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INFECTIONS WITH HIV-1 AND *MYCOBACTERIUM TUBERCULOSIS*; THE ROLE OF HLA CLASS II ALLELES AND HIV-1 PHENOTYPES

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It is not the strongest of the species that survive, nor the most intelligent, but the ones most responsive to change

- *Charles Darwin*

Dedicated to my parents and my lovely family

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ABSTRACT

Human Immunodeficiency Virus (HIV) and *Mycobacterium tuberculosis* (*M.tb*), are the two infectious diseases causing the greatest number of deaths globally. The burden of these infections is most felt in the developing world, particularly in sub-Saharan Africa. This is also a region where some of the studies in this thesis were conducted. The thesis explores i) host genetic factors, i.e. human leukocyte antigen (HLA) class II molecules and ii) pathogen factors in the form of HIV phenotypes in order to better understand the host-pathogen interaction in infection.

We investigated in paper I the frequency of HLA class II allele frequencies in Botswana and the association of the HLA class II alleles with susceptibility or control of HIV-1 infection. Certain HLA alleles were identified to be associated with protection from HIV infection whilst other alleles were found to be associated with better control of infection.

In paper II, we described a novel peptide microarray and used recombinant HLA class II monomers to identify peptides binding to different HLA class II monomers alleles. HIV-1 peptides binding to three common HLA class II alleles (HLA DRB1*0101, DRB1*1501 and DRB1*0401) were identified. Some of the HLA class II binding peptides were further explored using intracellular cytokine staining to determine if they could serve as T-cell epitopes and were able to elicit a functional CD4+ T-cell response.

We proceeded to implement the assay described in paper II to identify peptides from sixty-one *M.tb* proteins for binding to the three HLA class II alleles (HLA DR1, DR2 and DR4). The 61 *M.tb* proteins were then ranked by epitope density which helped to identify *M.tb* proteins that could serve as immunogens for CD4+ T-cells and ultimately as targets for *M.tb* vaccine design or the development of diagnostic assays.

In paper IV, we investigated antibody responses directed against 7466 *M.tb* peptides derived from 61 *M.tb* proteins. The peptides were printed on a peptide microarray chip. Antibody responses from a group of patients with clinical tuberculosis were compared with the humoral recognition pattern in a well-defined healthy control cohort. This allowed the identification of peptides differentially recognized in the two groups. *M.tb* peptides which were exclusively recognized in serum from TB patients could be further explored for the use in diagnostic assays.

We investigated in Papers V and VI HIV-1 subtype C coreceptor use in HIV patients in Botswana. Whilst paper V focused on coreceptor use in treatment naïve patients, paper VI focused on coreceptor use in patients failing antiretroviral treatment. Compared to other HIV subtypes, subtype C seems to have less variants that use CXCR4, although a few dualtropic (R5X4), as well as monotropic X4 strains, have been identified. Using single genome sequencing we found that population sequencing can underestimate the prevalence of CXCR4 using or non R5 strains. Even in HIV subtype C infections, it would be important to first evaluate the viral phenotype before treatment with HIV coreceptor inhibitors.

In conclusion, this work may advance our knowledge of host and pathogen factors that are helpful in improving or developing better interventions against HIV and *M.tb.*

SAMMANFATTNING PÅ SVENSKA

Infektion med humant immunbristvirus typ 1 (HIV-1) och *Mycobacterium tuberculosis* (*M.tb*) orsakar det största antalet avlidna människor globalt per år. Bördan av dessa infektioner är störst i utvecklingsländer, särskilt i Afrika söder om Sahara, varifrån några av studierna i denna avhandling har ägt rum. Avhandlingen undersöker i) genetiska faktorer hos värden, som humant leukocytantigen (HLA) och dess klass II molekyler och ii) faktorer hos patogenerna, som coreceptoranvänding hos HIV-1, för att bättre förstå värdparasitinteraktionen vid dessa infektioner.

I delarbete I undersökte vi frekvensen av HLA klass II alleler i Botswana och associationen mellan HLA klass II alleler och mottaglighet för eller försvar emot HIV-1 infektion. Vissa HLA alleler befanns vara knutna till ett försvar emot att bli infekterad och andra med en bättre kontroll av infektionen.

I delarbete II beskriver vi en ny mikroarraymetod med rekombinanta HLA klass II monomerer för att identifiera peptider, som binder till tre olika HLA klass II alleler. Många HIV-1 peptider identifierades, vilka band till vanliga HLA klass II alleler, som HLA DRB1*0101 (DR1), DRB1*1501 (DR2) respektive DRB1*0401 (DR4). Vissa av dessa peptider undersöktes vidare efter reaktion med levande T celler med intracellulär färgning av cytokiner för att se om de kunde vara T cells epitoper och hade förmåga att väcka ett funktionellt CD4+ T-cells svar.

I delarbete III implementerade vi mikroarraymetoden för att identifiera peptider från 61 *M.tb* proteiner som kunde binda till samma HLA klass II alleler som i delarbete II (HLA DR1, DR2 och DR4). De 61 *M.tb* proteinerna rankades efter epitoptäthet, som underlättade identifieringen av de *M.tb* proteiner, som kunde fungera som CD4+Tcellsimmunogener, och i slutändan som vaccinkandidater eller för diagnostiska ändamål.

I delarbete IV undersökte vi antikroppssvaret mot 7466 *M.tb* peptider från de 61 *M.tb* proteinerna, som fästes till mikroarray chips. Antikroppssvaret från en grupp patienter med tuberkulos jämfördes med antikroppssvaret hos en väl karakteriserad kontrollgrupp. Detta möjliggjorde identifieringen av peptider, som reagerade med antikropparna. De olika *M.tb* peptiderna gav ett specifikt mönster, så att vissa kändes igen bara av serum från tuberkulospatienterna och andra bara av serum från kontrollgruppen. *M.tb* peptider som kändes igen exklusivt av patienterna med tuberkulos skulle kunna användas i diagnostiska tester.

I delarbetena V och VI undersökte vi coreceptoranvändningen hos HIV-1 subtyp C från patienter i Botswana. Arbete V fokuserade på coreceptoranvändningen hos HIV-1 från patienter som aldrig fått antiretrovirala läkemedel, medan delarbete VI undersökte coreceptoranvändningen hos HIV-1 subtyp C från patienter, behandlade med antiretrovirala medel, som uppvisat behandlingssvikt. HIV-1 subtyp C förefaller ha färre varianter som använder CXCR4, som anses vara mer patogen än CCR5-användande virus (R5). Med sk ”single genome” sekvensering av HIV-1 genomet fann vi att populationssekvensering underskattade prevalensen av CXCR4-användande virus. Även hos patienter med subtype C infektion, vore det därför värdefullt att undersöka om det finns CXCR4 användande virus innan behandling med HIV coreceptor läkemedel sätts in.

Sammanfattningsvis ökar detta arbete vår kunskap om värd-, respektive agensspecifika faktorer, som kan förbättra vår förmåga att utveckla effektiva interventionsstrategier mot infektioner med HIV och *M.tb.*

For SG by AE

LIST OF PAPERS

I. Ndung'u T, **Gaseitsiwe S**, Sepako E, Doualla-Bell F, Peter T, Kim S, Thior I, Novitsky VA, Essex M. Major histocompatibility complex class II (HLA-DRB and -DQB) allele frequencies in Botswana: association with human immunodeficiency virus type 1 infection. *Clin Diagn Lab Immunol.* 2005 Sep;12(9):1020-8.

II. **Gaseitsiwe S**, Valentini D, Ahmed R, Mahdavifar S, Magalhaes I, Zerweck J, Schutkowski M, Gautherot E, Montero F, Ehrnst A, Reilly M, Maeurer M. Major histocompatibility complex class II molecule-human immunodeficiency virus peptide analysis using a microarray chip. *Clin Vaccine Immunol.* 2009 Apr; 16(4):567-73.

III. **Gaseitsiwe S**, Valentine D, Mahdavifar S, Reilly M, Ehrnst A, and Maeurer M. Peptide microarray – based identification of *Mycobacterium tuberculosis* epitope binding to HLA-DRB1*0101, DRB1*1501 and DRB1*0401. *Clin Vaccine Immunol.* 2009, Preliminary accepted.

IV. **Gaseitsiwe S**, Valentini D, Mahdavifar S, Magalhaes I, Hoft DF, Zerweck J, Schutkowski M, Andersson J, Reilly M, Maeurer MJ. Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from M. tuberculosis. *PLoS ONE*. 2008; 3(12):e3840.

V. Ndung'u T, Sepako E, McLane MF, Chand F, Bedi K, **Gaseitsiwe S**, Doualla-Bell F, Peter T, Thior I, Moyo SM, Gilbert PB, Novitsky VA, Essex M. HIV-1 subtype C in vitro growth and coreceptor utilization. *Virology*. 2006 Apr 10; 347(2):247-60.

VI. **Gaseitsiwe S**, Pramanik L, Mine M, Essex M, and Ehrnst A. Coreceptor use in HIV-1 subtype C strains from patients failing antiretroviral treatment in Botswana. *Manuscript.*

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LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
APC	antigen presenting cells
ARV	antiretroviral
BCG	Bacille Calmette-Guérin
CCR5	CC-Chemokine receptor 5
CXCR4	CXC-chemokine receptor 4
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FDA	food drug administration
GALT	gut associated lymphoid tissue
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
ICS	intracellular cytokine staining
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
M.tb	mycobacterium tuberculosis
NK	natural killer cells
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
RNA	ribonucleic acid
SIV	simian immunodeficiency virus
TB	tuberculosis
TCR	T-cell receptor
TNF	tumor necrosis factor

INTRODUCTION

Despite all the technological advances of the 21st century, infectious diseases cause about 25% of deaths globally and more than 40% of all deaths in low-income countries (World Health Organization WHO global burden of disease 2002 estimates www.who.int/healthinfo/bodestimates/en/). HIV and TB are the two leading causes of infectious disease associated mortality worldwide (1). Individually, HIV and TB are challenging to deal with. Together, they form a deadly liaison. Individually and synergistically, the two infections have resulted in untold human suffering and economic implications.

Mycobacterium tuberculosis (M.tb) has infected mankind for more than 4000 years. Early in human history, TB infection was sporadic but reached epidemic proportions in Western Europe in the 17th to 19th centuries during the industrial revolution (2). With improved living conditions in the late 19th and 20th century in Western Europe and the USA, the incidence of TB declined and continues to do so (3). This decline, however, has not been reciprocated in the developing nations, where the conditions of poor nutrition, overcrowding, and poor sanitation are not improving.

