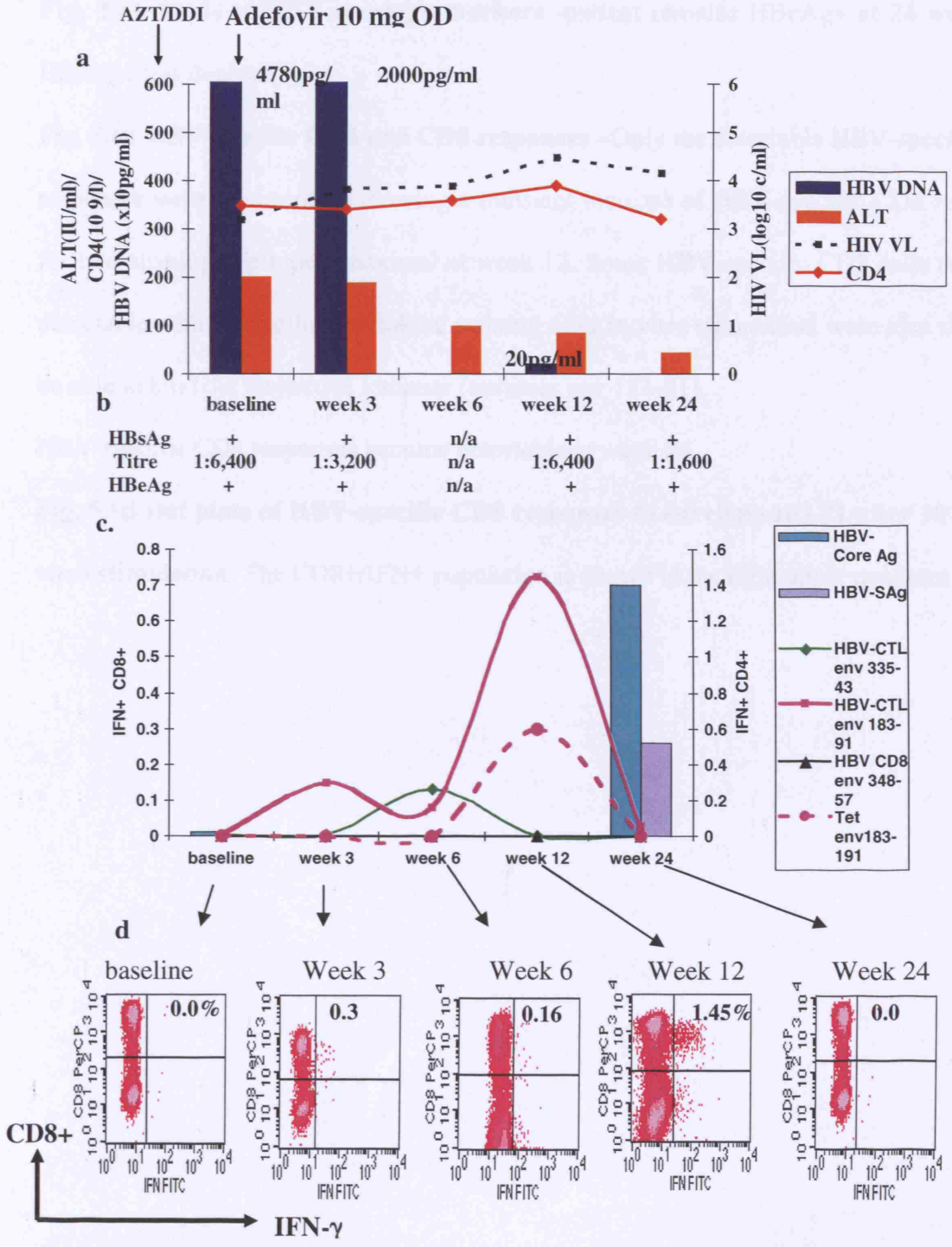


Fig.5.6a. Long-term follow-up of HBV DNA, ALT, CD4 counts and HIV-VL data at baseline and at 3, 6, 12 and 24 weeks after starting low dose adefovir. HBV DNA and ALT decline after staring HBV therapy.

Fig. 5.6b Table of HBV serology markers -patient remains HBeAg+ at 24 weeks but HBsAg titres decline.

Fig. 5.6c HBV-specific CD4 and CD8 responses –Only the detectable HBV-specific CD8 responses were represented, showing a transient increase of HBV-specific CD8 responses for two envelope epitopes, maximal at week 12. Some HBV-specific CD8 cells that were detectable with intracellular cytokine staining after in vitro stimulation were also shown to be able to bind the respective tetramer (tetramer env 183-91).

HBV-specific CD4 responses become detectable at week 24.

Fig. 5.6d Dot plots of HBV-specific CD8 responses to envelope 183-91 after 10 days in vitro stimulation. The CD8+/IFN+ population is shown in the right upper quadrant

5.3.4 Impact of HAART and HBV treatment on HBV-specific CD4 responses

We analyzed HBV-specific CD4 responses in a subgroup of patients: P1-on a non-lamivudine containing HAART regimen, P3 treated with lamivudine as part of HAART and P5 who was started on Adefovir alone for HBV treatment. We performed intracellular cytokine staining for IFN-γ and CD4 directly ex-vivo and after 10 days cell culture in the presence of core and surface antigens on samples prior to HAART or HBV treatment (week 0) and from the 24-week time point. *Fig 5.6c* shows reconstitution of HBV-specific CD4 responses in P5 for both core and surface antigens at 24 weeks after starting Adefovir, but no increase in functionally active HBV-specific CD4 cells was detected in P1 and P3 (not represented). Ex-vivo intracellular cytokine staining (data not shown) and 5 day proliferation assays with HB core and surface antigens showed equivalent results *(fig 5.7)*. .

Fig. 5.7 Proliferation assay results with HBV core and surface antigens in 3 representative HIV positive HBV chronic carrier patients (P1, P3 and P7).

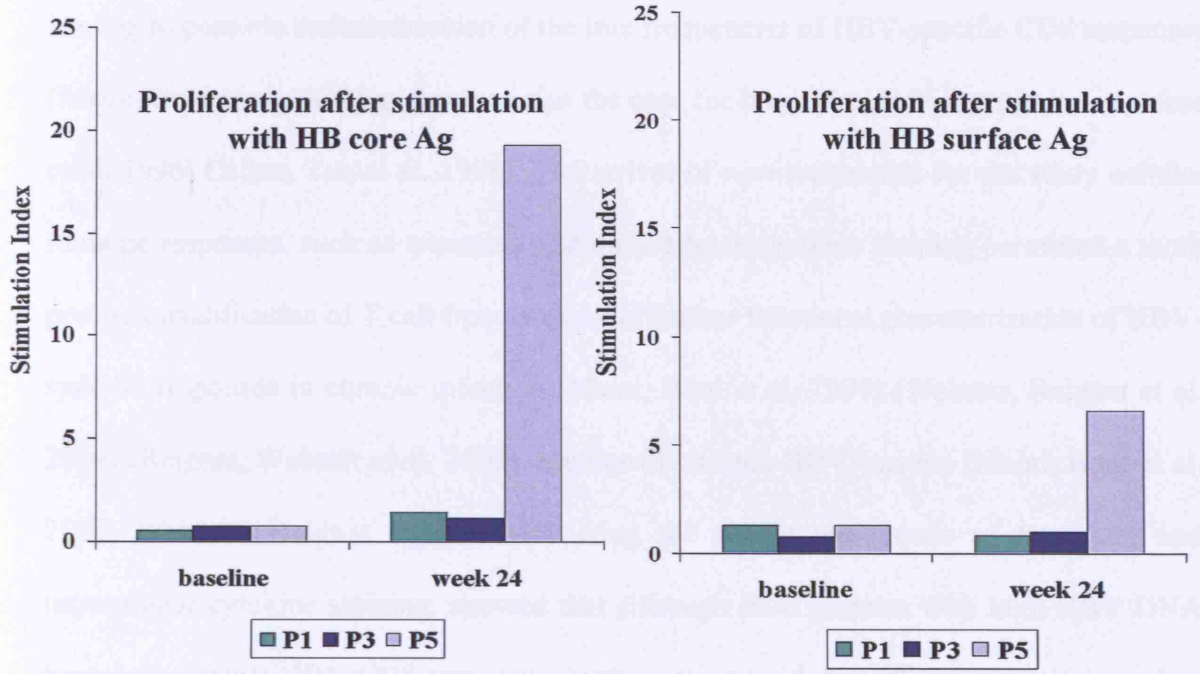


Fig.5.7 P1 was a HBV HIV co-infected patient who started HAART without any anti-HBV agent, P3 started a lamivudine containing HAART regimen and P5 started anti-HBV treatment with low dose adefovir whilst continuing AZT/DDI for HIV treatment.

P5 was the only patient to have a significant stimulation index for both core and surface antigens after 24 weeks of starting anti-HBV treatment with adefovir, consistent with results obtained with intracellular cytokine staining for CD4/IFN-γ after 10 days in vitro stimulation (fig. 5.6c).

5.4 Discussion

HBV-specific CD8 responses in chronic carriers have been previously described to be undetectable or of low frequencies, narrowly directed against single epitopes (Bertoletti, Costanzo et al. 1994) (Ferrari, Penna et al. 1990) (Jung, Spengler et al. 1991). Many studies were done with cytotoxic experiments, which required several rounds of in vitro expansion, leading to possible underestimation of the true frequencies of HBV-specific CD8 responses (Maini, Boni et al. 1999) as this was also the case for other viral infections (Altman, Moss et al. 1996; Callan, Tan et al. 1998). The arrival of new techniques for the study cellular immune responses, such as tetramers and intracellular cytokine staining permitted a more precise quantification of T cell frequencies and further functional characterization of HBV-specific responses in chronic infection (Maini, Boni et al. 1999) (Webster, Reignat et al. 2004) (Reignat, Webster et al. 2002). Studies of chronic HBV carriers (Maini, Boni et al. 2000; Webster, Reignat et al. 2004) using the recent techniques of tetramers and intracellular cytokine staining, showed that although most patients with high HBV DNA have undetectable HBV CD8 responses, many patients with low HBV DNA can have low level CTLs. The question of T cell hyporesponsiveness in chronic hepatitis B carriers in HIV negative patients was re-addressed by a recent study (Webster, Reignat et al. 2004) taking into account the marked heterogeneity of the clinical and virological picture of HBV. HBV-specific CD8 responses were detected in chronic carriers at low frequencies, usually less than 0.2% of total number of CD8 directly ex vivo. Most patients (76%) had some low level responses when tested with the same panel of HLA-A2 restricted peptides used in our study. Same study showed that patients with low HBV DNA (less than 10^7 copies/ml) had core 18-27-specific CD8 responses in all cases, with some patients displaying a multispecific response to envelope and occasionally polymerase epitopes. Patients with higher HBV DNA (more than 10^7 copies/ml) had undetectable responses to core 18-27 but

had occasionally low level or narrowly focused responses to the envelope epitopes, often with functional alterations and impaired tetramer binding (Reignat, Webster et al. 2002). This T cell hyporesponsiveness of chronic HBV carriers was associated with high HBV DNA levels, where exhaustion or deletion of CTLs may play a role (Rocha, Grandien et al. 1995).

Using the same techniques, I had the opportunity to study for the first time HBV-specific T cell responses in HIV/HBV infected patients, who were either HAART naïve or treated with or without an anti-HBV drug as part of their antiretroviral regimen. I first studied cross-sectionally HBV-specific CD8 responses in HIV positive patients, including 4 patients with low HBV DNA and normal ALT and 7 patients with high HBV DNA and high ALT. By contrast, our data in HIV positive HBV chronic patients showed that the 4 patients with low HBV DNA had no responses to core 18-27. Only one patient with a higher HBV DNA (70pg Digene or 2x 10^{8} c/ml) had an isolated low level envelope response. Although we only sampled the patients on one occasion, this would suggest a decreased HBV-specific response in the setting of HIV.

Treatment with lamivudine of HIV negative HBV chronic carriers has highlighted the potential for some recovery of CD4 and CD8 responses, which may have therapeutic implications (Boni, Penna et al. 2001; Boni, Penna et al. 2003). In this study we addressed the question of whether such hyporesponsiveness to HBV proteins characteristic of HBV chronic carriers with high level of viral replication could be overcome by HAART or anti-HBV agents even in the setting of immunosuppression due to HIV infection.

Overall in this study, out of the 4 patients in whom a therapeutic reduction in HBV viral load was achieved, 3 developed some detectable HBV-specific T cell responses. The fourth patient had no response at the only post-treatment time point available (24 weeks), but may have had a transient earlier response, as seen in a number of cases in the study of Boni et al

of HIV negative HBV treated patients. By contrast, the patient in whom HAART effectively reduced HIV viral load and increased CD4 counts but did not have any anti-HBV activity showed no recovery of HBV responses. These differential effects were further dissected by examining two patients in whom existing HAART regimes were augmented by drugs with specific anti-HBV activity. In both these cases HBV reactivity was only recovered following the introduction of drugs capable of substantially reducing HBV DNA levels. Our data reveal that reconstitution of some CD8 responses can occur in conjunction with a reduction in viral load in this HIV positive patient group. The recovery of HBV CD8 was always preceded by a reduction in HBV DNA and surface antigen titres, although only one patient underwent HBeAg seroconversion. Responses were maximal between weeks 12 and 24 after the start of therapy comparable with the data on treatment of HIV negative patients with chronic HBV infection (Boni, Penna et al. 2001), and were not associated with any ALT flares. In this study, HIV HBV coinfected patients were followed up for six months only, leaving open the question of maintenance of such responses long term. The recovered HBV-specific CD8 responses were however shown to be transient in HIV negative HBV chronic carriers treated with lamivudine in the longer term, displaying a biphasic behaviour with an early enhancement of T cell frequency and intensity, followed by a persistent decline from six months onwards (Boni, Penna et al. 2003). The transient nature of the immune reconstitution may be responsible for HBV reactivation once lamivudine is withdrawn.

This contrasts with the situation observed for HIV-specific CTLs, which are easily detectable in situations of high viral load, with 1-2% of total CD8 being specific for a dominant CD8 epitope (Ogg, Jin et al. 1998; Ogg, Kostense et al. 1999). This difference in the behaviour of CTLs during chronic HIV may be a reflection of a different mechanism of immune evasion in the context of active virus replication (Zajac, Blattman et al. 1998),

where HIV CTLs have been shown to exert immune pressure and lead to viral escape mutations (Borrow, Lewicki et al. 1997). HIV-specific CTLs have also been reported to have an impaired effector function with marked reduction in the levels of intracellular perforin, resulting in poor ex vivo killing of appropriate target cells (Appay, Nixon et al. 2000). Although HBV CTL escape mutations have been described (Bertoletti, Sette et al. 1994), they do not appear to be responsible for the failure of the immune control in the majority of patients with chronic infection (Chisari and Ferrari 1995). Interestingly, HIV CTLs have been shown to decline shortly after starting HAART (Ogg, Jin et al. 1998; Ogg, Kostense et al. 1999) whereas HBV CTLs are initially transiently restored followed by a persistent decline long term after starting anti-HBV therapy. The decline of HBV CTL after more than six months of anti HBV therapy (Boni, Penna et al. 2003) suggests that these CTLs are also dependent on antigenic stimulation, reminiscent of the situation with HIV CTL, although the latter seem to decline more rapidly in the absence of antigen.

The significance of the restored CTLs in the context of reduced HBV DNA merits further investigation. We showed that these HBV-specific CD8 cells were directed against envelope epitopes and were able to expand and produce interferon. We were able to demonstrate that some of the reconstituted CD8 responses specific for envelope 183-191 were able to bind the corresponding tetramer. It was previously shown that some HBV-specific CD8 responses to the envelope 183-91 epitope in untreated chronic HBV carriers have impaired tetramer binding inspite of being able to expand and produce interferon (Reignat, Webster et al. 2002). These tetramer negative cells could however be converted experimentally to an enhanced tetramer binding capacity after repetitive antigen stimulation, but even then they maintained a less differentiated phenotype when compared to the CD8 cells specific for the immunodominant core epitope. The CD8 responsiveness of these cells in the presence of high HBV load suggested that they did not exert an adequate

immunological pressure on the virus. In this context, it was interesting to note that the reconstituted envelope-specific CD8 cells in patients treated with anti-HBV agents appeared following a reduction in HBV DNA and were able to bind the tetramer (as was previously reported (Boni, Penna et al. 2001)). Although only tested in two patients, the levels of tetramer positive CD8 cells seemed to be lower than those detected with intracellular cytokine staining at the same time points. We did not find any tetramer positive cells directed against core 18-27 epitope.

The HBV-specific CD8 cells detectable in our cohort of co-infected patients were all envelope-specific, despite screening for responses to a panel of 10 peptides representing epitopes within core, polymerase and envelope proteins. Similarly, reconstituted responses in HIV negative patients were frequently envelope-specific and there was a notable absence of the core-specific CD8 cells that are immunodominant in the response to acute infection associated with control. This altered hierarchy of the CTL response is a feature of patients with chronic HBV (Webster, Reignat et al. 2004) and raises the possibility that a full re-constitution of the specificities required for efficient viral control may not be possible once chronicity is established, even with effective anti-viral treatment. On the other hand, one must take into account that the CD8 responses that play an important role in the course of acute infection may be different from those that exert an immunological pressure in the setting of chronic infection. This was shown for HIV, where responses to a p17 Gag epitope were associated with viral control in chronic HIV, but did not seem to play any role in the acutely infected patients (Goulder, Altfeld et al. 2001), suggesting that the contribution to viral control may differ according to epitope specificity.

Further phenotyping and functional characterization for production of different cytokines by the reconstituted CD8 responses needs more investigation, but I was unable to explore this further due to the relatively low frequencies of these cells. In the HIV negative setting,

these cells were found to be HLA-DR positive, suggesting an activated phenotype (Boni, Penna et al. 2001).

Thus the protective and pathogenic potential of the HBV-specific CD8 responses seen in the setting of anti-viral therapy both with and without concomitant HIV disease merit further investigation.

