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ROLE OF ALLOIMMUNITY IN SUSCEPTIBILITY TO HIV-1 INFECTION

Melinda Shelley Suchard

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I, Melinda Shelley Suchard, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It was submitted in April 2021 at this University and has been revised in line with recommendations by the examiners.



.....

14 December, 2021

Dedication

Time to work on a PhD degree at an advanced career stage is an academic indulgence not afforded to many. I appreciate all the support, professionally and at home, that made such an endeavour possible. I dedicate this work to my father Ronald Isaacs, who inculcated skeptism, questioning and debate; to my late mother Felicia Isaacs, who instilled confidence and a passion for learning and teaching; to my husband Rowan, who caught all the balls I dropped; and to my special children Talia, Adam and Amy, for whom I hope to model life-long learning. I thank Dave and Minette Suchard for their unwavering support.

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1. **Suchard MS**, Martinson N, Malfeld S, de Assis Rosa D, Mackelprang R, Lingappa J, Hou X, Rees H, Delany-Moretlwe S, Goldfein H, Morris L, Tiemessen CT and Savulescu DM. Alloimmunity to class 2 human leucocyte antigens is associated with reduced HIV-1 acquisition – a nested case-control study in HIV-1 serodiscordant couples. Manuscript submitted to Frontiers in Immunology, 2021.

2. Savulescu DM, Groome M, Malfeld SCK, Madhi S, Koen A, Jones S, Duxbury V, Scheuermaier K, de assis Rosa D and **Suchard MS**. HLA antibody repertoire in infants suggests selectivity in transplacental crossing. American Journal of Reproductive Immunology 2020, e13264 https://doi.org/10.1111/aji.13264

3. **Suchard MS***, Savulescu DM*, Neil Martinson, Susan Malfeld, Debbie de Assis Rosa, Romel Mackelprang, Jairam Lingappa, Xuanlin Hou, Helen Rees, Sinead Delany-Moretlwe, Hadassa Goldfein, Lynn Morris, Caroline T. Tiemessen and de Assis Rosa, D. Class 2 human leucocyte haplotypes reveal unusual linkage patterns HIV-1 serodiscordant South Africans. Manuscript in preparation. (*joint first authors)

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2. Savulescu DM, Groome M, Malfeld SCK, Madhi S, Koen A, Jones S and **Suchard MS**. Investigation of mother to child transmission of HLA antibodies. Poster presentation and International Union of Immunological Societies Congress, 19-23 October 2019

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Abstract

Allo-immunity is defined as immunity of one individual against another of the same species. In humans allo-immunity can be measured as antibodies against human leucocyte antigens (HLA), termed HLA antibodies. HLA antibodies are naturally occurring antibodies present in an individual with specificity against the HLA proteins of another individual. HLA antibodies may develop following exposure to HLA proteins of another individual. HLA antibodies may arise during pregnancy but may also be present in women who have never been pregnant as well as in men and children.

The overarching hypothesis of this thesis was that HLA antibodies may play a physiological role in the human immune system and protect against infection with certain pathogens. Enveloped viruses, including Human Immunodeficiency Virus-1 (HIV), incorporate host proteins such as HLA into their envelope. Pre-existing HLA antibodies may bind to these extraneous surface proteins and impact viral infectivity. Macaque studies using simian immunodeficiency virus have confirmed that xenoimmunization with HLA antigens protect against infection. Since HIV gp120 shows homology with class 2 HLA, including shared affinity for binding to CD4, antibodies against class 2 HLA proteins (class 2 HLA antibodies) have the potential to influence HIV acquisition via binding to gp120 on the viral envelope.

To explore whether naturally occurring HLA antibodies in humans protect against HIV acquisition, in the first part of this work I characterized the HLA allele and haplotype frequencies of HIV-1 serodiscordant couples. In the second part of the work I analysed class 1 and class 2 HLA antibodies in originally HIV-uninfected partners. I interrogated whether the prevalence of HLA antibodies varied between non-seroconverting and seroconverting individuals. First I assessed whether the prevalence of antibodies against any class 1 or class 2 locus varied between non-seroconverting and seroconverting individuals. I then analysed only those antibodies directed against the HLA type of the HIV-infected sexual partner, which I termed *matching antibodies*. I enquired whether *matching antibodie*s differed between non-seroconverters and seroconverters. In the third section of this work, I investigated the natural history of HLA antibody development in the physiological situations of pregnancy and infancy. I analysed whether maternal HLA antibody specificities were shared between mothers and their infants. This work serves as background for future studies in infants examining whether infant HLA antibodies impact the risk of viral infections in the infant.

Results from 143 HIV-serodiscordant couples showed that my cohort was representative of the wider South African black population, with a few minor variations. Dominant haplotypes were HLA-A*01 – HLA-B*81 – HLA-C*18 for class 1 and HLA-DPA*02 – HLA-DPB*01 – HLA-DQB*02 – HLA-DRB1*03 – HLA-DRB3*02 for class 2. For the less frequently investigated HLA-DRB3/4/5 loci, I found unusual HLA-DRB1-HLA-DRB3/4/5 haplotypes, which may have future relevance to vaccine design, disease association studies and transplantation medicine if confirmed by sequencing.

Using highly sensitive Luminex-based methodology, I characterized HLA class 1 and 2 antibodies in the originally HIV uninfected partner, in 115 highly exposed seronegative individuals and 28 individuals who went on to acquire HIV. Seventy-nine percent of highly exposed persistently seronegative healthy individuals had HLA antibodies; 56% against class 1 and 50% against class 2 alleles. The findings emphasize that HLA antibodies are common in healthy individuals.

Prior to seroconversion, significantly more highly exposed persistently seronegative individuals than seroconverters expressed class 2 HLA antibodies (50% versus 29%, p=0.05). These data are in keeping with the hypothesis that class 2 HLA antibodies may protect against HIV acquisition. In univariate logistic regression, class 2 HLA antibodies at the early timepoint were associated with significantly lower odds of HIV acquisition (OR 0.393, CI 0.159-0.969, P=0.04) and each log viral load in the index partner showed odds ratio of 2.192 (CI 1.137-4.225, P=0.02) for HIV acquisition by the partner who was HIV-uninfected at enrolment. Gender, age, ethnicity, number of children, history of unprotected sex and presence of class 1 HLA antibodies were not significantly associated with HIV acquisition. In multivariate regression, presence of class 2 HLA antibodies remained significantly associated with lower odds of HIV infection (odds ratio of 0.330, confidence interval 0.112-0.976, p=0.05). Incident HIV infection was a sensitizing event leading to *de novo* development of antibodies against HLA-A and HLA-B loci at later timepoints, but not against class 2 loci.

HLA antibodies were further analysed to assess if they matched the HLA types of the sexual partner, whether such antibodies were potentially autoreactive and if there were additional complement-fixing class 2 antibodies. Interestingly, at the early timepoint, there was no significant difference in *matching* HLA antibodies between highly exposed persistently seronegative individuals (controls) and those who later acquired HIV. We can thus conclude that that any protective effect of class 2 HLA antibodies is due to general specificities rather than specificities directed only at the HIV "donor". Following HIV

acquisition, seroconverters had a higher frequency of *matching* HLA-A antibodies than those who did not seroconvert, in keeping with the general trend of more HLA-A antibodies post-seroconversion than in controls at the later timepoint.

HLA autoantibodies, analysed against HLA genotypes at two-digit resolution, were not uncommon. At early timepoints, there was a trend to a higher frequency of class 2 HLA autoantibodies in controls (16% of controls compared with 0% of seroconverters, p=0.06), in keeping with the finding of more class 2 HLA antibodies generally in controls at the early timepoint. Seroconverters, following seroconversion, tended to have a higher frequency than controls of HLA-B autoantibodies (16.7% in seroconverters versus 5.1% in controls, p=0.07). There was no difference in frequency of complement-fixing class 2 HLA antibodies between seroconverters and controls.

In the third part, my published work analysed transplacental transfer of HLA antibodies from pregnant women to their infants. Infant HLA genotypes are partly paternally inherited, thus maternal HLA antibodies may act as autoantibodies in the infant. In a cross-sectional analysis of 30 mother-infant pairs and longitudinally in six pregnant women, HLA antibodies were measured and the HLA class 1 and class 2 loci in infant DNA were genotyped to assess whether maternal HLA antibodies were directed at infant specificities.

Results showed that 68% of mothers and 44% of infants expressed class 1 HLA antibodies, while 56% of mothers and 52% of infants expressed class 2 HLA antibodies. Infants shared up to 78% of antibodies with their mothers, suggesting that the remaining antibodies were self-made. Less than 25% of maternal HLA antibody specificities were detected in infants, possibly due to selection in transplacental crossing. Complement-fixing HLA antibodies were detected in mothers and at low levels in infants. In a third of pregnant subjects studied longitudinally, infant-directed HLA antibodies were detected.

In summary, I report a high frequency of natural HLA antibodies in healthy adults, healthy pregnant women and healthy infants. I found a higher frequency of class 2 HLA alloantibodies of IgG isotype in highly exposed seronegative individuals compared to those who went on to acquire HIV. This data provides epidemiological support to the hypothesis that class 2 HLA alloantibodies of IgG isotype may lower the risk of HIV acquisition, likely even if not directed at the sexual partner from whom HIV was transmitted. Potentially autoreactive HLA autoantibodies appear present in the general population at

low frequency without obvious health consequences. I have further provided evidence that HLA antibodies, well-known to occur in pregnancy, can cross the placenta to the foetus, but in a non-uniform manner such that certain antibody specificities in the infant are shared with that of the mother, but not all. Maternal antibodies included antibodies directed against the HLA genotype of the infant. Further, six week old infants had various HLA antibody specificities present that were not detected in the mother, suggesting *de novo* synthesis of HLA antibodies in young infants. The role of HLA antibodies in development of the healthy neonatal immune system and protection against infection warrants further exploration.

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This work was conducted while I was employed by the National Institute for Communicable Diseases, a division of the National Health Laboratory Service, South Africa. I acknowledge technical assistance of Dr Dana Manuela Savulescu, Dr Susan Malfeld, Ms Hadassah Goldfein and Dr Heena Ranchod, who were trained by myself and conducted the HLA typing and HLA antibody laboratory work. All antibody interpretations on the Match-It software, using the Luminex instrument, were performed by myself for both parts of this study.

For the first part of this work, namely presentation of allele and haplotype frequencies, I thank Debra de assis Rosa who assisted with use of Arlequin software and gave me the output files. I conducted the analysis on the output files. For the second part of the work, namely the investigation of HLA antibodies in HIV susceptibility; study design, statistical analysis of HLA antibodies and data interpretation and writing were performed by myself and are presented as a manuscript of which I am the first author. Data tables, analysis of matching compared with non-matching antibodies, autoantibodies and complement-fixing antibodies were performed by myself. I enlisted assistance of a statistician, Kennedy Otwombe, for the logistic regression. I also thank Dr de Assis Rosa for formative discussions, in addition to an early mentor, Professor Barry Mendelow.

For the third part of this work, namely the exploration of HLA antibodies in pregnant women and their infants, I designed the study, trained and supervised a post-doctoral fellow (DM Savulescu). The fellow conducted the laboratory work and performed the statistical analysis. She was therefore first author of the related peer-reviewed publication which has been included in this dissertation, and I was the senior author. I guided her analysis throughout, including critical review of figures, tables and interpretations as well as focused discussion.

I acknowledge the clinical investigators from the trial sites who facilitated access to the study samples, including Prof Neil Martinson, Prof Jairam Lingappa, Dr Romel Mackelprang, Prof Helen Rees, Prof Sinead Delany-Moretlwe and Dr David Coetzee. Thanks to Mercy Kamupira, Vania Duxbury and Xuanlin Hou for facilitating access to samples and data retrieval. I thank my supervisors, Prof Lynn Morris and Prof Caroline Tiemessen for their enduring support and constructive criticism.

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Abbreviations

Aa	Amino acid
AIDS	Acquired immunodeficiency syndrome
AMP	Antibody Mediated Protection
CD	Cluster of differentiation antigen
CDC	Complement-dependent cytotoxicity
CTL	Cytotoxic T cells
DNA	Deoxyribonucleic acid
***gag/*Gag**	HIV group-specific antigen gene/protein
***env/*Env**	HIV envelope gene/protein
FlowPRA	Flow cytometric panel reactive antibody
gp120	HIV glycoprotein 120
gp160	HIV glycoprotein 160
HIV	Human immunodeficiency virus type 1
HLA	Human leucocyte antigen
HLA antibody	Antibody against human leucocyte antigen
IgH	Immunoglobulin heavy chain
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
***pol/*Pol**	HIV polymerase gene/protein
***nef/*Nef**	HIV negative regulatory factor gene/protein
SHIV	Simian-human immunodeficiency virus
SIV	Simian immunodeficiency virus
PHRU	Perinatal Health Research Unit
RHRU	Reproductive Health Research Unit
TB	Tuberculosis
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation

1. CHAPTER 1 - Introduction

1.1 Introduction to HIV epidemiology and immunology

1.1.1 Global burden of HIV

The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that in 2019 there were 38 million people living with HIV-1 (HIV). Despite modes of HIV transmission being known for decades, in 2019 there were 1,7 million new HIV infections and 690 000 deaths from AIDS-related illnesses [1]. UNAIDS had set a goal for the year 2020 known as the 90-90-90 goal. The aim was for 90% of people living with HIV to know their status, for 90% of those who know their status to be on antiretroviral therapy, and for 90% of those on antiretroviral therapy to be virally suppressed. Achieving this target was crucial for curtailing transmission of the disease. Globally UNAIDS estimates that in 2019, 81% of people living with HIV knew their HIV status, 82% who knew their status were accessing treatment, of whom 88% had suppressed viral loads [2], thus falling short of the targets.

Estimates for HIV transmission per coital act are 0.08% (male to female) and 0.04% (female to male) in high income countries and 0.38% (male to female) and 0.3% (female to male) in low income countries [3] although the risk can be amplified by confounding factors such as genital ulcer disease and exposure route. Viral load is a major determinant of risk, with a 2.5 fold increase in risk described for every ten-fold increase in viral load [3]. Pregnancy is a risk factor and can raise the risk of acquisition to more than double that of non-pregnant women [3,4].

HIV-1 (HIV) genotypes vary globally. HIV is classified phylogenetically into groups M, N, O and P [3]. The group M pandemic strain comprises nine subtypes and dozens of circulating recombinant forms that differ up to 30% in their amino acid sequence from each other [3,5]. The major subtype in Southern Africa is subtype C [3]. Africa, particularly Southern Africa, bears a disproportionate burden of HIV globally. From a global perspective, one in five HIV infected adults live in South Africa [6].

1.1.2 HIV burden in South Africa

South Africa is disproportionately heavily affected by HIV. Stats-SA estimated in mid-2020 that the South African overall HIV prevalence was 13%, implying 7.8 million people living with HIV [7]. HIV prevalence in adults 15-45 years was 18.7%. There were more than 79 000 AIDS-related deaths in South Africa in 2020 [7]. The country has the highest number of people on antiretroviral therapy of any country, overburdening the health system [6]. In 2019, South Africa had 5 200 000 people on chronic antiretroviral therapy [2]. Estimates were that only 64% of those on antiretroviral therapy were virally suppressed [2].

The HIV epidemic in South Africa is intertwined with that of Tuberculosis (TB) [8]. TB reactivation rates are much higher in people who are HIV-infected than those who are HIV-uninfected [9]. The morbidity and mortality rates resulting from HIV are closely linked with those of TB [8]. Tuberculosis figures in areas of South Africa remain amongst the highest in the world, with incidence rates 520/100 000 in 2018 [10]. The twin epidemics of HIV and TB have posed an unprecedented health crisis for South Africa over the last decades [8]. Interventions to improve access to care for these chronic diseases require intense public health resources [8].

Huge strides have been made in expanding access to antiretrovirals; implementation of a strong prevention of mother to child transmission programme; interventions for populations at risk; scaling up of medical male circumcision and adoption of the “test and treat” strategy [6]. Behavioural interventions have been identified which, in combination, can decrease transmission rates in target populations [11]. Pre-treatment HIV drug resistance is, however, a growing problem, with studies reporting resistance in the Southern African region to non-nucleoside reverse transcriptase inhibitors at levels higher than 10% of patients initiating or re-initiating first line therapy [12].

A preventative vaccine against HIV is urgently required. Despite efforts over the last four decades, a licensed vaccine is not imminent. Improved understanding of HIV immunology is crucial to improving the likelihood of success for the HIV vaccine effort. In particular, correlates of risk and protection from HIV in naturally occurring cohorts may pave the way to improved vaccine design.

1.1.3 HIV life cycle

The human genome contains many endogenous retroviruses, belonging to beta, gamma or spuma retrovirus groups. The only retroviruses causing human disease are exogenous retroviral groups – delta or lentivirus groups [13]. HIV is a lentivirus with similar structure to other retroviruses, comprising *gag*, *pol* and *env* genes flanked by long terminal repeats. Additionally, HIV like other lentiviruses but not all retroviruses, contains accessory genes, including *vif, vpr, nef, tat, rev and vpu* [14]. Vif, VPR and nef proteins are incorporated into virions, while tat and rev proteins provide regulatory functions and vpu assists in assembly of virions [15]. Accessory proteins act to downregulate host proteins in non-dividing cells. The viral protein, nef, downregulates class 1 MHC on the cell surface, and vpu causes degradation of the cellular CD4 protein [16]. The viral protease can cleave Gag and Pol into smaller functional proteins, while host enzymes process Env. The *pol* gene codes for protease, reverse transcriptase and integrase enzymes[13]. The genome is packaged within a viral capsid and surrounded by a lipid envelope.

HIV infects cells expressing the CD4 receptor, including $CD4^+$ helper T cells, monocytes, macrophages, dendritic cells, follicular dendritic cells, Langerhans cells and microglia [15,17–20]. Virions first attach to the host cell non-specifically for example by Env interacting with heparin sulphate proteoglycans, Env binding α4β7 or host proteins incorporated into the virion envelope binding to their cellular counterparts [21]. Non-specific binding brings gp120 into close proximity with the host CD4 protein, allowing gp120 to bind to CD4. Dendritic cells may prompt infection of other cell types, spreading HIV through cell-cell transmission via virological synapses, in which viral gp120 binds to host CD4 [22], or via transporting HIV virions via lectins such as DC-SIGN without being infected themselves to the lymph nodes where they form infectious synapses with T cells [21,22].

The primary receptor for HIV is the CD4 protein [23,24]. Strains differ in their requirement for a co-receptor, either requiring CCR5 [25–28], CXCR4, or able to bind either co-receptor [21]. CD4 binding causes conformational changes in gp120 and allows co-receptor binding. Co-receptor binding allows exposure of the hydrophobic viral gp41 fusion peptide which facilitates opening and stabilization of a fusion pore [21]. Endocytosis, direct cell-cell transmission, as well as signalling through CD4 or co-receptors may also play a role in viral entry [21].

Viral DNA can be translocated into the nucleus and can integrate into host chromosomes. Viral reverse transcriptase facilitates conversion from single-stranded viral RNA into double-stranded DNA (21). The viral reverse transcriptase enzyme has RNA-dependent and DNA-dependent polymerase activity as well as RNase H activity. Following reverse transcription, a viral pre-integration complex is formed, comprising viral DNA and cellular components. The pre-integration complex is transported to the nucleus, where the viral integrase enzyme catalyses integration of viral DNA within the host DNA [29]. Most positions in chromosomal DNA can serve as integration sites, although there is preference for transcriptionally active sites [30]. Cellular machinery for DNA replication ensures that once integrated, viral DNA becomes a permanent fixture in the cell and also allows transcription of viral mRNA, with assistance of the viral Tat protein [29,30].

Viral proteins accumulate near the plasma membrane and bud off to form new virions. The virions acquire their lipid envelope and Env protein spikes as they bud from the plasma membrane [31]. Viral protease cleaves the Gag and Gag-Pro-Pol polyproteins to allow viral maturation [31]. Cell-free virions may be released, or virions may spread directly from cell-to-cell at points of cell-cell contact, termed the virological synapse [31]. Viral spread from macrophages seems to differ from that of lymphoid cells, in terms of clustering of virions in the virus-containing-compartment rather than budding from the plasma membrane [20].

Understanding the HIV life cycle has allowed development of antiviral agents acting at multiple points. The mainstay of treatment has been nucleoside or non-nucleoside reverse transcriptase inhibitors, usually given as combination therapy of three agents to avoid emergence of resistance mutations. Nucleoside reverse transcriptase inhibitors, such as zidovudine and tenofovir, are analogues of native substrates for reverse transcriptase, while non-nucleoside reverse transcriptase inhibitors, such as efavirenz, bind to a non-catalytic allosteric pocket on the enzyme [29]. Protease inhibitors, such as indinavir, prevent cleavage of the viral polyprotein, thereby preventing development of new infectious particles [29]. Newer drugs include integrase inhibitors such as raltegravir, entry inhibitors such as enfuvirtide, and CCR5 blockers such as maraviroc [21,29].

What happens to the infected cell is less clear than the other stages of the viral life cycle. The infected cell may die, through multiple postulated mechanisms. Cell death was initially thought to be directly due to viral infection, although the number of infected cells in peripheral blood was not high enough to

result in marked $CD4^+$ T cell count depletion. Apoptosis of bystander cells was a proposed mechanism of death of uninfected cells, through multiple pathways [19,32]. Certain infected cells may not die, however, and may survive as viral reservoirs. In particular, antigen presenting cells such as macrophages and microglia may survive following HIV infection [19]. Such infected cells may have alterations in their function, resulting in secondary defects in immune cells, for example infected macrophages may show impaired signalling at the immunological synapse and result in T cell dysfunction and apoptosis [32,33].Recent advances have found that $CD4^+$ T cell death may in fact be due to abortive viral infection in resting $CD4^+$ T cells, where the accumulation of single or double stranded viral DNA products within cells stimulate an active form of pro-inflammatory cell death known as pyroptosis [34,35]. Pyroptosis is stimulated by host innate sensing of the viral products[35]. Mitochondria appear to play a role in the HIV life-cycle, an effect independent of oxidative phosphorylation [36]. It has been suggested that viral RNA import into mitochondria may interfere with host mitochondrial function and result in cell death [37].

1.1.4 HIV immunology overview

Despite a well characterised viral life cycle, HIV infection of target cells (mainly $CD4^+$ T cells) does not explain many of the complexities of HIV disease progression. Individuals are heterogeneous in their manifestations of disease, with some progressing rapidly to AIDS and death, while some people maintain robust $CD4^+$ T cell counts (termed long term non-progressors) or low viral loads (termed elite controllers) or both.

Much pathology during HIV-1 infection seems to occur in the early weeks of disease, when there is widespread ablation of mucosal associated lymphoid tissue, particularly in the gastrointestinal tract. In acute simian immunodeficiency virus (SIV) infection, approximately half of memory CD4+ T cells are depleted from the gastrointestinal tract and other lymphoid compartments within a few days of infection [38–41]. Mucosal associated lymphoid tissue comprises at least 40% of the body's lymphoid tissue [42]. A potential cause of immune activation has been postulated to be translocation of bacterial products, such as lipopolysaccharide, from the gastrointestinal mucosa into the blood stream [43]. Early treatment of HIV during primary infection (seroconversion illness) can improve viral setpoint although in many cases does not seem to replace the lost gastrointestinal mucosal T lymphocytes [44].

Longer term changes reflect later in the peripheral blood, with a slow decline in $CD4^+$ T cell count. During late stage disease, $CD8^+$ T cell counts also decline and viral loads show a sharp increase, despite poor numbers of remaining $CD4^+$ T cells [21].

Activation of $CD4^+$ T cells facilitates HIV infection and replication, but immune activation is not limited to $CD4^+$ T cells only. Cell cycle markers such as Ki-67 show increased replication and turnover of $CD4^+$ T cell and $CD8^+$ T cells [45]. Patients often have marked polyclonal hyper-gammaglobulinemia, affecting predominantly IgG and IgM, but nevertheless show reduced B cell responses to stimulation with T-dependent or T-independent antigens (36, 37). Progressive HIV disease appears to result from an overactive immune system, including activation of $CD4^+$ T cells, $CD8^+$ T cells, B lymphocytes and the innate immune system (33, 37). High levels of immune activation markers (such as CD38 or HLA-DR) precede decline of $CD4^+$ T cells [42]. In fact, levels of activation markers are more closely correlated to rate of disease progression to AIDS than $CD4^+$ T cell count or viral load [48–52].

Both viral and host factors have been postulated to be responsible for inter-individual differences in disease severity. A strong argument for the importance of host factors is comparative biology using monkeys, the natural hosts of SIV. Species such as macaques can be infected with SIV in the laboratory and progress to AIDS-like disease and death, and are therefore often used in vaccine trials. Natural monkey hosts of SIV such as sooty mangabeys, however, seem to "ignore" their virus, manifesting high viral loads but low adaptive immune activation and remaining asymptomatic and disease free [42,53]. Host response to viral infection may therefore be the determining factor in disease progression. Viral factors thus interact with host factors to result in disease phenotype.

Host genes, in particular HLA genotypes, have been shown to be associated with acquisition of HIV and with disease progression and will be discussed in detail below. Another host gene conferring HIV resistance includes the *CCR5* gene. A homozygous delta-32 base-pair deletion in the gene for CCR5, the HIV co-receptor, confers absolute resistance to HIV acquisition with R5 tropic strains, while heterozygotes have slower disease progression [21]. A multitude of intrinsic host restriction factors, such as APOBEC3G, TRIM5α and tetherin, also play a role in defense against HIV infection in myeloid and lymphoid cell types [20,54].

Host adaptive immunity is a major role-player in viral control, with both T lymphocytes and B lymphocytes intensively investigated. Cytotoxic T lymphocytes (CTL) control HIV through recognition via their T cell receptors of viral peptides presented by HLA class 1 proteins. Appearance of virus-specific CTLs coincides with reduced viral replication during primary infection and establishment of a viral set-point [5]. The virus tries to evade the pressure of responding CTLs through predictable mutations [5].

$CD8^+$ T cells are thought to be an important factor preventing infection in highly exposed seronegative individuals [55,56]. T cells induced by exposure to sub-infectious doses of SIV may protect macaques from subsequent challenge with SIV [57]. Control may also be due to certain subsets of T cells. Lu *et al* demonstrated in elite controllers that regulatory $CD8^+$ T cells could control HIV replication [58].