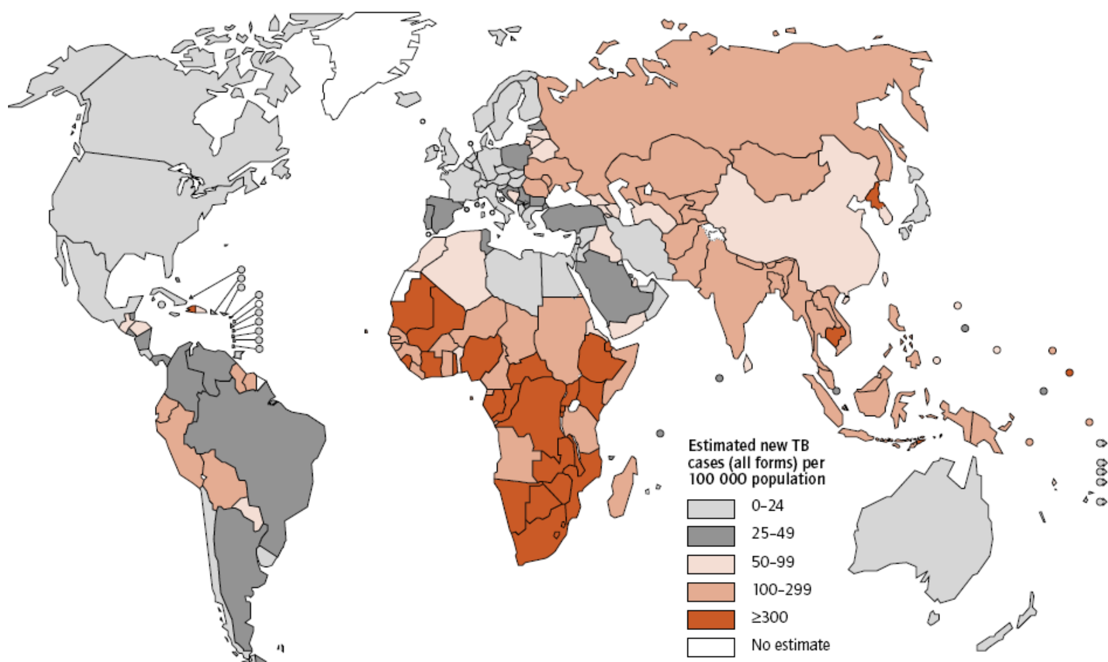

Figure 1 Global incidence of TB (per 100 000) in 2007 **Source WHO**

WHO estimates that 9.27 million new cases of TB occurred in 2007 (Global Tuberculosis Control 2009 http://www.who.int/tb/). The incidence of TB has

actually increased 5 to 10 fold over the past two decades in most countries in sub-Saharan Africa owing mostly to the HIV epidemic. An estimated 1.32 million HIV-negative people died from TB in 2007, and there were an additional 456 000 TB deaths among HIV-positive people. Figure 1 shows the global incidence of TB.

HIV has a shorter history. The virus was identified in 1985. It has been shown to have crossed over to humans around 1930 from wild monkeys. Currently, around 33.2 million people are infected with HIV, and in 2007 2.1 million people died from AIDS, of which 76% occurred in sub-Saharan Africa (UNAIDS 07 AIDS epidemic update http://data.unaids.org). Figure 2 shows the global prevalence of HIV.

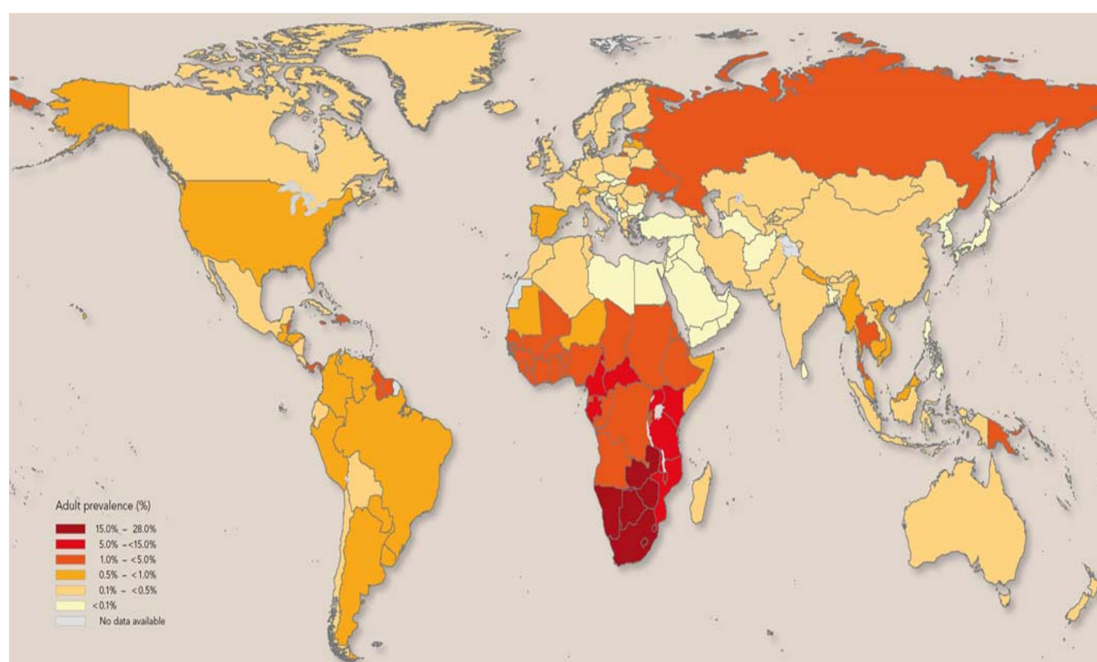

Figure 2 Global HIV prevalence in 2007 **Source UNAIDS**

Treatment strategies for TB and for HIV have become quite successful. TB is responsive to the treatment regimens and most individuals who are diagnosed with the infection can be cured. Multidrug resistant TB (MDR-TB) and also extremely drug resistant TB (XDR-TB) are a serious challenge to TB control. These *M.tb* strains have become more frequent and are not responsive to drugs. Most of the available drugs have been used for a long time. One of the pressing needs is to develop new drugs which can complement the current treatment options. The advent of highly active antiretroviral therapy (HAART) in 1996 resulted in a decline in the AIDS associated deaths in developed countries. These drugs have not been easily accessible, however, in resource-limited countries where they are

most needed as they are expensive. There is also a renewed interest in immune response modifiers for supplementing HIV and TB therapy (4-6). Coinfections with HIV and TB offer a challenge regarding the treatment strategies, as it has been shown that the drugs interfere with each other (7-9).

Vaccines have been successful in preventing many infectious diseases. The BCG vaccine is relatively successful in preventing extra-pulmonary TB in infants, but it is not useful in preventing TB in adults. As such, there is need to develop a better vaccine for TB and a number of vaccine trials with new TB vaccine candidates are underway. The search for an effective HIV vaccine has been disappointingly unsuccessful, with all vaccines that have been tried to date failing to prevent infection.

Better understanding of these pathogens and their interaction with the host may help to devise novel strategies for diagnosis, control, and prevention.

THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

MHC structure and genetics

The major histocompatibility complex (MHC), known in man as the human leucocyte antigen (HLA), is a large genomic region or gene family found in most vertebrates. The term 'major histocompatibilitiy' derives from the fact that the MHC was defined originally by mapping gene loci which had a major influence on graft rejection. The MHC genes are encoded for by a 4-MB region on the short arm of chromosome 6 at 6p21.3 (10). The MHC is divided into three regions, class I, class III, and class II (Figure 3). The classical MHC loci are the class I (HLA-A, -B, -C, -E, -F and –G) and class II (HLA-DR, -DQ, -DM, and –DP). These present antigens to CD8+ and CD4+ T-cells, respectively. The MHC class III genes have different functions as compared to that of MHC class I and class II. Since they have their loci between the other two, they are frequently discussed together. The focus here will be on the classical MHC loci, MHC class I and II.

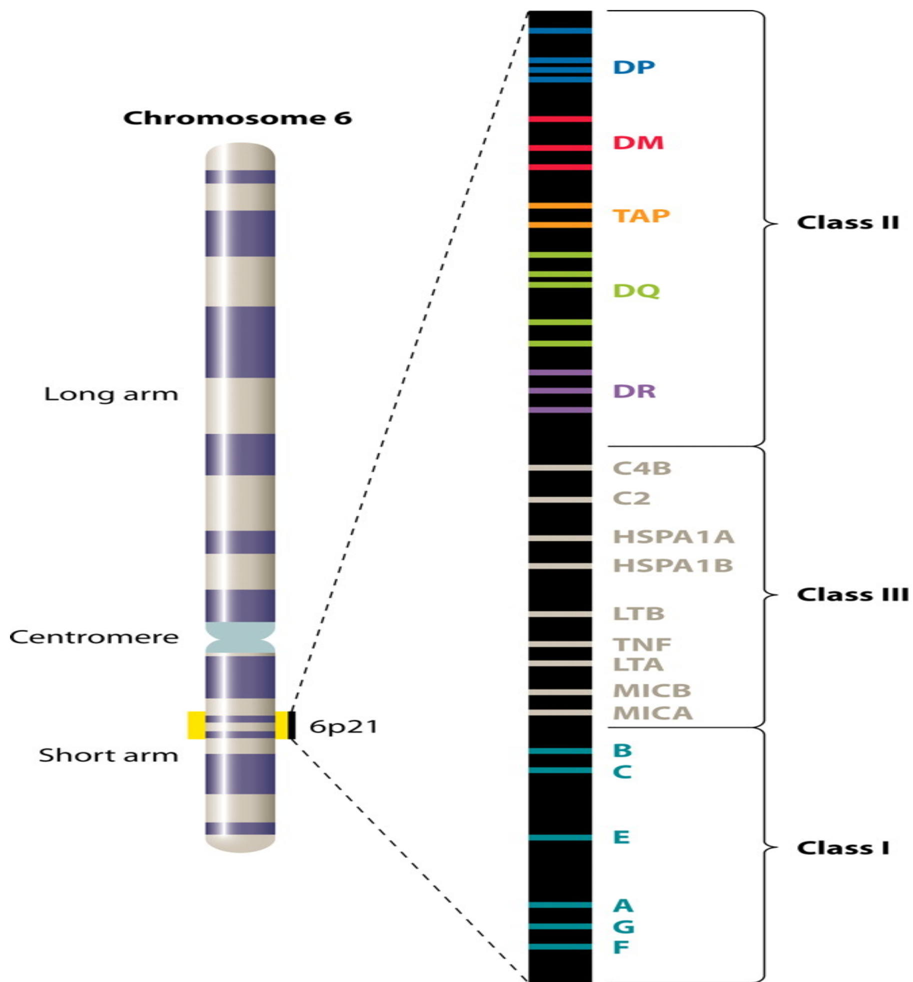

Figure 3 Location of the MHC genes **Adapted from Blackwell et al 2009 with permission**

Since there are three genes encoding HLA class I and three or four genes encoding HLA class II proteins on each chromosome, every individual will express at least three different HLA class I and HLA class II proteins on their cells. The various genes that can occupy a locus are known as 'alleles'. The latest number of HLA class I and II alleles, as provided by the HLA database, is 2 496 and 1 032, respectively (http://hla.alleles.org/nomenclature/stats.html).

Figure 4 shows the distribution of the HLA alleles by class and loci. The particular combination of HLA alleles found on a single chromosome is known as an HLA haplotype. The expression of HLA alleles is codominant, as protein products of both alleles are successfully translated into proteins and expressed at the cell surface.

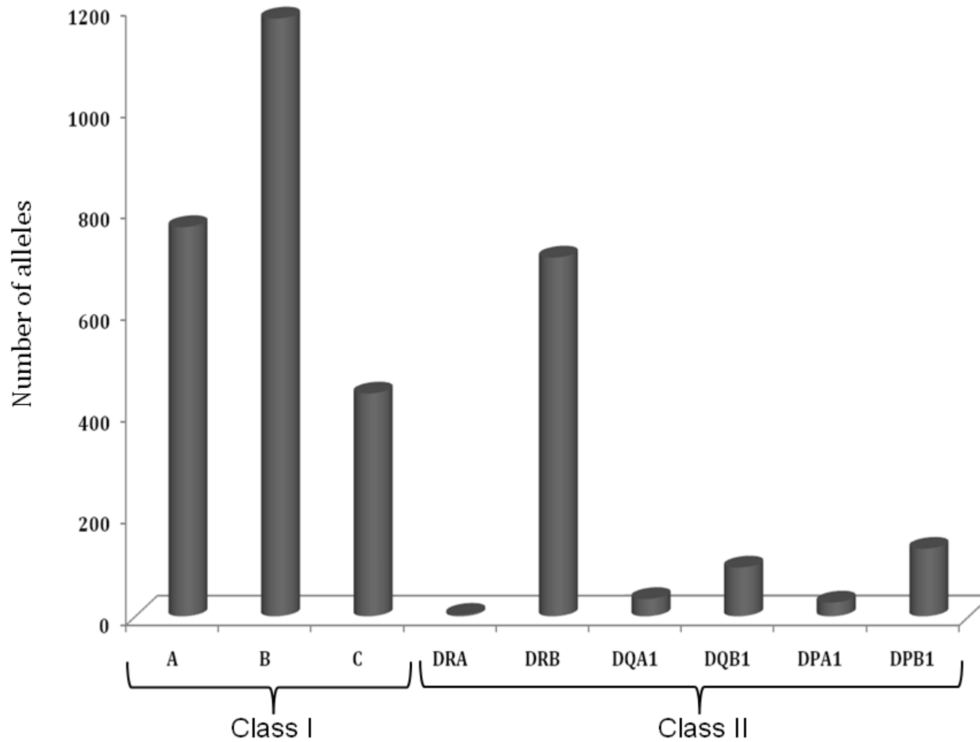

Figure 4 Frequency of HLA alleles for the different HLA loci

MHC class I molecules consist of an alpha or heavy chain, which is non-covalently associated on the cell surface with the second chain, which is β2-microglobulin. The alpha chain is the only one that spans the cell membrane. On the other hand, the MHC class II consists of two chains, α and β, both span the membrane (Figure 5).