The origin of the re-populating CD8 cells after lamivudine treatment in HIV negative HBV infected patients has been addressed by a recent study, showing that these cells are not merely an efflux of pre-existing cells from the liver but can originate from precursor cells in the lymph node (Malacarne, Webster et al. 2003). This would suggest a potential for a more complete reconstitution of the multispecific response associated with viral control, and it was encouraging to note that restoration of HBV-specific CD8 responses was possible in HIV infected patients.

We also studied the HBV-specific CD4 responses in the setting of HIV. An HBV-specific CD4 response to the core and surface HBV antigens became detectable in one out of the 3 patients tested, of whom 2 had an effective reduction in HBV load. Interestingly, one patient who had a marked reduction in HBV viremia on the addition of the novel anti-HBV drug adefovir dipivoxil (Benhamou, Bochet et al. 2001) showed reconstitution of HBV-specific CD4 cell responses to core and surface antigens. Although he was receiving only a suboptimal antiretroviral regimen (dual nucleoside analogue) and had incomplete suppression of HIV load, he was the only one who had never been significantly immunosuppressed and would therefore be expected to most closely mimic the behavior of an HIV negative patient with chronic HBV. Such CD4 responses may be important to maintain functionally active CTL, (Kalams and Walker 1998) and it is worth noting that the patient with a detectable CD4 response was the one with the highest peak CD8 response in the course of follow-up during treatment. In HIV negative patients with chronic HBV, CD4

responses predominantly to core and e antigen rather than surface antigen were detected by proliferative assays following the institution of treatment with lamivudine (Boni, Bertoletti et al. 1998). There are unpublished reports of adefovir improving HBV-specific T cell responses in HIV negative chronic HBV carriers (http://www.easl.ch/abstracts/647.doc), and it is encouraging to observe that this was also possible in the context of HIV infection with partial suppression of HIV viral replication as seen in our patient.

Lamivudine has been used as an anti-viral agent aspart of HAART, however its use is limited by low rates of eAg seroconversion and HBV replication, the rapid emergence of resistance and potentially severe hepatitis flares on discontinuation (Benhamou, Bochet et al. 1999) . One could speculate that potent combination therapy with more than one anti-HBV drug, leading to sharper falls in HBV DNA may further enhance HBV T cell recovery. Ideally, management of chronic HBV should aim not only to allow sustained suppression of HBV load but also to permit recovery of the specific immune responses associated with viral control. Our study suggests that such a goal could also be feasible in the increasingly common scenario of co-infection with HIV.

5.5 Summary conclusions and future directions

The experiments done in this work were undertaken between 1999- 2002 and at the time there was little published literature on the HBV-specific immune responses during HIV infection. New technologies such as tetramers and intracellular cytokine staining were becoming available for the study of HBV mono-infected patients, (Maini, Boni et al. 1999) providing a deeper understanding of the immunopathology of HBV.

I had the opportunity to use for the first time such sensitive techniques to analyse the HBV-specific T cell responses of HBV/HIV co-infected patients.

I first analysed (Chapter 3) HIV positive patients who have previously controlled HBV and showed a decrease in functionally active CD8 responses after HIV infection. Such reduction in HBV-specific CD8 responses may provide a mechanism that can contribute to the increased risk of reactivation seen in HIV infection and other conditions of immuno-suppression (Waite, Gilson et al. 1988; Chazouilleres, Mamish et al. 1994). Although the loss of HBV-specific CD8 responses in HIV positive patients with natural immunity to HBV may contrasts with the maintenance of memory to other persisting viruses, such as EBV memory populations during HIV, it can be accounted for by the initial lower frequencies of HBV-specific CD8 responses (Maini, Boni et al. 1999; Maini, Boni et al. 2000) compared to that EBV (Callan, Tan et al. 1998). EBV-specific CD8 responses have been shown to suffer a functional impairment during HIV infection, which is associated with an increased risk of progression to non-Hodgkin lymphoma (van Baarle, Hovenkamp et al. 2001).

Similar to studies of HIV co-infection with herpes viridae (Dalod, Dupuis et al. 1999; Rinaldo, Huang et al. 2000; Casazza, Betts et al. 2001; Wilkinson, Cope et al. 2002), I looked at the impact of HAART on HBV-specific CD8 responses in the HIV positive patients with resolved HBV. I showed that HAART can also restore functionally active

HBV-specific CD8 responses in some patients, which is associated with increased HBV-specific CD4 responses producing IL-2 and was maintained for up to 150 weeks of follow-up.

In Chapter 3 I also studied for the first time patients who had natural immunity to HBV without a prior history of acute infection and showed that a number of HIV negative patients with previous asymptomatic HBV can also preserve HBV-specific CD8 responses over time.

In Chapter 4, I had the opportunity to look at 2 patients who remained asymptomatic during acute phase HBV. I showed that HBV-specific CD4 responses were detectable and produced IFN-γ, suggesting a role for cellular immune responses even in the context of asymptomatic HBV.

Chapter 5 analysed a small group of HBV chronic carriers who were HIV infected and started treatment for HIV and/or HBV infection. At the time of this study, the choice of HAART in HIV positive HBV chronic carriers with high HBV loads did not necessarily include anti-HBV agents (lamivudine being the only licensed drug with dual anti-HIV and anti-HBV activity). This gave me the opportunity to compare HBV-specific responses with and without the addition of an HBV agent in the context of HAART. This data showed restoration of HBV-specific T cell responses can occur in the context of HIV immunosuppression but only in association with a decrease in HBV load. The number of patients studied was small and it would have been desirable to confirm these findings in a larger study, thus providing a molecular basis for the addition of an anti-HBV agent to HAART combinations in co-infected patients.

Although this was an entirely novel work at the time, there were some drawbacks.

HBV-specific responses had so far only been characterised in HLA-A2 positive patients, which may have represented a selection bias. The experience from HIV infection has

shown that studies using only HLA-A2 peptides or HIV-1 structural or envelope proteins did not always provide a representative measure of the total HIV-specific responses. At the time of study however, only HLA-A2 restricted epitopes had been defined for acute and chronic hepatitis B, and there was emerging data on cross-reactivity of the immunodominant epitope core 18-27 across the HLA-B51 class (Thimme, Chang et al. 2001). Ideally, the study should however have been done using an overlapping peptide library, which allows for detection of all HBV-specific CD4 and CD8 responses to all HBV gene products. A later study using overlapping peptides has confirmed our findings, showing a significant inverse correlation between HBV-specific T cell responses and HBV load. This study also showed that HIV infection has a significant effect on HBV-specific immune responses (Chang, Wightman et al. 2005).

I would like to expand this work by looking in more detail at the innate immune responses involved in controlling acute asymptomatic HBV infection in the two patients described in chapter 4. I would like to include enumeration as well as functional assays of NK cells. It may also be interesting assess the role of regulatory CD4+CD25+T cells in the pathogenesis of asymptomatic versus symptomatic HBV and look for a correlation with liver inflammation during acute hepatitis in the two groups.

With regards to HIV positive HBV chronic carrier patients described in Chapter 5, it would also be interesting to look further at the functionality of HBV-specific CD4 responses restored by HAART measuring IL-2 production. It would also be worthwhile including patients currently treated with dual anti-HBV agents (such as tenofovir/ lamivudine) as part of HAART and to assess whether a more significant HBV load reduction can increase the chances of restoration of HBV-specific T cell responses. The restored HBV-specific CD8 responses in the context of anti-HBV treatment merit further phenotypic and functional characterisation and longer follow-up overtime.

Overall, this study highlights the potential to reconstitute HBV-specific T cell responses in the context of HIV and antiretroviral treatment and the need for more in depth studies in the future.

6.Appendix

Publications

Reconstitution of Hepatitis B Virus (HBV)–Specific T Cell Responses with Treatment of Human Immunodeficiency Virus/ HBV Coinfection

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Liver-related mortality is an increasing problem in human immunodeficiency virus (HIV)/hepatitis B virus (HBV)–coinfected patients receiving highly active antiretroviral therapy (HAART). In HIV-negative patients, HBV chronicity is associated with a reduction in specific T cell responses that can be partially restored by treatment with lamivudine. We studied 5 HIV/HBV-coinfected patients treated with HAART, either with or without addition of a drug with specific anti-HBV activity. Our data show that reconstitution of some HBV-specific T cell responses can also occur in HIV-positive patients after a reduction in HBV load. This potential to recover T cell responses, which has been thought to be critical for HBV control, provides support for the addition of anti-HBV therapy in the treatment of HIV/HBV-coinfected patients.

A large proportion of patients infected with human immunodeficiency virus (HIV) have also been infected with hepatitis B virus (HBV) because of their shared transmission routes, and at least 10%–15% remain coinfected. Recent evidence has confirmed that HIV/HBV-coinfected patients, especially those with low CD4 cell counts, are at significantly increased risk of mortality due to liver disease [1]. Since the advent of highly active antiretroviral therapy (HAART), the overall improvement in survival of patients [2] has been associated with an apparent increase in liver disease–related mortality in coinfected patients [1, 3]. This increase in mortality has been attributed not only to potential hepatotoxicities of these drugs but also to prolonged survival, which allows time for slowly progressive viral hepatitis to become clinically relevant. Therefore, in the era of HAART, there is a need to reexamine the use of specific anti-HBV drugs in this coinfected population. It is unclear whether the immune reconstitution associated with HAART is sufficient to either restore control of HBV infection (as occurs with several other opportunistic infections) or allow specific anti-HBV therapy to be effective.

An important consideration is the effect that HAART regimens with and without an anti-HBV drug have on HBV-specific T cell responses in coinfected patients. Recent studies of HIV-negative patients have revealed that the hyporesponsiveness of both CD4 and CD8 T cells that is associated with HBV chronicity can be partially overcome by treatment with lamivudine [4, 5]. We studied patients coinfected with HIV and HBV to examine whether HAART with and without an anti-HBV drug is capable of inducing HBV-specific immune responses in the setting of HIV infection.

Patients and methods. Participating patients gave informed consent, the study was approved by the local ethical committee, and the experimentation guidelines of the authors' institution were followed. Twenty patients coinfected with HIV and HBV were screened to identify patients who expressed the HLA-A2 allele and allow assessment of HLA-A2–restricted CD8 cell responses; 11 patients were identified. Five of these patients were studied serially for up to 24 weeks after either the start of HAART or the addition of an anti-HBV drug to the HAART regimen. All patients were HIV-1 antibody–positive high-infectivity HBV carriers with alanine transaminase (ALT) levels elevated to >1.5 times normal. HBV DNA was quantitated by the Digene assay, HIV-1 loads were measured by the Quantiplex HIV RNA assay (bDNA; version 3.0; Chiron), and CD4 cell counts were monitored by flow cytometry by use of the Tritest program (Becton Dickinson) as part of the quality-controlled diagnostic service. Screening for the HLA-A2 haplotype was performed by staining peripheral blood mononuclear cells (PBMCs) with an anti–HLA-A2–positive monoclonal antibody (MAb; Incstar) followed by staining with a fluorescein isothiocyanate–conjugated sheep anti–mouse IgG second-layer MAb and flow cytometric analysis.

Peptides corresponding to the sequence of core 18–27; envelope 183–191, 335–343, 338–347, and 348–357; and polymerase 455–463, 502–510, 575–583, 655–663, and 816–824 regions of HBV genotype D were synthesized by Chiron Mimotopes and were found to be >90% pure. PBMCs were expanded in vitro

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with 1 μmol/L of the relevant HBV peptide for 10 days and then were subjected to peptide restimulation in the presence of brefeldin A for 6 h. Cells were stained with anti-CD8, were permeabilized with Cytofix/Cytoperm (BD Biosciences), and were stained with an anti–interferon (IFN)–γ MAb, as described elsewhere [6], to allow flow cytometric analysis of peptide-specific CD8 cells. HBV-specific CD4 cell responses were measured at baseline and at 24 weeks, by intracellular cytokine staining, after stimulation with either the hepatitis B surface antigen (HBsAg) or hepatitis B core antigen (HBcAg) for 10 days followed by restimulation for 6 h in the presence of brefeldin A. For all intracellular cytokine–staining experiments, HBV-specific responses were calculated by subtracting background IFN-γ production in a negative control well of cells not stimulated with peptide or antigen.

Results. To study HBV-specific CD8 cell responses across a wide range of epitopes, we used intracellular cytokine staining after 10 days of in vitro expansion with a panel of peptides representing commonly recognized HLA-A2–restricted cytotoxic T lymphocyte (CTL) epitopes. This technique allows sensitive detection of circulating HBV-specific T cells on the basis of their essential antiviral function of IFN-γ production [7]. HBV-specific CD8 cell responses were detected, at a low level, in only 2 of the 11 HLA-A2–positive, HIV/HBV–coinfected patients studied at baseline (data not shown), although most were not tested more than once before treatment. Table 1 shows the effect of antiviral therapy on HBV-specific T cell responses in relation to changes in HIV and HBV loads, CD4 cell count, and ALT level, in the 5 patients studied longitudinally. All patients were screened for CD8 cell responses to 10 epitopes at multiple time points for up to 24 weeks after either the start of HAART or the addition of an anti-HBV drug to the antiretroviral regimen. Of the 4 patients in whom a reduction in HBV load was achieved, 3 developed some detectable HBV-specific T cell responses at several time points (table 1). Patient 2 had no response at 24 weeks (the only posttreatment time point available) but could have had a transient earlier response, as reported in some HIV-negative patients treated for HBV infection [5]. Patient 3 reconstituted functional CD8 cell responses to 3 HBV epitopes on starting a lamivudine-containing treatment regimen, despite the presence of advanced immunosuppression and decompensated cirrhosis. By contrast, patient 1, in whom HAART effectively reduced HIV load and increased CD4 cell count in the absence of anti-HBV activity, showed no reconstitution of HBV responses.

These differential effects were dissected further by examining the 2 patients in whom existing antiretroviral drug regimens were augmented by addition of drugs with specific anti-HBV activity but without any effect on HIV load or CD4 cell count (patients 4 and 5). In both cases, HBV reactivity was augmented after the addition of drugs (lamivudine or adefovir dipivoxil) capable of reducing HBV DNA levels. To investigate whether the effect of these drugs was restricted to HBV-specific responses, an Epstein-Barr virus (EBV)–specific CD8 cell response was also studied longitudinally. In patient 4, the response to the HLA-A2–restricted GLCTLVAML epitope from the EBV BMLF1 lytic protein remained at a constant level after the addition of lamivudine (data not shown), excluding a generalized immunostimulatory effect of this drug.

HBV-specific CD4 cell responses were then analyzed in 3 of the patients, of whom 2 had a reduction in HBV load. Intracellular cytokine staining of CD4 cells showed reconstitution of HBV-specific CD4 cell responses in patient 5, in whom there was a marked reduction of HBV DNA level (table 1 and figure 1). These responses were measured by quantitation of IFN-γ production on restimulation with HBcAg and HBsAg, after 10 days of cell culture; ex vivo intracellular cytokine staining and 5-day proliferation assays showed equivalent results (data not shown).

The kinetics of HBV-specific CD8 cell responses were studied (exemplified by the results for patient 5; figure 1) and showed that recovery of T cell reactivity was preceded by a reduction in HBV DNA level and surface-antigen titers; in patient 3, the level of HBeAg also became undetectable. HBV-specific CD8 cell responses were maximal between 12 and 24 weeks after the start of treatment, which is similar to the time course in HIV-uninfected patients starting anti-HBV therapy [5]. Results derived from 10-day in vitro cultures do not necessarily represent circulating frequencies but are a reflection of the proliferative potential of these cells. Intracellular cytokine staining was therefore performed directly ex vivo in patient 5 and confirmed that identical CD8 cell specificities became detectable with the same time course as after 10 days of in vitro expansion (data not shown). Reductions in HBV load were accompanied by reductions in ALT level, and reconstituted CD8 cell responses were not associated with any "flares" of ALT (figure 1).

Discussion. Here we have presented the first analysis of HBV-specific T cell responses in HIV/HBV-coinfected patients in whom the effect of HAART with or without an anti-HBV drug can be examined. We have found evidence for reconstitution of functionally active HBV-specific CD8 cell responses when HIV/HBV-coinfected patients are treated. The fact that low-level responses were detected in only 2 of the 11 HIV/HBV–coinfected patients studied cross-sectionally adds weight to the increase in detectable responses after treatment. Our preliminary findings from this longitudinal study of 5 patients need to be confirmed in larger studies, but, from our sample, it appears that HAART alone may be insufficient for reconstitution of HBV-specific responses. However, some reconstitution of specific T cell responses can certainly occur with reduction of HBV load, even in the context of advanced immunosuppression or ongoing HIV viremia. These observations are consistent with the fact that HBV-specific T cell responses are difficult to detect in HIV-

Table 1. Hepatitis B virus (HBV)–specific T cell responses in human immunodeficiency virus (HIV)/HBV–coinfected patients with chan alanine transaminase (ALT) level, on starting the antiviral regimens indicated.

Patient	HBV-related clinical disease	HAART regimen (regimen change)	CD4 cell count, cells × 10^9/L		HIV-VL reduction at 24 weeks, $\log_{10}$ copies/mL	HBV DNA–load reduction at 24 weeks, $\log_{10}$ copies/mL	ALT-level reduction	I C resp Cl
			At baseline	At 24 weeks				
1	Mildly increased ALT level	ddI/ABC/NFV	100	480	4.28	0	No	
2	Hepatitis with fibrosis, membranous glomerulonephritis	ZDV/3TC/EFV	80	340	4.1	2.1	Yes	
3	Decompensated cirrhosis	ZDV/3TC/EFV	70	180	4.15	1.5	Yes	Envelo 338- 348-
4	Recurrent hepatitis flares	ddI/EFV/d4T (d4T switch to 3TC)	500	480	0	1.8	Yes	Envelo 338- 348-
5	Persistently increased ALT level	ZDV/ddI (addition of ADF[b])	360	320	−0.2	4.2	Yes	Envelo 335-

NOTE. 3TC, lamivudine; ABC, abacavir; ADF, adefovir; d4t, stavudine; ddI, didanosine; EFV, efavirenz; HAART, highly active antiretroviral therapy; HBcAg, hepa NFV, nelfinavir; VL, virus load; ZDV, zidovudine.