T cell responses in HIV may however be adversely affected by the virus. In late stages of HIV infection, T cells show poor responses to mitogens such as phytohaemagglutinin, but normal responses to antigens such as concanavalin A, pokeweed mitogen or alloantigens [46][59]. Patients with late stage disease (AIDS) show reduced T cell responses in the autologous mixed lymphocyte reaction, despite maintaining normal responses to allogeneic mixed lymphocyte reaction [46]. $CD8^+$ T cell and $CD4^+$ T cell poly-functionality (evidenced by ability to produce multiple cytokines simultaneously after in vitro stimulation,) as opposed to mono or pauci-functionality, is thought to be a factor associated with improved viral control and better prognosis [60–62]. During progressive disease, T cell exhaustion is thought to play a role in declining viral control. Exhaustion markers on T cells such as PD-1 are elevated in late stage disease [63].

During HIV infection, some but not all infected individuals develop antibodies that can neutralize a wide range of HIV viruses in vitro, termed “broadly neutralising antibodies”. Approximately half of infected individuals develop antibodies which can neutralize 50% of viral strains, while only approximately 10% of individuals develop a broader panel of antibodies able to neutralise 90% of viral strains [64–67]. Broadly neutralizing antibodies usually appear late after infection and therefore play only a minor role for the individual patient [68]. There appear to be common characteristics of broadly neutralising antibodies, such as long immunoglobulin heavy chain (IgH) complementarity determining regions. Some broadly neutralising antibodies are poly-reactive for non-HIV antigens such as cardiolipin [69–71] or other self antigens and may develop in patients with chronic HIV infection via defects in usual B cell self-tolerance pathways [68].

1.2 Introduction to Major histocompatibility complex (MHC) and Human Leucocyte Antigens (HLA)

1.2.1 Major histocompatibility genes

The genes of the major histocompatibility (MHC) complex encode proteins that play an integral role in immunity in mammals, with each species having its own nomenclature. The human MHC proteins are known as Human Leucocyte Antigens (HLA) [72]. HLA genes comprise multiple loci, at which the most polymorphic genes in the human genome are found [72,73]. The HLA genes are found in two clusters termed “classes” on chromosome 6; HLA class 1 comprising loci HLA-A, HLA-B and HLA-C and HLA class 2 comprising HLA-DRB1, HLA-DRB3/4/5, HLA-DQA, HLA-DQB1, HLA-DPA and HLA-DPB1 (Figure 1.1). There are additional interspersed less polymorphic genes, some of which are termed HLA-class 3 [72].

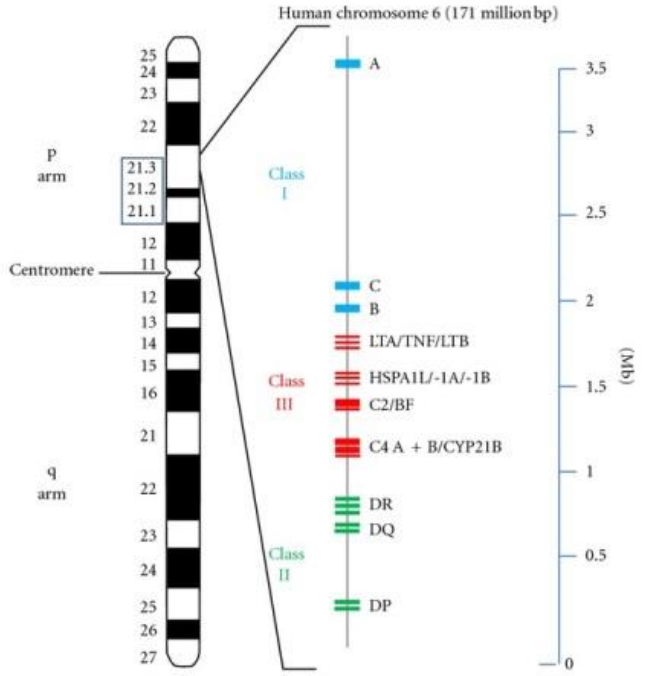


Figure 1.1 Schematic diagram of HLA loci on chromosome 6*. HLA-A; HLA-B and HLA C loci shown in blue, each comprising a single locus coding for a single protein chain, which associates with β2-microglobulin to form a functional HLA protein. HLA class 2 loci shown in green. HLA class 2 proteins comprise 2 protein chains (alpha and beta). Additional interspersed genes (termed HLA class 3 are shown in red). Figure taken from Relle et al, 2012* [74].

Each individual inherits two alleles, co-dominantly expressed, at each locus. Heterozygotes for a particular locus therefore express two different alleles on a particular cell, while homozygotes express only one HLA phenotype for that locus. For example, a heterozygote HLA-A*02:01/HLA-A*03:01 would express both the A2 and A3 proteins for the HLA-A locus on the same cell. There are at least 767 HLA-A, 1178 HLA-B, and 439 HLA-C alleles described in an ever-growing list. Of the more conserved HLA class 1 genes (termed HLA-1b genes) there are at least 9 HLA-E, 21 HLA-F and 43 HLA-G alleles described [72,75]. The HLA class 1 genes are in strong linkage disequilibrium and tend to be inherited *en bloc*, as are the class 2 genes.

For class 2 HLA genes, there are more than 500 HLA-DRB1 alleles described. The HLA-DQ region encodes at least 72 alleles and the HLA-DP subregion at least 118 alleles [73]. An updated list classifying alleles as common (above a frequency of 1 in 10 000), intermediate (above 1 in 100 000) or well-documented (at least 5 individuals) is available [76]. Certain HLA-DRB3, HLA-DRB4 and HLA-DRB5 alleles are pseudogenes and not consistently expressed. Linkage patterns between common HLA-DRB1 alleles and their corresponding HLA-DRB3, HLA-DRB4 and HLA-DRB5 partners are shown in Figure 1.2. Individuals may be hemizygous at the HLA-DRB3/4/5 loci if there is no functional DRB3/4/5 locus on the second chromosome. For example, at the HLA-DRB3 locus, individuals may be homozygous (eg HLA-DRB3*01/HLA-DRB3*01) or heterozygous (HLA-DRB3*01/HLA-DRB3*02) or hemizygous (HLA-DRB3*01/null) at the DRB3 locus. Similarly individuals may be homozygous, heterozygous or hemizygous at the HLA-DRB4 locus or the HLA-DRB5 locus.

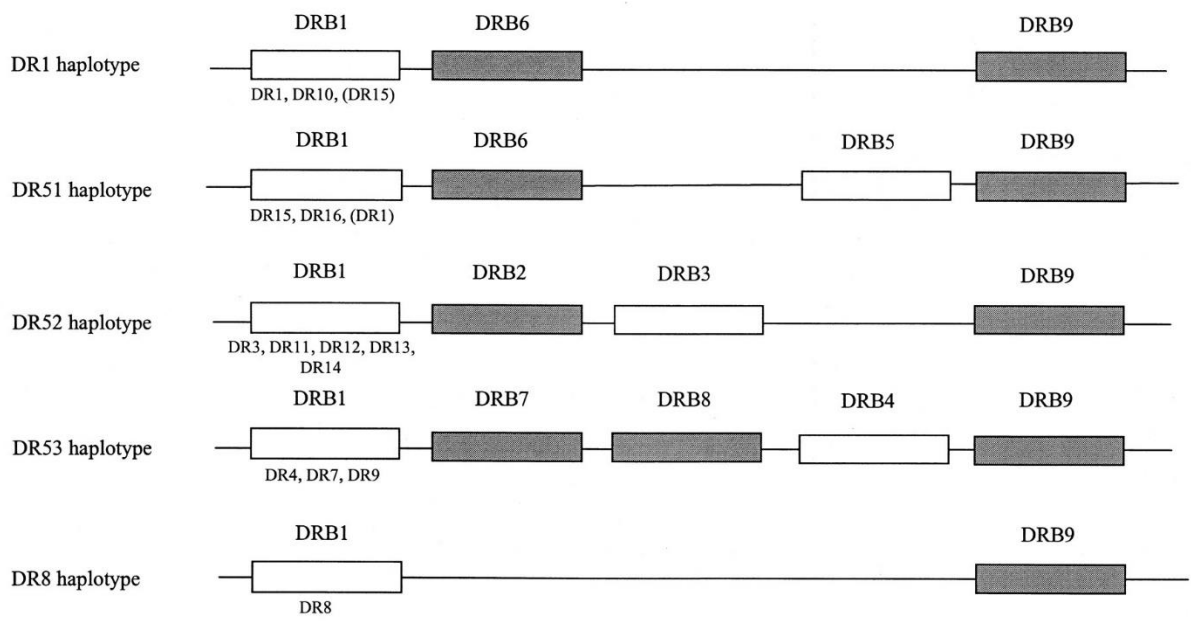


Figure 1.2 HLA-DRB3,4,5 associations with HLA-DRB1*. Open boxes indicate pseudogenes. Figure taken from Kotsch et al, 1999* [77].

HLA proteins can by typed by various methodologies including allele-specific oligonucleotides, allel-specific primers or sequence-based typing, amongst others. In this work an allele-specific oligonucleotide method was used, with detection via a Luminex bead-based instrument, illustrated in

Figure 1.3 The exons of interest are amplified in all individuals with the same primer set, followed by detection by hybridization to a matrix of allele-specific probes.

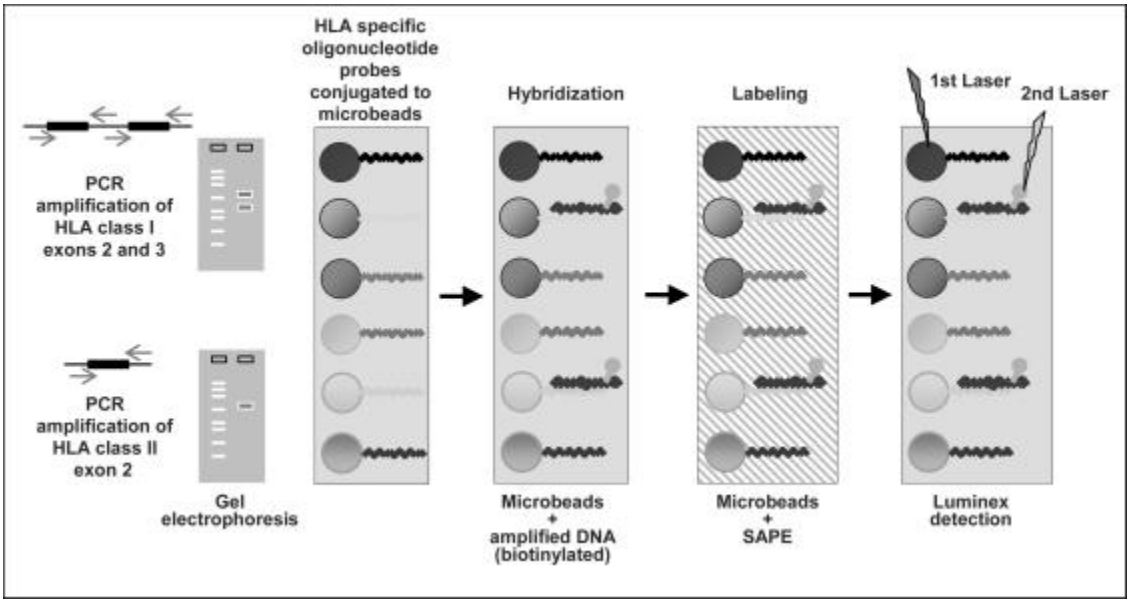


Figure 1.3 HLA typing using allele-specific oligonucleotide probes on Luminex beads. *Relevant exons (for class 1 exons 2 and 3, for class 2 exon 2) are first amplified by PCR to yield biotinylated PCR products, and then allowed to hybridize to allele-specific oligonucleotides on Luminex beads. Bound product is detected via fluorescence of Streptavidin-PE (SAPE). Gel electrophoresis of PCR product is optional. Figure from Heinemann, 2009* [78]

1.2.2 HLA proteins and the Immunological synapse

HLA proteins are transmembrane proteins, with most of their structure located extracellularly. They comprise folded proteins whose structure allows association with a peptide fragment that is not part of the HLA protein itself. HLA class 1 comprises a single alpha chain, bound to β2-microglobulin. HLA class 2 comprises 2 chains, alpha and beta. HLA proteins are unstable in conformation until such time as a peptide is bound in their peptide binding groove, and are held in conformation by chaperone proteins until they come into contact with a suitable peptide [73]. HLA class 1 and HLA class 2 proteins differ in the cellular location of their peptide ligands and the length of the peptide ligands which may be bound. HLA class 1 binds peptides of 8-15 amino acids in the endoplasmic reticulum of the host cell, and then carries these peptides to the cell surface [73,79]. These peptides may be fragments of newly synthesised host proteins, or may be proteasomally degraded remnants of ubiquitinated cellular components. Such degraded remnants are translocated to the endoplasmic reticulum of the cell via the Transporter

Associated with Antigen Processing (TAP) proteins. It is therefore often described that HLA class 1 genes present endogenous host peptides at the cell surface, displaying a snapshot of the intracellular constituents of that cell. HLA class 1 proteins are found on all nucleated host cells [73]. HLA class 2 proteins have their peptide binding groove blocked while in the endoplasmic reticulum of the cell and only expose their antigen binding sites while in the lysosomal component of the cell. HLA class 2 proteins therefore pick up peptide fragments resulting from endocytosis or phagocytosis of antigen into a cell. Once bound to peptide, HLA class 2 transports the peptide to the surface of the cell. Peptides binding to HLA class 2 are 14-17 amino acids in length. Cell types expressing HLA class 2 are the so-called "professional antigen presenting cells", namely B lymphocytes, macrophages and dendritic cells [73]. Activated T cells also express HLA-DR [80,81]. Schematic diagrams HLA class 1 and HLA class 2 structure are shown in Figures 1.4 and 1.5.

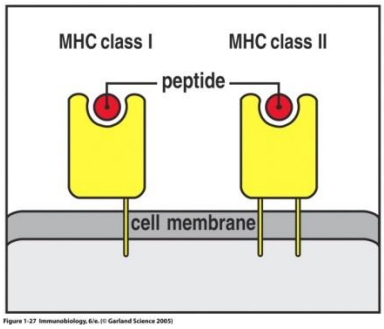


Figure 1.4 Simplified structure of HLA class 1 and HLA class 2 proteins*. HLA class contains one transmembrane domain while HLA class 2 contains 2 transmembrane domains. HLA proteins shown in yellow and presented peptide shown in red. Figure taken from Janeway et al, 2001* [82].

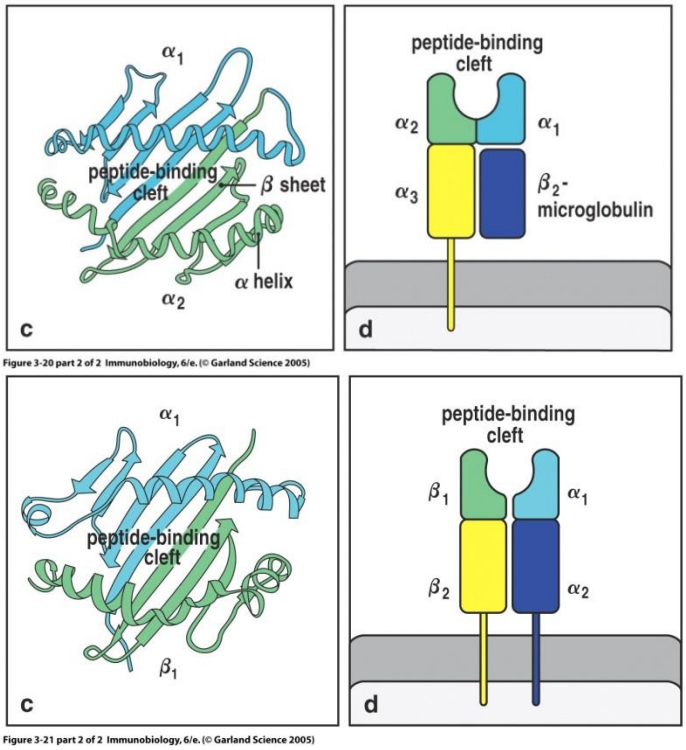


Figure 3-20 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)
Figure 3-21 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)

Figure 1.5 More detailed schematic of HLA class 1 and class 2 proteins*. Top row c and d: HLA class 1 comprises one chain which associates with beta2 microglobulin. Bottom row c and d: HLA class 2 comprises 2 chains (denoted alpa and beta, each with 2 segments). Figure taken from Janeway et al, 2001* [82].

HLA class 1 has affinity to bind with the protein CD8, expressed on cytotoxic T lymphocytes. HLA class 2 has affinity to bind with the protein CD4, expressed on a helper T cells. Binding of HLA proteins to CD4 or CD8 stabilises the binding of the antigen-presenting cell with the responding T lymphocyte [83,84]. The interaction of the antigen presenting cell and T lymphocte is termed the immunological synapse (Figure 1.6). Structural specificity of the T cell receptor determines whether a particular T cell binds to the the person's particular HLA protein presenting a particular peptide. Change of a single amino acid in the HLA protein presenting the peptide or the T cell receptor responding to the peptide may change the binding characteristics [85,86] There may aditional accessory molecules involved in the immunolgical synapse, often termed "second signals" [87]. A minimum of two successful signals at the immunological synapse allows both cells involved in the synapse to undergo further activation. For the T cell activation may entail cytokine production by $CD4^+$ T cells or secretion of perforin and granzymes by $CD8^+$ T cells. An infected cell expressing antigen via HLA class 1 is therefore thought to be killed or undergo apoptosis following engagment of a cytotoxic T cell at the immunological synapse. For a professional antigen presenting cell engaged by a $CD4^+$ helper T cell, the resulting consequences include oxidative burst by

macrophages or dendritic cells; or Immunoglobulin M (IgM) production by B cells, followed by class switch to other antibody isotypes. The immunological synapse wherein the B cell acts as the antigen presenting cell to a responding T cell is often termed “T cell help” for the B cell [88–90].

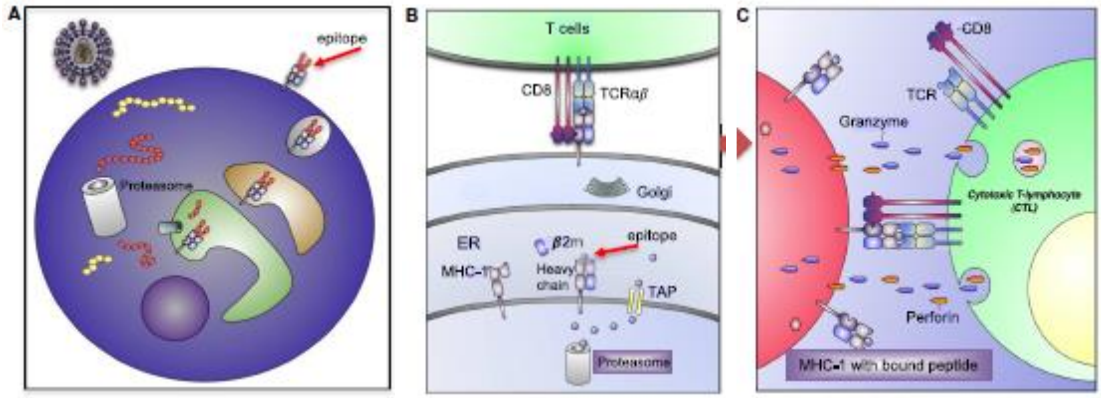


Figure 1.6 Antigen processing and presentation at the Immunological synapse*. Panel A shows viral protein being degraded by the proteasome, loaded via TAP transporter into lumen of endoplasmic reticulum and presented at the cell surface on HLA class 1. Panel B shows the immunological synapse first signal, where the T cell receptor binds to the HLA-presented peptide, and CD8 stabilises the binding to HLA class 1. Panel C shows result of the immunological synapse where the cytotoxic T cell releases perforins and granzyme to kill the infected cell. Figure taken from Crux et al, 2017* [72].

1.2.3 HLA and transplantation

Early work on transplant immunology showed the relevance of HLA to tissue matching between tissue donors and recipients [91]. Serological studies confirmed the importance of matching HLA-A, HLA-B and HLA-DRB1 genes for haemopoietic (blood and bone marrow) transplantation. For solid organ transplantation, such as kidney transplantation, serological work confirmed that pre-existing antibodies in the recipient directed against the HLA specificity of the donor (in particular HLA class 1 genes) could precipitate hyperacute rejection of the kidney [92,93]. Pre-existing antibody against HLA (HLA antibody) against donor HLA class 1 HLA alleles are therefore viewed as a contraindication to kidney transplantation. Matching of HLA alleles is not a pre-requisite for kidney transplantation, unlike HLA antibody testing, which is pre-requisite [94].

1.2.4 HLA associations with HIV

As with other infectious and autoimmune diseases, there has been intense scrutiny regarding whether HIV disease is linked with particular HLA alleles. HLA specificity plays a role in the likelihood of acquiring or transmitting HIV, as well as the rate at which HIV infection progresses to chronic disease.

1.2.4.1 HLA associations with HIV disease acquisition/transmission

Multiple studies over decades have identified various HLA alleles as being associated with decreased or increased risk of HIV acquisition or transmission. Decreased risk of HIV infection was associated with possession of alleles similar to HLA-A*02 (A2/6802 supertype comprising HLA-A*02:02; A*02:05, A*02:14 and A*68:02)) or with DRB1*01. The class 1 associations may point to cytotoxic T lymphocyte-mediated protective immunity, while the strong class 2 association highlights also a role for $CD4^+$ T cells in protection [95]. HLA-A*23:01 has been associated with increased risk of infection. Sharing of HLA alleles, in particular HLA-B, can increase risk of transmission of HIV from HIV infected partners to uninfected recipients [96]. Although HLA-G is a relatively conserved HLA type, associations of polymorphisms with HIV acquisition have been reported. [97]. Concordance at HLA class 1 loci between mother and child have been associated with increased mother to child HIV transmission [98]. HLA-A*02 positive newborns have been reported to have a 9 fold reduced risk of HIV infection compared with HLA-A*02 negative newborns [99]. Regarding class 2 alleles, in the Pumwani Kenyan cohort, HLA-DRB1*01:01:01, HLA-DRB1*01:02:01 and HLA-DRB1*11:02 were higher in HIV-resistant women than HIV-infected women. The alleles HLA-DRB1*03:02:01, HLA-DRB1*07:01:01 and HLA-DRB1*15:03 were more common in HIV infected women than HIV-resistant women.Similarly HLA-DRB5*01:01:01 was more common in HIV-infected women [100].

1.2.4.2 HLA associations with HIV disease progression

MHC variability is a key determinant of immunological heterogeneity within members of the same species. HLA variability amongst humans has been shown to impact rate of progression of HIV infection to AIDS and death.

Heterozygosity of HLA-alleles, rather than homozygosity, seems to be protective against disease progression [42] presumably by "doubling the chance" of harbouring suitable alleles to restrict viral peptides. Matching (concordance) of HLA-A and HLA-B supertypes between donor and recipient has inversely been associated with unusually severe primary HIV infection [101]. HLA-A or HLA-C concordance between the HIV donor and HIV recipient was associated with higher viral set point [102].

Multiple particular HLA specificities have been shown to impact host or viral fitness. Of the class 1 alleles, HLA-B gene alleles have shown strongest impact on viral set point and rate of progression of disease to AIDS [103–105]. Alleles associated with ***rapid progression*** of HIV infection to symptomatic disease include HLA-A*68; HLA-B8; HLA-B*35; HLA-B*53; Cw*04, and B*58:02 [85,101,103,106]. In African individuals, HLA-A*23:01 was the allele reported most strongly associated with higher viral set point, with HLA-A*30:01 and B*53:01 also associated with higher viral setpoints [102]. Class 2 alleles have also been associated with accelerated progression to AIDS. HLA-DR2, HLA-DR5 and the A1-B8-DR3 haplotype are associated with rapid progression [105,107,108].

Alleles thought to be beneficially associated with ***slower disease progression*** include HLA-B*27; HLA-B*57; HLA-B*58:01; B*14/Cw08:02; B*52 and HLA-A*25 [102,106,109–111]. In African individuals four alleles were associated with lowest viral set-point; HLA-B*27:03, HLA-A*33:03, HLA-B*14:01 and HLA-C*02:02. Multiple protective alleles can be grouped into the HLA-Bw4 serological group, sharing the Bw4 motif, including HLA-B*27 and HLA-B*57, potentially pointing to T cell or NK cell control of infection. Genome wide analysis of single nucleotide polymorphisms associated with spontaneous HIV control have also strongly implicated HLA genes [112]. HLA-E restricted $CD8^+$ regulatory T cells were found in nine of ten human elite controllers, all of whom also had an HLA-Bw4-80I genotype [113]. Thus even relatively non-polymorphic HLA alleles such as HLA-E may play a protective role in a therapeutic vaccine response to HIV, aimed to control rate of disease progression.

Aside from allele specificities, ***expression levels*** of certain alleles may also correlate with disease progression. HIV is reported to downregulate expression levels of HLA-A and HLA-B but not HLA-C [42]. Individuals with high expression levels of HLA-C seem to control disease more effectively and progress slower than those with low expression levels [114]. Higher HLA-A expression levels correlate with poor HIV control, potentially via association with HLA-E mediated effects on natural killer cells [115].

1.2.4.3 HLA associations with HIV vaccine efficacy

HLA associations may play a role in HIV vaccine efficacy. Gartland *et al* reported vaccine efficacy from the RV144 Thai trial (described later) as 54% in HLA-A2 positive individuals versus 3% in HLA-A2 negative individuals, generating the hypothesis that there may be an association between class 1 genoype and vaccine efficacy [116]. Prentice *et al* reported that protective antibodies in the RV144 trial were associated with HLA class 2 specificities. Benefical IgG responses against Env were associated with HLA-DPPB*13. IgA responses against Env, which were deleterious antibodies associated with increased risk of transmission, were associated with HLA-DQB*06 [117].