The crystal structures of MHC class I and II have been determined (11, 12)and show some similarities, although there are differences which are important concerning their functions related to peptide presentation and interaction with CD4+ or CD8+ T-cells. One of the crucial differences is that the peptide binding site for MHC class II is open at the ends. The presences of bulky tyrosine residues at both ends of the MHC class I peptide binding sites results in a closed binding groove. MHC class II can bind relatively longer peptides, 12-mers to 20mers, as compared to MHC class I which bind shorter peptides, 8-mers to 10-mers (13).

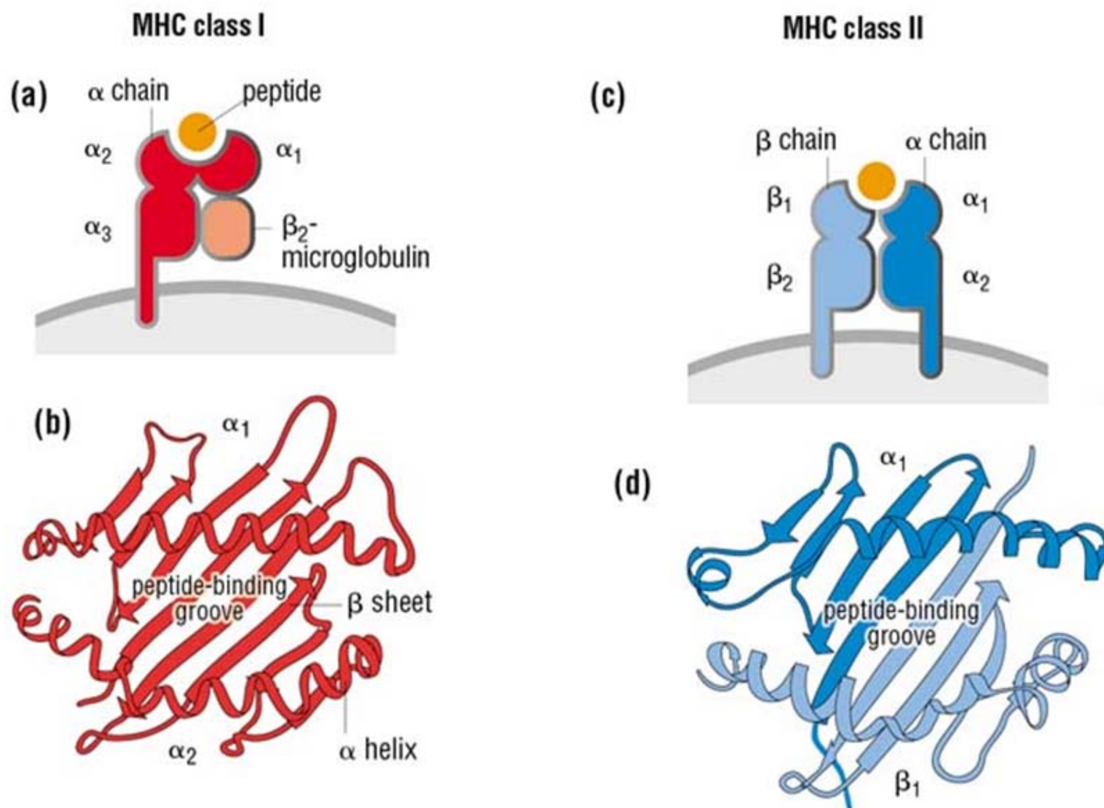

Figure 5 The structure of MHC class I and class II molecules **Adapted from Defranco et al Immunity**

MHC and antigen presentation

The MHC class I and II molecules play a crucial role in the immune system, they present antigens to CD8+ and CD4+ T-cells, respectively. T-cells are not able to recognize foreign antigens directly, but are only able to recognize their cognate antigen through their T-cell receptors (TCRs); the antigen is presented as short peptide fragments bound to MHC molecules. The crucial role of the MHC molecules was first described by Doherty and Zinkernagel in 1975 as the phenomenon of 'MHC-restricted recognition' of antigens. They were awarded the 1996 Nobel Prize in Medicine and Physiology for this work.

MHC class I and II molecules are distributed differently on cells and in tissues. MHC class I molecules are expressed by all nucleated cells. MHC class I molecules present predominantly intracellular antigens to CD8+ cytotoxic T-cells, which are endowed to kill their targets using perforin/granzyme. Since HIV and *M.tb* are both chronic intracellular infections, cytotoxic T-cells are expected to play a crucial role in the host response defense against both pathogens.

MHC class II alleles present predominantly extracellular antigens to CD4+ T-cells. Once activated, the CD4+ T-cells activate macrophages to acquire cytotoxic

functions. CD4+ T-cells may also stimulate B-cells to secrete antibodies and they also produce growth factors for CD8+ T-cells. MHC class II molecules are constitutively found on professional antigen presenting cells, such as B lymphocytes, dendritic cells, and macrophages. However, the expression of both MHC class I and II proteins is regulated by cytokines, produced in the immune response. Interferon-γ (IFN- γ) has been shown to increase the expression of both MHC class I and class II molecules and can induce the expression of MHC class II on nonprofessional APCs (14, 15).

The MHC class I heavy chain, a transmembrane glycoprotein of approximately 44 kDa, binds upon synthesis to the membrane-associated endoplasmic reticulum (ER) chaperone, calnexin. Folding and intra chain disulfide bond formation occurs at this stage. Upon dissociation from calnexin, the heavy chain binds β2-microglobulin and is incorporated into the peptide-loading complex. Peptides are transported into the ER from the cytosol via transport associated with antigen processing (TAP). If necessary, they are trimmed by an ER associated aminopeptidase (ERAAP or ERAP1) to 8–10 amino acids. This length is generally required for association with class I molecules (16-18). If the peptide has the appropriate sequence, it binds to the MHC class I- β2-microglobulin heterodimer, which is released from the peptide-loading complex. The fully assembled MHC class I molecule then leaves the ER and travels via the Golgi apparatus to the plasma membrane, where it becomes accessible to CD8+ T-cells.

MHC class II are synthesized in the ER, where they associate with the invariant chain (19). This association with the invariant chain has been shown to promote the proper folding of the class II molecules in the ER, avoid class II loading of peptides in the ER, and also to facilitate their transport from the ER through the Golgi complex to endosomes (20-23). The invariant chain and other MHC class II chaperones, such as DM and DO, regulate the peptide loading of the class II molecules (24-26).

MHC class II molecules bind peptides generated in endosomes. The complexes are transported to the cell surface for presentation to CD4 T-cells (27, 28). It has also been shown that endogenous proteins and peptides can bind MHC class II following transport into the endosomal pathway (27, 29).

MHC and infectious diseases

In many infections, the 'virulence' of the pathogen and 'environmental factors' are not solely able to explain different patterns of infections among different populations. Host factors influence the outcome of the infection as well. The MHC molecules have been shown to be major players among host genetic factors that can affect the infection outcome, they actively shape the quality and quantity of immune responses.

MHC diversity may explain some of the individual differences in the host response to infection. Thus, the evolutionary pressure attributed to the different frequency of pathogens in different populations has resulted in a selection of different MHC allele frequencies in different populations. The selected alleles are those that may confer protection from pathogens that are frequently encountered.

It has been difficult to provide conclusive associations between HLA and infectious diseases. Many factors, such as the sample size, clinical information, as well as the consideration of other genes in the disease process have to be accounted for (30). Co-infection with other pathogens may also impact on the association with HLA and disease outcomes. An association has to be confirmed in more than one study. As a result, a number of allele associations with some infectious diseases remain to be confirmed. Despite this caveats, some convincing HLA class I and class II associations with infectious diseases have been identified (31). HLA-B*53 has been associated with protection against severe malaria in West Africa. This may be responsible for the high frequency of HLA B*53 in this region (32). Also, HLA-DR2 has been associated with susceptibility to mycobacterial diseases (33, 34), while DQB1*0301 has been associated with immunological clearance of hepatitis C virus (35-37). Furthermore, DRB1*1302 has been associated with clearance of hepatitis B (38, 39).

These and other studies, which show HLA associations with infections, indicate that the HLA loci play a major role in susceptibility to infections. Further studies have shown that some of these HLA allele molecules are able to bind and present specific peptides to CD8+ T-cells and also to CD4+ T-cells. Identifying these peptides offers the possibility to design a more rational approach to diagnostics and vaccine design.

Identifying peptides binding to MHC

MHC molecules present peptides to T-cells with antigen specific T-cell receptors. Therefore, identifying the peptides that bind to MHC molecules will aid to describe targets of clinically relevant T-cell responses. A number of strategies have been developed over time to identify MHC-peptide binding.

The 'direct way' to identify peptides binding to MHC proteins is to isolate cells which express the MHC alleles of interest, followed by precipitation of MHC molecules with solid phase bound antibodies. The peptides are then dissociated from the MHC molecules by acid treatment. High phase liquid chromatography is used to separate the different peptides, the peptide sequences are then determined by mass spectrometry (40-42). The major challenge with this assay is the small number of individual peptides that are isolated from the cells. With an estimated number of 1000 different peptides per 100000 MHC alleles per cell (41), a high number of cells is needed to identify relevant individual peptides (43).

The use of peptide binding assays is a different way to identify peptides binding to the proteins of different MHC alleles. Alternate versions of peptide binding assays have been developed, but the principle is the same: The binding of the test peptide to an MHC allele is compared with that of a labeled control peptide with a biochemically defined binding affinity to the same MHC allele (44, 45). This assay has been proven to be popular, but it is limited to the testing of a few test peptides and MHC alleles at the same time.

With the increased knowledge of MHC structure and MHC/peptide interaction and specificity, a number of computer-based approaches for predicting peptide binding to HLA alleles have been developed (46-49). These may not always be correct (50), yet they are convenient as they do not require biological samples and they save time.

Once the binding of a peptide to MHC allele protein has been confirmed, it is important to determine if a T-cell repertoire exists that can recognize the MHC-peptide complex, e.g. by determining the corresponding cytokine profile produced upon the T-cell/MHC-peptide interaction. Functional assays such as enzyme-linked immunosorbent spot (ELISPOT), intracellular cytokine staining (ICS), or tetramer-guided staining are useful to link T-cell recognition with immune effector cell functions.

HUMAN IMMUNODEFECIENCY VIRUS (HIV)

Origin and classification

HIV is divided into two types, HIV type 1 and HIV type 2. From epidemiological studies, it has been determined that HIV-1 and HIV-2 were transmitted from two different African nonhuman primate species to humans. Since HIV-2 and simian immunodeficiency virus- sooty mengaby (SIVsm) share an identical genome organization (51, 52), it is assumed that HIV-2 originated from SIVsm, whose natural host is the Sooty Mengaby monkeys (51, 52).