[a] Only tested at 24 weeks.
[b] Ten milligrams once daily (therapeutic dose for HBV, not HIV).
[c] Percentage of interferon (IFN)–γ–positive CD8 cells, out of total CD8 cells.
[d] Percentage of IFN-γ–positive CD4 cells, out of total CD4 cells.

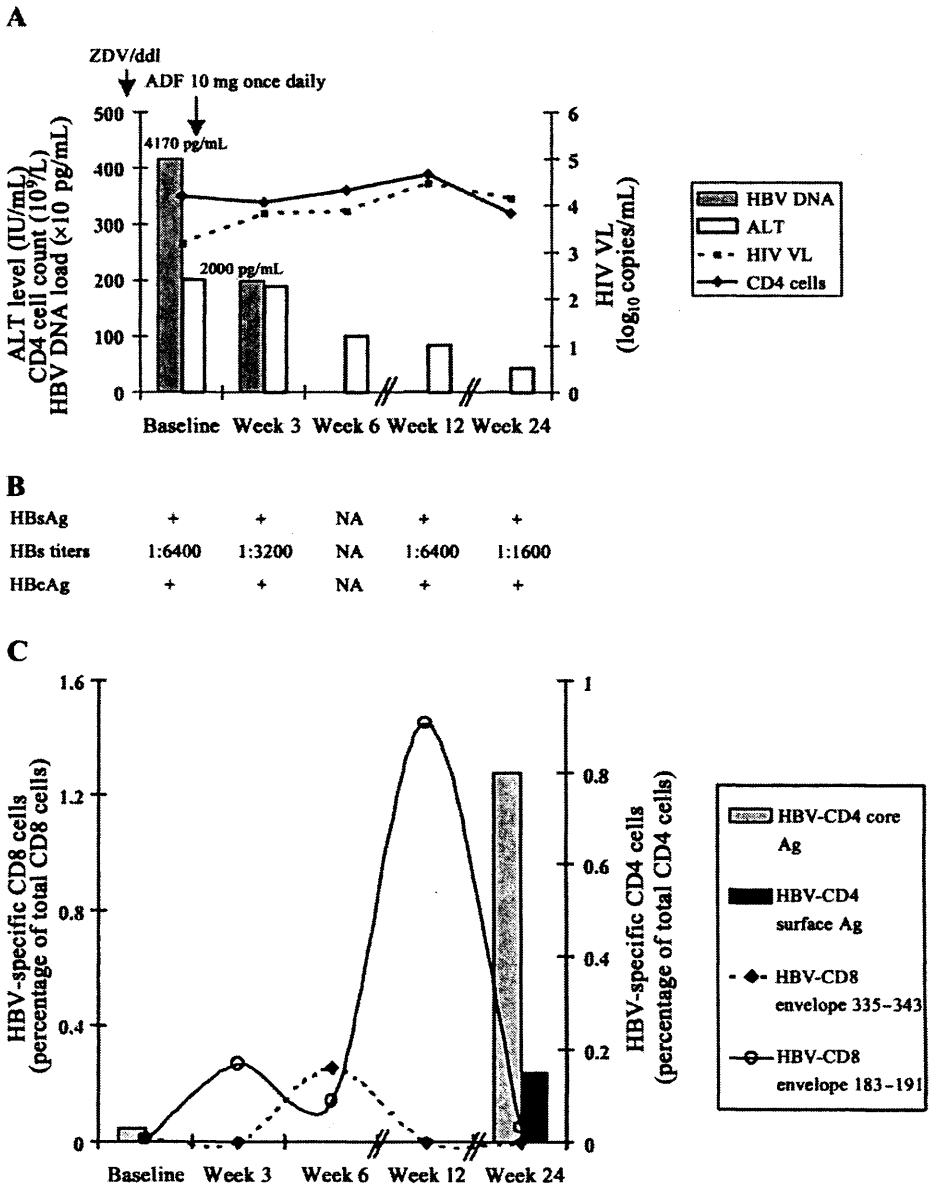

Figure 1. Kinetics of hepatitis B virus (HBV)–specific T cell responses *(A)* in relation to changes in HBV serostatus *(B)* and in human immunodeficiency virus (HIV)/HBV load, CD4 cell count, and alanine transaminase (ALT) level *(C)*, for patient 5 after addition of adefovir dipivoxil to existing antiretroviral therapy regimen. ADF, adefovir; ddI, didanosine; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; NA, not applicable; VL, virus load; ZDV, zidovudine.

negative patients with HBV infection [8] until HBV load is reduced [5]. The increase in HBV-specific CD8 cell responses after treatment contrasts with the situation observed for HIV-specific CTLs, which are easily detectable in patients with high virus loads and typically decrease after the reduction in virus load that is induced by HAART [9].

One patient who had a marked reduction in HBV viremia on the addition of the novel anti-HBV drug adefovir dipivoxil [10] showed reconstitution of HBV-specific CD4 cell responses to HBcAg and HBsAg. Although he was receiving only a dual nucleoside regimen and had incomplete suppression of HIV load, he was the only patient who had never been severely immunosuppressed and might therefore be expected to most closely mimic an HIV-negative patient with chronic HBV infection. Such CD4 cell responses are thought to play a critical role in maintaining functionally active CTLs [11], and it is of note that this patient also had the highest peak CD8 cell response while receiving treatment.

The reconstituted HBV-specific CD8 cell responses were mostly low frequency after 10 days of in vitro expansion, and all were envelope specific, even though all patients were screened repeatedly for responses to epitopes within core and

polymerase as well. Reconstituted responses in HIV-negative patients have also been reported to be frequently envelope specific [5], with a notable absence of the core-specific CD8 cells that are immunodominant in response to acute infection associated with viral control [12]. However, these responses were only measured from the peripheral blood; the recent identification of other CD8 cell specificities in secondary lymphoid organs during treatment with lamivudine in HIV-negative patients with HBV infection [6] suggests the potential for a more complete reconstitution of the multispecific response associated with viral control. Recent reports have highlighted the potential benefits of additional anti-HBV drugs, such as adefovir dipivoxil and tenofovir disoproxil fumarate, for HIV/HBV-coinfected patients. Whether more-potent anti-HBV therapy or therapeutic immunization will ultimately be able to further enhance reconstitution of HBV-specific T cell responses, even in the setting of HIV disease, remains to be established.

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MAJOR ARTICLE

Effect of HIV Infection and Antiretroviral Therapy on Hepatitis B Virus (HBV)–Specific T Cell Responses in Patients Who Have Resolved HBV Infection

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Coinfection with hepatitis B virus (HBV) is a common occurrence in human immunodeficiency virus (HIV)–positive patients and an increasing cause of morbidity and mortality. The $CD8^+$ T cell response is critical for long-term control of HBV in patients resolving acute infection. Here, we examine the effect of HIV on HBV-specific $CD8^+$ T cell responses in patients who have resolved HBV infection. A cross-sectional study showed a reduction in HBV-specific $CD8^+$ T cell responses in HIV-positive, HBV-immune patients, compared with those in HIV-negative, HBV-immune patients. A longitudinal study of a subgroup of patients examined whether this attrition could be reversed by effective antiretroviral therapy. The introduction of highly active antiretroviral therapy (HAART) resulted in reconstitution of some HBV-specific $CD4^+$ and $CD8^+$ T cell responses, in association with restoration of $CD4^+$ T cell counts. These data provide a mechanism to account for the observed impairment of control of HBV infection in the setting of HIV infection and support the ability of HAART to reconstitute functionally active T cell responses.

Coinfection with hepatitis B virus (HBV) is a frequent occurrence in HIV-positive patients and an increasing cause of morbidity and mortality in the context of the prolonged survival associated with highly active antiretroviral therapy (HAART) [1–4]. The proportion of HIV-positive patients with serological evidence of previous exposure to HBV ranges from 64% to 84% in published cohorts; 10%–15% of such patients are chronically infected with HBV [5, 6]. HIV-related immunodepletion influences the natural history of HBV infection. Epidemiological studies have revealed that HIV-positive patients are more likely to have a prolonged duration of acute illness after HBV infection and to have lower rates of clearance of hepatitis B e antigen [7]. They have higher circulating levels of HBV DNA and higher rates of reactivation of HBV infection [8]. These findings suggest impaired immune control of HBV infection during HIV infection.

In the present study, we explore, for the first time, the effect of HIV-related immunodepletion on HBV-specific $CD8^+$ T cell responses in patients who have resolved HBV infection. Recent data have directly demonstrated that $CD8^+$ T cells are critical effectors in the control of acute HBV infection [9]. HBV-specific $CD8^+$ T cell responses are difficult to detect in patients chronically infected with HBV [10], making them an unsuitable group in which to study the impact of HIV infection. We therefore studied patients who resolved HBV infection, since, in the absence of HIV, such patients maintain strong $CD4^+$ and $CD8^+$ T cell responses for many years after complete clinical and serological recovery [11, 12]. Patients without a history of jaundice found to have natural immunity to HBV (hepatitis B core antibody [HBcAb] plus/minus hepatitis B surface antibody [HBsAb]) as part of routine screening before HBV vaccination have never previously been studied

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Table 1. Clinical characteristics of HIV-positive patients.

Patient	$CD4^+$ T cell count nadir,[a] cells/μL	$CD4^+$ T cell percentage	HIV load, copies/mL	HAART status/combination at study entry	HAART combination started
P1	320	19.0	54,000	Naive	Stavudine/lamivudine/nevirapine
P2	700 (160)	24.1	<50	Stavudine/lamivudine/nelfinavir	...
P3	540	25.5	11,000	Naive	...
P4	480	12.8	118,600	Naive	...
P5	220	6.7	360,100	Naive	Zidovudine/didanosine/efavirenz
P6	290	22.3	2200	...	...
P7	260	20.0	296,700	Naive	Zidovudine/lamivudine/efavirenz
P8	280	13.2	141,400	Naive	Zidovudine/lamivudine/efavirenz
P9	510	25.0	8800	Naive	...
P10	310	24.2	50,000	Naive	...
P11	320 (230)	13.7	13,000	Naive	...
P12	350 (240)	18.0	500	Zidovudine/lamivudine	...
P13	190	15	426,000	Naive	...
P14	150	21.7	173,400	Naive	...
P15	640	22.0	800	Naive	...
P16	710	19.5	21,600	Naive	...

NOTE. HAART, highly active antiretroviral therapy.

[a] $CD4^+$ T cell count nadirs in parentheses are different from $CD4^+$ T cell counts at study entry.

immunologically, with regard to their HBV-specific immune responses, but are common among HIV cohorts [5, 13]. HBV-specific responses were compared in HIV-positive and HIV-negative patients, with a focus on those who had resolved HBV infection without symptoms. We used the sensitive technique of intracellular cytokine staining for interferon (IFN)–γ, to explore the breadth of functionally active HBV-specific $CD8^+$ T cell responses across a range of previously defined HLA-A2–restricted HBV epitopes [14–16].

To investigate to what extent any loss of HBV-specific immune responses could be reversed by antiretroviral therapy, a group of HBV-immune patients starting HAART were studied prospectively. There is a growing body of evidence suggesting that HAART may lead to successful restoration of specific immune responses to previously encountered pathogens [17–19]. A previous small study of HIV patients chronically infected with HBV suggested that HAART may be associated with restoration of HBV-specific responses once HBV load is reduced [20]. Natural immunity to HBV provided a good model system in which to further explore the potential for functional restoration of HBV-specific $CD4^+$ and $CD8^+$ T cell responses in the setting of controlled HBV replication.

PATIENTS, MATERIALS, AND METHODS

Patients and controls. Patients were recruited from the Mortimer Market Centre and provided written, informed consent, and the local ethics committee approved the study. All patients had HBV serologic test results confirming natural immunity to HBV (hepatitis B surface antigen [HBsAg] negative, HBcAb positive, and HBsAb positive) and were further categorized according to whether they had a history consistent with symptomatic acute hepatitis B.

Thirty-two HIV-positive gay men with natural immunity to HBV were screened, to identify 16 HLA-A2–positive patients. Two had a history of symptomatic acute hepatitis B, and 14 had no history of jaundice or other relevant symptoms. Patients had a median $CD4^+$ T cell count of 320 cells/μL and a median HIV load of 36,000 copies/mL (table 1). Fourteen patients were HAART naive, and 2 patients were already receiving antiretroviral therapy; 4 HLA-A2–positive, HAART-naive patients were followed up longitudinally after starting combination therapy. Twenty-five HIV-negative patients (tested within the previous year) with natural immunity to HBV were screened, to identify 14 HLA-A2–positive patients, of whom 3 had a history of symptomatic acute hepatitis B.

The HIV-positive and HIV-negative groups had similar demographic characteristics, with all but 1 patient who resolved asymptomatic HBV infection being gay men, and with all being white, apart from 1 Asian patient in the HIV-positive group. The HIV-negative group had a mean age of 38 years (median, 39 years), and the HIV-positive group had a mean age of 39 years (median, 39 years).

Tissue typing. Screening for the HLA-A2 haplotype was performed by staining peripheral blood mononuclear cells (PBMCs) with an anti–HLA-A2–positive monoclonal antibody (MAb) (Incstar), followed by fluorescein isothiocyanate–conjugated sheep anti–mouse IgG second-layer MAb and flow-cytometric analysis.

Synthetic HBV peptides and antigens. Peptides corresponding to the sequence of core 18–27; envelope 183–91, 335–43, 338–47, and 348–57; and polymerase 455–63, 502–10, 575–83,

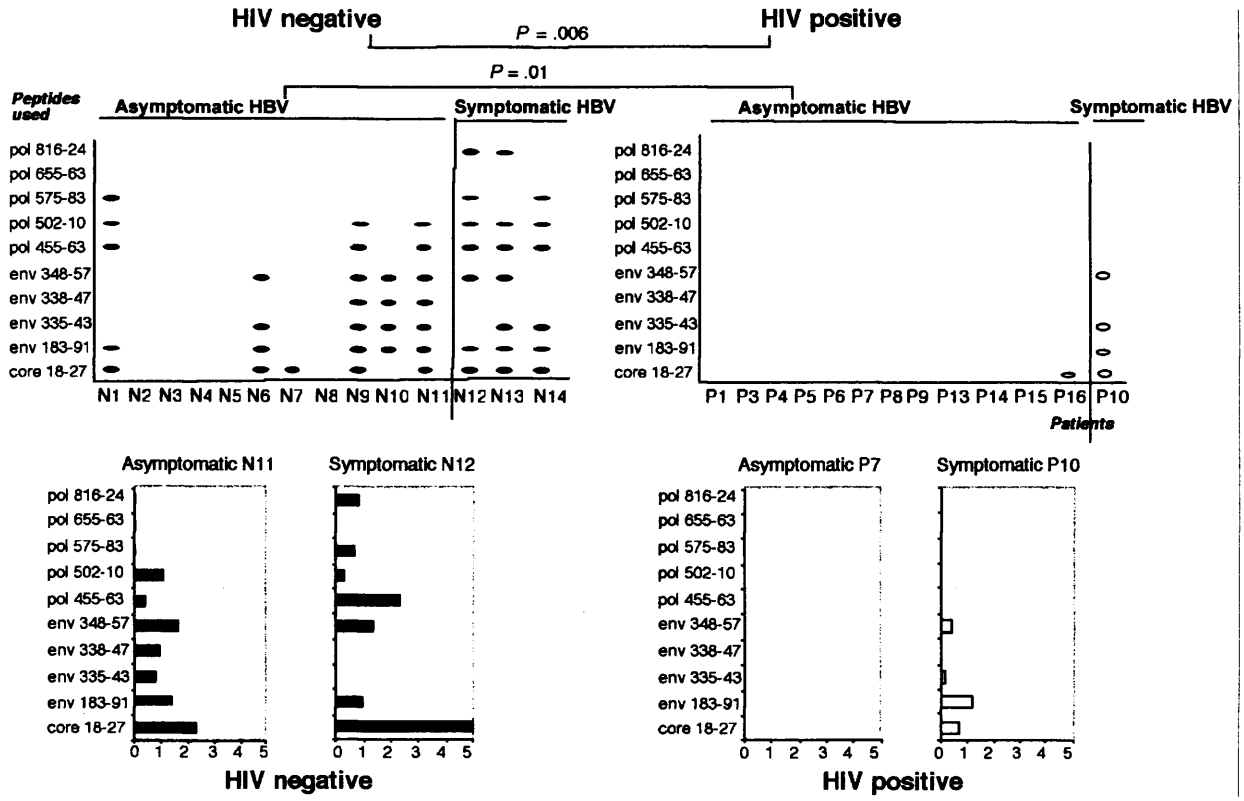

Figure 1. Comparison of CD8+ T cell responses in HIV-positive and HIV-negative patients with symptomatic or asymptomatic resolved hepatitis B virus (HBV) infection. Intracellular cytokine staining for interferon (IFN)–γ after 10 days of in vitro stimulation with 10 peptides representing HLA-A2–restricted HBV epitopes is shown for 14 HIV-negative patients (11 who resolved asymptomatic and 3 who resolved symptomatic HBV infection) and 13 HIV-positive patients (12 who resolved asymptomatic and 1 who resolved symptomatic HBV infection). The black marks indicate epitopes where the response was greater than the background level. In an analysis that took into account the no. and size of CD8+ T cell responses per patient, there were significantly more responses detectable in the HIV-negative group ($P = .006$, for all patients, Mann-Whitney U test; $P = .01$, for patients with asymptomatic HBV infection, Mann-Whitney U test). The histograms show representative examples of the magnitude of responses after expansion for patients from each category (frequencies for all responses for each patient are listed in table 2). HBV-specific CD8+ T cell responses are expressed as the percentage of total CD8+ T cells producing IFN-γ after stimulation with the appropriate peptide and after subtraction of background production of IFN-γ in unstimulated control wells. env, envelope; pol, polymerase.

655–63, and 816–24 regions of HBV genotype D were synthesized (Chiron Mimotopes). Hepatitis B core antigen (HBcAg) and HBsAg were produced in *Escherichia coli* strain K802 and were 90% pure. HBsAg was supplied by Rhein Biotech, and HBcAg was provided by G. Borisova (University of Latvia).