1.3 HIV vaccines – past, present, future

1.3.1 Human clinical trials of HIV vaccines

HIV Env is a highly glycosylated protein trimer, only transiently exposed during binding to CD4, hampering immunogen design. Multiple non-human primate models have been used for pre-clinical vaccine trials, with challenge viruses including simian immunodeficiency virus (SIV) and simian-human HIV (SHIV) (a construct in which the SIV envelope gene has been replaced with the HIV envelope gene). Humanized mice models are also in use for preclinical work [118]. Preclinical HIV vaccine studies have been reviewed [119,120] and challenges highlighted [121] and will not be discussed in depth. Prophylactic administration of neutralizing antibodies has been shown to prevent infection during subsequent challenge [122,123]. Additional antibody-mediated functions such as antibody-dependent cellular cytotoxicity or antibody-dependent cell-mediated viral inhibition may also play a role *in vivo* [54]. Vaccination strategies to elicit cytotoxic T cell responses have also shown promise in non-human primates using various vectors and antigens [54].

Despite intensive preclinical [119,120]and phase I trials[124], only a handful of HIV vaccine efficacy trials (phase IIb or phase III trials) have been completed in humans. Two phase 2b studies were conducted to assess a vaccine construct containing *gag/pol/nef* in a replication defective Adenovirus 5 vector. The adenoviral vector had been selected due to preclinical data suggesting its potential to induce strong cell-mediated immunity. The Step study (HVTN-504/Merck023) was conducted in areas where clade B infection and homosexual transmission is common (Americas, Caribbean, Australia) and the Phambili

study (HVTN-503) in South Africa where heterosexual transmission and clade C virus are predominant [125]. Interim analyses showed no efficacy and the studies were stopped prematurely. Post-hoc analyses showed a trend to increased HIV transmissions in one subgroup (men who were uncircumcised and had pre-existing immunity to the adenoviral vector)[125]. The RV144 trial was conducted in Thailand with a canarypox vector (ALVAC vaccine) containing *gag, pol and env*, followed by boosting recipients with gp120 recombinant protein. The trial, completed in 2009, showed an overall efficacy of 31.2% which could be stratified into 59.9% early efficacy at 12 months, which waned to zero efficacy by the end of the study period (42 months) [126–128]. The early efficacy has given hope that immunological factors associated with the early protection could be identified. Post hoc analyses identified that IgG against V1/V2 region of envelope was associated with protection, while IgA against Env seemsed associated with increased risk of infection [129–131]. Thus non-neutralizing antibodies binding to the V1/V2 region may have played a protective role in the RV144 trial [131,132]. The RV144 trial did not identify neutralizing antibody nor T cell immune responses as mechanisms of the observed protection, although $CD4^+$ T cell gamma responses were detectable in 41% of vaccinees studied, mostly targeting the V2 region of Env [133].

Others trials included the Vax003 and Vax004 (2003) trials evaluating protein subunit vaccines, which showed no efficacy in high risk groups in Thailand or in heterosexual transmission in the USA. Similarly the HVTN-505 trial (DNA prime with adenovirus boost) completed in 2013 in high risk groups in the USA also showed no efficacy. A further trial probing the Thailand results of partial efficacy was conducted in South Africa as the HVTN702 trial. HVTN702 evaluated ALVAC, the canary pox vector used in the Thai trial,,modified for HIV subtype C with an adjuvanted gp120 boost with a different adjuvant from that used in the Thai trial and multiple boosts. HVTN702 was stopped in 2020 for lack of efficacy [134].

Major challenges for HIV vaccine design include how to combat the antigenic variability of the virus, how to induce long lasting immunity, and the lack of knowledge regarding correlates (or ideally mechanisms) of protection [56,128]. These challenges are not unique to HIV and illustrate systemic gaps in our immunological knowledge, resonating with efforts to develop improved vaccines against malaria, TB and other pathogens. Many hope an immunogen can be designed which will stimulate production of antibodies (termed broadly neutralising antibodies) which will neutralise many viral clades and quasi-species. Immunogen design for induction of broadly neutralising antibodies is challenging immunologists to better define the process leading from naïve B cells to mature broadly-neutralising antibody

producers through the physiological processes of somatic hypermutation and affinity maturation [132,135,136]. Vaccines that aim to improve prognosis after infection or to decrease secondary transmission through lowering viral load of those infected have also been proposed [56].
The poor ability of our current immunological assays to predict efficacy of human vaccine studies emphasize that we are not yet measuring the factors strongly associated with protection from HIV infection. The Merck adenovirus 5 vaccine induced T cell interferon gamma responses in the majority of participants without providing protection [128].

Other approaches to vaccination include delivery of passive rather than active immunization. Passive transfer of neutralising antibody prevented SIV acquisition in the macaque model [137] and human trials evaluating repeated administration of neutralising antibody as a HIV prevention strategy (AMP trial) have been promising. In the AMP trial, the VRC01 antibody prevented infection with VRC01-sensitive HIV isolates, although not with other isolates, suggesting that a combination of monoclonal antibodies may be of value [138]. Further, animal trials have explored vectored immuno-prophylaxis as an alternative approach to administration of neutralising antibody. Vectored immuno-prophylaxis comprises an adenoviral vector used to administer genes for broadly neutralising antibodies which can then be synthesized in vivo [139].

Haynes and colleagues emphasized the need for additional insight into host-virus biology in order to develop a successful HIV vaccine [140]. T cell responses may enhance antibody responses, lowering the titre of neutralizing antibodies required to prevent infection [141]. Hansen and colleagues have shown that unconventional $CD8^+$ T cells, that were MHC2-restricted or HLA-E restricted, were important in clearance of SIV in Rhesus macaques after vaccination with a cytomegalovirus vector [142–145]. Andrieu and colleagues questioned the paradigm that a successful HIV vaccine needs to stimulate either antibody production or strong T cell immunity, showing in Chinese macaques and human elite controllers that a $CD8^+$ regulatory T cell subset suppressed CD4 and antibody responses to HIV and conferred sterilising immunity to SIV [58,113]. Their model was however not replicable in macaques of Indian origin [146]. Thus correlates of protection required by future HIV vaccines require further study and human cohorts of natural HIV exposure are of value.

1.4 Background to HLA antibodies and HIV

Early macaque vaccination studies reported protection against SIV challenge, with SIV grown in human cells, until it was noted that immunization with human cells alone conferred protection [147]. In four macaques used as a control group, immunization with cells and adjuvant (without virus) resulted in protection in 2 of 4 macaques. This was a model of xenoimmunization, that is immunization with cells or antigens from a different species (in this case, *macaques* immunised by *human* cells).

In subsequent work, immunising macaques with purified human leucocyte antigen(HLA), in particular the class 2 HLA protein HLA-DR alone, could protect against subsequent challenge with SIV grown in human, but not macaque cells. These results suggested that immunization with HLA-DR induced sterilising immunity to SIV by preventing the first infectious particle from entering macaque cells. The authors went on to suggest incorporation of HLA-DR into future HIV vaccines [148].

To explore xenovaccination as a potential strategy, Morner *et al* immunised macaques with recombinant HLA class 1 or 2 proteins, either alone or in combination with trimeric HIV gp120 and SIV p27 proteins and adjuvant [149]. Challenge was performed intravenously with heterologous simian-human immunodeficiency virus (SHIV) grown in cells expressing HLA types included in the vaccine. Complete or partial protection was achieved in macaques vaccinated with all 3 components and was transferrable by serum. The macaques developed anti-HLA antibodies which correlated inversely with viral load after challenge. Additionally macaques developed complement-dependent neutralizing activity. The authors did not note any protection using immunization with either SHIV alone or HLA proteins alone, although there was a trend to delayed viraemia in the HLA-immunised group. The adjuvant appeared to be a necessary component of the vaccine. To take this strategy further to develop a human alloimmunization strategy, Morner et al suggested that inclusion of four HLA class 1 alleles (HLA-A*01:01; HLA-A*02:01, HLA-A*03:01 and HLA-A*29:01) in a potential HIV vaccine could cover >90% of the HLA alleles in a sub-Saharan population [149].

The role of complement mediated lysis of HIV appears complex. Certain specificities of HLA antibodies, but not all HLA antibodies, could enhance complement-mediated HIV lysis *in vitro* in combination with anti-HIV antibodies [150]. Certain HLA specificities could, however, inhibit complement-mediated HIV lysis *in vitro*. HIV neutralizing activity was previously identified in sera of poly-transfused patients due to

antibody against HLA class 2 [151]. The neutralizing activity was enhanced in the presence of complement. The authors deduced that the neutralizing activity was likely due to increased steric hindrance by the complement-antibody complex bound to the virus, compared with antibody alone. HIV cultured in T lymphocytes appeared susceptible to complement mediated lysis, likely due to poor incorporation of complement regulatory proteins such as CD55 and CD59, while HIV cultured in peripheral blood mononuclear cells may be more resistant to complement due to incorporation of complement regulatory proteins [152].

Whether alloimmunisation (immunization against a second individual of the same species) rather than xenoimmunisation could be protective was unclear. Work showed that macaques could be protected by HIV grown in human cells but not macaque cells, again seeming to indicate that xenoimmunisation might be required [153]. Polyanskaya *et al* studied the effect of immunizing macaques with simian B cells, expressing a high level of MHC 1 and MHC 2 proteins, as a model of alloimmunization (rather than xeno-immunisation). Anti MHC 1 and antiMHC 2 proteins were induced but failed to protect against subsequent challenge [154]. Criticism of this approach was that the MHC was likely denatured rather than intact, and antibodies detected similarly may have reacted with denatured MHC [155].

The field of alloimmunisation as an approach to HIV vaccine design was discarded in favour of more traditional vaccine design approaches. In view of lack of success over 3 decades, the field of alloimmunity in HIV protection deserves intense scrutiny.

1.5 Introduction to HLA antibodies

HLA antibodies are antibodies that can bind at their F_{ab} region to an HLA protein. In other words, an HLA antibody is an antibody whose target antigen is an HLA protein. Presence of HLA antibodies has particular relevance in renal transplant medicine. Due to the extreme population variability in HLA antigen repertoire, potential effects of HLA antibodies are highly variable in different individuals and can preclude transplantation with kidney donors of particular HLA specificities. HLA antibodies were previously thought to be incidental findings in a few individuals exposed to a sensitising event during which they were exposed to foreign HLA antigens. Common sensitising events include situations such as pregnancy (including miscarriage) or blood transfusion. The frequency of detection of HLA antibodies in the general population however depends on the methodology used [156]. Recent advances in the field

have highlighted that many individuals with no history of previous sensitising events may harbour HLA antibodies. In addition to relevance of HLA antibodies for kidney transplantation, such antibodies may have relevance to infectious or autoimmune disease. A brief review of salient methods for HLA antibody detection is outlined below.

1.5.1 Methods for HLA antibody detection

Older methods of detection of alloantibody include complement-dependent cytotoxicity assays and ELISA assays. The information from these tests is often reported as "percentage panel reactive antibody" (%PRA), meaning the percentage of healthy donors in a large panel to whom the individual reacts positively. Such %PRA readouts, do not necessarily give information regarding whether the individual in question would be sensitized against one specific donor of interest. In a complement-dependent cytotoxicity assay, recipient serum is added to cells of known HLA type (or cells from a potential donor for a "crossmatch"). T cells are used as a cell type expressing HLA class 1 and B cells are used as a cell type expressing class 2 in addition to class 1. Complement is added, usually in the form of rabbit serum. Any relevant anti-HLA antibodies present will bind to HLA antigens, fix complement and result in cell lysis. The assay readout is % lysis, detected microscopically [157].

Complement dependent cytotoxicity assays detect only antibody which fixes complement, namely IgM and a portion of IgG [156]. Cell based assays such as the complement-dependent cytotoxicity assay may also detect cell directed antibodies directed at non-HLA antigens [158]. A flow cytometric assay using donor lymphocytes (T cells expressing HLA class 1, B cells expressing HLA class 1 and 2) is also available for a donor specific "crossmatch". Donor cells are incubated with recipient serum, allowing binding of HLA antibody to donor cells. Following a wash step, fluorescently labelled anti-IgG is added and stained cells are detected flow-cytometrically. This is known as a "flow-crossmatch". The result is interpreted as positive or negative and does not give information regarding which HLA alleles are reacting [159].

Newer solid phase immunoassays can be performed on a fluoroanalyser (usually a Luminex instrument, although a traditional flow cytometer can also be used, termed a FlowPRA assay). The Luminex bead based array system comprises polystyrene beads, impregnated with different ratios of two fluorescent dyes, allowing up to 100 distinguishable bead populations in a single run. Any antibody attached to the

bead can be detected by a reporter antibody and a third fluorescent label (Figure 1.7). The level of antibody is expressed as a mean fluorescence intensity (MFI). The commercial kits detect IgG only [156].

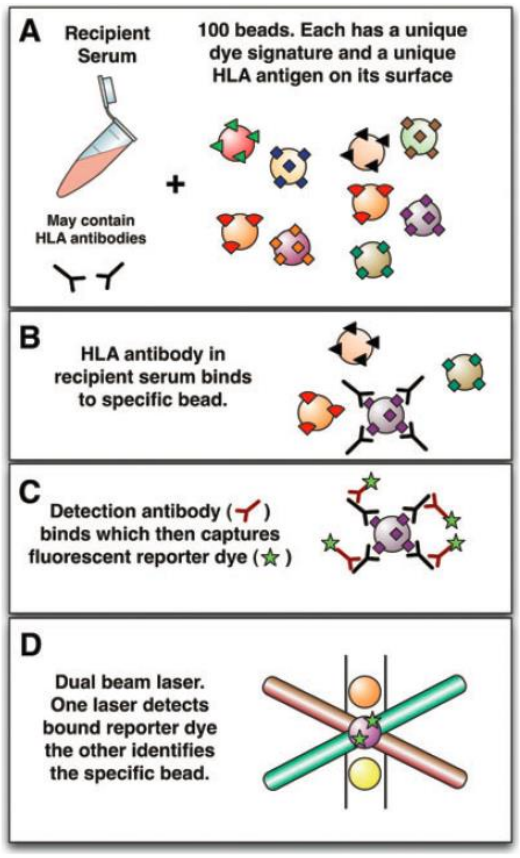


Figure 1.7 HLA antibody detection ("virtual crossmatch") by solid phase immunoassay on fluoroanalyser (for example Luminex assays). *Reproduced from Mulley et al, 2011* [157].

Commercially available HLA antibody kits come in three formats, according to power of resolution of antibody specificities and cost. The first is a screening kit, often named "PRA" for panel reactive antibody, which gives a "positive" or "negative" answer for the presence of any class 1 or class 2 antibodies. Beads are coated with affinity-purified HLA class 1 or 2 antigens, pooled from multiple cell lines. The second tier comprises beads coated with HLA class 1 or class 2 proteins, extracted from cell lines from individual donors. Each bead therefore contains proteins for two alleles at each locus and pattern interpretation is by matrix format. The third tier comprises "single antigen" beads, which contain usually only a single recombinant HLA allele per bead. Single antigen beads are useful in transplant laboratories for interpretation of donor-directed antibodies in individuals who have reactivity to high percentages of the population (patients with high %PRA). Luminex assays are more sensitive

than ELISA or complement-dependent cyotoxicity methods for detection of low titre antibody [156,160]. Antibodies detectable via Luminex even if not detectable by ELISA or complement-dependent methods have been shown to be clinically relevant and associate with higher risk of renal allograft loss [156,161].

The HLA antibody specificities in a recipient can be analysed through bead-based single antigen detection and then compared in silico with HLA types of a donor. This is termed a "virtual crossmatch"[156].

1.5.2 Prevalence of HLA antibodies in general population

When only complement-dependent and ELISA techniques were used, HLA antibodies were thought uncommon or absent without a history of a sensitising event. Recognised sensitising events included blood transfusion, pregnancy or miscarriage. Intimate exposure has also been postulated as a trigger for induction of HLA antibodies against the HLA proteins of a sexual partner. Newer technologies have, however, shown that even in healthy adult males with no history of sensitising events, HLA antibodies are often detectable. Hypotheses as to how they occur include as cross reactions with commensal flora, food antigens or allergens, although the occurrence of naturally occurring antibodies has been described [75,162]. Older literature had noted the presence of naturally occurring HLA antibodies of IgM isotype against HLA-A2, HLA-A3, HLAB7, HLAB8 and HLAB13 [163–166].

Using a flow cytometric method (FlowPRA) for antibodies against HLA class 1 antigens, 31% of healthy females and 12% of healthy males expressed HLA class 1 antibodies [163]. Using a microlymphocytotoxicity assay (a version of the complement-dependent cyotoxicity test), and PRA-Stat ELISA, Lusher *et al* reported the prevalence of antibodies to HLA class 1 as 12% in sex workers in Nairobi. The frequency of HLA antibodies did not differ in highly exposed seronegative sex workers compared with those who acquired HIV. The authors did not investigate antibodies to HLA-class 2 [167]. El Awar *et al* identified IgG antibodies against HLA class 1 in healthy male donors as well as in cord blood, suggesting that they do not arise due to alloantigen exposure. They further reported similarities in Japanese and Mexican individuals, suggesting conserved patterns of reactivity in diverse populations [168].

Using Luminex single antigen methodology Morales-Buenrostro [162] *et al* found HLA antibodies in 63% of healthy male blood donors. Antibodies to class 1 were found in 42%, to class 2 in 11% and to both in

12%. Five males retested 8 times over a 6-month period displayed similar reactivities over time. The antibodies reacted with specificities rare in the population, thereby suggesting that exposure was unlikely the trigger for antibody development [162]. Antibodies to the less polymorphic HLA class 1b alleles have also been documented. Ravindranath *et al* reported that in healthy adult males, antibodies to HLA-E are detectable, and cross-react with HLA class 1a proteins such as HLA-A or HLA-B [75].

1.5.3 Age of development of HLA antibodies

With Luminex-based assays pointing to the existence of HLA antibodies in large proportions of healthy adults without known sensitising events, one may ask at what age such antibodies usually develop. There is limited data on the prevalence of HLA antibodies in healthy infants or children. In 70 children prior to allogeneic haemopoietic stem cell transplantation for malignancy or other conditions, HLA antibodies were found in 44% using Luminex technology [169]. Luscher *et al* compared IgG to HLA class 1 in three groups of infants (0.7 years – 5.7 years); healthy infants, HIV infected and HIV exposed, uninfected infants using an ELISA based method. They found HLA antibodies in all three categories of infants. Depending on cutoffs used, the prevalence in healthy infants was 12-24%; for HIV negative exposed infants 12-38% and for HIV infected children 7-47%. They reported increasing frequency of antibodies with increasing age, suggesting that the IgG antibodies were of infant rather than maternal origin [170]. Using Luminex single antigen technology, Ravindranath *et al* reported presence of HLA antibodies in cord blood, in some cases differing in specificity from HLA antibodies in the infants mothers, suggesting HLA antibody development may occur from foetal life onwards [171].

1.5.4 Development of HLA antibodies in pregnancy

Pregnancy is a well described trigger for the development of HLA antibodies in women who previously had no detectable HLA antibodies [172]. There is a paucity of data on the frequency and timing of development of HLA antibodies during pregnancy. Even presence/absence of HLA antibodies is variable between different individuals, or in different pregnancies in the same individual. Forty-nine percent of multiparous women had detectable HLA class 1 or 2 antibodies using Flow PRA screening followed by Luminex single antigen technology, compared with only 19% of women with recurrent miscarriage [158]. Hebral *et al* used Luminex methodology to study kidney transplant recipients who later became

pregnant and were followed up for 1 year following delivery. Forty-four women had no HLA antibodies prior to pregnancy, of whom 42 had no HLA antibodies detectable following the pregnancy. Of 7 women with HLA antibodies prior to pregnancy, there was one development of *de novo* HLA specificities. Of the 3 women who developed *de novo* specificities, for 2 of these cases the paternal allele matched the original kidney donor. The relevance of this frequency of development of HLA antibodies cannot be extrapolated to a healthy population due to multiple immunosuppressant drug therapies and comorbidities in transplant patients. Additionally the authors did not discuss *de novo* development of antibodies against foetal or other HLA types but focused their report on donor directed specificities only [173].

HLA antibodies have been reported as elevated in women with recurrent miscarriage compared with healthy pregnancies. Using an ELISA method, HLA antibodies were noted in 32% of women with recurrent miscarriage compared with 10% in women with healthy pregnancies, with particular contribution by antibodies against HLA-C [174]. Using Luminex based methods, Lee *et al* showed an association of HLA class 1 and 2 antibodies present in second trimester with preterm birth, although antibodies were detectable also in healthy pregnancies. HLA antibodies were more common in subsequent pregnancies than privigravidae and the majority were directed against foetal antigen specificities [175]. Using ELISA and complement-dependent cytotoxicity methods Nielsen *et al* found HLA antibodies in 25% of multiparous women during first trimester of a healthy pregnancy, with higher frequencies in those with miscarriage. Thus HLA antibodies are a common, but not universal occurrence in pregnancy and their role in pregnancy complications remains debated.

1.6 Relevance of alloimmunity to HIV pathogenesis

1.6.1 Donor HLA incorporation into HIV envelope

Enveloped viruses bud from the host cell membrane and incorporate host proteins, including HLA, into the envelope [176]. Host proteins incorporated into the viral envelope include HLA class 1 (in particular HLA-C) [177], HLA class 2 (in particular HLA-DR) and others [151,178,179]. There appears to be some non-specific incorporation of host proteins, as well as Env-mediated selection in some cases [151]. Poon *et al* showed that the viral protein Env was required for efficient incorporation of HLA-DR into virions. Incorporation of class 1 HLA proteins, however, was not affected by Env [180]. HLA-DR incorporated into

the viral membrane enhanced infectivity of virions *in vitro* 1.6-2.3 fold [181,182]. The authors did not study different HLA-DR specificities but postulated that varying specificities may affect infectivity. There was more incorporation of HLA-DR into HIV envelope during chronic rather than acute infection [183]. HLA-C also associates with gp120 in the viral envelope and increases infectivity. HLA-C also increases ability of virions to induce syncytium formation in vitro [177].

HLA proteins are present in the HIV envelope in higher quantities than gp120 trimers [42]. Conceivably, therefore, pre-existing antibodies against HLA-DR or other HLA proteins may affect viral infectivity. Incorporation of host HLA within the viral envelope of an HIV "donor" implies that antibodies against HLA in the HIV "recipient" may play a role as non-neutralizing antibodies. Such antibodies could hypothetically enhance or inhibit HIV transmission to the recipient.

One of the earliest reports of cross-reactivity between HIV and to HLA was the demonstration in 1987 that HLA-DR4 proteins incorporated into virions grown in human cell lines was the cause of false positive ELISA assays for HIV (then called HTLV-3) [184]. Lakashe *et al* showed that HIV virions incorporated HLA proteins into the viral envelope at higher quantities than gp160 trimers. Their work estimated incorporation of 42 to 135 molecules of HLA class 1 per virion in comparison with approximately 14 envelope trimers. They cultured a primary HIV isolate in cells derived from husbands of multiparous women with known specificities of HLA antibodies directed at their husband's HLA type. The plasma of the women was found to contain antibodies that reacted with HIV when the HIV was cultured in cells with matching HLA antigens. The plasma of the women did not, however, neutralize viral infection, with or without addition of exogenous complement. Sample numbers were small (only 2 samples tested for neutralisation activity, showing 27 and 29% neutralisation activity respectively [152]. Contrastingly, Wilfingseder *et al* [151] showed that sera from poly-transfused patients could neutralize HIV *in vitro*, with the assistance of exogenous complement. The authors deduced that HLA class 2 and gp120/gp41 on the viral envelope or host cell are in close proximity. Antibody binding to HLA class 2 caused viral neutralisation due to steric hindrance that blocked interactions with CD4 or a co-receptor.

1.6.2 Homologies between HIV proteins and HLA

GP120 binds to CD4 and co-receptors to allow viral entry. The natural ligand of CD4 is MHC class 2. Multiple sequence homologies between gp120 and MHC class 2 have been identified, suggesting

speculatively that HIV may have evolved from MHC class 2 or by mimicry of MHC 2 [185]. HIV gp120 has even been shown to be able to present peptide as antigen, resulting in stimulation of specific T lymphocytes, analogous to peptide presentation by MHC class 2 [186]. Additional homologies between MHC class 2 and other HIV proteins (gp41 and Nef) and even between HIV proteins and MHC class 1 were identified. These homologies have been described in the text below and summarized in Table 1.1.

Gp120 binds to CD4 at a site overlapping the usual binding site of CD4 with MHC class 2 [187,188]. While it was first postulated that gp120 might compete with MHC class 2 for CD4 binding, it was subsequently shown that CD4 has two separate binding sites. MHC class 2 comes into contact with CD4 on a surface opposite the gp120 binding site [189–191].

During evolution, viruses may develop attachment sites that mimic the natural ligands of their cellular receptor [192]. Multiple homologies between MHC 2 and gp120 have been reported [192]. A third of HIV infected individuals harboured antibody to a region of gp120 (aa 837-844) cross reactive with HLA class 2 (aa 19-25) via ELISA. In the presence of the cross-reactive antibody, $CD4^+$ T cells exhibited dysfunction *in vitro*. The antibodies could also elicit antibody-dependent cytotoxicity *in vitro.* The authors hypothesized that molecular mimicry between HIV gp120 and MHC 2 could result in autoimmunity mediated by cross-reactive antibodies, resulting in the observed $CD4^+$ T cell dysfunction early in HIV disease.

The gp120 derived peptide aa254-263 shares homology with the HLA class 2 beta chain (found conserved among HLA class 2 proteins including HLA-DR, HLA-DQ and HLA-DP) [187,193,194]. This peptide is found in HIV-1 strains but not in the less pathogenic HIV-2 retrovirus, suggesting a role in pathogenesis of AIDS. The peptide is inhibitory to T cells when stimulated with PPD *in vitro* [187]. The gp120 derived peptide aa 261-270 also shares homology with HLA class 2 β chain [195]. Zaitseva *et al* reported that sera from 75% of HIV infected individuals contained antibody to MHC 2, using ELISA methodology and competitive binding to monoclonal HLA-DR. 28-48% of HIV positive sera contained antibody to homologous regions of gp120 and MHC 2 [196].

Homologies between viral proteins and MHC 2 imply that viral proteins may be replacing host proteins in the immunological synapse, with possible deleterious effects on immune responses to HIV or other antigens. T cells become anergic in the presence of gp120, even in the absence of whole virons [42].

Pugliese et al showed that 30% of HIV infected individuals harbour antibodies against the particular gp120 peptide aa254-263 which shares homology with HLA class 2, but none made antibodies to an irrelevant HLA-DQ peptide used as a control. Further, the gp120 peptide aa254-263 could increase T cell stimulation by purified protein derivative (PPD) *in vitro*, likely acting at the level of antigen presentation (as a homologue of MHC 2 binding to CD4) [194].