HIV-1 most probably originated from SIVcpz, which infected several geographically isolated chimpanzee communities in southern Cameroon (53). Three independent transmissions have been predicted from phylogenetic analysis to have occurred early in the 20th century, which led to the three HIV-1 groups; major (M, between 1915 and 1941), outlier (O), and nonmajor and nonoutlier (N) (53). Group O HIV-1 may have originated in gorillas in which the closest relatives to this group have been identified (54) while the other two groups M and N most likely evolved from strains found in chimpanzees (55). The earliest documented case of HIV-1 infection with a group M strain in humans has been traced to a blood sample from 1959 (56). A new HIV group, group P, linked to SIV from gorillas has recently been described in a patient from Cameroon (57).

Most of the global HIV epidemic is caused by the HIV-1 group M. HIV-1 group M has been divided into subtypes denoted with letters and sub-subtypes denoted with numerals. The subtypes and sub-subtypes currently recognized are: A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K. Intra-subtype genetic variation ranges from 15 to 20 %, whereas inter-subtype genetic variation ranges from 25 % to 35 %. Circulating and unique recombinant forms (CRFs and URFs, respectively) which are a result of recombination between HIV subtypes within a dually infected person, have also been identified. More complex recombinants (CPX) are also found and these are viral strains resulting from recombination of multiple subtypes. HIV-2 infections are rare and are confined to West Africa and India.

The global distribution of the different HIV-1 subtypes is shown in Figure 6. The dominant subtype in Western Europe and North America is subtype B. This consequently is the most studied HIV-1 subtype although it makes up only 20% of the global HIV-1 strains. The most prevalent HIV-1 subtype accounting for about

50% of the global HIV-1 is subtype C, which predominates in the ranging epidemic in southern Africa. HIV-1 subtype C is found also in the horn of Africa and India. HIV-1 subtypes may be associated with different rates of transmission and with different disease progression.

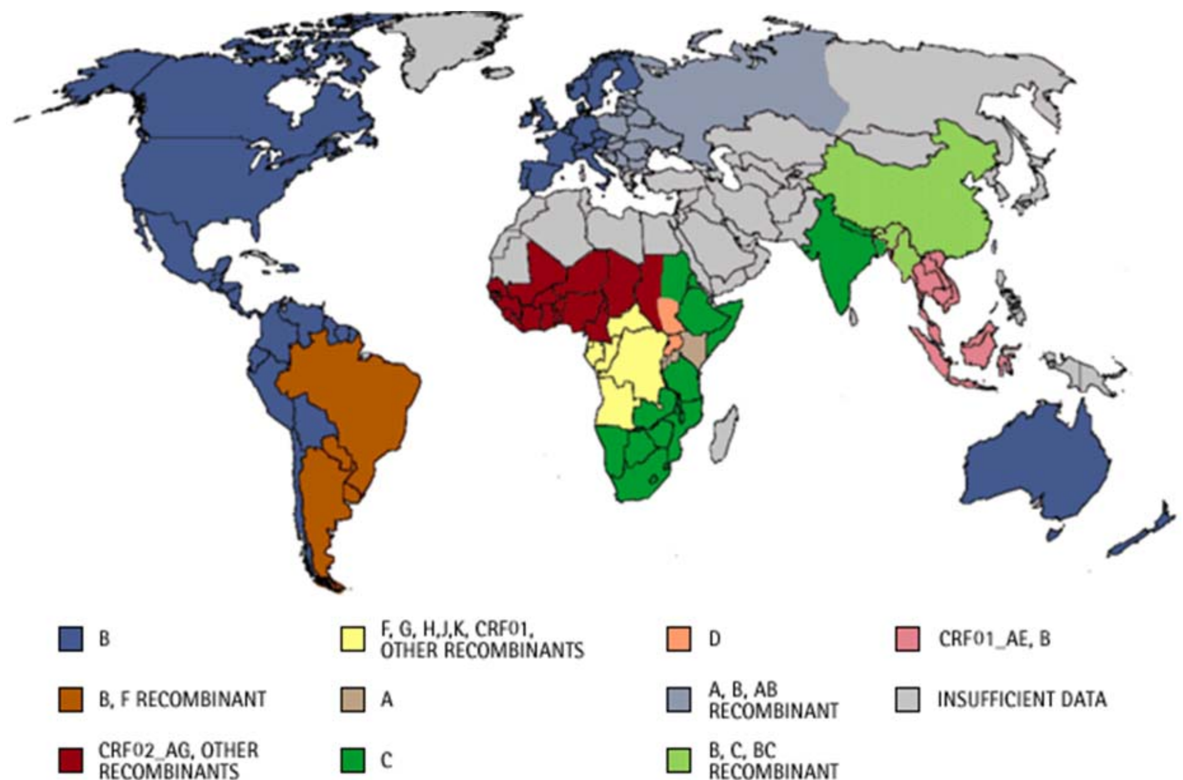

Figure 6 The global distribution of HIV subtypes

Structure of the virus

HIV belongs to the Lentivirus genus which belongs to the Retroviridae family. Lentiviruses have been known to infect other animal species such as cats, cattle, horses, goats, as well as nonhuman primates. HIV is an enveloped virus with a conical nucleocapsid and a diameter of about 110 µM. The virus has two copies of a positive–sense, single stranded RNA genome. The HIV genome is about 10 Kb and codes for the three main retroviral structural genes; envelope (*env*), group-specific antigen (*gag*) and polymerase (*pol*), regulatory genes; *tat*, and *rev*, as well as accessory genes; *nef*, *vif*, *vpr*, and *vpu*. Long terminal repeats (LTR) sequences are found at both ends of the genome. This contains enhancers, promoters, and other sequences that interact with host-cell transcription factors,

necessary for the success of the virus replication. Figure 7 and Figure 8 show the organization of the viral genome and an illustration of the virion, respectively.

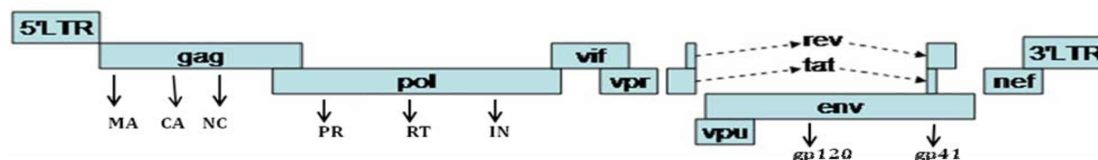

Figure 7 HIV-1 genome organisation

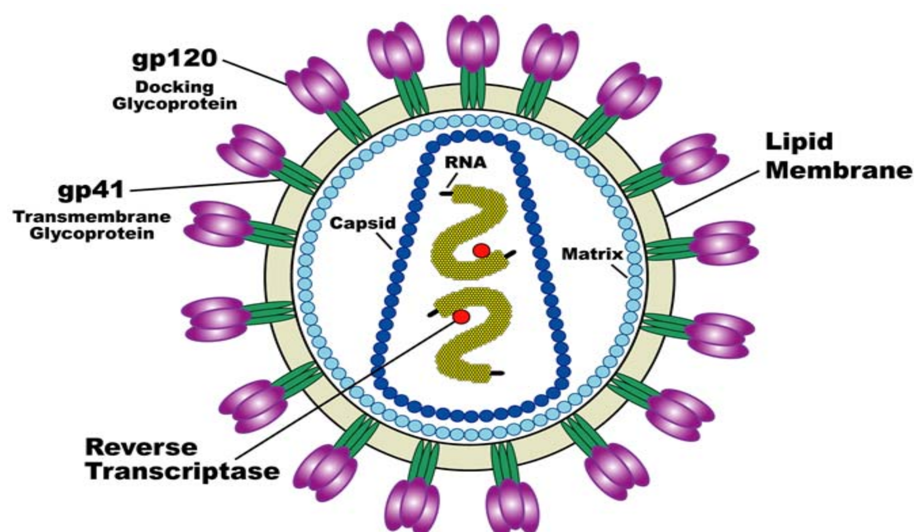

Figure 8 An illustration of the HIV-1 virion **Adapted from NIAD**

The HIV life-cycle

HIV enters the body through exchange of body fluids (mostly blood, semen, and breast milk). The virus targets T helper cells, macrophages, microglial and dendritic cells for infection. HIV binds the CD4 receptor through the gp120 protein. This results in that the viral envelope undergoes some conformational change, which facilitates the binding of the chemokine receptor, mostly CC-chemokine receptor 5 (CCR5) or CX-chemokine receptor 4 (CXCR4). The viral and cellular membranes fuse and the virus nucleocapsid and associated proteins are internalized. After being internalized, uncoating releases the single stranded RNA genomes, which are reverse transcribed by the viral reverse transcriptase to double-stranded DNA. The viral reverse transcriptase lacks proofreading activity and results in a mutation rate of about 3.4×10^{-5} mutations per base pair per replication cycle (58). As a result, millions of viral variants are produced in an infected person in a single day (59). The HIV reverse transcriptase is also known

to jump from one RNA strand to the other during the reverse transcription process. This results in recombination, the process that has resulted in the circulating recombinant forms (CRFs) of HIV (60, 61).

After the viral double stranded DNA has been generated, it is integrated into the host cell genome by the viral enzyme integrase, resulting in an LTR flanked provirus. The integrated provirus behaves like any other human gene and cellular activation increases the transcription level. The viral protein tat also increases the transcription level and ensures production of full-length viral transcripts (62, 63). Interestingly, tat was also found to impair the antigen processing and presentation through the MHC class II pathway (64, 65).

The regulatory protein *rev* together with the proteins tat and nef are synthesized early in infection. Rev facilitates the synthesis of the structural proteins and also ensures the availability of full-length genomic RNA to be incorporated into new virus particles (66). Once the late genes are transcribed, the spliced and unspliced transcripts are transported to the cytoplasm where they are translated into proteins that can be post-translationally modified.

After translation, the viral structural and enzymatic proteins travel to the plasma membrane where immature virions assemble in lipid rafts (67). The viral particles bud from the plasma membrane as immature noninfectious viruses with some uncleaved polyproteins. After the virions bud, the protease enzyme, which is autocatalytically released from the Gag-Pol polyprotein, starts the processing of the Gag and Gag-Pol polyprotein into mature proteins. The pol polyprotein is cleaved into the enzymes protease, integrase, and reverse transcriptase (68, 69). This together with the condensation of the inner core results in the formation of mature infectious HIV.

HIV coreceptor use phenotypes

To infect a cell, a virus must first be able to enter it. The primary cellular receptor for HIV entry is CD4. However, expression of CD4 on a target cell is necessary but not sufficient for HIV entry and infection. It has also been reported in a few cases that HIV can infecT-cells which do not express CD4 on their surfaces (70-72). The fact that CD4 alone was not sufficient for viral entry led to the search of coreceptors that are necessary for cell entry. Further support for the need for coreceptors for HIV entry was given by the observation that certain cell types,

such as monocytes and immortalized CD4+ T-cell lines, were only infected by certain strains of HIV and not by other. Viral isolates were generally categorized as M-tropic, T-tropic, or dual tropic isolates. The M-tropic isolates could replicate in primary monocytes only. T-tropic isolates could replicate in immortalized CD4+ T-cells only, while dual tropic isolates could replicate in both cell line (73).

A breakthrough came in 1996, when chemokine receptors were discovered to be the coreceptors for HIV (74-76). The main function for chemokine receptors is to interact with chemokines. Chemokine receptors are transmembrane receptors with an N terminus extracellular domain, seven membrane spanning domains, and a C terminus intracellular signaling domain which interacts with G-proteins. CCR5 and CXCR4 are the major chemokine co-receptors used by HIV to enter into cells. The natural ligands for CCR5 are Macrophage Inflammatory Proteins (MIP-1 α and β), and regulated upon activation, normal T cell expressed and secreted (RANTES) while the natural ligand for CXCR4 is stromal derived factor (SDF-1). Chemokines play an important role in the inflammatory response by inducing leukocyte migration to areas of infection by providing chemotaxis signals (77). These chemokines have all been shown to inhibit HIV-1 entry by binding to the chemokine receptors CCR5 and CXCR (78). Other chemokine receptors and related orphan receptors have been found to be used in some cases as HIV coreceptors, such as CCR2d, CCR3, CCR9, CX3C, BOB and BONZO (79).