Production of T cell lines and intracellular IFN-γ staining. PBMCs were seeded at a concentration of 3×10^6 cells/mL and stimulated with 1 μmol/L relevant peptide (the 10 peptides listed above). Recombinant interleukin (IL)–2 (10 IU/mL) (Boehringer Mannheim) was added on day 4 of cell culture. Cells were restimulated on day 10, with 1 μmol/L relevant peptide (5 h), the last 4 h with 10 μg/mL brefeldin A (Sigma-Aldrich). Cells were stained with anti-CD8+ (Pharmingen), permeabilized with Cytofix/Cytoperm (Pharmingen), stained with anti–IFN-γ mAb (R&D Systems), and analyzed by use of a FACScan flow cytometer with CELLQuest software (Becton Dickinson).

For enumeration of HBV-specific CD4+ T cells, PBMCs were suspended at a concentration of 3×10^6 cells/mL in RPMI 1640 and 5% human serum and stimulated with 1 μmol/L HBcAg or HBsAg for 6–16 h, with addition of brefeldin A after 1 h. Cells were washed, stained with anti-CD4+ phycoerythrin (Pharmingen), and subjected to intracellular cytokine staining with anti–IFN-γ or anti–IL-2 mAb (R&D Systems). For all intracellular cytokine staining experiments, responses were calculated by subtracting background production of IFN-γ or IL-2 in a negative control well without peptide or antigen restimulation. Stimulation with anti-CD3 and anti-CD28 MAb (1 μmol/L each) or phorbol 12-myristate 13-acetate (3 ng/mL) and ionomycin (100 ng/mL), for 6 h in the presence of brefeldin A, was used as a positive control.

Statistical analysis. In the cross-sectional study of HIV-negative and HIV-positive patients, the nonparametric Fisher's exact test was used to compare the number of patients with

Table 2. Cross-sectional study of CD8⁺ T cell responses after 10 days of in vitro stimulation.

Category, infection, patient	core 18–27	env 183–91	env 335–43	env 338–47	env 348–57	pol 455–63	pol 502–10	pol 575–83	pol 655–63	pol 816–24
HIV negative										
Asymptomatic HBV										
N1	0.7	0.3	0	0	0	0.1	0.1	0.1	0	0
N2	0	0	0	0	0	0	0	0	0	0
N3	0	0	0	0	0	0	0	0	0	0
N4	0	0	0	0	0	0	0	0	0	0
N5	0	0	0	0	0	0	0	0	0	0
N6	0.8	0.6	2.0	0	0.1	0	0	0	0	0
N7	0.4	0	0	0	0	0	0	0	0	0
N8	0	0	0	0	0	0	0	0	0	0
N9	14.8	1.5	1.3	0.8	6.3	6.6	0.2	0	0	0
N10	0	0.9	0.1	0.1	0.1	0	0	0	0	0
N11	2.3	1.4	0.8	0.9	1.6	0.4	1.1	0	0	0
Symptomatic HBV										
N12	5.0	1.0	0	0	1.4	2.4	0.3	0.7	0	0.8
N13	20.4	0.9	0.4	0	6.9	12.3	1.3	0	0	0.4
N14	1.1	1.2	0.5	0	0	7.9	0.6	0.5	0	0
HIV positive										
Asymptomatic HBV										
P1	0	0	0	0	0	0	0	0	0	0
P3	0	0	0	0	0	0	0	0	0	0
P4	0	0	0	0	0	0	0	0	0	0
P5	0	0	0	0	0	0	0	0	0	0
P6	0	0	0	0	0	0	0	0	0	0
P7	0	0	0	0	0	0	0	0	0	0
P8	0	0	0	0	0	0	0	0	0	0
P9	0	0	0	0	0	0	0	0	0	0
P13	0	0	0	0	0	0	0	0	0	0
P14	0	0	0	0	0	0	0	0	0	0
P15	0	0	0	0	0	0	0	0	0	0
P16	0.1	0	0	0	0	0	0	0	0	0
Symptomatic HBV, P10	0.6	1.1	0.1	0	0.4	0	0	0	0	0

NOTE. Data are the percentage of CD8⁺ T cells producing interferon-γ in response to each peptide. env, envelope; HBV, hepatitis B virus; pol, polymerase.

any HBV-specific CD8⁺ T cell response detectable. The nonparametric Mann-Whitney *U* (Wilcoxon rank sum) test was used to compare the groups, with the number and size of T cell responses for each patient taken into account.

RESULTS

Reduction of HBV-specific CD8⁺ T cell responses in HIV-positive, HBV-immune patients. We initially conducted a cross-sectional study of HIV-positive and HIV-negative patients with natural immunity to HBV, either with or without a history of acute symptomatic infection. All patients were screened for CD8⁺ T cells producing IFN-γ in response to a panel of 10 peptides representing frequently recognized HLA-A2–restricted HBV epitopes, and results were calculated after subtraction of background staining in wells without peptide restimulation. In 3 HLA-A2–positive, HIV-negative patients who had successfully resolved acute symptomatic HBV infection 3–5 years before, we found peptide-specific production of IFN-γ for 6 or 7 of the 10 epitopes tested (figure 1 and table 2). We found that HIV-negative patients who had resolved HBV infection without prior symptoms of acute infection also had detectable CD8⁺ T cell responses to at least 1 HLA-A2–restricted epitope in 6 of 11 cases (figure 1 and table 2). In some patients (e.g., N9, whose last risk exposure to HBV was >10 years before), responses had a level of multispecificity and expansion potential similar to those seen in patients with symptomatic acute infection, who have been the basis of all previous immunological studies of patients resolving HBV infection.

In a similar group of 12 HAART-naive, HIV-positive patients who also had serological evidence of past HBV infection without a history of symptoms, HBV-specific CD8⁺ T cell responses were markedly diminished. This group was matched with the HIV-negative, HBV-immune group for sex, age, ethnic origin, and likely route of acquisition of HBV infection, and all

Table 3. Cross-sectional study of CD8⁺ T cell responses directly ex vivo.

Category, infection, patient	core 18–27	env 183–91	env 335–43	env 338–47	env 348–57	pol 455–63	pol 502–10	pol 575–83	pol 655–63	pol 816–24
HIV negative										
Asymptomatic HBV										
N1	0.2	0	0	0.4	0.2	0.2	0	0	0.8	0
N7	0.3	0	0	0	0	0	0	0	0	0
Symptomatic HBV										
N12	0.1	0	0	0	1.2	1.2	0.1	0.5	0	0.7
N13	1.0	0.9	0.2	0.1	0.3	1.3	0	0	0	0
HIV positive										
Asymptomatic HBV										
P5	0	0	0	0	0	0	0	0	0	0
P7	0	0	0	0	0	0	0	0	0	0
Symptomatic HBV										
P10	0.8	0.8	0	0	0.7	0.6	0	0	0	0
P11	0	0	0	0	0	0	0	0	0	0

NOTE. Data are the percentage of CD8⁺ T cells producing interferon-γ in response to each peptide. env, envelope; HBV, hepatitis B virus; pol, polymerase.

were antiretroviral naive. HBV-specific CD8⁺ T cell responses were undetectable after 10 days of specific peptide stimulation, by both intracellular cytokine staining (figure 1) and tetramer staining (data not shown), apart from 1 low-level core 18–27 response in P16. In an HIV-positive patient with a history of acute symptomatic hepatitis B (P10; 8 years before), intracellular cytokine staining for IFN-γ after 10 days of in vitro stimulation showed recognition of 4 of 10 epitopes tested. The proportion of patients with any HBV-specific CD8⁺ T cell response and the total number and magnitude of responses was significantly greater for the whole HIV-negative group than for the whole HIV-positive group ($P = .018$, Fischer's exact test, and $P = .006$, Mann-Whitney U test, respectively). These differences remained significant in a comparison of the closely matched subsets in the HIV-negative and HIV-positive groups who resolved HBV infection without symptoms ($P = .027$, for the proportion of patients with at least 1 response, Fisher's exact test; $P = .01$, for the number and size of responses, Mann-Whitney U test) (figure 1).

Intracellular cytokine staining directly ex vivo, in a subgroup of 2 HBV-immune patients who resolved symptomatic infection and 2 HBV-immune patients who resolved asymptomatic infection, from both the HIV-negative and HIV-positive groups (table 3), confirmed that responses were detectable in all of the HIV-negative patients tested. In the HIV-positive group, responses were detected directly ex vivo in only 1 of the HIV-positive patients (in P10, who had a history of acute symptomatic HBV infection 8 years before, but not in P11, who had acute infection >15 years before, or in the patients with asymptomatic infection). In all patients studied by both methods, the breadth of the HBV-specific immune responses seen after direct ex vivo expansion was similar to that seen after in vitro expansion.

HBV-specific CD8⁺ T cell responses in HIV-positive patients receiving antiretroviral therapy. To investigate any potential for reconstitution of HBV-specific CD8⁺ T cell responses, we initially examined responses in 2 patients who had resolved HBV infection without symptoms and were already receiving treatment for HIV infection. P12 started zidovudine and lamivudine at a CD4⁺ T cell count nadir of 240 cells/μL; at the time that samples were obtained, he had been maintained on this regimen for 3 years (at which time his CD4⁺ T cell count was 350 cells/μL and his HIV load was 500 copies/mL). P2 had been receiving HAART for 6 months, but lamivudine was substituted for didanosine 1 month before samples were obtained. His CD4⁺ T cell count increased from a nadir of 160 cells/μL to 700 cells/μL, and his HIV load was suppressed to <50 copies/mL. Both of these patients had CD8⁺ T cell responses to 3 of the 10 HBV-specific, HLA-A2–restricted epitopes tested (figure 2), which, after 10 days of in vitro expansion, were present at a magnitude similar to that seen in the HIV-negative, HBV-immune patients. This contrasted with the lack of responses detectable in 11 of the 12 untreated HIV-positive patients who had an equivalent pattern of HBV immunity and no history of symptomatic acute infection (figure 2). HBV-specific CD8⁺ T cell responses were detectable in the treated HIV-positive patients but not in the untreated HIV-positive, HBV-immune patients tested directly ex vivo (figure 2).

Reconstitution of HBV-specific CD8⁺ T cell responses on longitudinal study of patients starting HAART. The cross-sectional data suggested a decrease in HBV-specific CD8⁺ T cell responses in HIV-positive, HBV-immune patients and a possible reconstitution of such responses in those receiving HAART. We therefore longitudinally studied the impact of HAART on HBV-specific immune responses. Four patients who had resolved

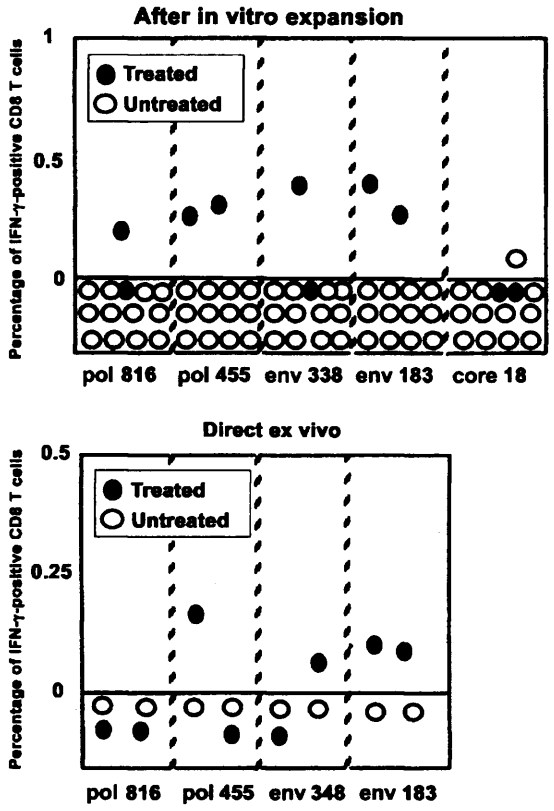

Figure 2. Comparison of breadth of $CD8^+$ T cell responses in HIV-positive patients with immunity to hepatitis B virus (HBV), after asymptomatic infection, with or without antiretroviral treatment. Intracellular cytokine staining for interferon (IFN)–γ after 10 days of stimulation with 10 HLA-A2–restricted peptides is shown for patients with immunity after asymptomatic HBV infection *(upper panel).* Results are shown for 14 HIV-positive patients: 12 highly active antiretroviral therapy (HAART) naive (P1, P3, P4, P5, P6, P7, P8, P9, P13, P14, P15, and P16) and 2 antiretroviral treated (P2 and P12). HBV-specific $CD8^+$ T cell responses are expressed as the percentage of total $CD8^+$ T cells producing IFN-γ after stimulation with the appropriate peptide and after subtraction of background production of IFN-γ in unstimulated control wells. Only the epitopes with any positive response are shown. *Lower panel,* Intracellular cytokine staining directly ex vivo in a subgroup of the above patients (2 HIV-positive, HAART-naive patients [P5 and P7] and 2 HIV-positive, antiretroviral-treated patients [P2 and P12]). env, envelope; pol, polymerase.

HBV infection (without a history of asymptomatic acute infection) and were starting HAART for progressive HIV infection were studied prospectively, and sequential blood samples were obtained before and during therapy. The drug regimens used are shown in table 1 and figure 3*A* and were selected by the clinician independently of participation in the present study. These patients were screened for functionally active HBV-specific $CD8^+$ T cell responses on 2–6 occasions by use of the same panel of 10 peptides applied in the cross-sectional studies.

The temporal relationship between increase in $CD4^+$ T cell count, suppression of HIV load, and reconstitution of HBV-specific $CD8^+$ T cell responses for each of the 4 patients is presented in figure 3*A*. Patients were screened up to 2 years after starting HAART, and, 3 years after starting HAART, samples were again obtained from any patients with reconstitution of HBV-specific $CD8^+$ T cell responses. In 2 patients, the increase in $CD4^+$ T cell count and suppression of HIV load were accompanied by detection of HBV-specific $CD8^+$ T cell responses by 24 weeks of antiretroviral treatment. The epitopes detected were from the envelope group; 1 patient also recovered a response to core 18–27 (figure 3*A*). HBV-specific $CD8^+$ T cell responses were still detectable when samples were again obtained from P1 and P7, 3 years after starting HAART (figure 3*A*), and, in P1, expanded to higher frequencies than at earlier sampling times. This is compatible with the finding of persistent $CD8^+$ T cell responses in the patients studied cross-sectionally 6 months to 3 years after starting antiretroviral therapy (figure 2).

Direct ex vivo analysis of P7 revealed reconstitution of HBV-specific $CD8^+$ T cell responses at week 24 (figure 3*B*), which is consistent in specificity and timing with the in vitro data (figure 3*A*), and a simultaneous decrease in levels of $CD8^+$ specific for an HIV-1–specific, HLA-A2–restricted epitope (gag 77–85; data not shown). This patient was also tested directly ex vivo for HBcAg-specific $CD4^+$ T cell responses, by intracellular IFN-γ staining, which revealed a parallel increase in HBV-specific $CD4^+$ and $CD8^+$ T cell responsiveness (figure 3*B*).

Parallel between reconstitution of HBV-specific $CD4^+$ T cell responses and HAART-induced increases in total $CD4^+$ T cells. To further investigate the potential role that enhanced $CD4^+$ T cell help plays in recovering HBV-specific $CD8^+$ T cell responses in these patients, we screened all the patients being longitudinally studied for HBV-specific $CD4^+$ T cell responses at each time point. In light of recent data highlighting the potential importance of IL-2–producing $CD4^+$ T cells in maintaining adequate antiviral $CD8^+$ T cell responses (reviewed in [21]), patients were tested for the presence of $CD4^+$ T cells able to produce IFN-γ and/or IL-2 on stimulation with HBcAg and HBsAg. The assay used to measure $CD4^+$ intracellular production of IFN-γ and/or IL-2, with or without stimulation with HBcAg or HBsAg, is shown in figure 4*A*. As illustrated by the fluorescence-activated cell sorter dot-plot profiles for this treated patient (P1; week 150), responses to overnight antigenic stimulation were easily detectable in some cases (IL-2–positive $CD4^+$ responding to HBcAg). In other examples (IFN-γ–positive $CD4^+$ responding to HBcAg and HBsAg), the responding populations were only slightly greater than background levels but could be confirmed by their ability to increase after a 10-day antigen-specific in vitro expansion (figure 4*A*).