Homologies between gp120 and MHC 2 may result in activation of either the direct or indirect pathways of antigen presentation. HIV gp120 can present peptide antigen, similarly to HLA-DR1, in *in vitro* systems [186]. Conceivably, the direct activation pathway could induce symptoms similar to those of graft versus host disease, or the indirect activation pathway could induce autoreactive T cell activation [186]. Wilson *et al* note that in early stages of HIV, the direct pathway of allo-antigen responses appears intact, while the indirect pathway appears to be impaired [197,198]. Wilson *et al* studied T cell responses to a peptide homologous between gp120 and MHC 2 (see Table 1.1). They reported that presentation of this peptide as an antigen (via the indirect pathway of allo-recognition) induced $CD8^+$ T cell lines which could suppress *in vitro* T cell responses to recall antigens such as tetanus toxoid or purified protein derivative (PPD). It has been pointed out that many symptoms and signs of HIV mimic those of graft versus host disease. HIV/AIDS and graft versus host disease are characterised by generalized immune stimulation, increased pro-inflammatory cytokines, opportunistic infections, dysfunction of numerous cell types and loss of function of antigen specific immune responses over a long period of time [42]. Thus allo-recognition may be playing a role in HIV disease progression.

Additional sequence homologies with MHC 2 and HIV proteins other than gp120 have been noted. Similarity has been noted between gp41 (aa 837-844) and the HLA class 2 β chain, a conserved region of all HLA class 2 [199]. Additionally, sequence similarities between the HIV *nef* gene and MHC class 2 have been observed [188]. It has been suggested that evolutionary pressures resulted in multiple homologies between viral and host proteins [188,200]. Zagury *et al* reported homology between gp120 (5 amino acids SLWDQ) and the same pentapeptide sequence in the CD4 protein [187]. The sequence is conserved among HIV-1 strains but absent from less pathogenic HIV-2 strains, implying that the sequence may be important in pathogenesis of AIDS.

Additionally, some homology between gp120 and MHC 1 have been recorded. There is homology between two non-contiguous stretches of gp120 (KYK and KAKR) and the alpha1 chain of HLA-C. The

KAKR stretch lies in an immune-dominant region of gp120 (APTKAKRRVVQREKR), to which the majority of HIV infected persons make antibodies [189]. The C5 terminus of HIV gp120 is an immune-dominant region of the virus to which many infected individuals produce antibodies. The region has not been pursued for vaccine design as antibodies to the C5 terminus are non-neutralizing. Antibodies against this region have however been associated with slower disease progression [42,201]. Non-neutralizing antibodies against the C5 region may thus play an important role in protecting the individual. Such antibodies may be cross-reactive with anti-HLA antibodies [42]. Overall gene structure of gp120 and gp41 is shown in Figure 1.8.

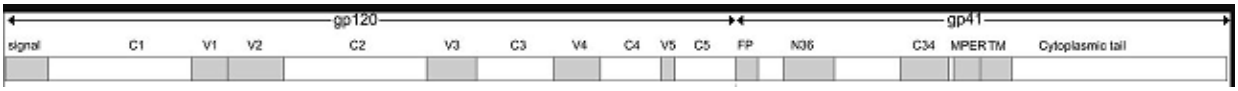


Figure 1.8 Gene structure of gp120 and gp41 [202]

Table 1.1 Reported sequence homologies between HIV and host proteins

Host protein	Host Amino acid sequence	HIV protein	HIV amino acid sequence	References
Homologies with gp120				
HLA class 2 beta chain HLA-DR, HLA-DP, HLA-DQ) aa 142-151	VVSTGLIQNG	gp120 (aa254-263)	VVSTQLLNG	[193,194,196]
HLA DP class 2 beta chain (aa 179-188)	VVSTNLIRNG	Gp120 (aa 254-263)	VVSTQLLNG	[187,203]
HLA-DR alpha chain (aa 28-40)	EEHVIIQAEFYLN		EEVVIRSANFTDN	[196]
HLA class 2 beta chain	Not specified	Gp120 (aa 261-270)	Not specified	[195]
HLA-DR β chain 3rd hypervariable loop (aa 69-81)	Not specified	Gp120 carboxy terminus	TKAKARRVVEREKR	[197]
CD4 (aa 60-64)	SLWDQ	Gp120 (aa 110-114)	SLWDQ	[187]
HLA class 1 α heavy chain (HLA-A, HLA-B, HLA-c) antigen binding site, as found on activated rather	KYKY ELYKYK KYKR (aa 66-69) and RKLR (aa 79-82)	Gp120 C5 domain (aa490-492 and 505-508)	KYK and KAKR (two non-contiguous stretches flanking a hydrophobic region involved in gp41 binding)	[189,197,203,204]

than resting T cells, not associated with β2 microglobulin				
Chemokines (MIP-1alpha, MIP-1beta, RANTES, SDF-1) 40's loop	Not specified	Gp120 V3 loop	Not specified	[205] Criticized in [206]
Homologies with gp41				
Amino terminus HLA class 2 beta chain (HLA-DR, HLA-DP, HLA-DQ) (aa 19-25)	NGTERVR	Carboxy terminus of Gp41 (aa 838-844)	EGTDRVI	[192,199]
HLA-DR alpha chain (aa169-183)	VEHWGLDQPL	Gp41 (aa 583-599)	VERYLKDQQL	[196]
Homologies with nef				
HLA class 2	Not specified	HIV nef	Not specified	[188]

Cross reactive antibodies to host MHC and viral proteins may arise through multiple mechanisms. Such mechanisms could include incorporation of MHC proteins into the infecting virion, with MHC from the viral "donor" acting as antigen in the viral "recipient". An alternate mechanism is through molecular mimicry (similarity) between viral antigens with host antigen, resulting in antibody against viral protein having cross-reactive effects in the host. Additional hypotheses include immune dysregulation induced by infection leading to loss of immune tolerance and generation of self-reactive antibodies; or massive destruction of lymphocytes with increased presentation of altered host MHC as antigen [207]. In rhesus macaques infected with SIV grown on human cells, there was no correlation in kinetics between development of anti-SIV and anti-MHC class 1 antibodies, suggesting that the cross-reactivity was not likely due to molecular mimicry between SIV antigens and MHC [207].

The multiple monkey and human data in which exposure to HLA preparations seem to have favourable effects on host resistance to HIV have two conceptual reasons. One reason is that exposure to allo-antigens may have resulted in non-specific host immune responses yielding beneficial effects, such as dampening down immune activation. An alternate reason is that induction of alloimmunity may have had specific cross-reactivity with HIV antigens [42].

1.7 Alloimmunity in HIV-infected humans

Discussion of HLA antibodies can be divided into studies of HIV infected populations and studies of individuals persistently exposed to HIV who remained HIV-uninfected.

1.7. 1 HIV-infected humans

In addition to homologies between HIV virions and host MHC, already discussed, additional studies have described HLA antibodies in HIV-infected individuals. Leite *et al* showed class 1 HLA antibodies in 32% of HIV- infected patients using a screening method (Quickscreen). The proportion with positive HLA class 1 antibodies did not vary according to disease stage or viral load. Muller *et al* showed that 75% of HIV infected patients made anti-lymphocyte antibodies, detectable flow cytometrically (while no HIV uninfected patients did). The antibodies were detectable bound to T cells, but the exact specificity of the antigen was not determined [208].

1.7. 2 Alloimmunity in highly exposed persistently seronegative humans

While HLA cross-reactive antibodies in HIV infected individuals may arise due to HIV infection itself, potentially affecting disease progression, it is necessary to look in HIV uninfected but exposed individuals in order to question whether such antibodies may play a role in susceptibility to HIV infection.

In highly exposed but persistently negative people, Brown *et al* showed antibodies in 30% that reacted with the C5 terminal region of gp120 via ELISA. Most antibody appeared to be of IgM class, although IgG was sometimes present. Additionally, 70% of the group had antibodies to HLA class 1. The authors postulated that the high frequency of HLA antibodies was due to a high proportion of multiparous females in the sample group. The highly exposed seronegative individuals did not have antibodies reactive with other regions of gp120 (including the region homologous with MHC 1). The authors hypothesized that anti-HLA class 2 or anti-HLA class 1 antibodies might be protective against HIV acquisition. They further comment that such antibodies may be surrogates of a successful T cell mediated anti-HIV response via T cell alloimmunity [203].

In a study of persistently HIV seronegative intravenous drug users [209], antibodies against HLA (cross-reactive with HIV) were present in 33% of the intravenous drug users compared with 4% of healthy controls. All patients with HLA antibodies also had detectable T cell responses to HIV. The authors hypothesized that the presence of HLA/HIV antibodies in the control groups at low frequencies could be due to exposure to other retroviruses or microbial glycoproteins [209].

In 19 highly exposed seronegative sex workers from Abidjan, Cote d'Ivoire, using a flow-cytometric PRA screening test for class 1 HLA antibodies and a modified mixed lymphocyte reaction for T cell responses, there was no difference in proportion with HLA class 1 antibodies between sex workers and female blood donors. The sex workers however showed lower allo-specific T cell responses than the controls. The authors discussed that unprotected sexual intercourse likely decreased alloimmune T cell responses and may be associated with immune tolerance to alloantigens [210]. Importantly, class 2 HLA antibodies were not analysed.

Luscher *et al* questioned whether naturally occurring anti-HLA class 1 antibody could be a protective factor against HIV. They reported anti-HLA antibody in 12% of highly exposed seronegative sex workers, with a similar frequency in healthy controls, using a micro-lymphocytotoxicity method and PRA-Stat assay (Sangstat medical corp, USA). Antibodies against HLA class 2 were again not tested [167]. Luscher *et al* further hypothesized that HLA antibodies may contribute to HIV resistance in infants born to HIV infected mothers. Using PRA-stat ELISA methodology, they investigated children aged 0.7-5.7 years born to HIV infected or uninfected mothers. The authors found HLA class 1 antibodies in more than 20% of the children. There was no significant difference in proportion of those with HLA class 1 antibodies between the HIV exposed uninfected, HIV infected and HIV unexposed groups. Individual loci were not directly compared, and class 2 antibodies were not investigated as there was no reliable assay for class 2 antibody detection at the time. [170].

1.7.3 Alloimmunity and *in vitro* HIV suppression

Some investigators have directly examined the effect of anti-HLA antibodies on HIV replication *in vitro*. Lopalco *et al* studied exposed seronegative individuals from discordant couples, and showed neutralising activity against HIV *in vitro* in 5 of 17 individuals, compared with none of 17 healthy blood

donors. In total, 11 of 17 exposed seronegative individuals had antibody against HLA class 1 compared with none in controls [211].

Leith *et al* tested sera from women undergoing lymphocyte immunotherapy for recurrent miscarriages. Immunotherapy was performed by one intravenous and 3 intradermal injection of 10^8 donor cells. Two of seven women tested made strong HLA antibody responses towards their partner's HLA type (detected using FlowPRA beads and ELISA). For one recipient, the donor-directed HLA class 1 and class 2 antibodies were able to neutralize strains of HIV *in vitro* [212].

In women following immunization with their partners lymphocytes for recurrent miscarriages [213]. HLA-DR antiserum (serum known to contain antibodies to HLA-DR) could bind to CCR5 and could directly interfere with viral replication in culture. Other HLA typing antisera also inhibited viral replication in vitro to varying degrees.

1.8 Relevance of alloimmunity to HIV vaccine trials

The concept of alloimmunity has relevance HIV vaccine trials from two perspectives. Firstly, alloimmunity debately remains a conceptual target of a novel HIV vaccine [214]. Secondly, for traditional models of HIV vaccines, alloimmunity might be an unintended side-effect. The relevance of unintentionally induced alloimmunity remains obscure and requires a better understanding of natural history of development and function of HLA antibodies.

In 2011 Boasso and Shearer published a report titled "alloantigen based HIV vaccine: revisiting a "rightfully discarded promising strategy" in which they argue that in the face of recent HIV vaccine trial disappointments, the promise shown by protection of >200 macaques through xenoimmunisation with human cells in trials in the early 1990s should be revisited and explored in depth [215]. They listed advantages and disadvantages of an alloimmunization vaccine strategy. HLA is one of the most immunogenic proteins known. Anti-HLA antibodies are likely to react against virions more efficiently than antibodies to envelope antigens. The HLA type from a potential HIV "donor" could not be predicted, which may require a strategy of engineering a group of multiple HLA specificities into a vector, such as a leucocyte cell line. This cell line could then be used as the vaccine, or as the cell type in which to culture HIV for production of vaccine. HLA carried by the vaccine would remain allogenic only

as long as infection did not occur. Such a vaccine would be required to induce sterilising immunity. As soon as infection occurred, newly produced viral particles would incorporate host HLA and no longer be allogeneic.

The call for investigation of alloimmunity as a potential HIV vaccination strategy has been echoed by other authors [214]. Advantages of alloimmunization as a HIV prevention strategy include avoidance of the issue of high variability and mutability of HIV. Potential disadvantages include the risk of induction of autoimmunity and limitations to future transplantation options in vaccinated individuals. It was noted however that in more than 2000 women who had received lymphocyte immunotherapy for induction of alloimmunity for fertility reasons, there were no reported deleterious consequences. The benefit:risk ratio of a successful HIV vaccine would likely outweigh concerns around organ transplantation for HIV endemic areas. Multiparous women and people who have received multiple transfusions represent natural examples of people with alloimmunity without obvious autoimmune side effects.

A working group consultation was convened by the National Institute of Health of the United States of America in 2012 to evaluate challenges and opportunities for investigation of alloimmunity in protection against HIV [216]. In the NIH working group consultation, one of the principal components of a strategy to advance the alloimmunization field was listed as "identification of human cohorts/HLA typing databases to complement the protective effect of alloimmunization with epidemiological studies" [216]. This work represents one such epidemiological study.

This epidemiological approach gives valuable insight into the natural history of HLA antibodies in a healthy population exposed to HIV and a healthy cohort of mothers and their infants.

1.9 Overarching hypothesis, aims and objectives of this study

The overarching hypothesis of this study was that HLA antibodies play a physiological role in the healthy immune human immune system and protect against certain infectious diseases, HIV in particular.

In the first part of this thesis, my aim was to characterise HLA genotypes of South African participants in HIV-serodiscordant couples, in preparation for the second part of the study. Results of part one are presented in chapter 3.

In the second part, my aim was to investigate whether alloimmunity, in the form of HLA antibodies, was epidemiologically associated with decreased risk of HIV acquisition from a heterosexual partner. Results are shown in chapter 4 as a manuscript for a peer-reviewed journal.

In the third part of this study, my aim was to explore the natural history of HLA antibodies in healthy pregnancy and healthy neonates. Results of part three are shown in chapter 5 as a published article.

1.9.1 Aims and Objectives of Part 1

The first aim was to HLA genotype each partner of all couples for multiple HLA class 1 and class 2 loci.

Specific objectives were:

a. To HLA genotype HIV-infected and uninfected partners at the HLA-A, HLA-B, HLA-C, HLA-DPA1, HLA-DPB1, HLA-DQB1, HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 loci
b. To describe allele and haplotype frequencies amongst the sample
c. To compare allele and haplotype frequencies with that published for the South African black population.

1.9.2 Aims and Objectives of Part 2

The next aim was to characterise HLA antibodies against HLA class 1 and class 2 antigens in HIV sero-negative individuals who remained HIV-uninfected despite a long term relationship with an HIV-infected partner, and compare them to individuals from the same cohort who acquired HIV (seroconverted). I aimed first to analyse ***all*** HLA antibodies and then specifically analyse HLA antibodies directed at the HIV-infected index parter ("**matching**" antibodies). My hypothesis was that non-matching HLA antibodies would show no difference in frequency between partners who remained HIV uninfected compared with those who went on to acquire HIV, but that *matching HLA antibodies* would be at higher frequency in the partners who remained HIV uninfected than in those who went on to acquire HIV.

Both of these analyses were firstly conducted using the complete seroconverter group (n=28) and then again using only seroconverters who acquired HIV from their index partner (linked seroconverters, n=19).

An additional aim was to describe the frequency of potentially autoreactive HLA antibodies in the general population and to look for *de novo* HLA antibodies and HLA autoantibodies following HIV acquisition. My hypothesis was that autoantibodies are present at low frequency in the general population and that HIV would increase the frequency of such antibodies.

I further aimed to investigate whether there were complement-fixing class 2 HLA antibodies, potentially belonging to the IgM subclass, that differed between those who did and did not later acquire HIV.

My specific objectives were:

a. To categorize the presence or absence of all allo-antibodies (termed "non-matching antibodes) in HIV-uninfected partners at the HLA-A, HLA-B, HLA-C, HLA-DP, HLA-DQB, and HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 loci at two timepoints, which in seroconverters included a timepoint prior and a timepoint after seroconversion. Both prior and after seroconversion I aimed
    - To compare the frequency of antibodies at each locus in those who acquired HIV compared to those who did not acquire HIV.
    - To compare the number of HLA antibody specificities per individual in those who did compared to those who did not acquire HIV.

b. To use the genotyping and antibody information to determine the presence or absence of "matching antibodies" in the HIV-uninfected parters against their partner's HLA type at two timepoints, which in serocoverters included a timepoint prior and a timpoint after seroconversion.

c. To explore whether autoantibodies against HLA were prevalent in healthy individuals (highly exposed seronegative partners). Using the data on HLA antibodies, we looked for autoantibody

specificities in partners originally HIV-uninfected, at two timepoints, and compared those who did and who did not acquire HIV.

d. To identify complement-fixing alloantibodies including those of IgM isotype in originally HIV-uninfected partners at the HLA-DPB, DQB, DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5, loci at the first timepoint

e. To identify whether HLA antibodies or *matching* HLA antibodies were epidemiologically associated with risk of HIV acquisition by the originally HIV-uninfected partner and describe the strenght of the association in comparison with traditional risk factors for HIV acquisition. This was performed through univariate and multivariable logistic regression.

1.9.3 Aims and Objectives of Part 3

In the third part of this study, the aim was to explore the natural history of HLA antibodies in healthy pregnancy and healthy infants and to assess whether HLA antibodies in mothers and infants are directed at the same specificities, or differ in their repertoire.

Specifically, the objectives were

a. To categorize the presence or absence of all allo-antibodies (termed “non-matching” antibodies) in pregnant women against HLA-A, HLA-B, HLA-C, HLA-DP, HLA-DQB, and HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 loci

b. To categorize the presence or absence of all allo-antibodies (“non-matching” antibodies) in six-week old infants against HLA-A, HLA-B, HLA-C, HLA-DP, HLA-DQB, and HLA-DRB1, HLA-DRB3, HLA-DRB4 and HLA-DRB5 loci

c. To determine whether HLA antibodies from mothers crossed the placenta to infants, by comparing the repertoire of HLA antibody specificities in mothers and infants directed against identical antigen specificities, termed “shared antibodies”.

d. in a subset of six pregnant women and their infants followed longitudinally on whom DNA was available from the infants, the objective was to describe the timing of antibody development and

whether antibodies present in the mother were directed at infant HLA genotypes (termed "matching" antibodies).

2. CHAPTER 2 – Methods

2.1 Study descriptions

2.1.1 HIV-serodiscordant couples

We accessed samples from two studies of HIV-serodiscordant couples, the Partners in Prevention and Couples Observational Study. The Partners in Prevention study, hereafter termed the "Partners" study, was a randomized, double blind, placebo-controlled trial in HIV-1 discordant couples investigating the role of treatment for genital Herpes Simplex Virus-2 (HSV-2) for preventing transmission of HIV [217–219]. The Partners study enrolled 3408 heterosexual HIV sero-discordant couples from seven countries (14 sites) in Southern and Eastern Africa between 2004 and 2007 and was funded via the University of Washington. For this study, only South African samples were included, comprising 28 couples in which the second partner acquired HIV ("seroconverting couples") and 115 matched control couples (where the partner did not acquire HIV).

In the Partners study, participants had been followed up longitudinally with 6 monthly study visits. Enrollment criteria required **HIV infected participants** to be dually infected with Herpes Simplex Virus -2 (HSV-2) with a $CD4^+$ T cell count above 250 cells/µl, age above 18 years, no conditions at enrolment of symptomatic AIDS and no antiretroviral therapy prior enrollment. For the initially **HIV uninfected partner**, age needed to be above 18 years of age. HSV-2 serostatus was not an inclusion/exclusion criterion. Couples required to have reported at least three episodes of vaginal sex in the three months prior to enrollment and the intention to remain together for at least 24 months. At every visit, couples were counselled regarding HIV risk reduction, provided with condoms, treated for any sexually transmitted infection and females tested for pregnancy. Pregnant women were excluded further from the trial. If participants met national criteria for antiretroviral therapy they received it from local clinics. HIV-infected participants were randomized to treatment for HSV-2 with acyclovir during the trial. Overall results showed that acyclovir decreased HIV viral load but did not affect HIV transmission. There were 132 total HIV transmissions within the 14 sites [217], of which 29 were amongst South African participants, 28 of which have been included in this thesis (for one couple no sample was available). In the Partners trial, couples had co-habited for a median of 5 years (range 2-9 years) with 28% reporting unprotected sexual intercourse in the month prior to enrolment [219]. The Partners study was closed and residual samples were made available for further analyses.

The Couples Observational Study ("COS" study) was conducted in Soweto, South Africa and Kampala, Uganda and enrolled 485 HIV-1 sero-discordant couples. Recruitment criteria were similar to the Partners study but had no restriction regarding eligibility based on $CD4^+$ T cell count or Herpes simplex virus infection. Couples were followed up quarterly for twelve months [220].

From the Partners and COS studies, 863 participants were previously genotyped by genome wide association study for single nucleotide polymorphisms (SNPs) associated with HIV transmission or HIV control. No statistically significant SNPs were identified [220], HLA typing of 510 participants with either seroprevalent HIV (at enrolment) or sero-incident HIV (during the studies), conducted in the USA, showed associations of certain HLA alleles with higher or lower viral setpoints [102]. Additionally, concordance of HIV donor and HIV recipient at HLA-A or HLA-C loci was associated with higher viral setpoint [102]. In a genome wide association study using samples from the Partners in Prevention trial, Lingappa *et al* found no single nucleotide polymorphisms associated with HIV acquisition risk after controlling for HIV exposure risk and multiple comparisons [220]. Class 2 HLA typing of Partners or COS participants has not been done previously.

2.1.1.1 Participating South African sites

There were three participating South African sites in the Partners Study. The Perinatal Health Research Unit (PHRU) was a participating site in Soweto, South Africa, with local principal investigator Guy De Bruyn. Additional sites included a site at Orange Farm run by Reproductive Health Unit (RHRU), University of the Witwatersrand with principal investigators Sinead Delaney-Moretlwe and Helen Rees as well as a University of Cape Town site with principal investigator David Coetzee. Only the Soweto site (PHRU) participated in the COS study. All seroconverters from these sites, with sufficient residual samples for analysis, were included (n=28). More information on selection of controls for inclusion in this nested study is given later in relevant methods section 4.9.1.

2.1.1.2 Linked transmissions

For both the Partners and COS studies, sequencing of HIV *gag* and *env* genes had been performed by collaborators at the University of Washington in couples with HIV transmission, in order to determine

whether the transmission was from the enrolled partner (linked) or a non-enrolled partner (unlinked). In the original Partners study, of the 132 HIV seroconversions globally, 84 were linked [221]. Of our 28 South African seroconverting couples, 19 were linked and 9 unlinked.

2.1.2 Mother-infant pairs

Thirty healthy mother-infant pairs available from a previously completed study on rotavirus vaccine immunogenicity (NCT0221522654) were used. The study, conducted in Soweto, South Africa in 2009-2010, explored the effect of at least an hour abstention from breast feeding at immunization visits on immune responses of infants to rotavirus vaccine. The study enrolled black South African mother-infant pairs who were human immunodeficiency virus (HIV)-negative, at their six week post-partum visit. Blood samples were drawn from all participants at enrolment, immediately before administration of the rotavirus vaccine to the infants. The serum fraction was separated by centrifugation and stored at -700C until further use. Infant DNA was not available from this cohort.

An additional twenty-nine women in their first trimester of pregnancy were recruited from collaborating gynaecologist practices (the Charlotte Maxeke Johannesburg Academic Hospital and Netcare private clinics in South Africa). Recruited patients were of both black and white South African ethnicity. All patients were HIV-negative, multigravida and over 18 years of age at enrolment. Blood samples were drawn from participants at routine visits to the clinic, including during the first (up to 13 weeks of pregnancy), second (14-26 weeks of pregnancy), and third (27-40 weeks of pregnancy) trimesters, and/or 8-11 weeks post-partum. Buccal swabs were collected from infants at the post-partum visit and later processed for DNA extraction. Mothers' serum was stored at -70^{0}C until further use. Samples of six participants with healthy and uncomplicated pregnancies, who had at least two visits to the clinic and for whom buccal swabs were successfully collected from their babies, were analysed and their results presented here as case studies. The remaining participants are not presented here due to missing data such as incomplete infant HLA typing results or maternal serum timepoints. We did not collect serum from infants in this study

2.4 Sample storage and retrieval

The samples (sera and dried blood spots) from the Soweto site had been previously stored by Contract Laboratory Services in South Africa and were transferred to us on dry ice. Upon delivery, sera samples were stored at -80°C, whereas dried blood spots were stored at room temperature.
Samples from the Orange Farm and Cape Town sites had been stored in the USA and were transported back to South Africa. The sera had been stored at -80°C and were transported to South Africa on dry ice. The dried blood spots were stored and transported at room temperature.

Samples of mother-infant pairs from the rotavirus vaccine study had been stored by the trial site (Respiratory and Meningeal Pathogens Unit, Baragwanath hospital, Johannesburg) at -80°C, were transferred on dry ice and stored again at -80°C. Samples from the longitudinal pregnancy study were stored in our laboratory (Centre for Vaccines and Immunology, National Institute for Communicable Diseases, Johannesburg) at -80°C since collection.