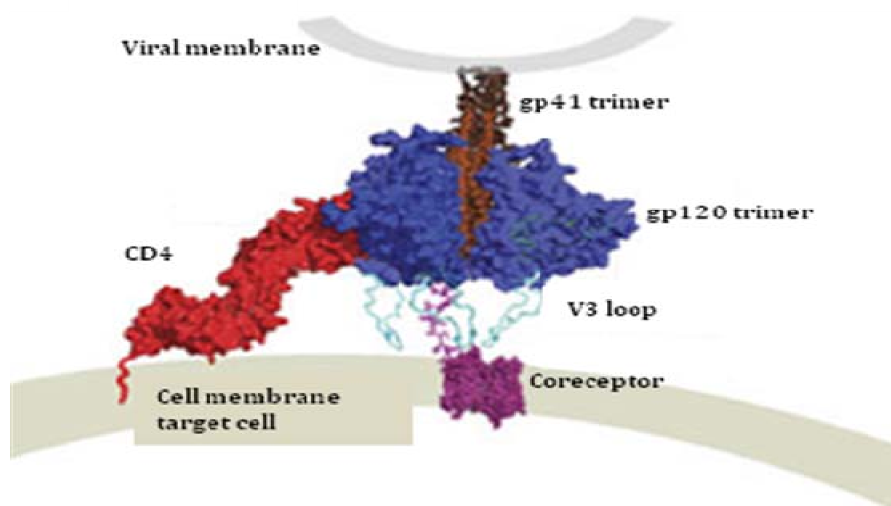

Figure 9 Schematic of HIV-1 envelope interacting with the CD4 receptor and the viral coreceptor of the host cell Adapted from Walker BD et al Science 2008

A new HIV classification was thus established in 1998 to take into account these developments (80). Virus isolates that use CCR5 exclusively as coreceptor are called R5 viruses, and those that use CXCR4 exclusively are called X4 viruses. Virus isolates that are capable of using both CCR5 and CXCR4 as coreceptors are called R5X4, or dual tropic viruses. In vitro, R5 viruses are usually nonsyncitium-inducing (NSI) in T-cell lines and are able to replicate in monocytes (M-tropic). In contrast, X4 strains are syncitium-inducing (SI) on T-cell lines and replicate preferably on T lymphocytes (T-tropic). There is not a complete synergy between NSI and R5 and SI and X4, respectively. Thus experiments performed with either system are not completely interchangeable.

The variable loop 3 (V3) region of gp120 is implicated in binding to the coreceptor, although other gp120 regions V1/V2 and C4 are also involved. The amino acid sequence of the V3 loop has been shown to predict the viral phenotype (81). Figure 9 shows the important features of the viral envelope interaction with CD4 and the coreceptor.

Infection and disease progression

Heterosexual transmission of HIV-1 is the leading means of viral transmission globally although in certain locations, intravenous drug use and homosexual sex are the leading causes of transmission. The rates of heterosexual transmission in high risk cohorts have shown transmission rates of about 1 infection per 100 coital acts (82, 83).

HIV infection is divided into three phases; primary infection, chronic infection and AIDS. The primary infection phase is marked by a burst of veremia, which is detected around day 7 and reaches a peak on the third week post exposure with viral load reaching about 10^7 virus particles per ml of plasma. Studies in rhesus macaques have helped understand the dynamics of the infection during primary infection phase (84, 85). An important event during primary infection is the spread of the infection to gut-associated lymphoid tissue (GALT), where large numbers of memory CD4+ T-cells reside. Up to 20% of the gut-associated CD4+ T-cells become infected and about 80% are destroyed (86). Viral load levels then begin to decrease, either due to saturation of the target cells, CD4+CCR5+T-cells (87), and/or because of the immune pressure (88, 89). The

viral load reaches the 'setpoint', which has been shown to be predictive of the rate of disease progression and clinical outcome (90-92).

The chronic infection phase follows, in which there is gradual decline in the CD4+ T-cell count, but the viral load remains around the set point. This phase of the infection can last for months to years.

Once the CD4+ T-cell count reaches around 200, the viral load steadily rises above the set point and the host is afflicted by an array of opportunistic infections, which indicate the onset of AIDS. In the absence of treatment, the host ultimately succumbs to these opportunistic infections.

Immune responses and the role of MHC

Innate responses

The innate immune response is the first defense against infection. The success of the innate immune response in abating HIV-1 infection remains an active field of research. Activated dendritic cells and natural killer cells are found once the patient becomes viremic (93, 94). Soluble β-chemokines such as MIP-1 α and β, and RANTES, produced by NK cells have been associated with resistance against HIV infection (95, 96).

T-cell mediated responses

Many studies from both primary and chronic infections show the important role played by T-cell mediated immune responses in HIV and SIV infections (97, 98). The CD8+ T-cells from HIV infected patients can inhibit HIV replication in autologous CD4+ T-cells. This inhibition is directed by both the direct cytotocixity and the production of soluble antiviral mediators such as CC-chemokine ligand 3 (CCL3), CCL4 and CCL5 (95, 99, 100).

The early control of viral load is associated with the emergence of virus specific CD8+ cytotoxic T lymphocytes, while high level of cytotoxic T-lymphocytes are also associated with good clinical status in chronic infection (101-103). Administration of an anti-CD8 antibody to monkeys resulted in lack of SIV replication control during primary infection and the animals died after a rapid disease progression (104, 105).

There is a CD4+ T-cell response to HIV-1 infection at the same time as the CD8+ T-cell response, though it is less in magnitude (106, 107). The CD4+ T-cell

response could be hampered by the higher susceptibility of HIV specific CD4+ T-cells to HIV infection (108). Recent studies, which showed the role of CD4+ T-cells in the quality of CD8+ T-cells and in immune memory formation, highlighted the importance of CD4 + T-cells in the immune response (109-111).

A number of vaccine trials have been conducted which aim at inducing T-cell responses against HIV, but they have all had disappointing results (112).

Humoral immunity

Neutralizing antibodies against the HIV envelope appear within weeks following the onset of infection (113). These neutralizing antibodies can bind the envelopes of autologous virus but are ineffective against heterologous viruses (113). It seems the antibodies fail to neutralize the virus, due to viral immune escape (113, 114). Monkeys depleted of B-cells showed normal virus clearance in the early stages of infection (115), indicating that antibodies may not play a pivotal role in the control of infection in the early stage of infection. Also, treatment of chronically infected patients with anti HIV-1 monoclonal antibodies with neutralizing activity in vitro has little effect on disease progression (116). However, broadly neutralizing human monoclonal antibodies from HIV infected persons targeting highly conserved epitopes have been identified (117). It is hoped that the characterization of these antibody epitopes can be used to design a vaccine able to induce broadly neutralizing antibodies.

The HIV vaccines that have been tested with the aim to elicit antibodies against HIV-1 have so far not been successful (117, 118). A phase 3 trial of a gp120 vaccine showed lack of efficacy as the vaccine failed to prevent HIV-1 infection and did not lower viral replication (119).

MHC and HIV

Data on MHC association with HIV infection and control is among the most convincing in human infection and offers some prospects of harnessing genetic association studies to ultimately develop new clinical interventions (120). MHC restricted CD8+ CTLs have been shown to control HIV and as such there is strong evolutionary pressure for the development of viral variants that escape the CTL response (121). The CTL escape is, however, constrained by the functional implications of the escape mutations. Mutations with limited fitness cost are more

tolerated than mutations with higher fitness cost (122). An example is HLA B*57, which recognizes a relatively conserved epitope in p24, and this is associated with significantly improved survival in HIV patients (123). Escape variants of these epitopes, if transmitted to non HLA B*57 hosts, are associated with better outcomes. This indicates that this variant epitopes come at a fitness cost (124). HLA B*27, which presents the conserved gag epitope KRWIILGLNK, is also associated with improved survival upon HIV infection (125-127). Interestingly, a recent study in which samples from five different continents were analyzed showed that HIV is evolving to escape immune responses orchestrated by the most abundant MHC class I alleles in the different populations (128). This indicates that the immunological control that is associated with certain MHC alleles might be lost as HIV adapts to the genetics of the different populations.

HLA class I alleles also interact with natural killer (NK) cells, which play an important role in the innate response against viral infections. NK-cells are regulated in part by inhibitory and activating signals through killer inhibitory receptors (KIRs), with specific KIRs interacting with specific HLA class I ligands. An activating HLA-KIR allotype results in increased NK-cell effector function, marked by release of perforin, granzyme, and gamma interferon (120, 129). A number of studies have identified HLA-KIR allotypes that are associated with HIV infection (130-132).

Most of the strong associations of HIV infection and HLA are with the HLA class I alleles. There are comparatively fewer studies making associations of HLA class II alleles with HIV infection. This could be due to the fact that CD8+ T-cells play a more direct role in the immune response against HIV, whilst the CD4+ T-cells play a more indirect role i.e. by providing T-cell help. Hence larger patient cohorts may be needed to visualize the association of HLA class II alleles and HIV infection. However, there have been some studies that found associations of certain HLA class II alleles with HIV infection. A study in highly exposed seronegative sex workers in Kenya identified HLA DRB1*01 to be associated with resistance to HIV infection (133). In Uganda, HLA DRB1*1302 was found least frequently in HIV infected patients (134) HLA DRB1*03 and DQB1*02 were found to be associated with development of symptomatic HIV infection in Italians, while HLA DRB1*15 was associated with faster disease progression (135).

Overall, it is clear that MHC exerts both independent and epistatic effects on HIV infection (130). A better understanding of these effects may result in a rational approach to vaccine design.

MYCOBACTERIUM TUBERCULOSIS (M.tb)

The bacterium

Mycobacterium tuberculosis (*M.tb*) was first described by Robert Koch in 1882 (136). It is the most frequent causative agent of human TB others are *M. africanum* and *M. bovis*. *M.tb* has infected mankind for over 4000 years, analysis of DNA samples from South America and Egyptian mummies from 3000-5000 years BC tested positive for *M.tb*. Bacteria in the genus Mycobacterium are thought to have initially lived in soil. Then some species evolved to live in mammals (137). *M.tb* was initially thought to have evolved from Mycobacterium bovis (138), but more recent data provides strong evidence for the independent evolution of both *M.tb* and *M.bovis* from precursor species, closely related to *M.canetti* (139).

The complete genome sequence and annotation of the H37Rv strain of *M.tb* was published in 1998 (140). In 2002, a re-annotation of the genome was conducted and subsequent re-annotations are presented on the public database TubercuList (http://genolist.pasteur.fr/TubercuList/) (141). The *M.tb* H37Rv genome consists of 4.4 x 10^6 bp and has about 4 000 genes (140). The functions of the genes have been subject to intensive research. Table 1 shows the number of genes in each functional group, as determined over the years.

An interesting feature of the *M.tb* genome is that a high number of genes are devoted to encoding enzymes for fatty acid metabolism. This may be related to the ability of *M.tb* to grow in the tissues of the infected host where fatty acids maybe the major carbon source (137). Another important and unique feature of the *M.tb* genome, which is shared with other members of the mycobacterium complex, is the presence of the unrelated (PE) and (PPE) families of acidic, glycine-rich proteins. The two protein families derive their names from Pro-Glu (PE) and Pro-Pro-Glu (PPE). Their sequences are located in the two conserved N-terminal regions in each of the protein families, which are about 110 and 180 amino acids long, respectively.