The temporal relationship between $CD4^+$ T cell responses and changes in $CD4^+$ T cell count and HIV load is shown in figure 4*B*. All 4 HAART-treated patients developed detectable $CD4^+$ T cell responses to both HBcAg and HBsAg from week 6 onward, which persisted for the duration of follow-up (at

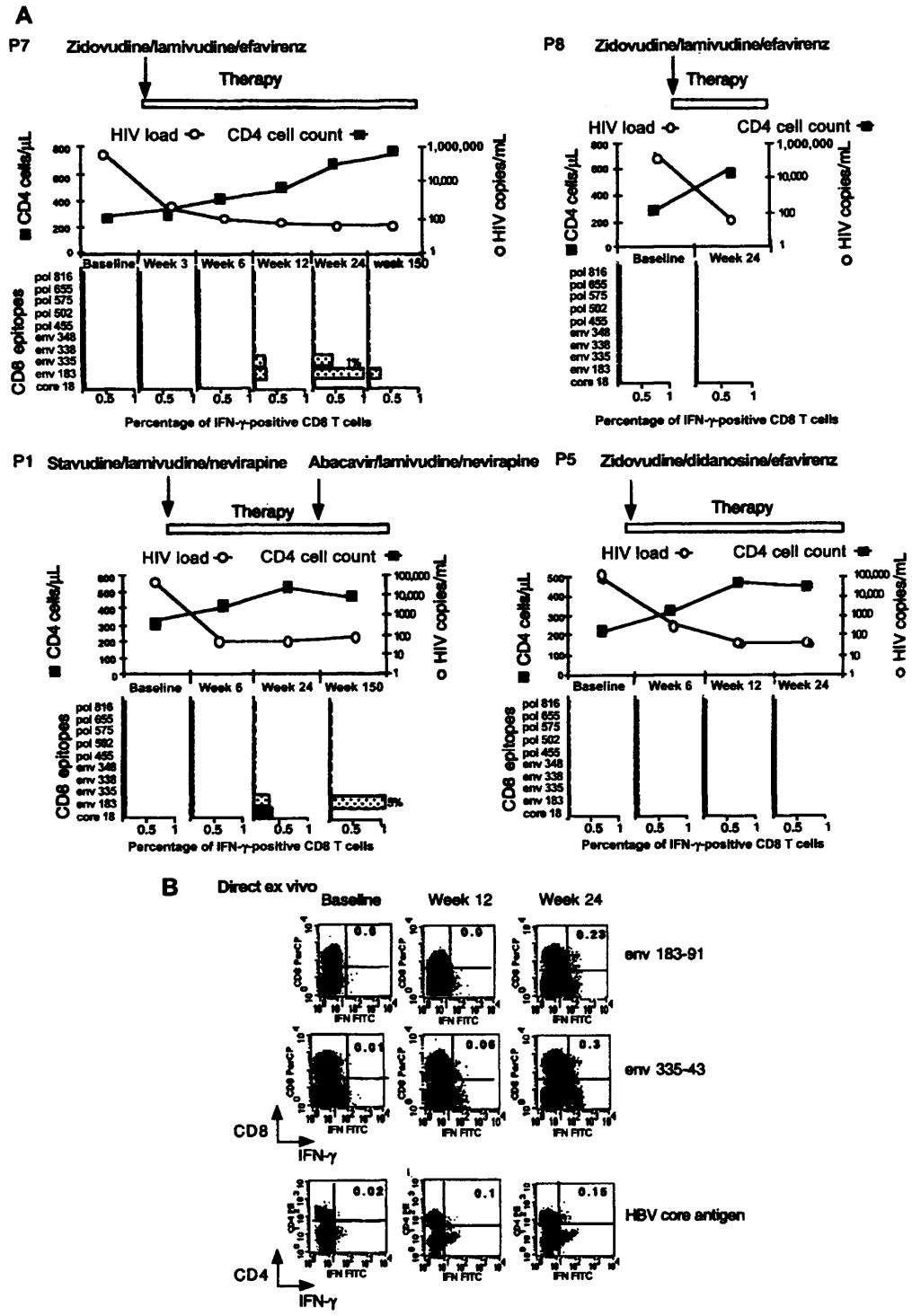

Figure 3. Hepatitis B virus (HBV)–specific CD8⁺ T cell responses in HIV-positive, HBV-immune patients starting highly active antiretroviral therapy (HAART). *A*, Longitudinal analysis of HBV-specific CD8⁺ T cell responses in 4 HBV-immune patients (P1, P5, P7, and P8) starting HAART. Drug regimens are shown for each patient above the changes in CD4⁺ T cell count and HIV load at each time point at which T cell responses were measured. The lower panel for each patient shows the results, for each time point, of intracellular cytokine staining of CD8⁺ T cells for IFN-γ after 10 days of in vitro stimulation with 10 HBV peptides. *B*, Fluorescence-activated cell sorter dot-plot profiles of intracellular cytokine staining of CD8⁺ T cells from P7 for IFN-γ, directly ex vivo. The epitopes for which response greater than the background level was detected are represented. Nos. in the right upper quadrants represent the percentage of CD8⁺ T cells responding to envelope (env) 183–91 *(upper panel)* and env 335–43 *(middle panel). Lower panel,* Flow-cytometric data from the same patient time points, showing intracellular cytokine staining of CD4⁺ T cells for IFN-γ, directly ex vivo, after stimulation with hepatitis B core antigen. FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP; peridin chlorophyll protein; pol, polymerase.

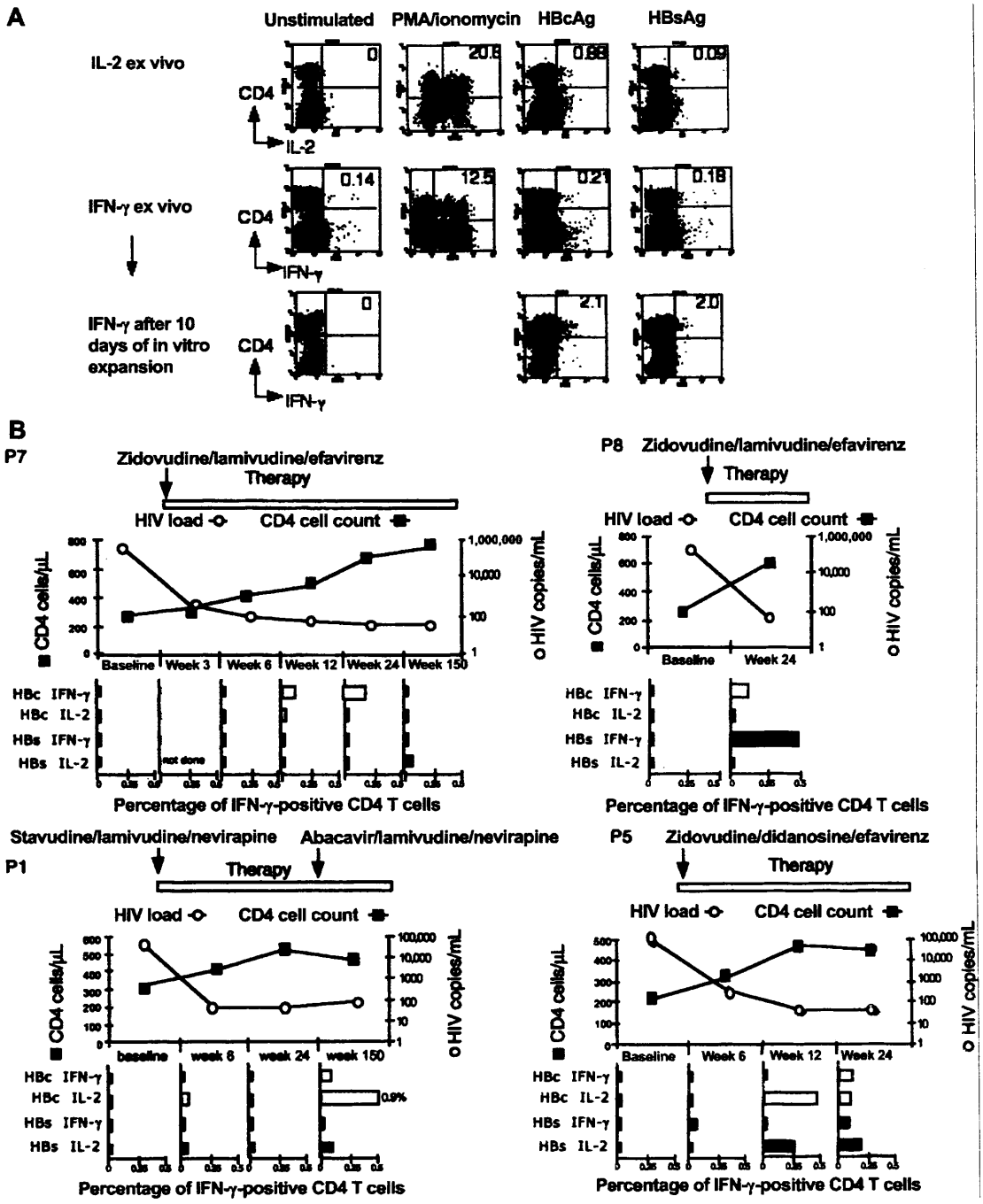

Figure 4. Hepatitis B virus (HBV)–specific CD4⁺ T cell responses in HIV-positive, HBV-immune patients starting highly active antiretroviral therapy (HAART). *A*, Fluorescence-activated cell sorter dot-plot profiles of intracellular cytokine staining of CD4⁺ T cells for interleukin (IL)–2 *(upper panel)* and interferon (IFN)–γ *(middle panel)* after overnight stimulation with hepatitis B core antigen (HBcAg) or hepatitis B surface antigen (HBsAg), compared with that of an unstimulated negative control and a phorbol 12-myristate 13-acetate (PMA)/ionomycin positive control (P1; week 150). The lower panel shows intracellular cytokine staining for IFN-γ with the same antigens after 10 days of antigen-specific in vitro expansion. Nos. in the right upper quadrants represent the percentage of CD4⁺ T cells responding to each stimulus. *B*, Longitudinal analysis of HBV-specific CD4⁺ T cell responses in 4 HBV-immune patients (P1, P5, P7, and P8) starting HAART. Drug regimens are shown for each patient above the changes in CD4⁺ T cell count and HIV load at each time point at which T cell responses were measured. The lower panel for each patient shows the direct ex vivo intracellular cytokine staining of CD4⁺ T cells with IFN-γ and IL-2 in response to HBcAg and HBsAg (not done, CD4⁺ T cell responses not analyzed at this time point). *C*, Cross-sectional comparison of HBV-specific CD4⁺ T cell responses in 5 HIV-positive, HAART-naive patients *(left chart)*, with the peak of responses seen while receiving HAART treatment in 4 of these patients *(middle chart)* and with the responses in 6 HIV-negative patients *(right chart)*. All patients had resolved asymptomatic HBV infection. The no. and size of responses was significantly greater in HIV-negative than in HIV-positive patients ($P = .005$, Mann-Whitney U test), and responses in HIV-negative patients were comparable with peak responses while receiving treatment in HIV-positive patients ($P = .67$, Mann-Whitney U test).

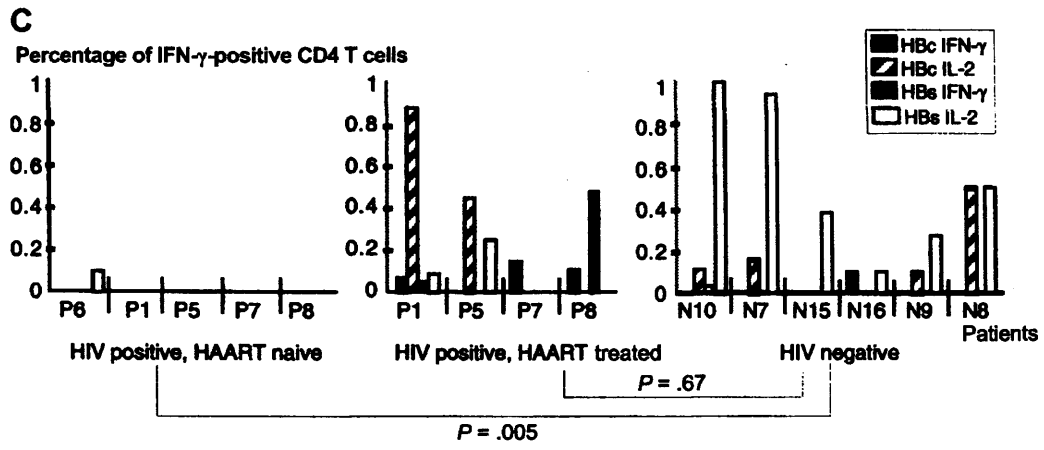

Figure 4. *(Continued.)*

least 6 months in 2 patients and at least 3 years in the other 2). Three of the 4 patients were given lamivudine-containing combinations; P5, who also developed strong HBV-specific $CD4^+$ T cell responses, was not treated with any drug with anti-HBV activity. All patients achieved adequate viral suppression and good total $CD4^+$ T cell count recovery/increases, but the patient with the highest $CD4^+$ T cell count nadir, lowest starting HIV load, and most rapid suppression of HIV load (P1) had the most effective reconstitution of both $CD4^+$ and $CD8^+$ T cell responses (figure 4*B*).

Figure 4*C* summarizes the lack of $CD4^+$ T cell responses to HBcAg and HBsAg in 5 HBV-immune patients with HIV-related $CD4^+$ lymphopenia ($CD4^+$ T cell count <350 cells/μL) before the start of antiretroviral therapy. This contrasts with the HBV-specific $CD4^+$ T cell responses in 6 HIV-negative patients who had also resolved asymptomatic HBV infection. The peak responses seen in the HIV-positive patients receiving HAART were comparable in frequency and magnitude to those seen in the HIV-negative patients (figure 4*C*).

DISCUSSION

In the present study, we explored the effect of HIV immunodepletion and HAART-related immune restoration on reshaping immune responses to HBV infection. We observed a decrease in functionally active HBV-specific $CD8^+$ T cell responses in association with HIV infection in HBV-immune patients. Accumulating data suggest that residual virus is kept under tight control by an ongoing immune response in such patients [11, 12, 22]. Thus, the reduction in HBV-specific T cell responses observed in the present study provides a mechanism that could contribute to the increased risk of reactivation of hepatitis B surface antigenemia seen in HIV infection and other situations of clinical immunodepletion [23, 24].

To date, immunological studies of HBV immunity after acute infection have only included patients presenting with symptomatic infection. However, a large proportion of patients are found to have immunity to HBV without having had any prior symptoms of acute infection. Although the duration since the initial exposure to HBV was not always known, a number of HIV-negative patients in the potentially heterogeneous group in the present study had preserved HBV-specific responses over the course of many years. Although we cannot exclude the possibility that HIV-positive patients had a longer interval between resolution of HBV infection and the time that samples were obtained, the efficient long-term preservation of HBV-specific $CD8^+$ T cell responses makes this unlikely to account for their lack of responsiveness.

Data describing the impact of HIV on responses to other viruses suggest that the persistence of detectable responses is related to the magnitude of antigen-specific response generated by each virus. For example, Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are both associated with stable high-frequency memory cytotoxic T lymphocyte (CTL) populations, which, although reduced by HIV infection, remain easily detectable [18, 25]. Since the responses generated in association with control of HBV infection are typically lower in frequency than those to viruses such as EBV and CMV, it is not surprising that few remain detectable in HIV-positive patients. A recent study [26] including long-term follow-up of HIV-positive patients revealed maintenance of numbers but functional impairment of the EBV-specific $CD8^+$ T cell response associated with subtle increases in EBV load and progression to non-Hodgkin lymphoma. Thus, careful long-term assessment of HIV-positive, HBV-immune patients would be required to examine the virological impact of the impaired HBV-specific $CD8^+$ T cell response in this group of patients. A reduction of HBV-specific $CD8^+$ T cell responses after HIV infection would be consistent with the demonstrated ability of HIV to induce apoptosis of CTLs of unrelated specificities through FasL-mediated [27] and tumor necrosis factor–mediated [28] counterattack. Similarly,

data from murine models show that heterologous viral infections can quantitatively and qualitatively alter the memory pool of existing antiviral $CD8^+$ [29, 30].

Our previous study of HIV-positive patients with ongoing HBV infection revealed the potential to recover some HBV-specific T cell responses in patients receiving HAART, in association with the reduction in HBV load induced by anti-HBV agents [20]. By contrast, in the present study, we investigated a group of patients in whom HBV load was already efficiently suppressed, to dissect the potential contribution of HAART-mediated immune reconstitution to the restoration of HBV-specific T cell responses. There is evidence from studies of the herpes viridae (EBV, CMV, and Kaposi sarcoma–associated herpesvirus) that HAART can restore specific T cell frequencies [17, 18, 31, 32], and this correlates with a decrease in end-organ disease [4, 33, 34]. The 2 patients studied cross-sectionally and 2 of the 4 patients studied longitudinally restored functionally active HBV-specific $CD8^+$ T cell responses that had expansion potential after in vitro stimulation. That $CD8^+$ T cell responses were reconstituted only in a proportion of these HBV-immune patients is in accordance with our findings in HIV-negative patients without a history of symptomatic HBV infection and are in line with those of a study of the impact of HIV infection and antiretroviral therapy on responses to *Mycobacterium avium* complex [35].

An important contribution to the reconstitution of HBV-specific $CD8^+$ T cell responses is likely to be a restoration of $CD4^+$ T cell help associated with the increasing $CD4^+$ T cell counts. T cell help is known to be important for maintaining functionally active $CD8^+$ T cell responses [36], and recent data suggest that IL-2–producing $CD4^+$ T cells play a key role in antiviral immunity [21, 37]. In both of the patients with restoration of HBV-specific $CD8^+$ T cell responses, we observed a concomitant reconstitution of IFN-γ–positive and IL-2–positive $CD4^+$ T cell responses to HBcAg and HBsAg after the start of HAART. These HBV-specific $CD4^+$ T cell responses were similarly observed in a patient receiving an antiretroviral combination that did not include lamivudine or other drugs with anti-HBV activity. Importantly, we demonstrated the potential for long-term maintenance of both $CD4^+$ and $CD8^+$ T cells during prolonged follow-up of 2 patients, in contrast to the rapid but short-lived effects of lamivudine on HBV-specific T cell function reported in patients with ongoing HBV infection [20, 38]. There is a temporal correlation between the reconstitution of T cell responses observed in the present study and that seen in other studies of HAART-mediated reconstitution of T cell function (reviewed in [39]), with initial reconstitution at ~3 months and subsequent reconstitution continuing gradually for at least 2 years [40]. These findings should be extended to a larger group of patients, since the present study was not powered to detect an effect of $CD4^+$ T cell count nadir, magnitude of changes in $CD4^+$ T cell count, or HIV load on the extent of HBV-specific T cell reconstitution. However, it is worth noting that the patient with the most effective reconstitution of HBV-specific $CD4^+$ and $CD8^+$ T cell responses was the one who started HAART with the highest $CD4^+$ T cell count nadir and the lowest HIV load and who achieved the most efficient containment of HIV to undetectable levels.

The findings of the present study could be extrapolated to suggest that, in patients treated for HBV/HIV coinfection, reconstitution of HBV-specific T cell responses may involve 2 distinct components: the reconstitution of responses associated with reduction in HBV load [20], as seen in treatment of HBV monoinfection [41, 42], and the more gradual, sustained reconstitution associated with the prolonged suppression of HIV viremia and reconstitution of HBV-specific $CD4^+$ T cell responses demonstrated here. Thus, antiretroviral therapy can lead to an increase in functional $CD4^+$ and $CD8^+$ T cell responses to HBV infection, supporting the potential of HAART to reconstitute immune responses to clinically important pathogens.