2.5 DNA extraction from dried blood spot

DNA was extracted from dried blood spots which had been collected at enrolment. The extraction was performed using a commercial kit (Gentra® Puregene® Tissue Kit, Qiagen) according to manufacturer's instructions. Filter paper containing 50µl of dried blood spot was cut out into small pieces with a single use scalpel and placed in to a 1.5ml microcentrifuge tube. Cell lysis solution (600µl) and 3ul Proteinase K (20mg/ml) (Gentra® Puregene® Tissue Kit, Qiagen) was added and incubated overnight at 55^0C with gentle shaking to complete cell lysis. On the following day, RNase A solution (3µl) (Gentra® Puregene® Tissue Kit, Qiagen was added to cell lysate and incubated at 37˚C for 15 minutes. The sample was cooled to room temperature by placing on ice for 1 minute. 200µl protein precipitation solution (Gentra® Puregene® Tissue Kit, Qiagen) was added, tube vortexed for 20 seconds, then incubated on ice for 15 minutes. The solution was centrifuged at 13000g for 3 minutes after which the precipitated proteins formed a pellet. Supernatant was poured into a clean microcentrifuge tube with 600µl isopropanol. 1µl Glycogen solution (30mg/ml) (Gentra® Puregene® Tissue Kit, Qiagen) was added and tube mixed by inverting, incubated on ice for 15 minutes and centrifuged at 13000g for five minutes. Supernatant was discarded. To the DNA pellet, 600µl 70% ethanol was added to wash the DNA, tube spun at 13000g for 1

minute and supernatant discarded. DNA was air dried for 15 minutes. 25µl of DNA hydration solution (Gentra® Puregene® Tissue Kit, Qiagen) was added and incubated at 65˚C for 1 hour to dissolve the DNA. To concentrate the DNA, 1:10 volume of 3M sodium acetate was added and 2.5 volumes of chilled 100% ethanol followed by incubation at -70˚C for 1 hour. Samples were centrifuged for 30 minutes at 4˚C. Supernatant was removed and pellet washed with 500µl 70% cold ethanol. Samples were centrifuged at 10 minutes at 4˚C and ethanol was discarded. Only samples with a concentration of over 25ng/µl were used. if less, another dried blood spot from the same patient was used to prepare DNA. The extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The DNA was further used in HLA typing experiments.

2.6 HLA typing

Commercial single stranded oligonucleotide probe kits (Lifecodes, Immucor Transplant Diagnostics, USA) were used according to manufacturer's instructions for typing the following HLA loci: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3,4,5, HLA-DQB1 and HLA-DPA/B. All kits allow for allele specific PCR amplification. By use of forward and reverse primers which are at differing concentrations, both single and double stranded labelled PCR product is generated. Single stranded product, and double stranded product following denaturation, is available for hybridisation with known oligonucleotide probes. Oligonucleotide probes in the kits are attached to microspheres detectable by Luminex instrument (Bioplex Biorad). Up to 100 different microspheres are detectable in any one run, each one of which can be conjugated to a different probe. Each kit contains a pattern of known oligonucleotide probes specific for typing a particular locus. The software analyses the pattern of microspheres with attached PCR product to generate a likely HLA typing based on common and well defined alleles listed in the software. The data obtained from the typing experiments was analyzed using Match-It DNA software versions 3.18-3.24 (GenProbe). The typing is reported at the two digit resolution, with some samples typed to four digit resolution.

In brief, 15ul of Lifecodes mastermix, 0.5ul Taq polymerase, nuclease free water and 5µl of PCR product containing 100-300 nanogram DNA was used to prepare a reaction mix. The reaction mix was placed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) with the following conditions for amplification:

1 cycle: 95°C for 5 minutes

8 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds

32 cycles of 95°C for 30 seconds, 63°C for 45 seconds, 72°C for 45 seconds

1 cycle of 72°C for 15 minutes

Probe mix (containing microspheres) was heated at 60°C on a heating block followed by high speed vortexing to solubilize the beads. 5µl PCR product was added to in a well of a 96 well plate together with 15µl of appropriate probe mix. Samples were hybridised in a thermocycler (GeneAmp PCR system 9700, Applied Biosystems) using the following conditions:

97°C for 5 minutes

47°C for 30 minutes

56°C for 10 minutes

56°C hold

Streptavidin-phycoerythrin (Strep-PE) was diluted according to manufacturer instructions, protected from light, and added to each well within 5 minutes of the 56°C 10-minute step. Theplate was then run on the Luminex instrument (xPonent® 3.1 software). Results were imported into MAtchIt software (Immucor, USA) for analysis.

For class 1 loci, where results were unavailable due to sample quality or quantity, results were requested from the Partners database (collaborator Romel Mackelprang, University of Washington) where available.

2.7 HLA allele and haplotype frequency analysis

HLA alleles were imported into Arlequin software (Version 3.5) for analysis. We analysed allele frequencies at each locus. We then analysed three-locus haplotypes for class 1 (HLA-A - HLA-B - HLA-C) and for class 2 we analysed two-locus haplotypes (HLA-DRB1 – HLA-DRB3/4/5) and five locus haplotypes (HLA-DPA1 – HLA-DPB1 – HLA-DQB1 – HLA-DRB1 – HLA-DRB3/4/5). Haplotypes were assigned by best fit alogrithms by the Arlequin software. We used the ELB (Excoffier-Laval-Balding) algorithm (Excoffier et

al. 2003) implemented in Arlequin, a pseudo-Bayesian approach aiming at reconstructing the gametic phase of multi-locus genotypes.

2.8 HLA antibody testing

Expression levels of antibodies against HLA proteins were measured from stored sera using Lifecodes LSAI and LSAII (Immucor Transplant Diagnostics, USA) kits with Luminex technology. These kits belong to the class of "single antigen beads" and contain one recombinant HLA specificity per bead. The beads are incubated with patient sera, allowing specific antibody to bind to the bead. Unbound antibody is removed through washing steps, and then bound antibody is detected by addition of phycoerythrin labelled anti-human IgG. Bead fluorescence is detected using a Luminex instrument. Florescence is analysed by the software in comparison with negative control beads included in the kit. HLA antibody strength is reported as mean fluorescent intensity (MFI). Software assigns a "positive", "weak", or "absent" result. User interpretation allows additional assignment of "weak" or "possible" antibodies according to patterns of reactivity. Highly reactive sera samples were purified by adsorption of non-specific antibodies using commercial "Sera clean" kit (Immucor Transplant Diagnostics, USA), followed by a second analysis of HLA antibodies.

Sera were heat inactivated at 56°C for 30 minutes and spun for 30 seconds in a micro-centrifuge. Wells of a 96 well filter plate were pre-wet with distilled water, which was removed by aspiration 2-5 minutes later by vacuum manifold. Beads were brought to room temperature, protected from light and well vortexed to resuspend them. We added 40µl beads and 10µl sera to each well and incubated for 30 minutes in the dark on a rotating platform (200 rotations per minute) at room temperature. 100µl wash buffer was added and wells aspirated with vacuum manifold. Following aspiration, 250µl wash buffer was added to each well and vacuum aspirated twice more to wash plates. Conjugate (phycoerythrin labelled anti human IgG) diluted in wash buffer was added to each well and incubated for 30 minutes in the dark on a rotating platform (200 rotations per minute). 150µl wash buffer was added to resuspend beads, after which plates were read using xPonent® 3.1 software (Luminex). Data was imported into Match-It software (Immucor Transplant Diagnostics, USA) for analysis. Default cut-off values were used (BCM cut off was 1500, and positivity was defined if at least 2 out of the 3 parameters were above cut off). Positive and negative control sera were included in each run.

For analysis of complement-fixing antibodies, the Lifecodes C3d detection kit (ImmuCor, USA) was utilized. The principle of the assay is patient serum is allowed to bind with the HLA-coated beads as in the class 2 procedure outlined above. Following incubation, negative serum reagent is added as a source of human complement, which can bind to bead-bound antibody, including that of IgG or IgM or other isotype. The sensitized beads are washed to remove unbound antibody. An anti-human complement component C3d antibody conjugated to phycoerythrin is used as a fluorescent detection reagent.

2.9 Statistics

Statistics are described in relevant sections of chapters 4 and 5.

2.10 Ethics

The Partners study was approved by institutional review boards in each national site including the US. During enrollment into the Partners study, patients signed informed consent including for genetic testing for host factors affecting HIV transmission. Ethical approval was granted by the University of the Witwatersrand Human Research Ethics Committee (HREC) for a study entitled “Host Genetic factors – Investigation of host genetic factors affecting HIV, HIV-2, other sexually transmitted diseases and viruses interacting with HIV in the Partners in Prevention Study” in 2006 (Ethics number 060808). HLA as a candidate gene for investigation was specifically mentioned, as were host proteins that interact with HIV. The COS study was approved by the HREC (060809) Individuals had signed informed consent for storage of genetic material. An additional ethics application was approved for this nested work from the University of the Witwatersrand HREC (M1411118).

The mother-infant study, conducted on samples from the previously published NCT02215226 study, was approved by the Human Ethics Research Committee of the University of the Witwatersrand with consent for storage and testing (M1611144). The pregnancy study was approved by the Human Ethics Research Committee of the University of the Witwatersrand (M130230).

3. CHAPTER 3 - HLA GENOTYPING FROM HIV-SERODISCORDANT COUPLES

3.1 Introduction to genotyping serodiscordant couples

Genotyping the second partner, in whom I analysed HLA antibodies, allowed interrogation of whether HLA antibodies were directed against self HLA genotypes (autoantibodies).

This work generated descriptive data of HLA genotype and haplotype frequencies from the South African black population. In this chapter, I aimed to describe the allele and haplotype frequencies and emphasize haplotypes which differ from previously published haplotypes.

3.2 Summary of enrolment sites and ethnic groups

A description of the sites and studies from which my participants were recruited is shown in Figure 3.1 and Table 3.1. There were 28 seroconverting couples (56 individuals) and 115 control couples (230 individuals) included (286 individuals in total). From the Partners study participants were included from all 3 South African sites. For the COS study only participants from the Soweto site were included.

The ethnic groups to which the participants identified are summarized in Table 3.2. There were nine ethnic groups to which participants self-identified. The majority of the participants belonged to the Xhosa, Zulu and Sotho ethnic groups.

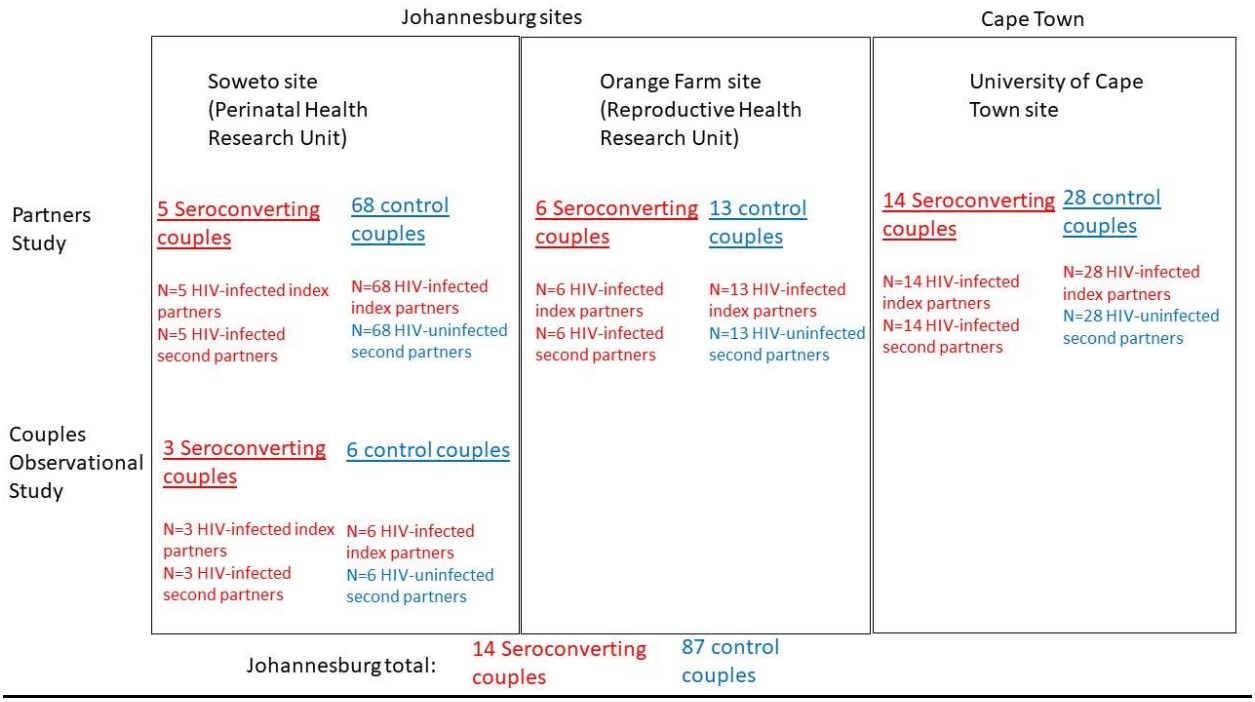


Figure 3.1 Schematic representation of participant enrollment sites

Table 3.1 Study and site of enrolment (n= 143 couples)

Cohort	Site	HIV-seroconverting couples		Highly exposed persistently seronegative control couples	
		Number of couples	% of seroconverter couples	Number of couples	% of control couples
COS	Soweto, Johannesburg	3	10.7	6	5.2
Partners	Soweto, Johannesburg	5	17.9	68	59.1
	Cape Town	14	50.0	28	24.3
	Orange Farm, Johannesburg	6	21.4	13	11.3
	Total	**28**	**100.0**	**115**	**100.0**

Number and proportion of participants from various geographic areas of South Africa for each study. The COS study enrolled participants from only 1 site. The Partners study enrolled participants from three sites. In the Partners study, 50% of the HIV-seroconverters (14 participants) were recruited from the Cape town site.

Table 3.2 Ethnic groups of study participants (n=286 participants)

	Controls		Seroconverters		Total (all 4 groups)	
Ethnic group	**Partner 1**	**Partner 2**	**Partner 1**	**Partner 2**	**N**	**%**
	HIV infected	**HIV uninfected**	**HIV infected**	**Became HIV infected**		
Xhosa	46	37	14	12	**109**	**38.1**
Zulu	23	32	7	8	**70**	**24.5**
Sotho	17	24	5	5	**51**	**17.8**
Tswana	14	5	0	2	**21**	**7.3**
Tsonga	5	9	0	0	**14**	**4.9**
Swati	6	1	1	1	**9**	**3.1**
Venda	3	0	0	0	**3**	**1.0**
Ndebele	0	1	0	0	**1**	**0.3**
Afrikaans	0	0	1	0	**1**	**0.3**
NA	1	6	0	0	**7**	**2.4**
Total	**115**	**115**	**28**	**28**	**286**	**100.0**

Ethnicity of each partner from 115 control couples (n=230 participants) and 28 seroconverting couples (n= 56 participants) are shown. Nine ethnic groups were self-reported by participants.

3.3 Methods for HLA allele frequency and haplotype frequency analyses

HLA alleles and haplotypes were analysed using Arlequin software (Version 3.5). Haplotypes were assigned by best fit alogrithms by the Arlequin software.

For reporting HLA-DRB3/4/5 allele frequencies, where the software assigned a homozygous result, I included the individual in my analysis. in some cases, the software was unable to assign the result for one allele. In such cases I was unable to differentiate missing alleles (hemizygous individuals), new (undescribed) alleles or homozygous results. I therefore excluded the individual in question from the calculation of allele frequencies. The number of results included for the frequency analysis (2n) has been reported individually for each locus.

3.4 HLA allele frequencies

I explored allele frequencies in the Partners participants to add additional data to that available for the South African population [222–229].

The HLA-A, HLA-B and HLA-C allele frequencies of the Partners participants are shown in Table 3.3. From 185 individuals with HLA-A typing available, there were 19 two-digit alleles present, with 14 noted at a frequency higher than 0.010. The most common alleles were HLA-A*30 (frequency of 0.215), HLA-A*02 (0.152) and HLA-A*68 (0.100). For HLA-B, from 177 individuals, there were 22 alleles present at two-digit resolution, with 15 present at a frequency higher than 0.010. The most common alleles were HLA-B*15 (0.189), HLA-B*58 (0.138) and HLA-B*07 (0.102). For HLA-C, from 106 individuals there were 15 alleles present, with 11 at a frequency greater than 0.010. The most common 2-digit HLA-C alleles were C*06 (0.179), C*07 (0.160) and C*04 (0.146). Differences between alleles for numbers of individuals typed related to DNA availability and quality. Prioritization was given to using available DNA for typing HLA-A and HLA-B before HLA-C, and technical issues resulted in inability to amplify HLA-A in certain sample batches.

Table 3.3 HLA-A 2-digit allele frequencies, sorted by highest to lowest frequency

HLA-A alleles	Frequency 2n=270	HLA-B alleles	Frequency 2n=354	HLA-C alleles	Frequency 2n=212
A*30	0.215	B*15	0.189	C*06	0.179
A*02	0.152	B*58	0.138	C*07	0.160
A*68	0.100	B*07	0.102	C*04	0.146
A*23	0.089	B*42	0.102	C*02	0.137
A*29	0.063	B*44	0.088	C*03	0.075
A*01	0.056	B*08	0.065	C*18	0.061
A*03	0.056	B*45	0.054	C*08	0.057
A*66	0.056	B*14	0.051	C*16	0.057
A*34	0.052	B*81	0.045	C*17	0.052
A*74	0.044	B*18	0.037	C*12	0.042
A*43	0.037	B*13	0.023	C*05	0.014
A*32	0.026	B*53	0.023	C*15	0.009
A*24	0.019	B*35	0.020	C*09	0.005
A*26	0.015	B*57	0.020	C*13	0.005
A*33	0.007	B*39	0.011		
A*11	0.004	B*27	0.008		
A*31	0.004	B*49	0.008		
A*39	0.004	B*41	0.006		
A*80	0.004	B*38	0.003		
		B*40	0.003		
		B*51	0.003		
		B*67	0.003		

For class 2 HLA frequencies, allele frequencies are reported in Table 3.4 and Table 3.5. For HLA-DPA, in 155 individuals with available typing, there were 4 alleles present, all at frequencies above 0.010. The commonest was DPA*02 (0.519) followed by DPA*01 (0.310) and DPA*03 (0.148). For HLA-DPB1, from 63 individuals, there were 17 alleles, of which nine were present at greater frequency than 0.010. Commonest were DPB*01 (0.317), DPB*105 (0.190) and DPB*02 (0.111). For HLA-DQB1, from 147 individuals there were 5 alleles, all present at frequency greater than 0.010. The commonest were HLA-DQB1*06 (0.333), HLA-DQB1*03 (0.252) and HLA-DQB*02 (0.170).

For HLA-DRB1, from 209 individuals there were 13 alleles, with 12 at frequency above 0.010. The most common 2-digit allele was HLA-DRB1*13 (0.220) followed by DRB1*03 (0.163) and DRB1*15 (0.146). For

HLADRB3/4/5, from 219 individuals, there were nine alleles/haplotypes, all present at frequencies above 0.010. The commonest was HLA-DRB3*02 (0.283) followed by HLA-DRB3*01 (0.253) and HLA-DRB4*01 (0.151). The frequency of individuals with no HLA-DRB3 or DRB4 or DRB5 locus was 0.027. Differences between alleles for numbers of individuals typed related to DNA availability and quality and prioritization for using available DNA for typing HLA-DRB1 and HLA-DRB3/4/5, then HLA-DQB1, before HLA-DPA1 or HLA-DPB1.

Table 3.4 HLA-DRB1, DPA1, DP1 and DQB1 two-digit allele frequencies, sorted from highest to lowest frequency

DPA1 alleles	Frequency 2n=310	DPB1 alleles	Frequency 2n=126	DQB1 alleles	Frequency 2n=294	DRB1 alleles	Frequency 2n=418
DPA1*02	0.519	DPB1*01	0.317	DQB1*06	0.330	DRB1*13	0.220
DPA1*01	0.310	DPB1*105	0.190	DQB1*03	0.252	DRB1*03	0.163
DPA1*03	0.148	DPB1*02	0.111	DQB1*02	0.170	DRB1*15	0.146
DPA1*04	0.023	DPB1*13	0.063	DQB1*05	0.133	DRB1*11	0.144
		DPB1*18	0.063	DQB1*04	0.116	DRB1*07	0.086
		DPB1*04	0.056			DRB1*04	0.074
		DPB1*17	0.056			DRB1*01	0.043
		DPB1*11	0.048			DRB1*12	0.041
		DPB1*03	0.032			DRB1*08	0.038
		DPB1*106	0.008			DRB1*10	0.017
		DPB1*14	0.008			DRB1*09	0.014
		DPB1*15	0.008			DRB1*14	0.012
		DPB1*173	0.008			DRB1*06	0.002
		DPB1*358	0.008				
		DPB1*39	0.008				
		DPB1*516	0.008				
		DPB1*84	0.008				

Table 3.5 DRB3,4,5 allele frequencies, sorted from highest to lowest frequency

Frequency 2n=438	DRB3 allele	DRB4 allele	DRB5 allele		Frequency 2n=438	DRB3/4/5 allele
0.283	DRB3*02	DRB4*0	DRB5*0		0.283	DRB3*02
0.253	DRB3*01	DRB4*0	DRB5*0		0.253	DRB3*01
0.151	DRB3*0	DRB4*01	DRB5*0		0.151	DRB4*01
0.146	DRB3*03	DRB4*0	DRB5*0		0.146	DRB3*03
0.100	DRB3*0	DRB4*0	DRB5*01		0.100	DRB5*01
0.027	DRB3*0	DRB4*0	DRB5*0		0.027	no DRB345
0.014	DRB3*04	DRB4*0	DRB5*0		0.014	DRB3*04
0.014	DRB3*0	DRB4*02	DRB5*0		0.014	DRB4*02
0.011	DRB3*0	DRB4*0	DRB5*02		0.011	DRB5*02

Haplotypes are displayed as combination of three individual loci on the left hand side; or as allele frequency in one column on the right hand side.

3.5. Comparison of allele frequencies with published allele frequencies

I compiled a table of allele frequencies from my cohort in comparison with that previously published for the South African population (Table 3.6). I utilized supplementary data published by Loubser *et al*, which showed a composite of frequencies detected in black participants in the Eskom cohort [223] and black participants in a study published by Thorstenson *et al* from Cape Town [225]. The Eskom cohort enrolment was country-wide with ethnicity of black participants comprising Zulu (21%), Xhosa (17.5%), Pedi (17.5%), Tswana (11%), South Sotho (9.5%), Swati (9%), Ndebele (6.5%) and Tsonga (2.5%)[230]. Ethnicities in the Cape Town study were not recorded.

I found similar allele frequencies with minor deviations from expected, mostly in infrequent alleles. HLA-A*66 was found at higher frequency in my cohort than the reference cohort (0.056 versus 0.026, p=0.05) while HLA-A*33 was at lower frequency (0.007 versus 0.03, p=0.04). B*07 was more common in my cohort (0.102 versus 0.064, p=0.04) as was B*44 (0.088 versus 0.051, p=0.04). C*17 was less common in my group (0.052 versus 0.141, p<0.01) and HLA-C*02 was more common (0.137 versus 0.079, p=0.02). Variation was noted amongst less common DPB alleles. Not all reported DPB alleles were detected in my cohort, and I found some low frequency DPB alleles not detected in the reference cohort. DRB1*03 was less common in my group (0.163 versus 0.217, p=0.04).

Table 3.6 Comparison of frequencies in this study (Partners and COS) to published frequencies for the South African black population, sorted from highest to lowest frequency in this study.

HLA-A			HLA-B			HLA-C			DPB1			DQB1			DRB1		
Allele	Partners 2n=270	Reference 2n=534	Allele	Partners 2n=354	Reference 2n=534	Allele	Partners 2n=212	Reference 2n=533	DPB1 allel	Partners 2n=126	Reference 2n=534	DQB1 allel	Partners 2n=294	Reference 2n=538	DRB1 allel	Partners 2n=418	Reference 2n=534
A*30	0.215	0.262	B*15	0.189	0.176	C*06	0.179	0.169	DPB1*01	0.317	0.303	DQB1*06	0.330	0.349	DRB1*13	0.220	0.193
A*02	0.152	0.142	B*58	0.138	0.169	C*07	0.160	0.203	DPB1*105	0.190	0.155	DQB1*03	0.252	0.212	DRB1*03	0.163	0.217
A*68	0.100	0.116	B*07	0.102	0.064	C*04	0.146	0.113	DPB1*02	0.111	0.142	DQB1*02	0.170	0.154	DRB1*15	0.146	0.125
A*23	0.089	0.082	B*42	0.102	0.116	C*02	0.137	0.079	DPB1*13	0.063	0.071	DQB1*05	0.133	0.123	DRB1*11	0.144	0.148
A*29	0.063	0.090	B*44	0.088	0.051	C*03	0.075	0.071	DPB1*18	0.063	0.058	DQB1*04	0.116	0.162	DRB1*07	0.086	0.064
A*01	0.056	0.028	B*08	0.065	0.060	C*18	0.061	0.032	DPB1*04	0.056	0.049				DRB1*04	0.074	0.058
A*03	0.056	0.056	B*45	0.054	0.079	C*08	0.057	0.062	DPB1*17	0.056	0.007				DRB1*01	0.043	0.064
A*66	0.056	0.026	B*14	0.051	0.047	C*16	0.057	0.079	DPB1*11	0.048	0.024				DRB1*12	0.041	0.036
A*34	0.052	0.026	B*81	0.045	0.041	C*17	0.052	0.141	DPB1*03	0.032	0.036				DRB1*08	0.038	0.047
A*74	0.044	0.034	B*18	0.037	0.028	C*12	0.042	0.017	DPB1*106	0.008	0.011				DRB1*10	0.017	0.021
A*43	0.037	0.037	B*13	0.023	0.017	C*05	0.014	0.008	DPB1*14	0.008	0.021				DRB1*09	0.014	0.011
A*32	0.026	0.013	B*53	0.023	0.034	C*15	0.009	0.021	DPB1*15	0.008	0.006				DRB1*14	0.012	0.015
A*24	0.019	0.024	B*35	0.020	0.015	C*09	0.005	.	DPB1*173	0.008	.				DRB1*06	0.002	
A*26	0.015	0.013	B*57	0.020	0.032	C*13	0.005	.	DPB1*358	0.008	.				DRB1*16	.	0.002
A*33	0.007	0.030	B*39	0.011	0.032	C*14	.	0.008	DPB1*39	0.008	.						
A*11	0.004	0.007	B*27	0.008	0.002				DPB1*516	0.008	.						
A*31	0.004	.	B*49	0.008	0.004				DPB1*84	0.008	.						
A*39	0.004	.	B*41	0.006	0.017				DPB*34	.	0.019						
A*80	0.004	0.006	B*38	0.003	.				DPB*55	.	0.019						
A*35	.	0.006	B*40	0.003	0.006				DPB*104	.	0.019						
			B*51	0.003	0.009				DPB*665	.	0.013						
			B*67	0.003	.				DPB*131	.	0.011						
			B*37	.	0.002				DPB*835	.	0.007						
			B*47	.	0.002				DPB*558	.	0.006						
									DPB*40	.	0.004						
									DPB*654	.	0.004						
									DPB*16	.	0.002						
									DPB*23	.	0.002						
									DPB*30	.	0.002						
									DPB*51	.	0.002						
									DPB*58	.	0.002						
									DPB*417	.	0.002						
									DPB*584	.	0.002						
									DPB*61	.	0.002						

Comparison of my results to reference frequencies as per supplementary data of Loubser et al [223]*, comprising cumulative frequencies found by Loubser et al in black South Africans of the Eskom cohort* [223] *and frequencies in black South Africans in Cape Town reported by Thorstenson et al, supplementary data* [225]*. Column heading "Partners" represents "Partners and Cos studies".*

3.6 Haplotype frequencies

I went on to characterize the class 1 and class 2 haplotypes in my study sample. For class 1, I analysed the three-loci HLA-A – HLA-B – HLA-C haplotype. From 55 individuals with available typing at all loci, there were 65 haplotypes at two-digit resolution. The most frequent haplotype was HLA-A*01 – HLA*B*81 – HLA-C*18 at a frequency of 0.055. The full list of class 1 haplotypes are shown in Table 3.7.