The function of these protein families remains a subject of research. The fact that they are located in the cell membrane and cell wall and the characteristics of their antigenicity suggests that some of these proteins may play a role in antigenic variation during *M.tb* infection (142).

Table 1. Classification of *M.tb* genes by function over time

Class	Function	1998	2002	2009
0	Virulence, detoxification, adaptation	91	99	226
1	Lipid metabolism	225	233	238
2	Information pathways	207	229	232
3	Cell wall and cell processes	516	708	751
4	Stable RNAs	50	50	50
5	Insertion sequences and phages	137	149	147
6	PE and PPE	167	170	168
7	Intermediary metabolism and respiration	877	894	898
8	Proteins of unknown function	606	272	15
9	Regulatory proteins	188	189	193
10	Conserved hypothetical proteins	910	1051	1148

M. tuberculosis (M.tb) Infection

M.tb is spread by aerosol particles, released from the lungs of patients with pulmonary or laryngeal disease. The first contact is thought to be with resident macrophages and possibly also with alveolar epithelial type II pneumocytes. These have been shown to be more abundant than macrophages in alveoli (143). Dendritic cells also have been shown to play an important role in the early stages of the infection (144-146).

M.tb is phagocytosed in a process that is initiated by contact with macrophage mannose and or component receptors (147). On successful entry into the macrophage, *M.tb* resides in the phagosome. The mature phagosome fuses with the lysosome to form the phagolysosome. This is characterized by a hostile environment, that includes acid pH, reactive oxygen intermediates, lysosomal enzymes, and toxic peptides (137).

Mycobacteria and other intracellular bacteria have evolved various ways to avoid or withstand the hostile environment of the phagolysosome (148, 149). *M.tb* and other pathogenic mycobacteria have been shown to block the phagosome-lysosome fusion (150, 151). In this way they avoid the hostile environment of the phagolysosome. It has also been recently shown that *M.tb* through the use of a serine protease, encoded by the Rv3671c locus, is able to withstand the acidic environment of the phagolysosome (152).

Infected macrophages attract inactivated monocytes, lymphocytes, and neutrophils through production of chemokines (153). *M.tb* infected macrophages are gradually killed as cellular immunity develops, resulting in the formation of the granuloma. This is a caseous centre, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes (154).

The strength of the immune response at this stage determines how the infection progresses. With strong cell mediated immune responses, the infection may be arrested permanently at this point. The granuloma heals, leaving small fibrous and calcified lesions (137). If the immune response is 'weak', as in the cases of immune compromised patients, viable bacteria can escape from the granuloma and spread within the lungs, resulting in pulmonary TB. The bacteria can also spread via the lymphatic system and the blood. However, extrapulmonary TB results most likely from the spread of *M.tb* in the course of the primary infection.

Immune Response to *M.tb* infection and role of MHC

M.tb is a classic example of a pathogen for which the protective response relies on cell mediated immunity. The organism lives primarily within cells, usually macrophages. Murine studies have shown by antibody depletion of CD4+ T-cells, that the CD4+ T-cells are required for infection control (155). This is further validated by the fact that HIV infection, which is associated with loss of CD4+ T-cells, greatly increases susceptibility to both acute and reactivated TB. HIV infected patients with a positive TB skin test were shown to have 8–10% annual risk of developing active tuberculosis, whilst HIV uninfected patients with a positive TB skin test have a 10% life time risk (156).

Studies indicated that CD4+ T-cells from infected subjects produce IFN-γ in response to a wide variety of mycobacterial antigens. The primary effector function of CD4+ T-cells is believed to be production of IFN-γ and possibly other

cytokines (IL-2, TNF-α). These activate macrophages, which can control or eliminate intracellular organisms.

M.tb, an intracellular pathogen, has been associated with a Th1 cell response characterized by the production of IFN-γ, IL-2 and TNF-α, but not IL-4 and IL-5 (157). A Th2 response, characterized by IL-4 and IL-5 production, favoring humoral immunity (158) is not perceived to play an important role in anti-*M.tb* immune responses, it may rather be detrimental. This is now being challenged as the role of humoral immunity in *M.tb* infection is becoming more appreciated (157, 159-161).

The complex role of CD4+ T-cell subsets and cytokines in the response to *M.tb* is becoming increasingly understood (162-164). Subsets of functional T-cells producing IL-17 and IL-22 have recently been described in *M.tb* exposed mice and human (165). More than 20% of specific cytokine–producing CD4+ T-cells in peripheral blood of healthy TB-exposed adults expressed IL-17 or IL-22 in a study conducted in South Africa (166) suggesting that these 'IL-17/IL-22 T-cells' may play an important role in clinically relevant immune responses to *M.tb.*.

In addition to CD4+ T-cells, CD8+ T-cells, macrophages, DCs and NK cells also contribute to the immune response against *M.tb* (167).

The role of the MHC *in M.tb infection*

The significance of MHC in *M.tb* infections is shown by studies in mice where MHC class II knockout mice had a deficiency in IFN-γ production early on in the infection (168, 169). Although the mice were able to increase IFN- γ production later in the infection, they succumbed to TB. Deficiency in MHC class I does not have influence in the acute infection phase, but has influence on the chronic infection phase (170, 171).

In human, a number of MHC class II alleles have been shown to be associated with *M.tb* infection. HLA-DR2 is the one MHC class II allele that has been most consistently associated with *M.tb* infection in different populations (172-176). In South Africa, individuals with HLA DRB1*1302 and HLA DQB1*0301-0304 were more associated with having active TB as compared to control individuals lacking these MHC alleles (177). HLA DQB1*0601 and HLA DRB1*0803 were found to be associated with TB disease progression, disease severity and development of drug

resistance in Koreans (178). Prevalence of HLA DRB1*0401 and HLA DRB1*0801 was significantly decreased in Mexican patients with pulmonary TB as compared to their prevalence in healthy controls (179).

HIV AND *M.tb* COINFECTIONS

As discussed in the previous sections, HIV and *M.tb* are difficult infections to deal with individually, however, the difficulty is even more when they infect the same person at the same time. In most of the sub-Saharan African countries where HIV prevalence is the highest in the world, more than half of all individuals with active TB have concurrent HIV infection (180).

It was realized earlier on, that the two infections have a synergistic effect, 10% of HIV and *M.tb* coinfected individuals will develop active TB within a year of coinfection, as compared to the 10% lifelong risk in individuals infected with *M.tb* alone. HIV infected patients are more susceptible to both reactivation and primary *M.tb* infection even at moderate levels of immune suppression (181). The high susceptibility of HIV infected individuals to active TB is associated with the CD4+ T-cell counts decline that is the hallmark of HIV infection.

M.tb infection also has an impact on HIV-1 infection. A number of *in vitro* studies have shown that *M.tb* infection can enhance HIV replication (182-184). This effect is through the increased production of tumor necrosis factor-alpha (TNF-α) in *M.tb* infection. TNF-α has been shown to increase HIV replication and also up regulate CXCR4 expression (185). It has also been shown that the mutation of HIV-1 strains in HIV and *M.tb* coinfected individuals is two or threefold greater than in individuals only infected with HIV (186). This is a result of HIV-1 strains in the *M.tb*-infected pleural space leaking into the systemic circulation (187). It is also interesting to note that the risk of death in HIV and *M.tb* coinfected patients is twice that in HIV only infected patients with matched CD4+ T-cell counts, with most deaths caused by progressive HIV infection, rather than TB (188).

The immune compromised state of HIV patients makes the TB diagnosis challenging (189). Also, there is increased extrapulmonary TB in most HIV coinfected individuals. Isolated extrapulmonary localizations are described in

53–63% of TB cases in HIV infected patients (189). Most of these cases would not test positive with the acid fast staining of sputum.

BCG vaccine is given to almost all infants in sub-Saharan African countries at birth or in the perinatal period. The vaccine is effective in reducing disseminated TB in HIV uninfected infants. There is very little data on the efficacy of BCG in HIV infected infants. Recent data from South Africa show little efficacy of BCG in HIV infected infants (190). This combined with the risk of adverse events linked to BCG vaccination in HIV infected infants puts into question the need for BCG vaccination in this population (190-192).

AIMS OF THE THESIS

- To determine the frequency of MHC class II alleles in the Botswana population and explore their association with HIV infection.

- Develop an assay to identify peptides binding MHC class II alleles, using peptide microarray and recombinant HLA class II monomers.

- Identify HIV-1 and *M.tb* peptides binding to MHC class II alleles and determine if these provide CD4+ T-cell epitopes.

- Identify *M.tb* peptides targeted by antibody responses in patients infected with TB and healthy controls since B-cell epitopes and CD4+ T-cell targets are often closely related.

- Characterize the HIV-1 subtype C *in vitro* growth and coreceptor use in treatment naïve and patients failing treatment.

MATERIALS AND METHODS

The materials and methods for each paper are detailed in the papers. I provide here a brief summary of the patients' samples and the essentials of the methods implemented in the experiments.

PATIENTS SAMPLES

Healthy controls and HIV-1 positive patients used in paper I

Samples were collected from 55 HIV-negative and 74 HIV-infected consenting adults. The participants were part of various Botswana–Harvard School of Public Health AIDS Initiative Partnership studies and the Botswana sentinel surveillance study. The participants HIV serostatus was confirmed by two standard enzyme-linked immunosorbent assays (ELISA) and viral load was determined, using the automated COBAS Amplicor. CD4 cells were enumerated using a four-parameter FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, US). DNA for HLA typing was isolated from buffy coat or peripheral blood mononuclear cells.

HIV-1 positive patients used in papers V and VI

For paper V, 42 samples were collected in 2003 and 11 samples were collected in1996 from patients at different stages of HIV infection. The patients had not been treated with any antiretrovirals and were thus treatment naïve. The patients studied in 2003 were from differerent Botswana–Harvard School of Public Health AIDS Initiative Partnership studies, while the patients, studied in 1996, were attending the Botswana government health service centres.

For paper VI, 24 patients were recruited, who were undergoing antiretroviral treatment and experienced virologic failure in the Botswana national antiretroviral treatment programme.

TB patients and healthy controls used in paper IV

Three groups of participants were involved. Thirthy four patients who tested positive for active pulmonary TB, as defined by positive acid fast staining bacteria in sputum, were recruited from the Medical Yerevan State university hospital in Armenia. They had all received routine BCG vaccination in childhood.

Six patients with active TB, as defined by positive acid fast staining bacteria in sputum and *M.tb* culture, were recruited from the Karolinska Hospital Huddinge in Sweden. The patients had not received BCG vaccination. Thirty five healthy individuals with no previous BCG vaccination, testing negative for the tuberculin skin test and the Quantiferon-test at Saint Louis University, USA were enrolled in a TB vaccine study.

METHODS

HLA Class II typing

The HLA-DRB BigDye Terminator Sequencing-Based Typing kit (Applied Biosystems, Foster City, Calif.) was used for the DRB locus typing. Low-resolution sequence-specific primer PCR (SSP-PCR) was performed, based on allele group-specific motifs in the first hypervariable region of exon 2. SSP-PCR positive reactions were sequenced using BigDye terminator cycle sequencing chemistry on the ABI PRISM 3100 Genetic Analyzer automated sequencer (Applied Biosystems). Allele assignment was performed using MatchTools software and MTNavigator software, provided in the Matchmaker Allele Identification software package.

For DQB locus genotyping, we used the low-resolution Micro SSP HLA DNA typing trays (One Lambda, Inc, Canoga Park, CA, US) or the DQB1 SSP UniTray kit (Pel-Freez Clinical Systems, LLC, Brown Deer, WI, US). These systems use SSP-PCR after which the PCR products are run on a gel and the products visualised. The gel results were interpreted according to the manufacturers' instructions and the relevant HLA DQB alleles assigned.