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Greater CD8$^+$ TCR Heterogeneity and Functional Flexibility in HIV-2 Compared to HIV-1 Infection[1]

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Virus-specific CD8$^+$ T cells are known to play an important role in the control of HIV infection. In this study we investigated whether there may be qualitative differences in the CD8$^+$ T cell response in HIV-1- and HIV-2-infected individuals that contribute to the relatively efficient control of the latter infection. A molecular comparison of global TCR heterogeneity showed a more oligoclonal pattern of CD8 cells in HIV-1- than HIV-2-infected patients. This was reflected in restricted and conserved TCR usage by CD8$^+$ T cells recognizing individual HLA-A2- and HLA-B57-restricted viral epitopes in HIV-1, with limited plasticity in their response to amino acid substitutions within these epitopes. The more diverse TCR usage observed for HIV-2-specific CD8$^+$ T cells was associated with an enhanced potential for CD8 expansion and IFN-γ production on cross-recognition of variant epitopes. Our data suggest a mechanism that could account for any possible cross-protection that may be mediated by HIV-2-specific CD8$^+$ T cells against HIV-1 infection. Furthermore, they have implications for HIV vaccine development, demonstrating an association between a polyclonal, virus-specific CD8$^+$ T cell response and an enhanced capacity to tolerate substitutions within T cell epitopes. *The Journal of Immunology,* 2003, 171: 307–316.

Human immunodeficiency virus-2 is a lentivirus related to HIV-1 with up to 60% sequence homology, but with markedly different epidemiological features. HIV-2 infection has a relatively limited geographical distribution and is much less transmissible than HIV-1 by both horizontal (1) and vertical (2) routes. In addition, most patients infected with HIV-2 have a more prolonged clinically asymptomatic phase, with no reduction in survival in many of those infected and an overall mortality only twice as high as that of the uninfected population (3). These differences may result from an inherent reduced pathogenicity of HIV-2 and/or from a more effective immune response to the virus. Recent data do not support the notion that HIV-2 has an attenuated phenotype with less pathogenic potential, because it has a similar in vitro cytopathicity to HIV-1 (4). Plasma RNA levels are much lower in asymptomatic HIV-2-infected patients with high CD4 counts than in similar HIV-1-infected patients, despite comparable levels of proviral DNA in the two infections (5, 6). The lack of correlation between cellular proviral DNA and plasma viremia in HIV-2 infection (6) could reflect reduced production of virions by infected cells as a result of better immune control.

The CD8$^+$ T cell response is one of the critical correlates of viral control in HIV infection. One mechanism increasingly recognized to be of importance in the failure of CTL control of both SIV and HIV-1 infections is viral acquisition of mutations allowing CTL escape (Refs. 7–9; N. Jones, X. Wei, D. Flower, M. Wong, F. Michor, M. Saag, B. Hahn, M. Nowak, G. Shaw, and P. Borrow, manuscript in preparation). Interestingly, such escape mutations have yet to be demonstrated in the context of HIV-2, even though CTL are often detectable directly ex vivo (10). This may be one determinant contributing to the better long-term control of viral replication in HIV-2-compared with HIV-1-infected individuals.

One factor that may limit the propensity to CTL escape mutations is the polyclonality of the virus-specific CD8$^+$ T cell response. A single CD8$^+$ T cell clone may be able to recognize a number of variations of a peptide/MHC complex due to the inherent flexibility of the TCR and the small area of contact between the TCR and MHC/peptide complex (11–13). Despite this, single amino acid mutations within critical TCR contact sites have been shown to abrogate or functionally alter T cell recognition. A CTL response composed of a broad variety of TCRs can enhance recognition of amino acid changes within the peptide/MHC complex, because different TCRs can contact different amino acid residues within the same peptide and have differing susceptibilities to mutations (13–16).

In this study, we investigated whether there is a correlation between the diversity of TCRs expressed by virus-specific responses and their capacity to tolerate substitutions within the epitope. We present the first analysis of clonality of CD8 cells in HIV-2, which is compared with that in HIV-1 infection by both molecular and functional approaches. We find that restricted TCR usage in the CD8 response to HIV-1 at both the global and epitope level is associated with limited ability to recognize amino acid changes within these epitopes. The CD8 response to HIV-2 infection appears to be

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more polyclonal, correlating with enhanced cross-recognition of epitope variants.

Materials and Methods

Patients

Patients attending the Mortimer Market Center in the U.K. and the Medical Research Council clinic in The Gambia were initially tested by a combined HIV-1 and HIV-2 enzyme immunoassay (Wellcozyme 1 + 2; Murex Diagnostics, Dartford, Kent, U.K.). The diagnosis of HIV-2 infection was made on the basis of repeatedly negative HIV-1 and positive HIV-2 competitive ELISAs following a positive combined test (Murex Diagnostics). HIV-1- and HIV-2 infected patients were all clinically asymptomatic (with no HIV-related symptoms or signs) and had never received any antiretroviral therapy. Patients had repeated CD4 counts >350 cells/μl determined by flow cytometry using the Tritest program (BD Biosciences, San Diego, CA; data not shown). Viral loads were measured by the Chiron Quantiplex HIV RNA assay (b DNA) version 3.0 for HIV-1 and as described previously for HIV-2 (5). For HLA typing, DNA was extracted from blood samples using the Puregene kit (Flowgen, Ashby Park, Leicestershire, U.K.). Between 200 and 500 ng of DNA was used for HLA typing by a molecular PCR method that used 144 sequence-specific primer mixes (PCR-SSP). The study was approved by both local research ethics committees and all patients gave informed consent.

Heteroduplex analysis

This was performed as previously described (17), with modifications as specified below. Where required, CD4- or CD8-purified cells were obtained by positive selection using the Minimacs system (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions (>95% purity on mAb staining). RNA was extracted from 0.5×10^6 PBMC (or CD4- or CD8-purified cells) using the Dynabeads mRNA Direct Kit (Dynal Biotech, Oslo, Norway) according to manufacturer's instructions. Twenty-six RT-PCRs across the β-chain complementarity-determining region 3 (CDR3)[3] were conducted for each analysis, in a final volume of 50 μl and using 3% of the cDNA per reaction. Twenty microliters of the heteroduplex reactions and of the nondenatured C region control were loaded on a 12% nondenaturing polyacrylamide gel (National Diagnostics, Atlanta, GA), with a 4% stacking gel, run at 10 mA for 16 h at 4°C. Heteroduplex gels were visualized on a FluorS MultiImager (Bio-Rad, Hercules, CA) following ethidium bromide staining, and densitometrically analyzed using Quantity One software (Bio-Rad). Gels were analyzed without the usual Southern blotting and carrier hybridization (17) to reduce the resolution of the technique and limit the detection of small clones. Individual TCRVβ tracks were classified as "oligoclonal" if they had three or fewer clones detectable (six or less heteroduplex bands, since each clone is usually represented by a pair of bands). Heteroduplex bands were only counted if they were of equal or greater intensity than that of a standard dilution of the PCR product across the C region of the β-chain (C region control).

Production of T cell lines and intracellular IFN-γ staining

PBMCs were seeded at 0.3×10^6/200 μl/well in 96-well round-bottom plates in the presence of 0.5 μM of the relevant index or analog peptide. Peptides were purchased from Chiron Mimotopes (Clayton, Victoria, Australia). Purity of peptides was >90% by HPLC analysis. Recombinant IL-2 (10 IU/ml; Boehringer Mannheim, Mannheim, Germany) was added on day 3. Cells were restimulated on day 10 with 0.5 μM index or substituted peptides for 5 h, the last 4 h with 10 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO). Cells were then stained with anti-CD8 mAb (BD PharMingen, San Diego, CA), permeabilized with Cytoperm-cytofix (BD PharMingen), before staining with anti-IFN-γ mAb (R&D Systems, Minneapolis, MN) and analyzing on a FACScan (BD Biosciences) using CellQuest software. For Vβ analysis, the cells were incubated before restimulation with a panel of 17 FITC-conjugated anti-TCRVβ mAb (Immunotech, Marseille, France).

Staining with HLA-A2/peptide tetrameric complexes

The HLA-class I tetramer (HLA-A2/gag 77–85) was supplied by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Bethesda, MD). Tetramer staining of PBMCs was conducted for 20 min at 37°C followed by staining with anti-CD8 mAb and the panel of anti-Vβ mAb.

Selection of IFN-γ-positive cells

Nine million PBMCs from a patient previously shown to have a response to the gag 77–85 peptide were cultured for 10 days as described above. Ag-specific cells were isolated using the IFN-γ Secretion Assay Cell Enrichment and Detection kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, day 10 cultures were restimulated with the gag 77–85 peptide (0.5 μM) for 5 h, followed by addition of the IFN-γ catch reagent (5 min, on ice). This was left for 45 min at 37°C with the cells maintained in constant motion followed by labeling with anti-IFN-γ mAb (10 min, ice). Subsequently, anti-PE microbeads were bound to the labeled cells and purified using Minimacs MS^+RS^+ columns.

TCR sequencing

The Vβ5.1 PCR product was cleaned-up by a PCR Purification kit (Qiagen, Valencia, CA) according to manufacturer's instructions before sequencing. Nucleic acid was eluted in Tris-HCl and sequenced in both directions using Vβ5.1- and Cβ-specific primers (Wolfson Sequencing Unit, University College London, London, U.K.).

Generation of peptide-MHC models

The HLA-A2 crystal structure has previously been published (18). Structures were predicted for the HLA-B57 and -B58 molecules based on the published crystal structures of a number of MHC class I molecules available in the Research Collaboratory for Structural Bioinformatics protein data bank (19). Both protein models were built using the program modeler (20). Peptides were then modeled into the peptide-binding groove of each of these different HLA alleles and were energy-minimized in a solvent bath using the molecular mechanics program AMBER (University of California, San Francisco, CA).

HLA binding assay

Peptide binding to HLA-A2 was assessed using a FACS-based MHC stabilization assay (21) with modifications as described below. Briefly, T2 cells were incubated in 96-well flat-bottom plates at $1–1.2 \times 10^5$ cells per well in a 200 μl of volume of AIM V medium (Life Technologies, Paisley, U.K.) with human β_2-microglobulin at a final concentration of 100 nM (Scipac, Sittingbourne, U.K.) with and without peptides at concentrations between 200 and 0.04 μM for 16 h at 37°C. Cells were then washed and surface levels of HLA-A2 were assessed by staining with FITC-conjugated A2.1-specific mAb BB7.2 (BD Biosciences, Oxford, U.K.) or a FITC-conjugated isotype control Ab (BD Biosciences). Cells were fixed at 4°C in 1% paraformaldehyde and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. Results are expressed as fluorescence index (FI) values, calculated as the test mean fluorescence intensity (MFI) minus the no peptide isotype control MFI divided by the no peptide HLA-A2-stained control MFI minus the no peptide isotype control MFI.

Results

Molecular dissection of the global ex vivo TCR repertoire shows more oligoclonal $CD8^+$ T cell expansions in HIV-1- than HIV-2-infected individuals

Cryopreserved PBMC from 10 patients attending the outpatient clinic at the Medical Research Council (The Gambia) and 2 patients attending the outpatient clinic at University College Hospitals were used for this initial part of the study. All had asymptomatic HIV infection (six with HIV-1, six with HIV-2), with well-preserved CD4 counts and were antiretroviral naive. The RT-PCR-based heteroduplex technique (17) was used to compare the overall breadth of TCR usage of the CD8 response in HIV-1 or HIV-2 infection directly ex vivo, regardless of epitope specificity. Dissection of overall clonality within each Vβ family resulted in large expanded clones being detectable as bands with unique migration patterns, whereas polyclonal populations formed multiple heteroduplex bands resulting in a smear pattern on ethidium bromide-stained gels (Fig. 1*A*). As exemplified in Fig. 1*A*, HIV-1 patients had oligoclonal expansions represented by prominent bands in most Vβ tracks, whereas HIV-2 PBMC showed a predominant smear pattern in the majority of tracks.

To investigate whether the expansions detected represented CD8 clones, PBMC from four HIV-1 and four HIV-2 patients were fractionated into $CD4^+$ and $CD8^+$ subsets (purity >97%) and

[3] Abbreviations used in this paper: CDR3, complementarity-determining region 3; FI, fluorescence index; MFI, mean fluorescence intensity.

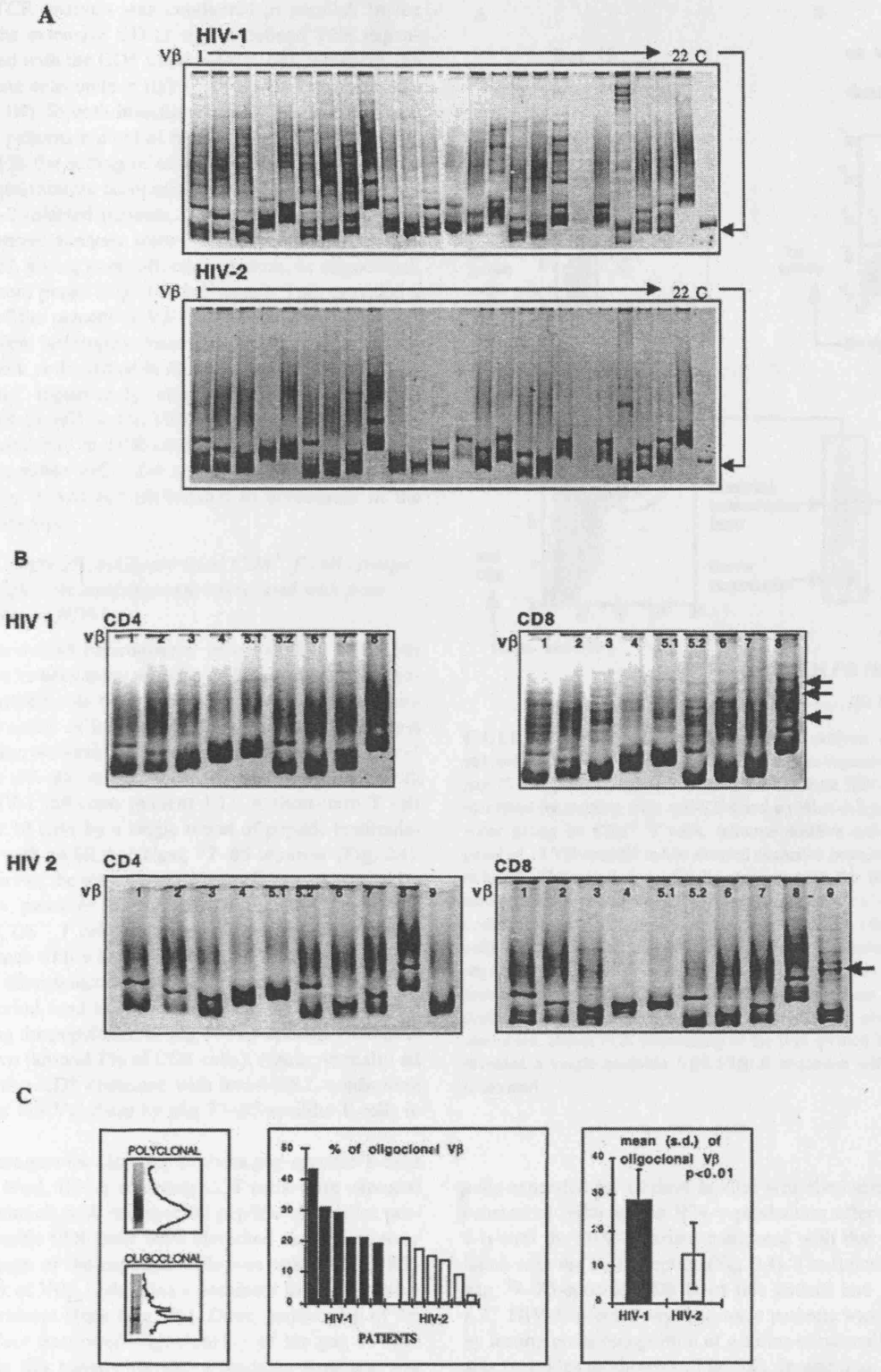

FIGURE 1. Global heteroduplex analysis of TCR heterogeneity in HIV-1 and HIV-2 infection. *A*, Ethidium bromide-stained gels show RT-PCR heteroduplex samples Vβ1–22 from a representative HIV-1 and HIV-2 patient (with the TCRVβ primer and carrier used indicated above the tracks). In the HIV-1 patient, there are distinct heteroduplices showing a specific migration pattern above the two dense homoduplex bands in most tracks, whereas in the HIV-2 patient polyclonal smear patterns are demonstrated above the homoduplex bands within most Vβ. The input of total TCR β-chain mRNA was similar, as suggested by the PCR control across the Cβ region (track C, arrowed). *B*, Heteroduplex analysis of purified $CD4^+$ and $CD8^+$ T cells are shown for Vβ1–8 for an HIV-1 and Vβ1–9 for an HIV-2 patient, demonstrating segregation of all heteroduplex bands with the $CD8^+$ subset (examples arrowed in the last track). *C*, Densitometric profiling was applied to categorize each Vβ track into polyclonal (*upper left*) or oligoclonal (*lower left*) patterns. The bar chart (*left*) shows the percent of Vβ tracks with an oligoclonal pattern for six HIV-1 patients and six HIV-2 patients, with the mean and SD for each group plotted (*right*). Differences between the two groups of patients were analyzed using the Mann-Whitney *U* test.

their molecular TCR analysis was conducted in parallel. In the HIV-1 patients, the extensive CDR3 region defined TCR expansions all segregated with the CD8 fraction (Fig. 1*B*). Similarly, the few clones that were detectable in HIV-2 were also restricted to the CD8 subset (Fig. 1*B*). In both infections, the CD4 subset retained polyclonal smear patterns typical of healthy controls, as was also recently observed in the setting of acute EBV infection (17).

To attempt a quantitative comparison of the CD8 clonality in HIV-1- and HIV-2-infected patients, densitometric profiling and blinded dual observer analysis were applied to categorize each track as polyclonal, giving a smooth curve pattern, or oligoclonal, producing prominent peaks (Fig. 1*C*, *left panel*). This provided a summary figure of the percent of Vβ tracks with oligoclonal patterns for each patient (arbitrarily defined as three or fewer detectable clones per track as described in *Materials and Methods*). This analysis confirmed significantly more oligoclonally restricted TCRVβ responses overall in the HIV-1 than the HIV-2 cohort (Fig. 1*C*). The variability in TCR clonality within each group of patients did not correlate with CD4 count or viral load (data not shown), suggesting it was not attributable to differences in the level of viral stimulation.