Table 3.7 Class 1 three-locus (HLA-A – HLA-B – HLA-C) haplotypes, sorted from highest to lowest frequency

Freq 2n=110	HLA-A - HLA-B - HLA-C haplotype		
0.055	A*01	B*81	C*18
0.045	A*30	B*08	C*07
0.045	A*02	B*45	C*16
0.036	A*02	B*15	C*04
0.036	A*30	B*15	C*02
0.036	A*30	B*14	C*08
0.036	A*68	B*58	C*06
0.027	A*30	B*42	C*17
0.027	A*30	B*44	C*04
0.027	A*02	B*58	C*07
0.027	A*02	B*15	C*02
0.027	A*68	B*14	C*08
0.027	A*30	B*45	C*16
0.018	A*29	B*13	C*06
0.018	A*68	B*15	C*03
0.018	A*02	B*58	C*06
0.018	A*23	B*08	C*02
0.018	A*03	B*07	C*07
0.018	A*34	B*44	C*04
0.018	A*29	B*45	C*06
0.018	A*23	B*42	C*17
0.009	A*34	B*81	C*04
0.009	A*68	B*15	C*02
0.009	A*01	B*35	C*04
0.009	A*23	B*57	C*09
0.009	A*68	B*14	C*05
0.009	A*01	B*58	C*03

Freq	HLA-A - HLA-B - HLA-C haplotype		
0.009	A*30	B*18	C*07
0.009	A*32	B*58	C*06
0.009	A*02	B*14	C*08
0.009	A*23	B*53	C*06
0.009	A*31	B*07	C*07
0.009	A*43	B*58	C*07
0.009	A*23	B*07	C*04
0.009	A*23	B*07	C*18
0.009	A*33	B*53	C*08
0.009	A*23	B*39	C*15
0.009	A*30	B*15	C*04
0.009	A*74	B*15	C*12
0.009	A*66	B*42	C*05
0.009	A*66	B*35	C*04
0.009	A*02	B*53	C*04
0.009	A*74	B*15	C*02
0.009	A*32	B*58	C*03
0.009	A*23	B*07	C*02
0.009	A*30	B*18	C*05
0.009	A*32	B*27	C*02
0.009	A*01	B*42	C*17
0.009	A*43	B*58	C*06
0.009	A*34	B*08	C*07
0.009	A*30	B*49	C*07
0.009	A*03	B*15	C*04
0.009	A*02	B*35	C*04
0.009	A*74	B*42	C*17
0.009	A*66	B*58	C*06
0.009	A*74	B*15	C*03
0.009	A*30	B*07	C*07
0.009	A*30	B*81	C*04
0.009	A*66	B*15	C*02
0.009	A*01	B*15	C*02
0.009	A*03	B*58	C*07
0.009	A*24	B*44	C*04
0.009	A*68	B*57	C*18
0.009	A*66	B*57	C*18
0.009	A*66	B*42	C*17

For class 2 haplotypes, I first explored the two-loci (HLA-DRB1 – HLA-DRB3/4/5) haplotypes in 199 individuals with available data for both loci. I found the haplotype with the highest frequency to be the

combination of DRB1*11 with DRB3*02 (corresponding to the DR52 nomenclature) at a frequency of 0.101. There were 54 combinations apparent (Table 3.8) from the Arlequin analysis with many unusual combinations not typically described in Caucasian populations (Table 3.9). The unusual combinations displayed at the individual level are listed in Table 3.10.

Table 3.8 HLA-DRB1 – HLA-DRB3/4/5 two-locus haplotypes, sorted from highest to lowest frequency

Frequency 2n=398	DRB1	DRB3/4/5
0.101	DRB11	DRB3*02
0.098	DRB03	DRB3*01
0.088	DRB13	DRB3*03
0.075	DRB15	DRB5*01
0.073	DRB13	DRB3*01
0.063	DRB07	DRB4*01
0.063	DRB03	DRB3*02
0.053	DRB04	DRB4*01
0.043	DRB13	DRB3*02
0.038	DRB11	DRB3*03
0.025	DRB15	DRB3*01
0.020	DRB12	DRB3*02
0.018	DRB12	DRB3*01
0.018	DRB01	DRB3*02
0.013	DRB15	DRB4*01
0.013	DRB08	DRB5*01
0.013	DRB15	DRB3*03
0.013	DRB04	DRB3*02
0.010	DRB08	no DRB345
0.010	DRB10	no DRB345
0.010	DRB09	DRB4*01
0.010	DRB01	DRB5*01
0.010	DRB13	DRB4*01
0.008	DRB08	DRB3*01
0.008	DRB14	DRB3*02
0.008	DRB13	DRB3*04
0.008	DRB15	DRB5*02
0.008	DRB07	DRB3*01
0.005	DRB01	no DRB345
0.005	DRB01	DRB3*03
0.005	DRB08	DRB3*02
0.005	DRB09	DRB3*01

Frequency 2n=398	DRB1	DRB3/4/5
0.005	DRB12	DRB4*01
0.005	DRB11	DRB4*02
0.005	DRB10	DRB3*02
0.005	DRB04	DRB4*02
0.005	DRB08	DRB3*03
0.003	DRB10	DRB3*03
0.003	DRB06	DRB3*03
0.003	DRB04	DRB3*01
0.003	DRB01	DRB3*01
0.003	DRB01	DRB4*01
0.003	DRB14	DRB3*04
0.003	DRB13	DRB5*02
0.003	DRB03	DRB5*01
0.003	DRB13	DRB5*01
0.003	DRB11	DRB5*01
0.003	DRB15	no DRB345
0.003	DRB11	DRB4*01
0.003	DRB13	DRB4*02
0.003	DRB03	DRB3*03
0.003	DRB07	DRB4*02
0.003	DRB07	DRB3*02
0.003	DRB07	DRB3*04

Table 3.9 Summary of Expected and Observed HLA-DRB1 – HLA-DRB3/4/5 haplotypes

DRB1 allele	Serological nomenclature based on DRB1 allele	Expected DRB3/4/5 allele	Observed DRB3/4/5 allele	Frequency 2n=398
DRB1*01	DR1 or DR51	no DRB345 or DRB5*XX	DRB3*02	0.018
			DRB5*01	0.010
			no DRB345	0.005
			DRB3*03	0.005
			DRB3*01	0.003
			DRB4*01	0.003
DRB1*03	DR52	DRB3*XX	DRB3*01	0.098
			DRB3*02	0.063
			DRB5*01	0.003
			DRB3*03	0.003

DRB1 allele	Serological nomenclature based on DRB1 allele	Expected DRB3/4/5 allele	Observed DRB3/4/5 allele	Frequency 2n=398
DRB1*04	DR53	DRB4*XX	DRB4*01	0.053
			DRB3*02	0.013
			DRB4*02	0.005
			DRB3*01	0.003
DRB1*06			DRB3*03	0.003
DRB1*07	DR53	DRB4*XX	DRB4*01	0.063
			DRB3*01	0.008
			DRB4*02	0.003
			DRB3*02	0.003
			DRB3*04	0.003
DRB1*08	DR8	no DRB345	DRB5*01	0.013
			no DRB345	0.010
			DRB3*01	0.008
			DRB3*02	0.005
			DRB3*03	0.005
DRB1*09	DR53	DRB4*XX	DRB4*01	0.010
			DRB3*01	0.005
DRB1*10	DR1	no DRB345	no DRB345	0.010
			DRB3*02	0.005
			DRB3*03	0.003
DRB1*11	DR52	DRB3*XX	DRB3*02	0.101
			DRB3*03	0.038
			DRB4*02	0.005
			DRB5*01	0.003
			DRB4*01	0.003
DRB1*12	DR52	DRB3*XX	DRB3*02	0.020
			DRB3*01	0.018
			DRB4*01	0.005
DRB1*13	DR52	DRB3*XX	DRB3*03	0.088
			DRB3*01	0.073
			DRB3*02	0.043
			DRB4*01	0.010
			DRB3*04	0.008
			DRB5*02	0.003
			DRB5*01	0.003
			DRB4*02	0.003
DRB1*14	DR52	DRB3*XX	DRB3*02	0.008
			DRB3*04	0.003
DRB1*15	DR51 or DR1	DRB5*XX or no DRB345	DRB5*01	0.075

DRB1 allele	Serological nomenclature based on DRB1 allele	Expected DRB3/4/5 allele	Observed DRB3/4/5 allele	Frequency 2n=398
			DRB3*01	0.025
			DRB4*01	0.013
			DRB3*03	0.013
			DRB5*02	0.008
			no DRB345	0.003

Expected allele frequencies as per Kotsch et al [77]. *Observed allele frequencies are my results based on phased output from Arlequin software. Yellow highlights represent unexpected observed DRB3/4/5 alleles not matching those of Kotsch et al* [77].

Table 3.10 Unanticipated HLA-DRB1 – HLA-DRB3/4/5 combinations (unphased, per individual participant)

ID	HLA-DRB1	Expected HLA-DRB3/4/5	Observed HLA-DRB3/4/5	Homozygous DRB3/4/5 result
1020319	DRB1*04 DRB1*06	DRB4*XX	DRB3*01 DRB3*03	No
1000115	DRB1*15 DRB1*15	DRB5*XX or no DRB345 DRB5*XX or no DRB345	DRB3*01 DRB5*01	No
1000900	DRB1*03 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*01 DRB3*02	No
1005501	DRB1*03 DRB1*09	DRB3*XX DRB4*XX	DRB3*01 DRB3*01	Yes
1017303	DRB1*11 DRB1*12	DRB3*XX DRB3*XX	DRB4*01 DRB4*02	No
2003012	DRB1*13 DRB1*13	DRB3*XX DRB3*XX	DRB4*01 DRB4*01	Yes (homozygous DRB1 also)
2010118	DRB1*03 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*01 DRB3*02	No
2003403	DRB1*13 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*02 DRB3*03	No
2011706	DRB1*12 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB4*01 DRB4*01	Yes
2011714	DRB1*01 DRB1*04	DRB5*XX or no DRB345 DRB4*XX	DRB3*02 DRB5*01	No
2008509	DRB1*01 DRB1*04	DRB5*XX or no DRB345 DRB4*XX	DRB3*02 DRB3*02	Yes
2009604	DRB1*07 DRB1*15	DRB4*XX DRB5*XX or no DRB345	DRB3*01 DRB5*01	No

ID	HLA-DRB1	Expected HLA-DRB3/4/5	Observed HLA-DRB3/4/5	Homozygous DRB3/4/5 result
2009006	DRB1*03 DRB1*04	DRB3*XX DRB4*XX	DRB3*01 DRB3*02	No
2009014	DRB1*03 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*02 DRB4*01	No
2002003	DRB1*03 DRB1*13	DRB3*XX DRB3*XX	DRB5*01 DRB5*01	Yes
1901407	DRB1*07 DRB1*13	DRB4*XX DRB3*XX	DRB3*01 DRB3*02	No
1906605	DRB1*07 DRB1*11	DRB4*XX DRB3*XX	DRB3*01 DRB5*01	No
1906610	DRB1*08 DRB1*15	no DRB345 DRB5*XX or no DRB345	DRB3*03 DRB4*01	No
1902504	DRB1*11 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*02 DRB4*01	No
1904803	DRB1*13 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*03 DRB3*04	No
1904815	DRB1*01 DRB1*13	DRB5*XX or no DRB345 DRB3*XX	DRB4*01 DRB5*01	No
1901910	DRB1*04 DRB1*15	DRB4*XX DRB5*XX or no DRB345	DRB3*01 DRB3*02	No
1009819	DRB1*09 DRB1*12	DRB4*XX DRB3*XX	DRB3*02 DRB3*01	No
1023109	DRB1*13 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*01 DRB3*02	No
1015608	DRB1*11 DRB1*15	DRB3*XX DRB5*XX or no DRB345	DRB3*01 DRB3*03	No
1904101	DRB1*08 DRB1*11	no DRB345 DRB3*XX	DRB4*01 DRB5*01	No
2002401	DRB1*04 DRB1*15	DRB4*XX DRB5*XX or no DRB345	DRB3*02 DRB3*03	No
2002412	DRB1*13 DRB1*13	DRB3*XX DRB3*XX	DRB4*01 DRB4*02	No
1901709	DRB1*03 DRB1*11	DRB3*XX DRB3*XX	DRB3*02 DRB4*02	No
1904906	DRB1*07 DRB1*15	DRB4*XX DRB5*XX or no DRB345	DRB3*01 DRB3*02	No
1904208	DRB1*01 DRB1*13	DRB5*XX or no DRB345 DRB3*XX	DRB3*02 DRB3*03	No
1904213	DRB1*07 DRB1*13	DRB4*XX DRB3*XX	DRB3*03 DRB3*04	No

Individual results of DRB1 and DRB3/4/5 alleles shown, unphased per individual (n=32). Each individual is represented by one row. Only 5 individuals had homozygous results at the DRB3/4/5 locus (highlighted) which could potentially represent software misinterpretation of hemizygous results with an absent DRB3/4/5 second allele. Of these five, a hemizygous DRB3/4/5 result would explain the unexpected linkage in only 2 samples, highlighted green. The remaining 30 samples have atypical haplotypes.

For class 2, we then described five locus haplotypes (DPA1 – DPB1 – DQB1 – DRB1 – DRB3/4/5) in 58 individuals with typing available at all loci. There were 79 haplotypes identified, with the commonest being HLA-DPA*02 – HLA-DPB1*01 – HLA-DQB1*02 – HLA-DRB1*03 – HLA-DRB3*02 at a frequency of 0.052 (Table 3.11). We did not explore haplotypes combining HLA class 1 and HLA class 2 due to insufficient sample numbers with available typing information for eight loci.

Table 3.11 Class 2 five-locus haplotypes (HLA-DPA1 - HLA-DPB1 - HLA-DQB1 - HLA-DRB1 - HLADRB3/4/5) sorted from highest to lowest frequency

Frequency 2n=116	DPA1	DPB1	DQB1	DRB1	DRB3/4/5
0.052	DPA*02	DPB*01	DQB*02	DRB*03	DRB3*02
0.043	DPA*01	DPB*02	DQB*06	DRB*13	DRB3*03
0.034	DPA*02	DPB*01	DQB*03	DRB*11	DRB3*02
0.034	DPA*02	DPB*01	DQB*06	DRB*11	DRB3*02
0.034	DPA*02	DPB*13	DQB*03	DRB*11	DRB3*02
0.034	DPA*02	DPB*01	DQB*04	DRB*03	DRB3*01
0.026	DPA*03	DPB*105	DQB*02	DRB*07	DRB4*01
0.017	DPA*02	DPB*01	DQB*06	DRB*12	DRB3*02
0.017	DPA*01	DPB*03	DQB*06	DRB*13	DRB3*03
0.017	DPA*04	DPB*105	DQB*03	DRB*08	no DRB345
0.017	DPA*02	DPB*01	DQB*02	DRB*07	DRB4*01
0.017	DPA*01	DPB*04	DQB*06	DRB*13	DRB3*03
0.017	DPA*02	DPB*01	DQB*05	DRB*12	DRB3*01
0.017	DPA*03	DPB*105	DQB*03	DRB*04	DRB4*01
0.017	DPA*02	DPB*105	DQB*03	DRB*04	DRB4*01
0.017	DPA*01	DPB*03	DQB*05	DRB*12	DRB3*02
0.017	DPA*02	DPB*11	DQB*06	DRB*15	DRB5*01
0.017	DPA*02	DPB*17	DQB*02	DRB*07	DRB4*01
0.017	DPA*01	DPB*02	DQB*06	DRB*15	DRB5*01
0.017	DPA*02	DPB*13	DQB*03	DRB*11	DRB3*03
0.017	DPA*01	DPB*04	DQB*06	DRB*13	DRB3*02
0.009	DPA*04	DPB*105	DQB*04	DRB*03	DRB3*01

Frequency 2n=116	DPA1	DPB1	DQB1	DRB1	DRB3/4/5
0.009	DPA*03	DPB*105	DQB*06	DRB*15	DRB3*01
0.009	DPA*02	DPB*11	DQB*06	DRB*13	DRB3*03
0.009	DPA*01	DPB*18	DQB*06	DRB*13	DRB3*03
0.009	DPA*02	DPB*01	DQB*04	DRB*04	DRB4*01
0.009	DPA*01	DPB*02	DQB*05	DRB*10	DRB3*03
0.009	DPA*03	DPB*105	DQB*03	DRB*11	DRB3*02
0.009	DPA*02	DPB*01	DQB*03	DRB*13	DRB3*01
0.009	DPA*03	DPB*105	DQB*05	DRB*10	no DRB345
0.009	DPA*03	DPB*105	DQB*03	DRB*11	DRB3*03
0.009	DPA*02	DPB*18	DQB*03	DRB*11	DRB3*02
0.009	DPA*02	DPB*17	DQB*05	DRB*01	no DRB345
0.009	DPA*02	DPB*17	DQB*05	DRB*10	no DRB345
0.009	DPA*02	DPB*14	DQB*02	DRB*07	DRB4*01
0.009	DPA*01	DPB*04	DQB*03	DRB*04	DRB4*01
0.009	DPA*02	DPB*17	DQB*06	DRB*13	DRB3*03
0.009	DPA*02	DPB*17	DQB*06	DRB*15	DRB5*01
0.009	DPA*02	DPB*11	DQB*06	DRB*13	DRB3*01
0.009	DPA*01	DPB*84	DQB*05	DRB*10	no DRB345
0.009	DPA*01	DPB*39	DQB*03	DRB*11	DRB3*03
0.009	DPA*02	DPB*173	DQB*03	DRB*13	DRB3*01
0.009	DPA*02	DPB*01	DQB*06	DRB*13	DRB3*03
0.009	DPA*02	DPB*358	DQB*06	DRB*13	DRB3*01
0.009	DPA*01	DPB*18	DQB*03	DRB*08	DRB3*01
0.009	DPA*01	DPB*02	DQB*05	DRB*12	DRB3*01
0.009	DPA*02	DPB*11	DQB*03	DRB*03	DRB3*01
0.009	DPA*03	DPB*105	DQB*02	DRB*03	DRB3*02
0.009	DPA*04	DPB*105	DQB*04	DRB*15	DRB3*01
0.009	DPA*01	DPB*105	DQB*02	DRB*03	DRB3*02
0.009	DPA*01	DPB*02	DQB*02	DRB*11	DRB3*02
0.009	DPA*02	DPB*01	DQB*05	DRB*12	DRB3*02
0.009	DPA*02	DPB*13	DQB*04	DRB*08	DRB3*02
0.009	DPA*01	DPB*04	DQB*06	DRB*11	DRB3*02
0.009	DPA*03	DPB*105	DQB*05	DRB*12	DRB3*01
0.009	DPA*03	DPB*105	DQB*03	DRB*12	DRB3*02
0.009	DPA*02	DPB*106	DQB*06	DRB*13	DRB3*02
0.009	DPA*02	DPB*01	DQB*04	DRB*04	DRB4*01
0.009	DPA*01	DPB*02	DQB*02	DRB*03	DRB3*02
0.009	DPA*02	DPB*17	DQB*05	DRB*10	DRB3*02
0.009	DPA*02	DPB*13	DQB*03	DRB*13	DRB3*02
0.009	DPA*02	DPB*01	DQB*02	DRB*09	DRB3*01
0.009	DPA*02	DPB*01	DQB*06	DRB*13	DRB3*01

Frequency 2n=116	DPA1	DPB1	DQB1	DRB1	DRB3/4/5
0.009	DPA*02	DPB*01	DQB*03	DRB*08	no DRB345
0.009	DPA*02	DPB*01	DQB*03	DRB*03	DRB3*02
0.009	DPA*02	DPB*11	DQB*06	DRB*15	no DRB345
0.009	DPA*03	DPB*105	DQB*03	DRB*15	DRB3*01
0.009	DPA*02	DPB*01	DQB*03	DRB*08	DRB3*03
0.009	DPA*01	DPB*02	DQB*04	DRB*03	DRB3*01
0.009	DPA*01	DPB*02	DQB*03	DRB*04	DRB4*01
0.009	DPA*03	DPB*105	DQB*03	DRB*08	DRB5*01
0.009	DPA*01	DPB*04	DQB*06	DRB*15	DRB5*01
0.009	DPA*02	DPB*105	DQB*03	DRB*11	DRB3*03
0.009	DPA*01	DPB*02	DQB*06	DRB*15	DRB3*01
0.009	DPA*01	DPB*18	DQB*06	DRB*13	DRB3*01
0.009	DPA*03	DPB*105	DQB*06	DRB*11	DRB3*02
0.009	DPA*01	DPB*18	DQB*03	DRB*08	no DRB345
0.009	DPA*01	DPB*18	DQB*06	DRB*11	DRB3*02
0.009	DPA*03	DPB*105	DQB*04	DRB*03	DRB3*01

3.7 Discussion of HLA genotypes in South African black HIV-serodiscordant couples

In chapter three I have analysed a large number South African black individuals for multiple class 1 and class 2 alleles and haplotypes at the two-digit resolution. Diversity in African populations can be expected to be much more diverse than that of Caucasian individuals as Africa is a continent of variable linguistic and cultural phenotypes comprising more than 200 ethnolinguistic groups [231]. Africa is a continent of diverse habitats from the largest deserts to tropical rainforests, swamps and savannas. Infectious diseases have played a large role in shaping genetic diversity amongst Africans[231].

My data adds to the body of knowledge regarding prevalence of alleles and haplotypes in South African black individuals, who represent diverse ethnic groups including Xhosa, Zulu, Sotho, Tswana, Tsonga, Swati and others. I found similar frequencies to that published, with minor variations. The allele-specific oligonucleotide method probe method cannot detect previously untyped, alleles and I have not described any new alleles. Interestingly I noted multiple unusual DRB1-DRB3/4/5 haplotypes which have not been previously discussed. While neither the DRB1 nor DRB3/4/5 alleles are novel, the linkage between the DRB1 and DRB3/4/5 allele are unanticipated and conflict with expected combinations shown in Figure 1.2. This finding is relevant to diagnostic transplant laboratories who sometimes infer

the DRB3/4/5 allele from predicted linkages with the DRB1 allele rather than typing both loci. My results suggest that for African individuals, transplant laboratories may incorrectly infer the presence or absence of a particular DRB3/4/5 allele if they do not type the DRB3/4/5 loci.

HLA-DRB3/4/5 loci are much less frequently studied than other class 1 or class 2 alleles and therefore my haplotypes may be due to my analysis of a large, ethnically diverse Black African population, in whom more variable HLA haplotypes can be expected than in Caucasian populations. Indeed, Thorstenson *et al* also described unusual HLA-DRB1 – HLA-DRB3/4/5 haplotypes using next generation sequencing method, namely HLA-DRB1*15 with an absent HLA-DRB3/4/5 locus, and HLA-DRB1*10 linked with HLA-DRB5*[225], neither of which are amongst my reported unusual combinations. These findings may be relevant to various contexts such as vaccine design or transplantation diagnostics. In many diagnostic transplant laboratories, the HLA-DRB3/4/5 allele is assumed from the HLA-DRB1 allele, according to common haplotypes, rather than typed. Should my data be confirmed, the practice in transplant laboratories of inferring the DRB3/4/5 allele from the DRB1 allele should be discouraged, or the common haplotypes require updating for the African setting**.**

Further work with this data may include comparison of HLA alleles amongst highly exposed seronegative controls in comparison with HIV-infected participants, but such analysis has been performed in many other cohorts, including the wider Partners study, and fell outside the scope of this study. Rather HLA genotyping was a prerequisite to interpretation of matching HLA antibody specificities and autoantibodies and was performed as a prelude to my primary aim, analysis of HLA antibodies.

3.8 Strengths and weaknesses of Luminex-based HLA typing

Strengths of this work include inclusion of multiple loci for HLA genotyping and a relatively large sample group. Inclusion of multiple ethnic groups of South African black individuals is another strength. Weaknesses include use of one HLA typing methodology without confirmation of alleles using a second method. Luminex based HLA typing usually gives a 2-digit resolution typing, and in some cases a 4-digit result. We have presented 2-digit HLA typing throughout for consistency and for easy of interpretation of whether HLA antibodies in the second partner matched the HLA type of the index partner. The Match-It software kits are not optimized to differentiate homozygous from hemizygous DRB3/4/5 results (personal communication, Jamie Smolin, Immucor), leading to the possibility of spurious

DRB3/4/5 results may have been reported by the software as homozygous when individuals were in fact hemizygous. Only very few of the unexpected individual results were however homozygous, and therefore software interpretation issues cannot explain the majority of the unanticipated results. I therefore conclude that unusual linkages between DRB1 and DRB3/4/5 alleles are due to genetic diversity in this African cohort which is greater than previously described in Caucasian populations, rather than due to technical software algorithms. A technical limitation was the limited quantity of DNA available from dried blood spots. Where DNA is available, unusual DRB1-DRB3/4/5 haplotypes will be confirmed by sequencing prior to publication of haplotype frequencies.