Peptide microarrays (papers II, III and IV)

Peptide microarray are glass slides with linear peptides printed at specific positions on the slides. The technology for peptide printing has advanced and allowed to print thousands of peptides on chips with appropriate biochemically activated surfaces. This makes it possible to explore the reactivity of a broad array of candidate target peptides within a single experiment. In principle, an entire proteome from any pathogen can be displayed as linear peptide streches on microarray chips. Although confirmational epitopes may not be covered, the intrinsic problem of batch-to-batch variation associated with folding of

recombinant proteins is avoided. This is a valuable feature when there is a need to explore all the genomic data that has been generated from whole genome sequencing of different pathogens, since an entire pathogen genome can be displayed as the corresponding proteome.

The peptide microarrays were produced at JPT (Berlin, Germany). The protein sequences which were used to design the overlapping peptides for the HIV slides were extracted from the Los Alamos HIV database. The *M.tb* protein sequences were extracted from the swiss-prot database.

Virus Isolation (Paper V)

Two million peripheral blood mononuclear cells (PBMCs) from an HIV-positive individual were co-cultured with 5 μg/ml phytohemagglutinin (Sigma, St. Louis, MO) stimulated HIV-uninfected PBMCs in the presence of 20 U/ml of interleukin-2. Cultures were sampled and fed with 50 % of replacement of the total culture volume at days 4, 7, 10, and 14. Viral growth was monitored by detection of p24 by ELISA.

Biological phenotyping (Paper V)

U87.CD4 cells expressing chemokine receptors CCR1, CCR2b, CCR3, CXCR4, or CCR5 were used for biological phenotyping of the virus isolates. PBMC culture supernatants, corresponding to 2000 pg of p24 antigen per well, were used to infect the U87.CD4 cells that expressed different chemokine receptors. Sampling was done by replacing half of the culture medium at days 4 and 7. Viral growth was again monitored by p24 ELISA.

Population sequencing (Paper V and VI)

In both paper V and VI, nested PCR was run with primers to amplify the V3 loop and part of the flanking C2 and C4 regions. The primers used in the two papers were different, but the products covered the entire V3 loop. The PCR products were then directly sequenced without cloning, resulting in population sequences, which in both papers were edited using the majority rule to obtain the amino acid sequence. If the sequence was used for phylogenetic analysis, correction was performed with ambiguity codes.

Single genome sequencing (Paper VI)

In paper VI, we used single genome sequencing to capture the diversity of the viral strains within the patients. Single genome sequencing has been shown to be more sensitive in detecting viral diversity in patients undergoing antiretroviral treatment. This is because compared to bulk or population sequencing, in single genome sequencing, the sequence that is generated is from one genome. This is made possible by dilution of the template to a point where ≤ 30% of the replicas become PCR positive. The resulting PCR products are sequenced and the sequences are also checked to make sure that there are no ambiguities, as a sign that the amplicon is not amplified from a single genome.

Phylogenetic analysis (Paper V and VI)

Phylogenetic analysis is the use of genetic information to gain understanding of the organism's evolutionary relationships. Most of the input data for phylogenetic analysis is a DNA sequence. The output is in the form of a phylogenetic tree, which shows relatedness of the input DNA sequences. In paper V and VI, phylogenetic analysis was used to identify the viral subtype as well as to determine the relationship between the viral genomes.

After the DNA sequences were edited and the consensus sequences generated Sequencher, the sequences were alligned using ClustalX version 1.81. The alignment was manually adjusted using BioEdit. The neighbor-joining tree and bootstrap values were generated in CluxtalX and visualised using Figtree.

Phenotype prediction using Geno2pheno(Paper VI)

Although biological phenotyping remains the golden standard for determining the coreceptor use of viral isolates, it is time consuming and expensive. Strategies of viral phenotype , which are based on the viral genotype are improving and are now being used instead of biological phentoyping. The geno2pheno method (193, 194) is one such method. It is freely available for use and has been used by others successfully (195).

In paper VI we used the geno2pheno method to predict the phenotype of the 294 single genome sequences and also of the population sequences.

RESULTS AND DISCUSSION

Paper I

The MHC complex represents the most polymorphic gene products in the human genome. Different populations have different MHC allele frequencies, in part due to different antigenic exposure. Since the MHC is crucial in determining the selection of antigenic peptides presented to the cellular immune system, it is important to increase our knowledge in the frequencies of MHC alleles in different populations. This may aid to better design and monitor T-cell based vaccines.

Previous work in the Botswana population had determined the MHC class I allele frequencies and found that these were different from those of other populations (196). In the current study, we sought to determine the HLA class II allele frequencies in the Botswana population and determine the associations of the different alleles with HIV infection and control. The 129 study participants were recruited from blood donors in the capital city of Botswana, Gaborone, whose population is quite diverse and represents the diversity of people in Botswana.

Comparing the allele frequencies in our study population to allele frequencies reported for north American Caucasians, there were statistically significant differences in the frequencies of the following alleles; HLA DRB1*04, DRB1*07, DRB1*08, DRB1*11, DRB1*13, and DRB1*14. There were also statistically significant differences in the frequencies of HLA DRB1*02, DRB1*07, DRB1*09, DRB1*11, and DRB1*14 compared to the allele frequencies in Afro-Americans. Importantly, our data confirmed the high prevalence of HLA DRB1*11 and DRB1*13 in the African populations.

To determine the association of the different MHC alleles with HIV infection, we compared the allele frequencies in the healthy and the HIV infected participants. HLA DRB1*01 was found to be overrepresented in the healthy participants in a statistically significant way ($P = 0.002$). The same allele was found to be associated with protection from HIV infection in a study in Kenya, which followed up highly HIV exposed sex workers (133). It remains to be determined how the HLA DRB1*01 allele could be protective, it may be possible that HLA DRB1*01 binds and presents a conserved HIV epitope that the virus cannot mutate without incurring a high fitness cost.

In the haplotype analysis, DRB1*01-DQB1*03 and DRB1*01-DQB1*04 were significantly more common among the HIV-negative participants than in the HIV-positive participants with P values of 0.013 and 0.042, respectively. None of these haplotypes have previously been associated with protection against HIV infection.

For the HIV-1 positive participants, we determined their CD4+ T-cell counts as well as their RNA viral loads. This enabled us to determine if any of the MHC alleles were associated with better control of infection. We compared these infection control associated parameters in those participants lacking the alleles with those individuals testing positive for the alleles. Of all the alleles, only HLA DRB1*08 was associated with a low mean viral load (P = 0.011).

Our results provided the MHC class II allele frequency in the Botswana population and showed that the allele frequencies were different from well characterized western cohorts. We also identified MHC alleles which were associated with HIV infection and HIV control i.e. HLA DRB1*01 and HLA DRB1*08 respectively. Future studies are needed to determine the mechanisms of these associations.

Paper II

The main function of the MHC class II alleles in the immune system is to bind peptides and present them to the CD4+ T-cell receptors. In paper II, we sought to design an assay that could be used to identify peptides binding to HLA class II alleles. We used this assay to identify HIV-1 peptides, binding to three common HLA class II alleles, HLA DRB1*0101, HLA DRB1*0401, and HLA DRB1*1501.

The peptide microarrays with 552 overlapping peptides derived from the HIV-1 subtype B consensus were incubated with recombinant, soluble HLA DRB1*0101, HLA DRB1*0401, and HLA DRB1*1501 molecules. The formation of the MHC class II peptide complex was detected by an anti-HLA DR antibody which was tagged with a fluorochrome Cy5. After slide scanning and quality data extraction, we identified 208/552 peptides binding to DRB1*0101, 163/552 peptides binding to DRB1*1501, and 162/552peptides binding to DRB1*0401 in at least one of the spots in which the peptides were printed.

Focusing on more reproducible responses, we identified 78, 91, and 101 peptides that bound to DRB1*0101, DRB1*1501, and DRB1*0401, respectively, in forty percent or more of the spots in which the peptides were printed. The

position of the HLA DR binding peptides within the HIV-1 gp160 and nef proteins were determined. Most of the peptides were within areas already described in the literature as CD4+ T-cell epitope rich areas. It was, however, difficult to make a good comparison with the published CD4+ T-cell epitopes, since most of the published epitopes were not reported with the exact identity of the presenting MHC class II molecules.

To evaluate whether the HIV peptides we identified in this study could act as CD4 + T-cell epitopes, we used four of the peptides, i.e. SLYVTVATLYCVHQRIEV $gag_{77\text{-}94}$, FRKQNPDIVIYQYMDDLYVG $RT_{171\text{-}190}$, ALFYKLDVVPINDNTSYRL $gp160_{174\text{-}193}$ and EKLWVTVYYGVPVWKEATTT $gp160_{32\text{-}51}$ in an *in vitro* assay to select for peptide specific CD4+ T-cells in HLA class II matched donor PBMCs, using intracellular cytokine staining. Compared to unmatched and unstimulated PBMCs, the peptide stimulated PBMCs produced more TNF-α, IFN-γ and IL-2. This supports the notion that MHC class II and peptide specific CD4+ T-cells exist in the T-cell repertoire which recognize these epitopes identified in the MHC class II-peptide binding assay.

Paper III

The full *M.tb* genome has been sequenced (140). The challenge is to translate the genomic data into biological functions that can be harnessed for intervention strategies against *M.tb* infection. A recent review showed that only 4% of the *M.tb* proteome has been explored for immune epitopes (197). Given the size of the *M.tb* proteome, more innovative strategies are needed to explore the proteome for potential vaccine targets.

In paper III we used the assay described in paper II to explore selected proteins from the *M.tb* proteome for peptides binding to three common MHC class II alleles. The aim was to identify potential CD4+ T-cell epitopes that could be useful in vaccine design and immune monitoring. The *M.tb* peptide arrays were incubated with the soluble HLA class II monomers as described for paper II.

Of the 7466 peptides, 1282, 674, and 1854 peptides formed stable complexes with HLA-DR1, -DR2 and -DR4, respectively. Five hundred and forty-four (544) peptides bound to all three MHC class II molecules, 609 only to two, and 756 bound only to a single MHC class II molecule. Figure 1 paper III, shows a cluster analysis of the peptides and the binding patterns for the three HLA DR

alleles which were quite distinct although there were peptides that bound to all the three alleles.

Calculation of the protein epitope densities enabled us to rank the proteins without bias for the protein size. Immunogenic protein MPT63 (Rv1926c) was the protein with the highest epitope density amongst the 61 proteins printed on the slides. This is interesting because Rv1926c has been shown to be highly recognized by PBMCs of HLA DR heterogeneous donors (198). Comparison of our data with that of Mustafa A et al (199) gave a strong concordance concerning the Antigen 85B epitopes with 10/13 epitopes previously identified. This shows that our results are comparable to data generated using biological, functional assays. Therefore the assay may aid to rationally test for the most immunogenic proteins/protein-regions based on MHC class II binding.

We also identified peptides that were binding to the three HLA DR alleles that were exclusively recognized by sera from TB patients (Paper IV). These peptides, and the corresponding proteins, may be attractive candidates for vaccine design as B cell targets are usually predictive for CD4+ T-cell targets (200, 201).

Paper IV

Antigen specific B cells are able to process and present antigens to CD4+ T-cells via MHC class II (202). As such, antigens that are targeted by antibodies have been used to identify CD4+ and CD8+ T-cell epitopes in the cancer field (203, 204). In paper IV, we sought to identify B cell targets in the *M.tb* proteome which could be predictive of CD4+ T-cell epitopes. The role of antibodies in *M.tb* infection is not well defined, yet there is renewed interest in defining B-cell epitopes in *M.tb* infection: B-cells may act as antigen presenting cells, presenting *M.tb* targets to CD4+ T-cells.