TCR usage for a single HLA-A2-restricted CD8$^+$ T cell epitope demonstrates a highly focused response associated with poor functional flexibility in HIV-1

The differences in overall heterogeneity of responding CD8 cells highlighted by the heteroduplex analysis of HIV-1 and HIV-2 patients could be attributable to differences in the multispecificity and/or the polyclonality of individual CTL responses. To address the latter possibility, we initially studied the response to the HIV-1 p17 gag epitope 77–85 in an HLA-A2-positive patient with asymptomatic HIV-1 infection (patient 1.1). A short-term T cell line expanded for 10 days by a single round of peptide restimulation was stained with an HLA-A2/gag 77–85 tetramer (Fig. 2*A*). The TCR repertoire of the tetramer-positive cells was dissected by costaining with a panel of 17 anti-Vβ-specific mAbs. All the epitope-specific CD8$^+$ T cells stained with the anti-Vβ5.1 mAb (Fig. 2*A*), consistent with a highly focused TCR usage.

To exclude in vitro selection for Vβ5.1 clonotypes during the limited culture period used to expand the CTL, the same analysis was repeated using the population of gag 77–85-specific CD8 cells identifiable ex vivo (around 1% of CD8 cells). Again, virtually all the tetramer-positive CD8 costained with anti-Vβ5.1, confirming restricted usage of this Vβ chain by gag 77–85-specific T cells in vivo (Fig. 2*B*).

To further investigate the clonality of these gag-specific T cells at the molecular level, IFN-γ-secreting CD8 cells were captured following restimulation with the specific peptide. A similar proportion of Ag-specific CD8 cells were identified as with tetramer staining. TCR usage of the captured cells was analyzed by RT-PCR heteroduplex of Vβ5.1, showing a dominant band suggestive of a single predominant clone (Fig. 2*C*). Direct sequencing of the Vβ5.1 PCR product confirmed oligoclonality of the gag 77–85-specific T cells in this patient because a readable sequence was obtained without the need for PCR cloning (Fig. 2*C*). Surprisingly, this sequence was identical (even across the highly variable CDR3 region) to that identified from a gag 77–85-specific CTL clone in a previous study on an unrelated patient (22). This would suggest the potential for a marked degree of conservation between individuals in this TCR response, as noted in the response to an HLA-B8-restricted EBV epitope (23).

The highly oligoclonal CD8 cells specific for the gag 77–85 epitope in this patient (1.1) were then tested for their ability to cross-recognize the naturally occurring HIV-2 epitope variant. T

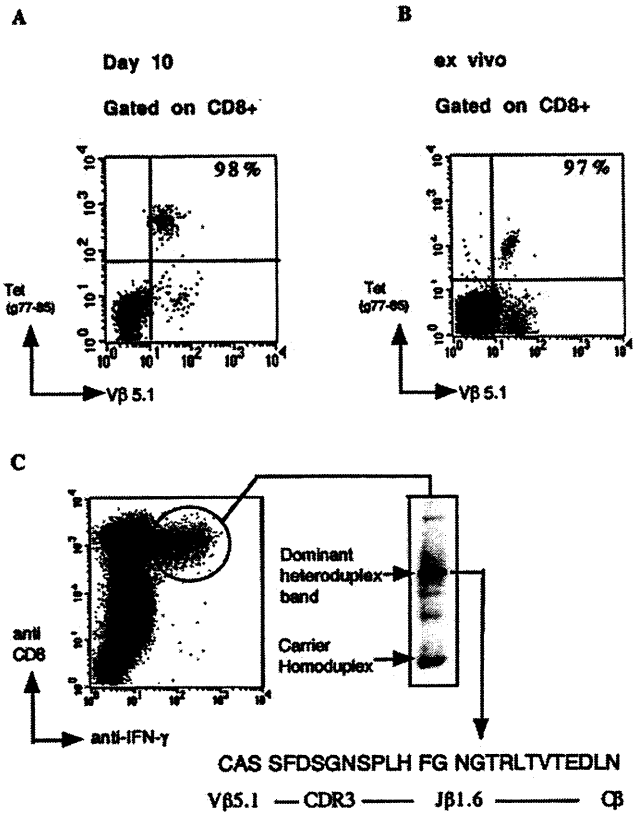

FIGURE 2. Cellular and molecular TCR analysis of an HLA-A2-restricted gag response in HIV-1. *A*, After 10 days expansion in vitro with the gag 77–85 peptide, epitope-specific T cells from HIV-1 patient 1.1 were identified by staining with anti-CD8 and an HLA-A2/gag 77–85 tetramer. After gating on CD8$^+$ T cells, tetramer-positive cells costained with a panel of 17 Vβ-specific mAbs showed exclusive costaining with the Vβ5.1 mAb. *B*, The population of cells staining with the HLA-A2/gag 77–85 tetramer after gating on CD8$^+$ T cells directly ex vivo were also all costained by the Vβ5.1 mAb and not by any of the other Vβ mAbs. *C*, T cells specific for the gag 77–85 epitope were selected using the IFN-γ secretion assay cell enrichment kit and heteroduplex analysis was performed on the Vβ5.1 RT-PCR product. A prominent heteroduplex band (indicating oligoclonality, arrowed) was visualized above the carrier homoduplex. Direct PCR sequencing of the IFN-γ catch Vβ5.1 PCR product revealed a single readable Vβ5.1/Jβ1.6 sequence with CDR3 region as illustrated.

cells expanded for 10 days in vitro with the index peptide showed substantial reduction in IFN-γ production after restimulation for 6 h with the HIV-2 variant compared with that seen on restimulation with the index peptide (Fig. 3*A*). The functional flexibility of gag 77–85-specific CD8 from this patient and four other HLA-A2$^+$ HIV-1-infected asymptomatic patients was further evaluated by testing cross-recognition of a series of naturally occurring variants (peptides as shown in Fig. 3*B*). In addition, alanine scanning mutagenesis was used to test the recognition of a series of peptides with single successive alanine substitutions in potential TCR contact sites (derived from the computer-generated model of the peptide bound to HLA-A2; Fig. 3*B*). There was poor cross-recognition of the peptide representing the HIV-2 variant in all cases, and in some patients even of the A clade variant with a single Y to F conservative substitution (Fig. 3*C*). Similarly, the alanine-substituted variants of the HIV-1 gag 77–85 epitope substantially abrogated recognition, apart from the conservative mutation V to A at position 82 (Fig. 3*C*). The highly restricted CD8 cells from patient

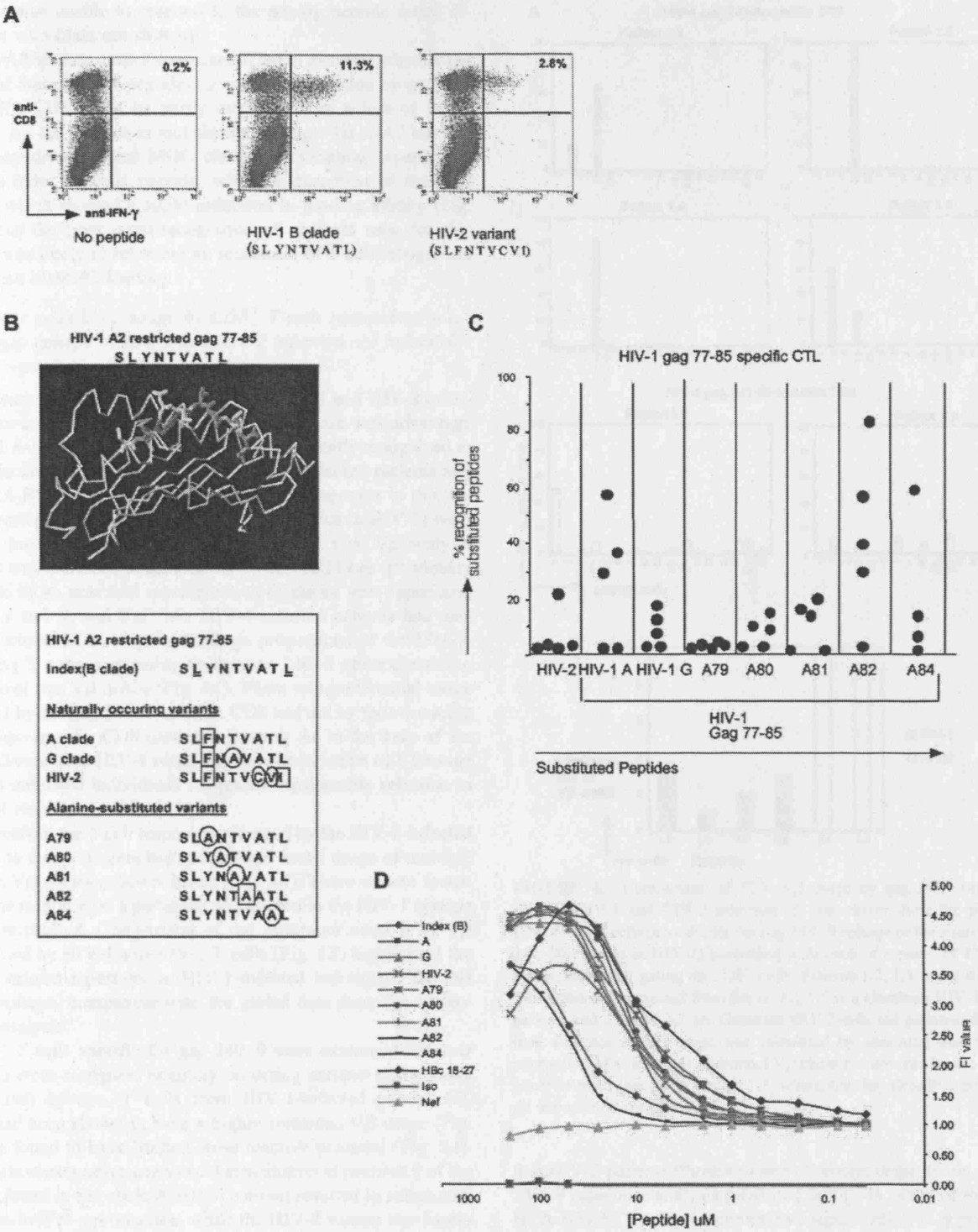

FIGURE 3. Ability of T cells responsive to an HLA-A2-restricted epitope in HIV-1 to cross-recognize naturally occurring and alanine-substituted epitope variants. *A*, The percent of gag 77–85-responsive CD8 cells (from patient 1.1) producing IFN-γ on restimulation with the indicated peptides are shown in the *upper right quadrants* of the FACS plots. *B*, Computer-generated model of the HIV-1 clade B consensus gag 77–85 peptide bound to HLA-A2. The epitope peptide is in an N to C orientation, with hydrophobic aliphatic amino acids (ALA, LEU, VAL) shown in cyan, hydrophobic aromatic residues (TYR) in green, and small polar amino acids (ASN, SER, THR) in yellow. The HIV-1 gag 77–85 index, naturally occurring and alanine-substituted analog sequences are listed, with anchor residues underlined, conservative amino acid substitutions boxed, and nonconservative substitutions circled. *C*, Functional flexibility of HIV-1 gag 77–85-specific CD8 from five HLA-A2$^+$ asymptomatic HIV-1 patients. The ability of index peptide-responsive short-term T cell lines to produce IFN-γ in response to restimulation with the variant peptides shown in (*B*) are represented as a percentage of the response to the index (unmutated) peptide after subtraction of background responses with no peptide. *D*, Comparison of the binding of the HIV-1 gag 77–85 epitope peptide and naturally occurring and alanine-substituted analogs to HLA-A2 using a T2 cell MHC stabilization assay. T2 cells were incubated overnight with the HIV-1 gag 77–85 index peptide, indicated variants (as in *B*), or with an HLA-B58-restricted epitope peptide from HIV-1 Nef (Nef; KAAVDLSHF) or an HLA-A2-restricted epitope peptide from the hepatitis B virus core protein (HBc 18–27; FLPSDFFPSV) as negative and positive controls, respectively. Surface HLA-A2 expression was assessed by staining with an A2-specific mAb and nonspecific binding with an isotype control mAb (iso). The results are expressed as FI values, calculated as described in *Materials and Methods*.

1.1 were also unable to respond to the analog peptide series directly ex vivo (data not shown).

HLA-A2 binding assays were conducted to evaluate whether the ability of these variant peptides to escape recognition by gag 77–85-specific CD8 could be partly attributable to a loss of MHC binding. All the variants in fact showed stronger HLA-A2 binding in T2 peptide-dependent MHC class I stabilization experiments than the index B-clade peptide, with the exception of the A82 variant, which showed a slight reduction in binding affinity (Fig. 3*D*). Thus the poor cross-recognition of variants seen for this epitope was likely to represent an inhibition of T cell recognition rather than of MHC binding.

Differences in TCR Vβ usage by CD8$^+$ T cells responding to a shared gag epitope in HIV-1 and HIV-2 infection are reflected in their capacity for cross-recognition

To compare the breadth of TCR usage in HIV-1 and HIV-2 infection at the level of a single CD8$^+$ cell response, we took advantage of an HLA-B57/58-restricted gag epitope frequently recognized in both infections. Four HLA-B57/58$^+$ HIV-1-infected patients and two HLA-B58 HIV-2-infected patients with responses to the gag 240–9 epitope (or its equivalent 241–50 sequence in HIV-2) were studied. Insufficient cells were available for ex vivo Vβ analysis and this was therefore conducted on 10 day cell lines (previously shown to be an unbiased representation of the ex vivo repertoire; Fig. 2, *A* and *B*, and Ref. 24). HIV-1-infected patients had narrowly focused TCR usage, with large proportions of the IFN-γ-producing T cells responding to the gag 240–9 epitope staining with one or two Vβ mAbs (Fig. 4*A*). There was preferential usage of Vβ5.1 by the gag 240–9-specific CD8 and not by the remaining nonepitope-specific CD8 (data not shown). As in the case of the HLA-A2-restricted HIV-1 response, this conservation of Vβ usage between unrelated individuals suggested considerable selection in the TCR response to this epitope.

By contrast, the T cell responses mounted by the HIV-2-infected patients to the equivalent epitope showed broad usage of multiple different Vβ chains at lower levels (Fig. 4*A*). There was no focusing of the response on a particular Vβ as seen in the HIV-1 epitope responses studied. Comparison of the minimum number of Vβ chains used by HIV-1 and HIV-2 T cells (Fig. 4*B*) highlighted the more restricted repertoire in HIV-1-infected individuals for this shared epitope, compatible with the global data from the heteroduplex analysis.

CD8$^+$ T cells specific for gag 240–9 were examined for their ability to cross-recognize naturally occurring variants of the index (unmutated) epitope. T cells from HIV-1-infected patient 1.2, which had been shown to have a highly restricted Vβ usage (Fig. 4), were found to have limited cross-reactive potential (Fig. 5*A*). The single conservative amino acid substitution at position 9 of the epitope found in the clade A HIV-1 variant resulted in substantial reduction in IFN-γ production, while the HIV-2 variant was barely recognized by the index epitope-responsive CTL (Fig. 5*A*). Serial peptide titrations showed that cross-recognition was not enhanced at alternative concentrations, including when using a 10-fold higher concentration (data not shown). By contrast, the T cells responding to the equivalent epitope from HIV-2 patient 2.1, expressing a broad repertoire of TCR (Fig. 4), showed efficient production of IFN-γ on restimulation with both the A and B clade HIV-1 variants, despite the presence of three mutations (one nonconservative) at potential TCR contact sites (Fig. 5*A*). Consistent data were obtained on testing for cross-recognition directly ex vivo (data not shown).

Fig. 5*B* shows the summary of testing for cross-recognition by gag 240–9-specific T cells as described above for five HIV-1 and

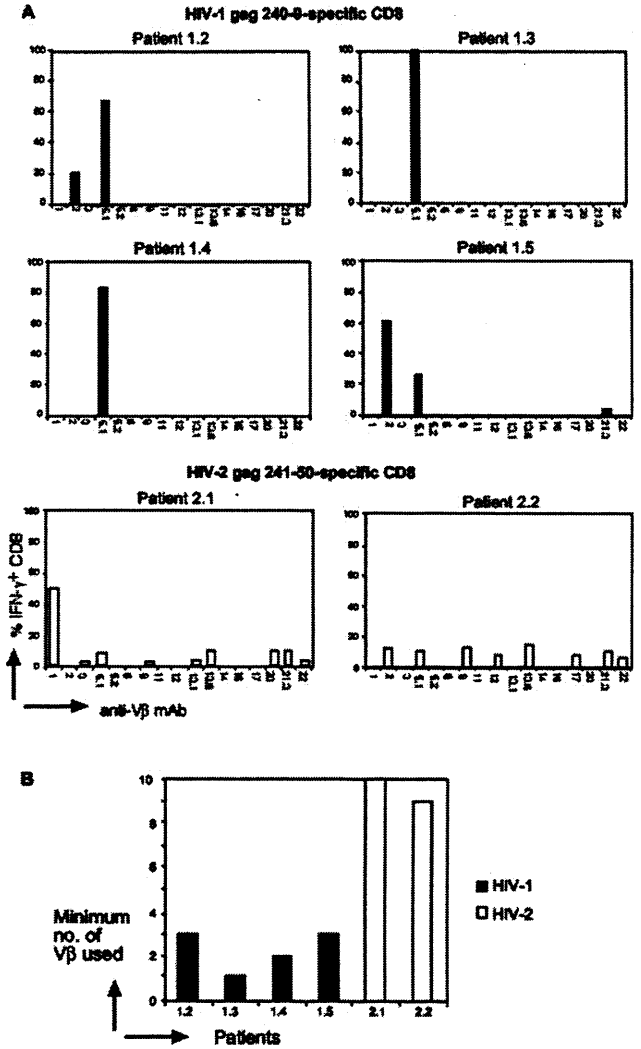

FIGURE 4. Comparison of TCR Vβ usage by gag 240–9-specific T cells in HIV-1 and HIV-2 infection. *A*, Bar charts show the percent of IFN-γ$^+$ CD8 cells (specific for the gag 240–9 epitope or the equivalent gag 241–50 epitope in HIV-2) costaining with each of a panel of 17 anti-Vβ mAbs, following gating on CD8$^+$ cells. Patients 1.2, 1.3, and 1.4 are HIV-1-infected and recruited from the U. K., 1.5 is a Gambian HIV-1-infected patient, and 2.1 and 2.2 are Gambian HIV-2-infected patients. *B*, A minimal estimate of Vβ usage was calculated by assuming that there was expression of at least one additional Vβ chain not covered by the available panel of mAb (except in patient 1.3, where staining already accounted for all the epitope-specific CD8).

five HIV-2 patients (three and two of whom, respectively, had gag 240–9 repertoire analysis presented in Fig. 4). None of the HIV-1 HLA-B57/58$^+$ patient samples had significant IFN-γ production on exposure to the HIV-2 variant at this epitope (Fig. 5*B*). However, five of six of the HLA-B58$^+$ HIV-2 patient samples showed efficient cross-recognition of the HIV-1 variant epitope (Fig. 5*B*). In all these cases, responses to the variant epitope were seen at low peptide concentration (0.5 μM), indicating that the cross-reactive interaction was of a similar high avidity to the response to the HIV-2 peptide. Thus the ability to recognize the extensive amino acid changes between the HIV-1 and HIV-2 epitope correlated with the broader TCR Vβ usage by these T cells in HIV-2 patients (Fig. 4).