4. Chapter 4 - HLA antibodies in HIV-serodiscordant couples

4.1 Introduction to HLA antibody analysis in HIV-serodiscordant couples

Having performed HLA typing on both partners of HIV-serodiscordant couples, I analysed HLA antibodies in the originally HIV-uninfected partner of each couple. I analysed whether individuals who remained HIV-uninfected throughout the study differed from those who acquired HIV.

I then determined whether such antibodies were directed against the index partner, that is whether they were *matching antibodies* against the HLA genotype of the index partner. For interpretation of *matching antibodies*, I analysed the cohort twice – once including all seroconverter couples and the next time analyzing only *linked* couples where HIV transmission was from the index partner enrolled in the study.

The reason for analyzing HLA antibodies from all seroconverters, even those in whom HIV transmission occurred from a different individual, was to analyse whether HLA antibodies originating though allo-recognition between sexual partners may precipitate development of certain HLA antibody specificities that confer against HIV gp120 protein itself. Inclusion of as large a sample size of seroconverters as possible, prior to HIV acquisition, gives greater power to detect common antibody patterns. The reason for analyzing *linked* seroconverters separately from *unlinked* seroconverters was that HLA antibodies matching the HIV "donor" may be the only important antibody subset, if the mechanism of action is to bind human HLA proteins incorporated within the viral envelope during exposure to HIV virions.

I then analysed whether participants harboured *autoantibodies* and whether *autoantibodies* differed amongst individuals who did and did not acquire HIV.

I went on to investigate whether *complement-fixing* class 2 HLA antibodies differed between individuals who did and did not acquire HIV.

I have presented the work related as a manuscript which been submitted for publication. Supplementary Figures and Tables are included in the appendix.

4.2 Title of Manuscript

Alloimmunity to class 2 human leucocyte antigens may reduce HIV-1 acquisition – a nested case-control study in HIV-1 serodiscordant couples

4.3 Authors

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4.6 Abstract

Enveloped viruses, including Human Immunodeficiency Virus-1 (HIV), incorporate host proteins such as human leucocyte antigens (HLA) into their envelope. Pre-existing antibodies against HLA, termed HLA antibodies, may bind to these surface proteins and impact viral infectivity. Related evidence includes macaque studies which showed that xenoimmunization with HLA antigens may protect against simian immunodeficiency virus infection. Since HIV gp120 shows homology with class 2 HLA, including shared affinity for binding to CD4, class 2 HLA antibodies may influence HIV acquisition via binding to gp120 on the viral envelope.

We conducted a nested case-control study on HIV serodiscordant couples, comparing the frequency of HLA antibodies among highly exposed persistently seronegative controls and those who went on to acquire HIV (HIV-seroconverters). We first performed low resolution HLA typing on 143 individuals who were HIV-infected at enrollment (index partners) and their corresponding sexual partners (115 highly exposed persistently seronegative individuals and 28 HIV-seroconverters). We then measured HLA class 1 and 2 antibodies in the highly exposed persistently seronegative individuals and HIV-seroconverters at early and late timepoints. We analyzed whether such antibodies were directed at HLA specificities of

their HIV-infected index partners, and whether autoantibodies or complement-fixing class 2 HLA antibodies were present.

Seventy-nine percent of highly exposed persistently seronegative individuals had HLA antibodies; 56% against class 1 and 50% against class 2 alleles. Significantly more highly exposed persistently seronegative individuals than HIV-seroconverters, prior to seroconversion, expressed class 2 HLA antibodies (50% versus 29%, p=0.05). HIV infection was a sensitizing event leading to *de novo* development of antibodies against HLA-A and HLA-B loci, but not against class 2 loci. HLA autoantibodies were present in 27% of highly exposed persistently seronegative individuals. Complement-fixing class 2 HLA antibodies did not differ significantly between highly exposed persistently seronegative individuals and seroconverters. In multivariable regression, presence of class 2 HLA antibodies at early timepoints was associated with a reduced odds ratio of 0.330 (confidence interval 0.112-0.976, p=0.045) for HIV acquisition.

These epidemiological data show that pre-existing class 2 HLA antibodies were associated with reduced odds of HIV acquisition.

4.7 Keywords (6)

HLA, antibody, heterologous, highly exposed persistently seronegative, MHC, non-specific

4.8 Introduction

Enveloped viruses derive their envelope from the host cell membrane, incorporating host proteins within the viral envelope. Such host proteins include polymorphic variants such as the human major histocompatibility antigens (MHC) called human leucocyte antigens (HLA) [151][178][179][181][182]. The presence of antibodies against HLA could therefore impact susceptibility to natural infection with enveloped viruses. Alloimmunity is immunity of an individual to other individuals of the same species, often mediated via antibodies against HLA specificities (HLA antibodies), while xenoimmunity is immunity of one individual against another of a different species. Despite intense interest in allo- and xenoimmunity for HIV vaccine development in the early 2000's [215] there remains no coordinated effort to explore whether intentional induction of HLA antibodies may indeed reduce HIV acquisition.

Cynomolgus macaque vaccine SIV challenge studies done in the laboratory of Dr James Stott initially showed protection from challenge associated with an SIV vaccine and challenge virus grown in human cell lines. They determined that the correlate of protection was antibody against the cell line, specifically human MHC and not HIV-specific immune responses that were responsible for protection [147,232]. This was a model of xenoimmunization, whereby macaques were immunized by human cells. Immunizing macaques *with purified HLA-DR protein alone* could protect against subsequent SIV challenge with SIV grown in human, but not macaque, cells [148]. The authors suggested that immunization with HLA-DR induced sterilizing immunity to SIV by preventing the first infectious particle from entering macaque cells, and suggested incorporation of HLA-DR into future HIV vaccines [148]. Additional relevant examples include immunization of macaques with recombinant HLA class 1 or 2 proteins, either alone or in combination with HIV and SIV proteins and adjuvant [149]. Complete or partial protection was achieved in macaques vaccinated with all three components and was transferrable by serum. The macaques developed anti-HLA antibodies which correlated inversely with viral load after challenge, as well as complement-dependent neutralizing activity. The authors did not

identify any protection using immunization with either SHIV alone or HLA proteins alone, although there was a trend to delayed viremia in the HLA-immunized group [149].

Published work investigating the phenomenon of human HLA incorporation into the viral envelope has shown selective incorporation of HLA class 1 and 2 proteins [233]. Expression of HLA-C, a class 1 HLA protein, in the viral envelope increases infectivity of virions and affects sensitivity to neutralization [177,234]. HLA-DR, a class 2 HLA protein, is incorporated into viral envelope in approximately one fifth the concentration of HIV gag protein, although HLA-DP and HLA-DQ are reportedly not incorporated [176,181]. HLA-DR in the viral envelope has been shown to be intact and functional and could stimulate T cells via superantigen presentation [235].

Aside from human HLA incorporation into viral membrane, HIV gp120 protein has sequence homology with class 2 HLA proteins (summarized in Supplementary Table 1). Both proteins bind to the CD4 receptor *in vivo*, suggesting structural homology. There are thus multiple conceptual reasons as well as suggestive animal studies that support the idea that pre-existing antibody to HLA proteins may affect HIV acquisition risk.

There has been limited epidemiological investigation exploring whether HIV-susceptible individuals prior to HIV seroconversion have pre-existing HLA antibodies that influence HIV acquisition risk. It is clear that pre-existing HLA antibodies occur in healthy individuals. Multiparous women often have detectable HLA class 1 or 2 antibodies as pregnancy is a well-described sensitizing event for their development [158][174][175][236]. Newer solid phase immunoassays, with improved sensitivity over older complement dependent cytotoxicity (CDC) or enzyme-linked immunosorbent assay (ELISA) methods [156][158][160], have shown that individuals with no history of previous sensitizing events also harbor HLA antibodies [163] [237] [75]. The natural history of HLA antibody development remains obscure and children, even neonates, can harbor HLA antibodies [169] [238] [170]. Proposed mechanisms for induction of the antibodies include cross reactions with commensal flora, food antigens or allergens [75][162][163][165][166].

In highly HIV-exposed, persistently seronegative people, 30% had antibodies that reacted with gp120 and 70% had antibodies to class 1 HLA, leading the authors to hypothesize that antibodies against HLA might be protective against HIV acquisition [203]. In persistently HIV seronegative intravenous drug users, antibodies against HLA (cross-reactive with HIV) were present in 33%, but in only 4% of healthy controls [209]. In HIV-exposed seronegative sex workers, there was no difference in proportion with HLA class 1 antibodies between sex workers and controls; however class 2 HLA antibodies were not analyzed [210][167].

To more conclusively associate presence of HLA antibodies with risk of HIV infection, we compared highly exposed persistently seronegative individuals to those who went on to acquire HIV (HIV-seroconverters). For this work, we have used South African samples collected during the Partners in Prevention HSV/HIV Transmission Study, hereafter termed the “Partners” study, and from the Couples Observational Study or “COS”, that prospectively followed couples serologically discordant for HIV and accurately documented HIV seroconversion. Antiretroviral therapy was consistent with national guidelines at the time of the study. We hypothesized that partner-directed HLA antibodies would be associated with lower odds of HIV acquisition. We found the presence of pre-existing class 2 HLA antibodies associated with lower odds of HIV acquisition.

4.9 Methods

4.9. 1 Study samples

The Partners study was a randomized, double blind, placebo-controlled trial in HIV discordant couples investigating the role of genital herpes simples 2 virus (HSV-2) suppression with daily acyclovir to prevent transmission of HIV from the HIV-infected partner (index partner) to their enrolled initially HIV-uninfected partner [217]. The Partners study enrolled 3408 heterosexual couples from seven countries in Southern and Eastern Africa between 2004 and 2007 who had co-habited for a median of five years. and reported three or more episodes of vaginal intercourse over the prior three months [217]. Enrolment criteria included age above 18 years and that the HIV infected partner (index partner) was seropositive for both HIV and HSV-2 with a $CD4^+$ T cell count above 250 cells/µl. Participants were followed every three months. At every visit, couples were counselled regarding HIV risk reduction, provided with condoms, treated for any sexually transmitted infection and females tested for pregnancy. If participants met the national criteria for antiretroviral therapy at the time, they received it from local clinics. There were 132 total HIV transmissions within the 14 sites [217]. The final results of the Partners study showed that acyclovir decreased the level of plasma HIV RNA in the index partner but did not reduce HIV transmission [217], thus study drug arm was not included in our analysis for determinants of HIV acquisition.

The COS study was conducted in South Africa and Uganda and enrolled 485 HIV serodiscordant couples. Recruitment criteria were similar to the Partners study but had no restriction based on $CD4^+$ T cell count or HSV-2 infection [220]. Couples were followed up quarterly for twelve months. A sampling of participants from both Partners and COS cohorts was included in an earlier genome wide association study [220] and HLA alleles associated with HIV transmission and plasma viral setpoints have been reported [102].

We accessed samples from the South African Partners and COS study sites in Johannesburg (Soweto and Orange Farm sites) and Cape Town. Originally, we included only the Soweto site of the Partners study, retrieving all samples, which comprised only 5 seroconverting couples and 68 couples who did not transmit HIV. To increase our numbers of seroconverters for meaningful statistical analysis, we requested all additional seroconverters from the Cape Town and Orange Farm sites as well as from the COS study at the Soweto site, with persistently seronegative control samples in a 2:1 ratio. Our enrollment sites are summarized in Supplementary Table 2. In total there were 28 couples who experienced a HIV-seroconversion event across these three sites, which were all included in our analysis. In addition, 115 non-transmitting couples with a highly HIV-exposed, persistently seronegative partner, hereafter referred to as a “control”, were included. Samples were analyzed for HLA antibodies at two time points for each participant who was HIV-uninfected at enrolment. The first time point was at 0-6 months from enrolment. The second timepoint was at least 6 months from enrolment and in HIV-seroconverters was selected to be after seroconversion.

4.9.2 Linked versus unlinked seroconversions

For both the Partners and COS studies, sequencing of a limited region of HIV *gag* and *env* genes had been performed in couples with HIV transmission, in order to determine whether the transmission was from the enrolled partner (linked) or a non-enrolled partner (unlinked) [217][221]. In our subset of South African samples from the Partners and COS studies, two thirds (19/28; 68%) of seroconversions were linked.

4.9.3 Ethics

The Partners and COS studies was approved by institutional review boards at each site in each country. Participants gave informed consent, including consent for genetic testing and future research on factors associated with HIV acquisition risk. New approval for this nested case-control study was granted by the University of the Witwatersrand Human Research Ethics Committee (M1411118).

4.9.4 HLA antibody testing and reporting

To degrade IgM, sera from HIV-seroconverters and highly exposed persistently seronegative individuals were heat inactivated at 56°C for 30 minutes after which expression levels of antibodies against HLA proteins were measured using Lifecodes single antigen LSAI and LSAII kits (Immucor, USA) containing one recombinant HLA specificity per bead. The beads were incubated with patient sera, allowing specific antibody to bind to the bead. Unbound antibody was removed through washing steps, and then bound antibody was detected by addition of phycoerythrin labelled anti-human IgG. Bead fluorescence was detected using a Luminex instrument (LX-200, Millipore, Milliplex, Germany). Highly reactive sera samples were purified by adsorption of non-specific antibodies using commercial "Sera clean" kit (Immucor, USA), followed by a second analysis. Data was imported into Match-It software (Immucor, USA) for analysis. We used manufacturer default settings for assignment of positive, negative, or weak antibodies, and manual review to confirm the instrument assignments.

Presence or absence of antibody (all antibodies or matching antibodies only) at each locus was recorded in a binary fashion in a spreadsheet (Excel, Microsoft). The number of individuals with and without antibodies were then calculated for each locus. For HLA A, B, C, DRB-1, and DRB3/4/5 loci we also reported the number of antibody specificities per individual at the four-digit resolution (for example, if an individual had antibodies against HLA-A*02:01, HLA-A*02:02 and HLA-B*27:01, the individual was considered to have a total of three class 1 antibody specificities). We could not perform this analysis for the other class 2 loci where even single antigen kits comprise more than one specificity per bead.

For analysis of complement-fixing antibodies in HIV-seroconverters and highly exposed persistently seronegative individuals, we utilized the Lifecodes C3d detection kit (ImmuCor, USA). The principle of the assay is that patient serum is allowed to bind with the HLA-coated beads as in the class 2 procedure outlined above. Following incubation, negative serum reagent is added as a source of human complement, which can bind to bead-bound antibody, including that of IgG, IgM or other isotypes. The sensitized beads are washed to remove unbound antibody. An anti-human complement component C3d antibody conjugated to phycoerythrin is used as a fluorescent detection reagent.

4.9.5 DNA extraction and HLA typing from dried blood spot

DNA was extracted from dried blood spots (DBS), collected from index participants, HIV-seroconverters and highly exposed persistently seronegative individuals at enrolment. The extraction was performed using a commercial kit (Gentra® Puregene® Tissue Kit, Qiagen, Germany) according to instructions. The extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Commercial single stranded oligonucleotide probe kits (Lifecodes, Immucor, USA) were used for typing the following HLA loci: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3,4,5, HLA-DQB1 and HLA-DPA/B. All kits allow for allele specific PCR amplification. Oligonucleotide probes in the kits are attached to microspheres detectable by Luminex instrument (LX-200, Milliplex, Merck), Germany. The data obtained from the typing experiments was analysed using Match-It DNA software versions 3.18-3.24 (GenProbe, USA). The typing was reported at the two-digit resolution.

4.9.6 Statistics

Demographic data and the difference in proportion of individuals with and without antibodies between the HIV-seroconverters and highly exposed persistently seronegative individuals was assessed using Fisher's exact test. Each locus was assessed independently. No adjustments were made for multiple comparisons. Number of antibody specificities in HIV-seroconverters compared with highly exposed persistently seronegative individuals was compared using a Mann-Whitney test for non-parametric data. P values less than or equal to 0.05 were considered significant and p values less than 0.1 were taken to suggest a trend to significance.

For group comparisons, means and standard deviations (SD) were determined for continuous measures, whereas frequencies and their percentages were determined for categorical measures. The continuous measures were compared by HIV status of the partner using the student t-test whereas the categorical measures were compared using chi-square analysis or Fisher's exact test as appropriate. Risk factors for HIV transmission were determined by univariate and multivariate logistic regression. Factors analyzed included gender, age, cohort, ethnicity, recruitment site, number of children at enrolment, history of unprotected sex during the study (more detail below) injectable contraception, laboratory-confirmed sexually transmitted infection at enrollment (C*hlamydia trachomatis*, *Trichomonas vaginalis* or *Neisseria ghonorrhoeae*), genital ulcer disease on history or examination and viral load (earliest recorded, maximum recorded and median over study period). There were three variables related to unprotected sex – the variables "number unprotected sex" and "proportion unprotected sex" referred to the number *of visits* and proportio*n of visits* in which the participant reported unprotected sex in the month prior to the visit, while "no condom sex" refers to the number *of unprotected sex acts* in the prior month. Regarding the cultural practice of dry sex, only one individual reported the practice, we therefore did not include the variable further in our analysis.

In the multivariate regression model, we applied a two-step process by first running a full multivariate (including all variables) model using the elastic net selection method that bridges the least absolute shrinkage and selection (LASSO) and ridge regression approaches. This approach identifies potential variables for consideration in the multivariate logistic regression model. The second step involved re-modelling the variables identified in step 1 to arrive at the final multivariate results. Model fit was assessed by the Hosmer-Lemeshow goodness of fit statistics. Regression results are presented as odds ratios (ORs) together with their 95% confidence intervals (95% CI). Statistical analysis was conducted in SAS Enterprise Guide 7.15 using the procedures PROC GLMSELECT and LOGISTIC (SAS Institute Inc., Cary, NC, USA).

4.10 Results

4.10.1 Gender and study site of cases and controls

We analyzed 143 index partners, who were HIV-infected at enrollment, and their corresponding sexual partners, comprising 28 HIV-seroconverters who were HIV-uninfected at enrolment but acquired HIV during the study and 115 highly exposed persistently seronegative controls who remained HIV uninfected throughout the study (286 individuals in total). Of the 143 HIV-infected index partners, 73% of index partners to highly exposed persistently seronegative controls and 75% of index partners to HIV-seroconverters were female. Amongst couples identified with linked HIV-seroconversion (19 couples, 38 individuals), 74% of HIV-infected index partners were female. In couples where the HIV-uninfected partner at enrolment remained highly exposed persistently seronegative, the median time interval

between first and second timepoints was 15 months (range 6-24 months), which was not different from the time interval between first and second timepoints in couples with a HIV-seroconversion event (median 14.5 months, range 6-31 months, p=0.900). In HIV-seroconverters, the second timepoint was a median of 9.5 months (range 2-19 months) following HIV acquisition.

Of control couples, 64.3% were from the Soweto site (59.1% from the Partners and 5.2% from COS) and of seroconverting couples 28.6% were from Soweto (17.9 from Partners and 10.7% from COS). Study site at enrolment is shown in Supplementary Table 2.

4.10.2 Presence of HLA antibodies at the first timepoint

HLA antibodies in each individual participant ranged from absent to having one or multiple specificities at one or more loci. A sample of patient-level data is shown in Supplementary Table 3.

In highly exposed persistently seronegative individuals, at the first timepoint, HLA antibodies were detected in 79% of highly exposed persistently seronegative individuals; 56% of highly exposed persistently seronegative individuals expressed antibodies against HLA class 1 and 50% against HLA class 2 (Table 3). The frequency of highly exposed persistently seronegative individuals expressing antibodies at individual class 1 or 2 loci ranged from 9% against HLA-C to 28% against HLA-A specificities (Table 1). At the first timepoint, prior to HIV-seroconversion, we noted a trend that fewer HIV-seroconverters than highly exposed persistently seronegative controls expressed any class 1 or 2 HLA antibodies (61% versus 79%, p=0.08, Table 1). **Importantly, significantly fewer HIV-seroconverters than highly exposed persistently seronegative controls expressed HLA class 2 antibodies at this pre-seroconversion timepoint** (29% versus 50%, p=0.05, Table 1, Figure 1). For each individual class 1 or 2 locus, there was no significant difference between proportion of HIV-seroconverters compared with highly exposed persistently seronegative controls expressing antibodies (Table 1).

Repeating the analysis in linked HIV-seroconverters, after exclusion of couples in whom there had been HIV-seroconversion unlinked to the index partner, there was no significant difference in proportion of HLA antibodies overall or at any locus between linked HIV-seroconverters and highly exposed persistently seronegative controls (Table 1).

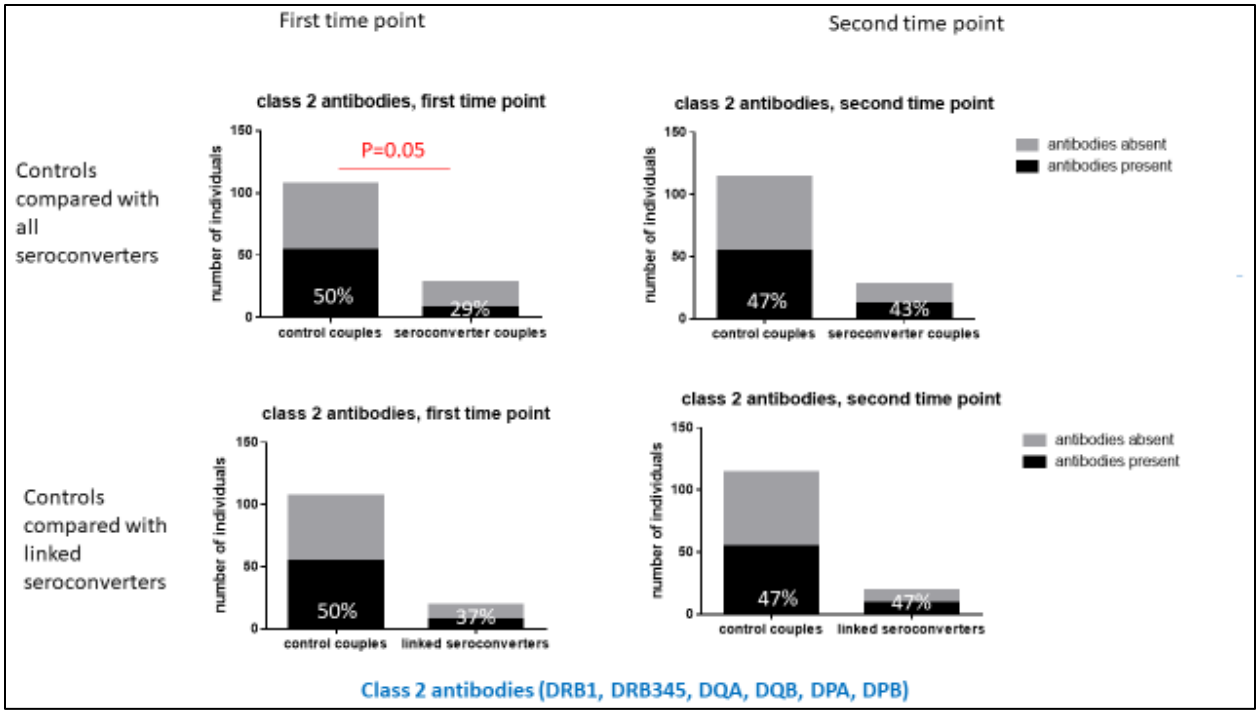


Figure 4.1. Class 2 HLA antibodies *in HIV-seroconverters compared with highly exposed persistently seronegative controls, at the first and second timepoints. Upper panel shows comparison of highly exposed persistently seronegative controls with all HIV-seroconverter couples. Lower panel shows comparison of highly exposed persistently seronegative controls with only linked HIV-seroconverter couples. At the first timepoint, a significantly higher proportion of highly exposed persistently seronegative controls than individuals who went on to acquire HIV expressed class 2 HLA antibodies.*

4.10.3 Presence of HLA antibodies at the second timepoint

At the second timepoint, in highly exposed persistently seronegative controls, 79% of highly exposed persistently seronegative individuals expressed HLA antibodies (Table 1). Half of the highly exposed persistently seronegative individuals expressed antibodies against class 1 and 47% against class 2 specificities (Table 1). The frequency of highly exposed persistently seronegative individuals expressing antibodies at individual class 1 or 2 loci ranged from 6% for HLA-C to 33% for HLA-A (Table 1). In HIV-seroconverters following HIV-seroconversion, 86% of HIV-seroconverters expressed HLA antibodies - 75% against class 1 and 43% against class 2 alleles. **After HIV-seroconversion, there were significantly more HIV-seroconverters than highly exposed persistently seronegative controls with class 1 HLA antibodies** (75% versus 50%, p=0.02, Table 1, Figure 2). In particular, there were significantly more HIV-seroconverters than highly exposed persistently seronegative controls with antibodies against HLA-A (57% versus 33%, p=0.03, Table 3) and HLA-B (50% versus 26%, p=0.02, Table 1).

Repeating the analysis after restriction to linked HIV-seroconversions only, the same pattern was apparent. Following HIV-seroconversion, more linked HIV-seroconverters expressed class 1 antibodies than highly exposed persistently seronegative controls (79% versus 50%, p=0.03, Table 1, Figure 2). Notably there were more linked HIV-seroconverters than highly exposed persistently seronegative controls who expressed HLA-A antibodies in particular (63% versus 33%, p=0.02, Table 1).