The *M.tb* peptide microarrays were incubated with sera from well defined cohorts; 34 patients who tested positive for active pulmonary from Armenia, 35 healthy individuals testing negative in the tuberculin skin test (TST) and the Quantiferon-test from USA, and 6 patients with pulmonary TB from Sweden. The peptide-antibody complexes were detected by an anti human IgG or IgA antibody conjugated to a fluorochrome. The fluorescence was then detected using a scanner, the images were analyzed and after data normalization quality data were extracted.

Comparing the peptide responses in the 34 TB patients with the 35 healthy individuals, three patterns of IgG reactivity were identified: 89/7446 peptides were differentially recognized (in 34/34 TB patients and in 35/35 healthy individuals) by SAM and PAM analysis and were highly predictive of the division into TB+ and TB-. Other targets were exclusively recognized by TB patients (e.g. Rv3286c) but not in any of the healthy individuals. A third peptide set was recognized exclusively in healthy individuals (35/35) but no in TB patients.

The segregation between TB infected and TB uninfected did not cluster into specific recognition of distinct *M.tb* proteins, but into specific peptides epitope 'hotspots' at different locations within the same protein. There was great concordance in the antigen recognition pattern profiles in serum from TB patients from Armenia versus patients recruited in Sweden. This showed that IgG-defined *M.tb* epitopes were very similar in individuals with relatively different genetic background. This raises the hope that the targets we identified in our cohort might also be important targets in the *M.tb* endemic areas, despite the genetic differences. This, however, needs to be confirmed by testing these peptides in well defined cohorts in *M.tb* endemic areas.

For the patients recruited from Sweden, the IgA responses were also analyzed. There were peptides that were recognized by both IgA and IgG and also peptides that were exclusively recognized by either.

The peptides that are exclusively recognized in the TB patients only are also interesting as potential diagnostic tools. A recent review by Doherty et al 2009 shows that antibody screening assays are some of the more promising tools for improved TB diagnosis (205).

Paper V

In addition to the CD4 which is the main cellular receptor used by HIV, the virus needs the presence of a coreceptor for viral entry into the host cell. The main HIV-1 coreceptors have been identified as CCR5 and CXCR4. HIV-1 subtype C is the most prevalent of all the HIV subtypes, causing close to 40% of the global HIV epidemic. Since this subtype predominates in developing countries, it has been less studied than HIV-1 subtype B which predominates in the developed countries. It is not clear if the relative success of HIV-1 subtype C spreading is related to viral factors or to host factors or to possible environmental factors. It is most likely

though that all these factors play a role. One of the interesting features of HIV-1 subtype C has been that there have been few documented cases of the virus evolving to use CXCR4 as the main coreceptor as is documented for other subtypes.

In paper V, we explored the in vitro growth characteristics of HIV-1 subtype C and determined the coreceptor use of HIV-1 subtype C isolates from Botswana patients. The viral isolation was successful for 75% (21/28) of the samples, collected from patients with CD4+ T-cell counts less than 200 and 53.8 % (7/13) of the samples collected from patients with CD4+ T-cell counts above 200. This did not represent a statistically significant difference (chi-square = 1.83, P = 0.18; Fisher's Exact test, P = 0.28).

Using U87.CD4 glioma cells, expressing different coreceptors, we explored the coreceptor use of the viral isolates. All the twenty-nine viruses studied from 1996 and 2003 that grew used CCR5 as a coreceptor for cell entry. One viral isolate from 1996 and two viral isolates from 2003 used CXCR4 more efficiently than they used CCR5. Six isolates used CXCR4, but less efficiently than they used CCR5. These results show that for HIV-1 subtype C, even in these patients who have low CD4+ T-cell counts, CCR5 is still the main coreceptor used by the viral isolates.

None of the viral isolates used any other coreceptors other than CCR5 and CXCR4. Overall, there was no difference in the coreceptor use for the 2003 viral isolates compared with the 1996 viral isolates, showing that in Botswana there is no evolution of HIV-1 subtype C to use CXCR4 more as postulated by Cilliers and colleagues (206) for the HIV-1 subtype C epidemic in South Africa.

Paper VI

There have been a number of reports indicating that patients who are failing ARV treatment are more likely to harbor CXCR4 using viral strains compared to treatment naïve patients (207, 208). Other groups have, however, failed to confirm this finding (195).

With Maraviroc, a CCR5 inhibitor, licensed by the FDA as one of the drugs that can be used in combination with others to treat HIV, the viral coreceptor use becomes an important factor to consider in the treatment of HIV patients, since Maraviroc is not active against CXCR4 using strains. In paper V we sought to

compare coreceptor use in treatment naïve patients and in patients failing treatment. For the patients failing ARV treatment we also compared population sequencing and single genome sequencing.

To determine the HIV coreceptor use, we sequenced the viral V3 loop and used Geno2pheno to predict the coreceptor used by the virus. From the population sequences of the 24 patients failing ARV treatment, five were predicted to harbor CXCR4 using strains and 19 carried CCR5 strains only. In the 23 treatment naïve controls, 2 patients harbored X4 or R5X4 strains, both able to use CXCR4, while 21 harbored R5 strains only (P>0.2).

Population sequencing has previously been shown to underestimate the frequency of drug resistant HIV variants in patients undergoing ARV treatment compared to single genome sequencing. We then used single genome sequencing for the 24 patients failing treatment to see if the results would differ from population sequencing. Of the 24 patients, 11/24 (45.8 %) of the patients failing treatment had at least one clone (>5%) that was predicted to be able to use CXCR4, P = 0.04, as compared to the 5/24 (20.8 %) detected by population sequencing. This shows that population sequencing does underestimate the viral population diversity and in the process underestimates the frequency of viral strains that may use CXCR4.

Samples from patients analyzed in this study carried HIV-1 subtype C, which is known to rarely use CXCR4. Still, failing antiretroviral treatment with drugs that do not involve coreceptor inhibitors was associated with a large number of patients that harbored viral strains with the ability to use CXCR4. It could be argued that in most of the patients these strains able to use CXCR4 were a minority. However, recent results (209, 210) show that CXCR4 strains at 1% before commencing Maraviroc treatment could be amplified to become the predominant variants and result in Maraviroc resistance. Patients failing treatment therefore ought to have the viral phenotype determined before Maraviroc treatment is provided.

CONCLUSIONS

The outcome of an infection is the result of the complex interplay between the host, the pathogen and environmental factors. Understanding this complex interplay can lead to better intervention strategies. HIV and *M.tb co*-infections represent also an increasing health problem and the definition of MHC class II in both infections may aid to devise new treatment strategies.

- Through exploring the MHC class II allele frequencies and their association with HIV infection, we identified alleles at high prevalence in the Botswana population and MHC alleles, which were associated with prevention of HIV infection.
- We developed an assay, using peptide microarrays and recombinant soluble MHC class II monomers useful for identifying peptides capable of binding to MHC class II alleles. We used this assay to identify HIV and *M.tb* peptides, binding to three common HLA class II alleles. The identified peptides could be useful for exploring immune responses to HIV and *M.tb* in the context of designing subunit vaccines and immune monitoring.
- We identified peptides that are differentially and exclusively recognized by serum antibodies in *M.tb* infection, using a peptide microarray assay. These peptides could be important in the diagnosis of TB. Also since B-cell epitopes predict CD4+ T-cell targets, the proteins from which the peptides are derived, could be important targets to explore for CD4+ T-cell epitopes and immunogenic targets.
- HIV-1 subtype C is the most predominant circulating HIV-1 subtype. We found that viral isolates from treatment naïve patients in Botswana preferentially used CCR5 as the main coreceptor, regardless of the disease stage. This was true for patients from early in the epidemic, 1996, and later on, in 2003. It is still not clear why there is a low frequency of CXCR4 use in subtype C, compared to other HIV-1 subtypes like D and B. In patients who were failing antiretroviral treatment, using single genome sequencing, we found that a large number harbored strains that were predicted to use CXCR4. CCR5 inhibitors would probably not work in these patients and we therefore recommend that before starting patients on coreceptor

inhibitors, blood from the patients should be screened for the presence of HIV virus strains that could be resistant to the drug.

FUTURE PERSPECTIVES

In sub-Saharan Africa, infections with HIV and *M.tb* are noticeable parts of everyday life. In most families someone is affected by either or both infections. The existence of valuable drugs to decrease the disease burden is jeopardized by costs and the development of drug resistance. Multi drug resistant *M.tb* thrives and spreads in patients, infected by HIV. In a country like Botswana HIV infection has become endemic. The risks of developing complications by coinfections with *M.tb* are a reality. All aspects of understanding how the two infections could be coped with in a better way are important.

I emphasized in my thesis the immune recognition of HIV and *M.tb* peptides, imposed by the structural constraints of the MHC class II molecules. This included the analysis of HIV phenotypes which may be important for drug failure as well as differential binding to MHC molecules and therefore escape from immune recognition. As is the case in most research work, the work carried out here leads to more interesting questions. Some of the questions that need to be addressed are mentioned below.

Regarding the association of HLA DRB1*01 and HLA DRB1*08 with HIV infection and control respectively, it will be important to determine the mechanisms. It is interesting that HLA DRB1*01 was also found to be associated with reduced HIV-1 infection in a longitudinal study involving commercial sex workers (133, 211). The most probable mechanism is that these MHC alleles bind certain conserved epitopes, which lead to a strong CD4+ T-cell response, as indicated by production of cytokines.

Peptide binding experiments to identify the peptides binding to these alleles can be done and then the peptides identified can be used in intracellular cytokine staining assays to determine the cytokine profile in HLA DR matched HIV infected patients. It will also be important to do the MHC class I typing in the same cohort and determine if the HLA DR effects we found are a result of the HLA DR alleles acting as a haplotype with some already described MHC class I alleles or not.

Recent data from studies in rhesus macaques (212) show that the presence of certain CD4+ T-cell responses in concert with CD8+ T-cell responses might result in better control of SIV replication.

It would also be important to determine the association of MHC class II alleles with *M.tb* infection in this population. A number of studies have identified alleles that are associated with *M.tb* infection or disease progression, but such work has not been carried out in the Botswana population.

The peptides we identified to bind to the three common HLA DR alleles need to be tested using patient samples to determine how immunogenic they are and to determine if recognition of any of the peptides is associated with better outcomes as has been shown with certain epitopes. If the peptides are associated with better outcome, they can be considered for a subunit vaccine, potentially in a boost-setting.

The differentially and exclusively recognized peptides need to be tested in well defined cohorts to explore their diagnostic potential. It would be interesting to find out if the exclusively recognized peptides can still differentiate the TB patients from healthy individuals in TB endemic areas, where there is increased exposure to environmental mycobacteria as well as universal BCG vaccination. Also, further work is needed to locate the epitope sites in the protein structures to help determine why certain epitopes are recognized.

The impact of HIV phenotypes on immune recognition is an interesting area that needs to be addressed. It is has been shown that the emerging HIV X4 strains are easier to neutralize than R5 strains (213). This has been used as one of the reasons why X4 strains mostly appear when the patients are immune compromised as they are easier targets for the immune system. Knowing the important role of CTL and T-helper cells in the immune response, it is tempting to hypothesis that X4 strains could be easier targets of T-cells compared to R5 strains.

Our preliminary data interestingly show that X4 strains had peptides that were binding to more MHC class II alleles compared to R5 strains from the same patient. This needs to be further analyzed by testing these peptides in patient cells to determine if the X4 peptides are ‘better’ recognized. For these experiments, it becomes necessary to design many variants of V3 loop peptides, rather than many peptides from larger portions of the proteins, since variation in the V3 loop

determines if the strains are CCR5 using or able to use CXCR4. Knowledge is accumulating, regarding the V3 loop peptide patterns of R5 and X4 strains, respectively. We could also use the collected information in papers V and VI of such peptides for future characterization of binding of R5 and X4 peptides to the HLA monomers.

When I return to Botswana after my dissertation, I hope that it will be possible to continue research along the lines discussed here to promote programs to reduce the impact of these infections.

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