The HIV-1 responses exhibited restricted Vβ usage and lack of cross-reactivity even when sampled from HIV-1-infected patients with well-preserved CD4 counts and low viral loads (e.g., patient

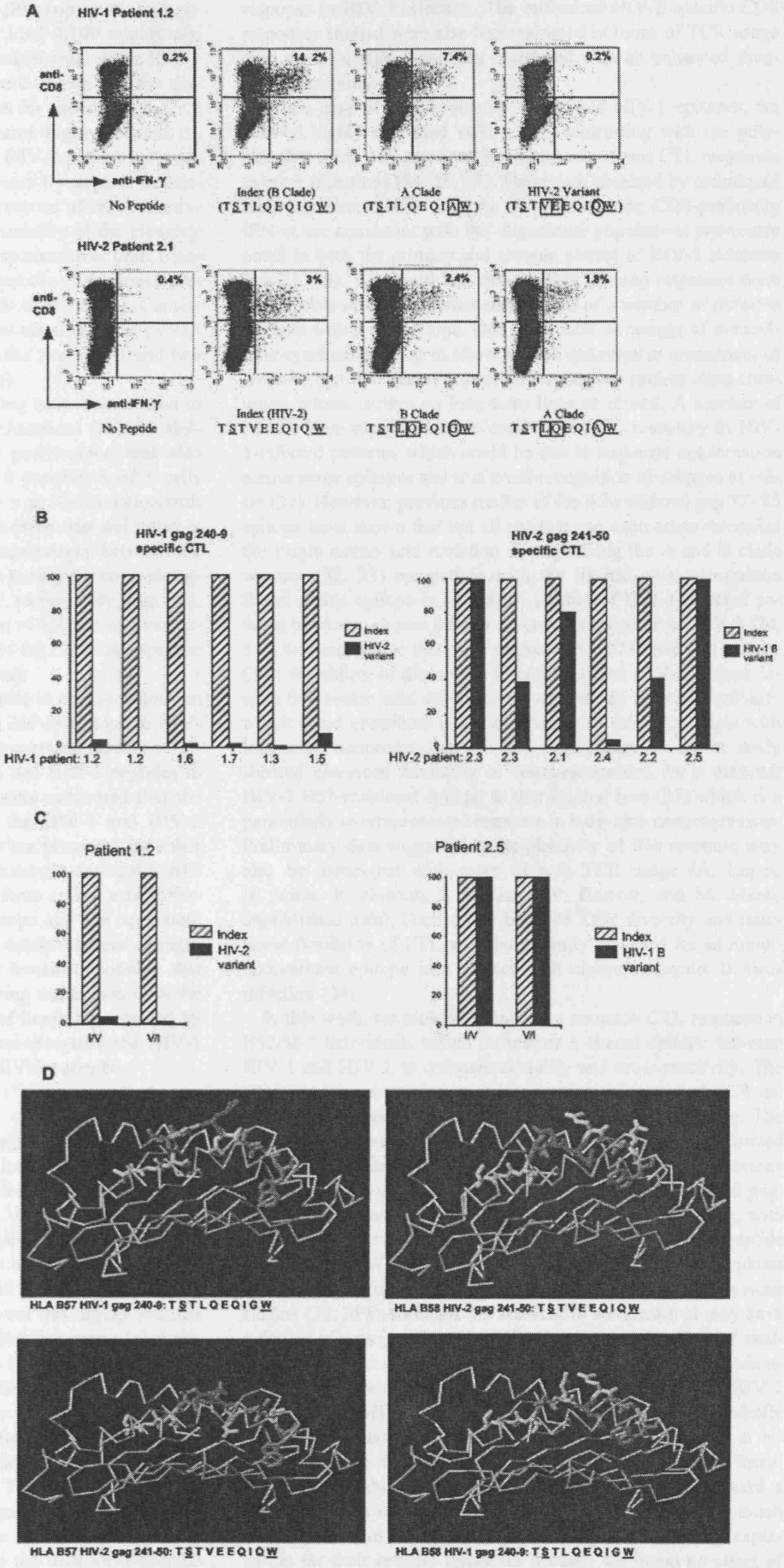

FIGURE 5. Comparison of the ability of T cells from HIV-1- and HIV-2-infected individuals responsive to a shared gag epitope to cross-recognize peptides from different HIV types. *A*, Responsiveness of T cells from an HIV-1-infected patient (1.2) and an HIV-2-infected patient (2.1) to HIV-1 and -2 epitope variants. The percent of CD8 cells producing IFN-γ on restimulation with the indicated peptides are shown in the *upper right quadrants* of the FACS plots. The peptide sequences are shown, with anchor residues underlined, conservative amino acid substitutions boxed, and nonconservative substitutions circled. *B*, Cross-recognition of the HIV-2 ROD-variant by HIV-1-responsive T cells (*left bar chart*) and of the HIV-1 consensus B clade variant by HIV-2-responsive T cells (*right bar chart*). The recognition on restimulation with the optimal index peptide is defined as 100%, and the proportion of these T cells able to produce IFN-γ on restimulation with the variant peptide is represented as a percentage of this (following subtraction of the background IFN-γ production on restimulation in the presence of brefeldin A without peptide). The optimal peptide response generated for HIV-1 patient 1.5 recruited from The Gambia was the A clade variant with which he was likely to have been infected. Repeat experiments on samples obtained on follow-up are shown for HIV-1 patient 1.2 (12 mo later) and HIV-2 patient 2.3 (30 mo later). *C*, Ability of HIV-1 gag 240–9-specific T cells and HIV-2 gag 241–50-specific T cells to expand on stimulation with the HIV-2/1 variant peptides, respectively, and cross-recognize the index peptide at day 10, expressed as a percentage of expansion induced by the index peptide (designated V/I, presented with results obtained as in *B*, designated I/V). *D*, Computer-generated models of the HIV-1 clade B consensus gag 240–9 peptide and the HIV-2 ROD gag 241–50 peptide bound to HLA-B57 (*left*), compared with their orientation when bound to HLA-B58 (*right*). The epitope peptides are in an N to C orientation, with hydrophobic aliphatic amino acids (LEU, ILE, VAL) shown in cyan, hydrophobic aromatic residues (TRP) in green, acidic residues (GLU) in red, small polar amino acids (GLN, SER, THR) in yellow, and sterically constrained residues (GLY) in orange.

1.2: CD4 count 680 cells/μl, viral load 1,500 copies/ml; and patient 1.6: CD4 count 450 cells/μl, viral load 3,100 copies/ml); HIV-specific CD4 responses were not tested. Several of the HIV-1 patients recruited from the U.K. clinic were known to have disparate durations of infection (e.g., >7 years for patient 1.6 and <2 years for patient 1.7) but exhibited the same highly focused responses (Fig. 5*B*). HIV-1 patient 1.2 and HIV-2 patient 2.3 were tested at two time points separated by at least 1 year, and demonstrated temporal stability of the lack or presence of cross-reactivity, respectively (Fig. 5*B*). This suggests stability of the clonality and resultant cross-reactive potential of responses over time, compatible with the long lifespan of HIV-1-specific CD8 clones previously demonstrated in asymptomatic infection (22, 25). Consistent with this, heteroduplex analysis showed stability of the overall clonal pattern over a year of follow-up in the two HIV-1 and two HIV-2 patients examined (data not shown).

Because variable levels of TCR triggering have been shown to be required to elicit different CD8 effector functions (26), the ability of mutated epitopes to induce CD8 proliferation was also tested. The ability of variants to expand a population of T cells directly ex vivo capable of producing IFN-γ on restimulation with the index peptide was again compared with expansion and restimulation with the index peptide. There was consistency between the results obtained with this method and that testing for cross-recognition at 10 days (indicated as V/I and I/V, respectively; Fig. 5*C*). Similar results of flexible cross-recognition of epitope variants using both of these approaches were observed for CD8 cells specific for another HIV-2 epitope (data not shown).

To further examine the striking differences in cross-recognition of the HIV-1/2 variants of the shared gag 240–9 epitope in HIV-1/2-infected patients, we used computer-generated models to visualize the binding of the HIV-1 clade B and HIV-2 peptides to HLA-B57 and B58 (Fig. 5*D*). These models confirmed that the three amino acid substitutions between the HIV-1 and HIV-2 epitopes would be at TCR binding sites when presented by either HLA-B57 or B58. These models of the three-dimensional MHC/peptide structure also predicted that the three amino acid differences between the HIV-1 gag 240–9 epitope and the equivalent epitope from HIV-2 would result in major conformational changes at the TCR contact surface. Although it remained possible that peptide conformations altered in vivo during interaction with the TCR, these data supported the high level of flexibility required by the HIV-2-specific CD8 to efficiently cross-recognize the HIV-1 variant as demonstrated in a number of HIV-2 patients.

Discussion

There is mounting evidence in favor of the key role of the CD8 T cell response in successful control of HIV infection. However, loss of viral control and disease progression clearly can occur despite ongoing strong, functionally active CTL. Even though such responses are often multispecific, viral mutants still appear to be a frequent means of escape. Therefore, we addressed whether the clonality of individual CD8 responses could be one factor affecting their plasticity and hence potential to control this highly variable virus. A previous study had suggested a link between global oligoclonality and poor viral control in HIV-1 infection (27). In this study, we explored a possible mechanism for this association, suggesting a link between the clonality of a CTL response and its ability to cross-recognize variant HIV epitopes. We found a limited capacity for cross-recognition of variant epitopes by HIV-1-specific CD8 with oligoclonal TCR usage. This contrasted with the findings in HIV-2-infected individuals, a generally well-controlled retroviral infection. A molecular analysis of TCR heterogeneity identified fewer oligoclonal expansions in the total virus-specific response to HIV-2 infection. The individual HIV-2-specific CD8 responses studied were also less restricted in terms of TCR usage than those in HIV-1 and this correlated with an enhanced functional flexibility.

In the case of two frequently recognized HIV-1 epitopes, we showed highly restricted TCR usage, contrasting with the polyclonality often demonstrated for immunodominant CTL responses in other infections (24, 28, 29). These data obtained by techniques allowing direct TCR analysis of virus-specific CD8-producing IFN-γ, are consistent with the oligoclonal populations previously noted in both the primary and chronic phases of HIV-1 infection (22, 25, 30). We found that these highly focused responses were not capable of efficient cross-recognition of a number of different variants within the epitope. Our study took advantage of intracellular cytokine staining to allow a more quantitative assessment of cross-recognition than was possible in previous studies using chromium release assays on long-term lines or clones. A number of studies have suggested some cross-clade CTL reactivity in HIV-1-infected patients, which could be due to sequence conservation across some epitopes and true cross-recognition of changes at others (31). However, previous studies of the A2-restricted gag 77–85 epitope have shown that not all patients can even cross-recognize the single amino acid mutation distinguishing the A and B clade variants (32, 33) compatible with the limited cross-recognition found at this epitope in our study. Studies of HIV-1-infected patients have also shown little or no cross-recognition of HIV-2 (34, 35). An exception to this is the response to a B27-restricted epitope (36); the ability of these CTL to recognize the HIV-2 variant despite five amino acid substitutions suggests an unusual flexibility which could contribute to the association of this HLA allele with long-term nonprogression of HIV-1 infection. A recent study showed enhanced flexibility of cross-recognition for a different HIV-1 B57-restricted epitope to that studied here (37) which is a particularly overrepresented response in long-term nonprogressors. Preliminary data suggest that the plasticity of this response may also be associated with more diverse TCR usage (A. Lopes, N. Jones, P. Newton, I. Williams, P. Borrow, and M. Maini, unpublished data). Such a link between TCR diversity and functional flexibility of CTL was also recently observed for an immunodominant epitope in a patient with chronic hepatitis B virus infection (24).

In this work, we took advantage of a common CTL response in B57/58$^+$ individuals which represents a shared epitope between HIV-1 and HIV-2 to compare clonality and cross-reactivity. The HIV-2 responses studied were less restricted in terms of TCR usage and this correlated with enhanced functional flexibility. The efficient cross-reactivity of T cells specific for the B58-restricted gag 241–50 epitope in HIV-2 extends the data from a previous study in HIV-2-infected patients, the majority of whom had gag-specific CTL lines capable of cross-recognizing HIV-1 gag, with some having lytic responses to the gag 240–252 region on peptide mapping (38). We observed some variability between individuals in the amount of cross-recognition as suggested from previous studies (38, 39). Although the differences we identified may have a degree of epitope selectivity (40, 41), the global molecular analysis points to an overall reduction in large oligoclonal expansions in the total virus-specific response to HIV-2 compared with HIV-1 infection. The HIV-1 and HIV-2 patients tested were all clinically HIV asymptomatic and had similar ages and CD4 counts at recruitment, but seroconversion dates were often not known; therefore it is possible that the HIV-2 sample was biased toward a longer duration of infection because they typically have a much slower decline in CD4 numbers. This would be an unlikely explanation for their broader responses because we found no effect of

duration of infection in the cases examined, and the existing literature points to a maintenance or narrowing of CD8 repertoires on prolonged or repeated pathogen exposure (16, 17, 23, 42, 43).

Perhaps the most plausible explanation for the differences in breadth and functional flexibility of $CD8^+$ T cell responses observed in HIV-1- and HIV-2-infected individuals is a difference in the availability of HIV-specific CD4 help in the two infections. Limited availability of CD4 help during CTL priming could result in expansion of a narrower CD8 response. This might be restricted to T cell clones bearing high affinity TCR, which would be consistent with the tendency to select highly conserved TCRs in the HIV-1 responses we studied and in a recent analysis of an immunodominant SIV response (43). CD4 help has been shown to be critical for the persistence of functionally active CTL (reviewed in Ref. 7), so it is possible that paucity of CD4 help may also limit the maintenance of polyclonal, broadly cross-reactive CTL. HIV-2-specific CD4 proliferative responses are well-preserved and broadly cross-reactive (44), whereas HIV-1-specific CD4 responses are depleted early in infection (45), even when CD4 numbers remain well-preserved. Thus it will be important to test whether greater availability of HIV-specific CD4 help can enhance the breadth and plasticity of individual CTL responses. In this context, a recent study identified several epitope-specific clonotypes able to recognize a variant containing two amino acid substitutions in an HIV-1 patient undergoing intermittent antiretroviral therapy (16).

We demonstrated efficient cross-recognition of the HIV-1 epitope by HIV-2-specific CD8 for a frequently recognized epitope (38) in individuals expressing a common Gambian HLA allele. Recent data confirm that this type of broad response capable of cross-recognizing mutated epitopes can be generated de novo after exposure to the wild-type sequence alone (46). This ability of HIV-2-specific CTL to cross-recognize HIV-1 variants could contribute to a degree of cross-protection to HIV-1 proposed to result from pre-existing HIV-2 infection or exposure (reviewed in Ref. 44). Although the epidemiological evidence for protection against HIV-1 by prior HIV-2 infection has been disputed (47), it is supported by animal studies showing long-term protection against SIV-induced disease in macaques vaccinated with a live, attenuated HIV-2 vaccine (48). Cross-reactive virus-specific CTL have been proposed to account for the apparent resistance to HIV-1 infection of seronegative sex workers who may have been initially exposed to HIV-2 (41), and for the protection against mucosal SIV infection in some HIV-2-exposed seronegative macaques (49).

The greater flexibility of HIV-2-specific CTL to tolerate variations within the epitope could also play a role in enhancing viral control by limiting the successful development of escape mutations. Many studies confirm the important role of mutational escape in the HIV and SIV models (7), but thorough prospective studies are now required to investigate whether such escape mutations are less likely to be selected in HIV-2 infection. Our data indicate that escape by loss of TCR interaction (rather than by inhibition of processing or MHC binding) may be reduced in HIV-2 infection, as many amino acid changes arising within the HIV-2 CTL epitope studied might not affect the capacity of the epitope both to expand and be recognized by the original population of CTL. By contrast, in HIV-1 infection, similar mutations would create a window with loss of viral recognition while switching to a new response; such new responses to variant epitopes are proposed to be unlikely because of the phenomenon of "original antigenic sin" (50). Although the multispecificity of the CTL response should allow viral control to be exerted through the response to an entirely different epitope, this is likely to weaken the overall response through forcing constant shifts to potentially subdominant epitopes and allowing temporary bursts of viral replication (51).

In summary, we show for the first time that there can be greater flexibility of recognition associated with the broader TCR usage seen in the CD8 responses in HIV-2-infected patients than the limited cross-recognition possible with a highly focused TCR usage in HIV-1 infection. Recent data have highlighted how a single gag CTL escape mutation arising in the SIV/HIV model can limit the protective efficacy of an otherwise promising env/gag plasmid DNA vaccine approach (52). This work suggests that future HIV-1 vaccine strategies may need to address the polyclonality in addition to the multispecificity of CTL elicited.

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