Table 4.1. All HLA antibody specificities in highly exposed persistently seronegative controls compared with HIV-seroconverters (all seroconverters or only linked seroconverters), at first and second timepoints

(n=115 highly exposed persistently seronegative controls, 28 HIV-seroconverters and 19 linked HIV-seroconverters)

ALL ANTIBODIES	controls			seroconverters (all)				seroconverters (linked)			
Locus	% controls with Abs present	# individuals with Abs present	# individuals with Abs absent	% seroconverters with Abs present	# individuals with Abs present	# individuals with Abs absent	p value compared with controls	% linked seroconverters with Abs present	# individuals with Abs present	# individuals with Abs absent	p value compared with controls
Time point 1											
HLA-A antibodies	28%	30	76	39%	11	17	0.36	42%	8	11	0.28
HLA-B antibodies	26%	39	68	32%	9	19	0.82	37%	7	12	1.00
HLA-C antibodies	9%	10	97	4%	1	27	0.46	5%	1	18	1.00
Any HLA class 1 antibodies	56%	60	47	54%	15	13	0.83	58%	11	8	1.00
HLA-DRB1 antibodies	17%	18	89	11%	3	25	0.56	16%	3	16	1.00
HLA-DRB3/4/5 antibodies	11%	12	95	4%	1	27	0.30	5%	1	18	0.50
HLA-DQB1 antibodies	25%	27	80	11%	3	25	0.13	16%	3	16	0.56
HLA-DPA1 antibodies	12%	13	94	4%	1	27	0.30	0%	0	19	0.21
HLA-DPB1 antibodies	18%	19	88	7%	2	26	0.24	5%	1	18	0.30
HLA-DQA1 antibodies	23%	25	82	7%	2	26	**0.07**	11%	2	17	0.36
Any HLA class 2 antibodies	50%	54	53	29%	8	20	**0.05***	37%	7	12	0.32
Any HLA antibodies	79%	84	23	61%	17	11	**0.08**	63%	12	7	0.15
Time point 2											
HLA-A antibodies	33%	37	76	57%	16	12	**0.03***	63%	12	7	**0.02***
HLA-B antibodies	26%	30	85	50%	14	14	**0.02***	**42%**	8	11	0.17
HLA-C antibodies	6%	7	108	7%	2	26	1.00	**5%**	1	18	1.00
Any HLA class 1 antibodies	50%	58	57	75%	21	7	**0.02***	79%	15	4	**0.03***
HLA-DRB1 antibodies	18%	20	94	14%	4	24	0.75	21%	4	15	0.74
HLA-DRB3/4/5 antibodies	10%	11	103	14%	4	24	0.69	11%	2	17	1.00
HLA-DQB1 antibodies	24%	27	87	11%	3	25	0.20	11%	2	17	0.25
HLA-DPA1 antibodies	11%	12	102	0%	0	28	0.12	0%	0	19	0.21
HLA-DPB1 antibodies	13%	15	99	7%	2	26	**0.07**	**11%**	2	17	1.00
HLA-DQA1 antibodies	24%	27	87	11%	3	25	0.20	11%	2	17	0.25
Any HLA class 2 antibodies	47%	54	60	43%	12	16	0.83	47%	9	10	1.00
Any HLA antibodies	79%	89	24	86%	24	4	0.60	84%	16	3	1.00

The proportion of highly *exposed persistently seronegative controls, HIV-seroconverters and linked HIV-seroconverters with antibodies at each specific locus are indicated as percentages. The number of positive and negative individuals analyzed at each locus are shown. p values were calculated using Fisher's exact test and apply to within-row comparisons; Abs: antibodies; bold figures indicate trend to significance p<0.01; asterisk indicates significant p values<0.05. Vertical numbers are not cumulative as one individual may express antibodies to more than one HLA locus, and number of analyzable results differed per locus.*

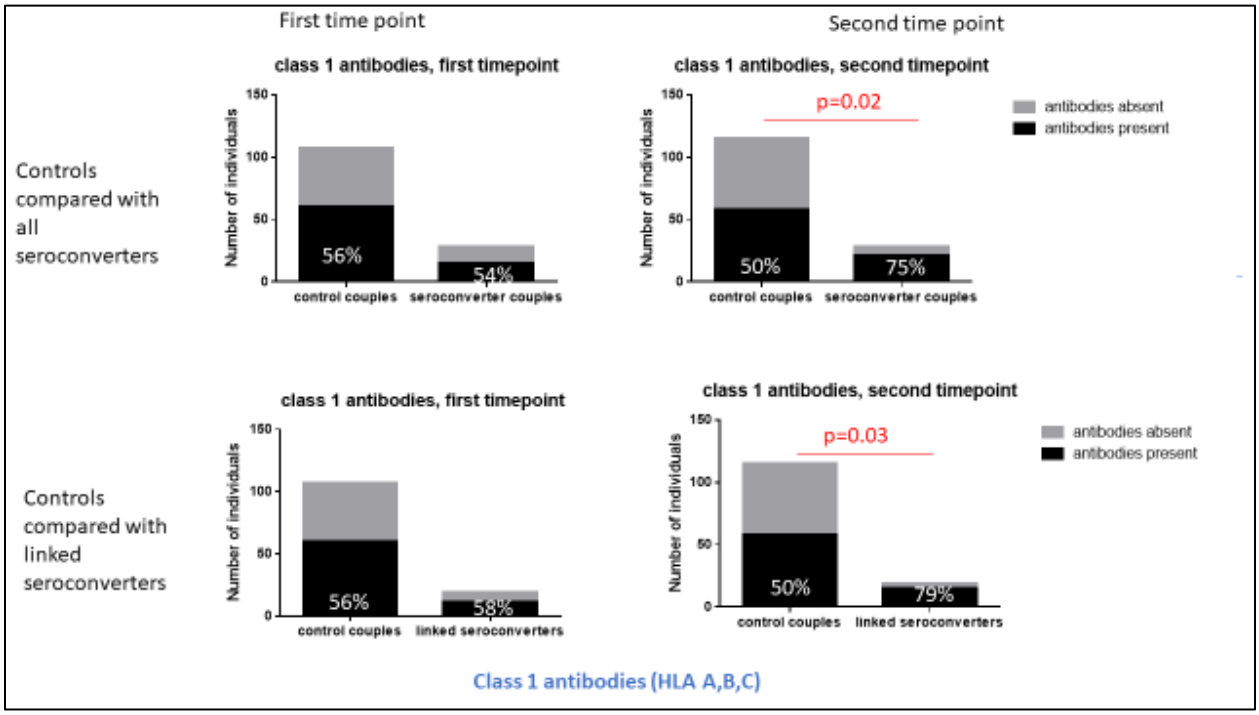


Figure 4.2. Class 1 HLA antibodies *in HIV-seroconverters compared with highly exposed persistently seronegative controls, at the first and second timepoint. Upper panel shows comparison of highly exposed persistently seronegative controls with all HIV-seroconverter couples. Lower panel shows comparison of highly exposed persistently seronegative controls with only linked seroconverters. At the second timepoint, significantly more HIV-seroconverters (all seroconverters or linked seroconverters) than highly exposed persistently seronegative controls expressed class 1 HLA antibodies.*

4.10.4 Presence of matching antibodies at the first timepoint

At the first timepoint, 45% of highly exposed persistently seronegative individuals expressed HLA antibodies matching their HIV-infected index partner's HLA type, hereafter referred to as "matching antibodies". (Table 2). This figure included 13% of highly exposed persistently seronegative controls who expressed matching HLA class 1 antibodies and 32% who expressed matching HLA class 2 antibodies against their index partner's HLA type. At individual loci, the frequency of highly exposed persistently seronegative controls with class 1 or class 2 antibodies matching their index partner ranged from 1% for HLA-C to 13% for HLA-DQB (Table 2). Prior to HIV-seroconversion, 30% of HIV-seroconverters expressed matching HLA antibodies of either class to their index partner's HLA type, not significantly different from highly exposed persistently seronegative controls. 23% of HIV-seroconverters expressed matching HLA-class 1 antibodies and 13% expressed matching HLA class 2 antibodies against their index partners HLA type (Table 2). **There was a trend to fewer HIV-seroconverters than highly exposed persistently seronegative controls expressing matching HLA class 2 antibodies against their index partner's HLA type at this pre-seroconversion timepoint** (13% versus 32%, p=0.07, Table 2, Figure 3). In HIV-seroconverters, the frequency of matching antibodies at each class 1 or 2 locus ranged from 0% (HLA C, HLA-DRB3/4/5 and HLA-DPB1) to 18% for HLA-A (Table 2).

Restricting analysis to seroconverters with linked HIV-transmissions only, 36% of linked HIV-seroconverters expressed matching HLA antibodies against their index partner, 29% against class 1 and 13% against class 2 specificities (Table 2). At each individual class 1 or 2 locus, antibodies ranged from 0% (HLA-C, HLA-DRB3/4/5, HLA-DPA1, HLA-DPB1) to 18% at HLA-A.

There was no significant difference in class 2 antibodies matching the index partner HLA type between HIV-seroconverters with linked HIV-transmissions and highly exposed persistently seronegative controls (13% versus 32%, p=0.22, Table 2, Figure 3) nor significant differences at any particular class 1 or class 2 locus.

Table 4.2. HLA antibodies which matched the HLA genotype of the HIV-infected index partner ("matching antibodies") in highly exposed persistently seronegative controls compared with HIV-seroconverters (all seroconverters or linked seroconverters) at first and second timepoints
(n=115 highly exposed persistently seronegative controls, 28 HIV-seroconverters and 19 linked HIV-seroconverters)

MATCHING ANTIBODIES ONLY	controls			seroconverters (all)				seroconverters (linked)			
Locus	% controls with abs	Abs present	Abs absent	% seroconverters with Abs	Abs present	Abs absent	p value compared with controls	% linked seroconverters with Abs	Abs present	Abs absent	p value compared with controls
Time point 1											
Matching HLA-A antibodies	5%	5	90	18%	4	22	0.10	18%	3	14	0.10
Matching HLA-B antibodies	8%	7	83	8%	2	24	1.00	12%	2	15	0.63
Matching HLA-C antibodies	1%	1	97	0%	0	27	1.00	0%	0	18	1.00
Matching HLA class 1 antibodies	13%	10	70	23%	6	20	0.21	29%	5	12	0.13
Matching HLA-DRB1 antibodies	8%	8	97	4%	1	25	0.68	6%	1	16	1.00
Matching HLA-DRB3/4/5 antibodies	8%	8	98	0%	0	27	0.36	0%	0	18	0.60
Matching HLA-DQB1 antibodies	13%	13	86	4%	1	25	0.30	6%	1	16	0.69
Matching HLA-DPA1 antibodies	8%	9	97	4%	1	27	0.69	0%	0	19	0.35
Matching HLA-DPB1 antibodies	1%	1	94	0%	0	27	1.00	0%	0	18	1.00
Matching HLA class 2 antibodies	32%	29	62	13%	3	21	**0.07**	13%	2	13	0.22
Any matching HLA antibodies	45%	36	44	30%	7	16	0.24	36%	5	9	0.57
Time point 2											
Matching HLA-A antibodies	4%	4	94	16%	4	21	**0.05***	19%	3	13	**0.06**
Matching HLA-B antibodies	7%	7	93	12%	3	23	0.43	12%	2	15	0.62
Matching HLA-C antibodies	1%	1	109	0%	0	27	1.00	0%	0	18	1.00
Matching HLA class 1 antibodies	11%	9	76	28%	7	18	**0.05***	31%	5	11	**0.04***
Matching HLA-DRB1 antibodies	6%	6	102	8%	2	23	0.64	13%	2	14	0.28
Matching HLA-DRB3/4/5 antibodies	4%	4	108	4%	1	26	1.00	0%	0	18	1.00
Matching HLA-DQB1 antibodies	15%	16	92	4%	1	25	0.19	6%	1	17	0.46
Matching HLA-DPA1 antibodies	3%	3	108	0%	0	28	1.00	0%	0	19	1.00
Matching HLA-DPB1 antibodies	1%	1	102	0%	0	26	1.00	0%	0	17	1.00
Matching HLA class 2 antibodies	23%	22	73	19%	4	17	0.78	23%	3	10	1.00
Any matching HLA antibodies	40%	32	48	48%	10	11	0.62	54%	7	6	0.38

The proportion of highly exposed persistently seronegative controls, HIV-seroconverters and linked HIV-seroconverters with matching antibodies against the index partner genotype at each specific locus are

indicated as percentages. The number of positive and negative individuals analyzed at each locus are also shown. p values were calculated using Fisher's exact test and apply to within-row comparisons; Abs: Antibodies, bold figures indicate trend to significance p<0.01; asterisk indicates significant p values<0.05. Vertical numbers are not cumulative as one individual may express antibodies to more than one HLA locus, and number of analyzable results differed per locus.

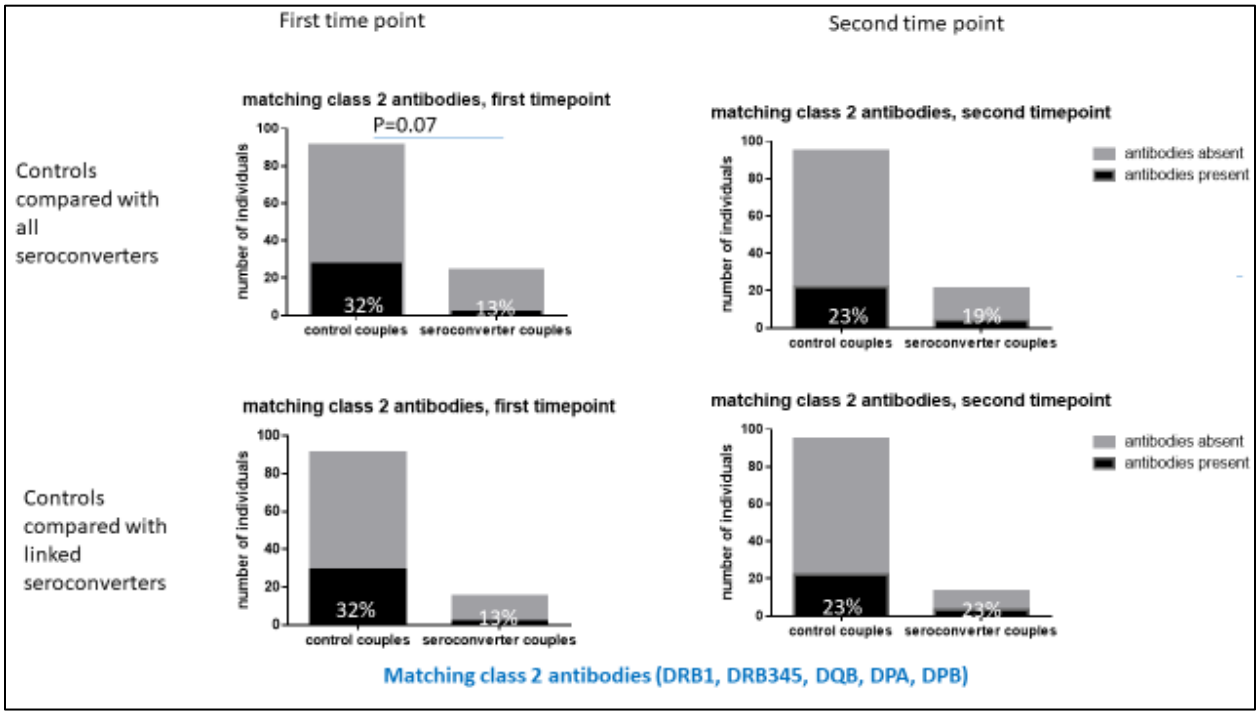


Figure 4.3. Matching class 2 HLA antibodies *in HIV-seroconverters compared with highly exposed persistently seronegative controls, at the first and second timepoints. Upper panel shows comparison of highly exposed persistently seronegative controls with all HIV-seroconverter couples. Lower panel shows comparison of highly exposed persistently seronegative controls with only linked HIV-seroconverter couples. At the first timepoint there was a trend to more matching class 2 HLA antibodies in highly exposed persistently seronegative controls than in those who went on to acquire HIV.*

4.10.5 Matching antibodies at the second timepoint

At the second timepoint, 40% of highly exposed persistently seronegative controls expressed matching class 1 antibodies against their index partners, 11% against HLA class 1 and 23% against class 2 specificities (Table 2). Post HIV-seroconversion, 48% of HIV-seroconverters expressed matching HLA antibodies against their index partners, 28% against class 1 and 19% against class 2 specificities. **Significantly more HIV-seroconverters, after seroconversion, expressed matching class 1 HLA antibodies than highly exposed persistently seronegative controls** (28% versus 11%, p=0.05, Table 2, Figure 4), particularly at the HLA-A locus (16% versus 4%, p=0.05, Table 4).

Restricting analysis to linked HIV transmissions only, 54% of HIV-seroconverters expressed matching HLA antibodies against their index partners, 31% against HLA class 1 and 23% against HLA class 2 specificities.

Similarly, there were significantly more linked HIV-seroconverters, post seroconversion, with class 1 HLA antibodies than highly exposed persistently seronegative controls (31% versus 11%, p=0.04, Table 4, Figure 4), with a trend to more linked seroconverters than highly exposed persistently seronegative controls with HLA-A antibodies (19% versus 4%, p=0.06, Table 4).

Table 4.3. Characteristics of the index partners (partners who were HIV-infected at enrolment)
(n=115 index partners to highly exposed persistently seronegative controls and 19 index partners to linked seroconverters)

Characteristics of Index Partners (excluding unlinked seroconverters)			
Variable	**HIV non-transmitters**	**HIV transmitters**	**p value**
Mean (SD) age at enrolment (years)	33.34 (7.53)	34.00 (9.72)	0.7355
Gender n (%)			
Female	84 (73.04)	14 (73.68)	1.0000
Male	31 (26.96)	5 (26.32)	
Cohort n (%)			
Cos	6 (5.22)	3 (15.79)	0.1169
Hsv	109 (94.78)	16 (84.21)	
Ethnicity n (%)			
Other	29 (25.22)	2 (10.53)	0.4003
Sotho	17 (14.78)	2 (10.53)	
Xhosa	46 (40.00)	9 (47.37)	
Zulu	23 (20.00)	6 (31.58)	
Site n (%)			
CT	28 (24.35)	8 (42.11)	0.1595
JHB	87 (75.65)	11 (57.89)	
Mean (SD) number of children at enrolment	1.75 (1.40)	1.16 (0.96)	0.0809
Ever unprotected sex n (%)			
No	51 (44.35)	2 (10.53)	**0.0049***
Yes	64 (55.65)	17 (89.47)	
Mean (SD) Proportion unprotected sex	0.15 (0.22)	0.14 (0.13)	0.8767
Mean (SD) Number unprotected sex	2.47 (4.26)	2.05 (2.46)	0.6789
Mean (SD) No. of no condom sex acts	18.14 (56.72)	8.05 (12.84)	0.4429
Contraception injection n (%)			
0	69 (60.00)	11 (57.89)	1.0000
1	46 (40.00)	8 (42.11)	
Chlamydia n (%)			
Negative	99 (93.40)	15 (93.75)	1.0000
Positive	7 (6.60)	1 (6.25)	
Trichomonas n (%)			
Negative	79 (74.53)	11 (68.75)	0.7608
Positive	27 (25.47)	5 (31.25)	

Genital ulcers n (%) No Yes	 85 (73.91) 30 (26.09)	 12 (63.16) 7 (36.84)	 0.4064
Any STI n (%) Negative Positive	 76 (71.70) 30 (28.30)	 11 (68.75) 5 (31.25)	 0.7744
Mean (SD) Earliest Log VL	4.16 (0.66)	4.71 (0.73)	**0.0017***
Mean (SD) Maximum Log VL	4.55 (0.76)	4.97 (0.78)	**0.0294***

STI sexually transmitted infection, VL viral load

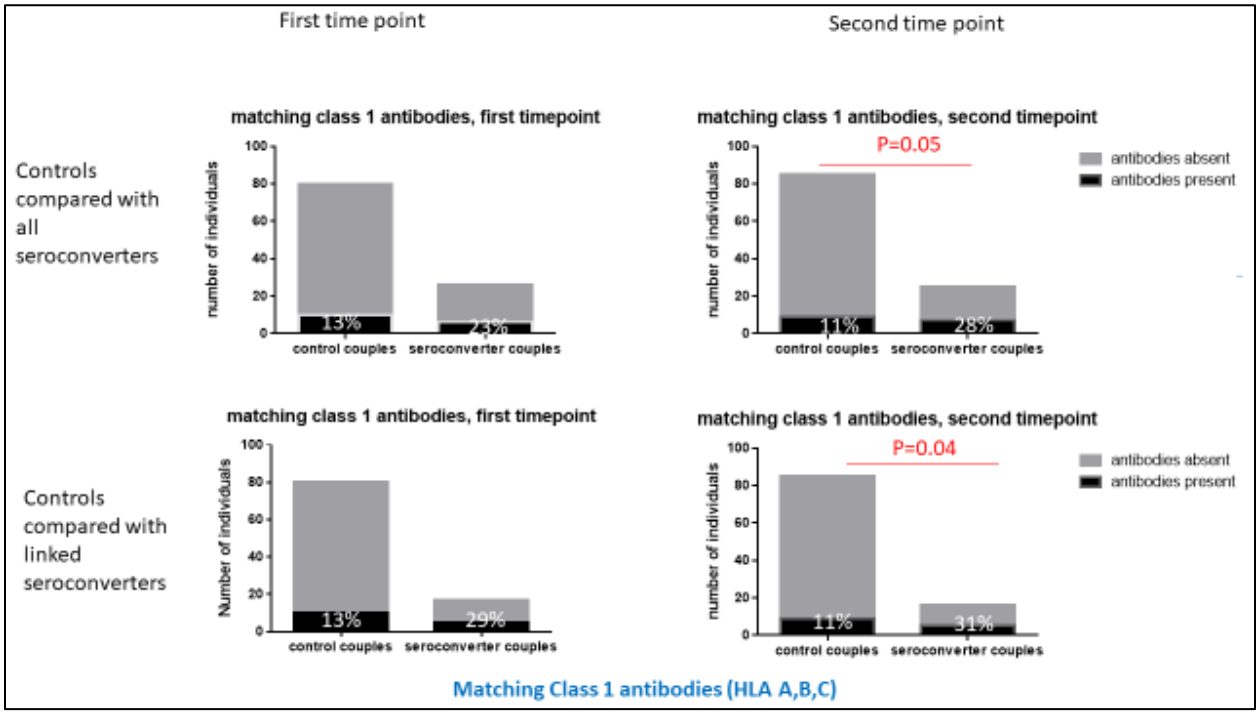


Figure 4.4. Matching class 1 HLA antibodies *in HIV-seroconverters compared with highly exposed persistently seronegative controls, at the first and second timepoints. Upper panel shows comparison of highly exposed persistently seronegative controls with all seroconverter couples. Lower panel shows comparison of highly exposed persistently seronegative controls with only linked HIV-seroconverters. At the second timepoint, a significantly higher proportion of HIV-seroconverters, following HIV seroconversion, than highly exposed persistently seronegative controls expressed matching class 1 HLA antibodies.*

4.10.6 Number of antibody specificities

At the first timepoint, the number of class 1 antibody specificities per highly exposed persistently seronegative individual ranged from 0 to 73 (median 1, IQR 3), not differing significantly from HIV-seroconverters who had a range of 0-24 specificities per individual (median 1; IQR 2, Figure 5). For class 2 specificities, number of antibody specificities per individual ranged from 0 to 35 (median 0, IQR 0) in

highly exposed persistently seronegative controls, also not differing from HIV-seroconverters, who had a range of 0 to 2 (median 0, IQR 0) specificities per individual.

At the second timepoint, the number of class 1 specificities per individual ranged from 0 to 40 (median 0, IQR 3) in highly exposed persistently seronegative controls, significantly lower than in HIV-seroconverters post seroconversion (range 0 to 25; median 2; IQR 4.75, p<0.01, Figure 5). For class 2, highly exposed persistently seronegative controls had 0 to 35 (median 0, IQR 0) specificities per individual, not differing from HIV-seroconverters post seroconversion who had a range of 0 to 9 (median 0, IQR 1) class 2 specificities per individual (Figure 5).

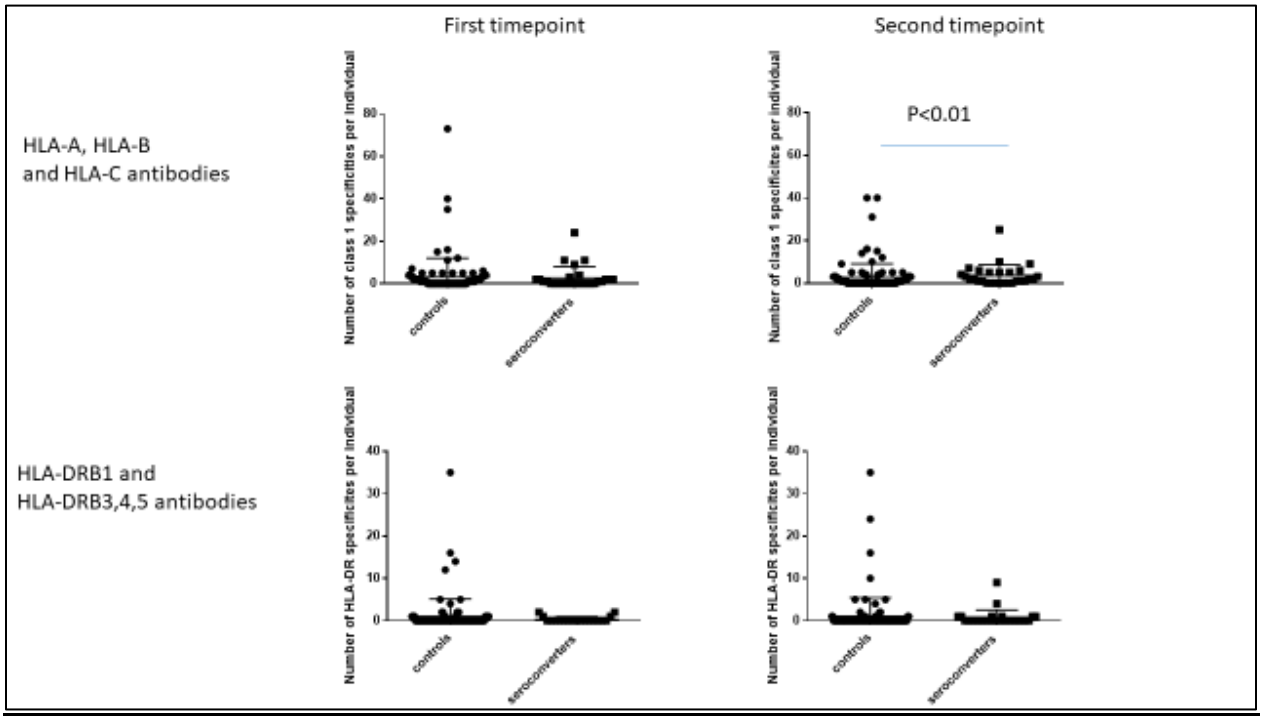


Figure 4.5. Number of HLA antibody specificities per individual, *at the first and second timepoints. The left hand graphs show HLA-A, HLA-B and HLA-C antibody specificities. The right hand graphs show HLA-DRB1 and HLA-DRB3,4,5 antibody specificities. At the second timepoint, HIV-seroconverters had a median of two antibody specificities per person compared with highly exposed persistently seronegative controls who had a median of one antibody specificity per person. There was no other significant difference noted.*

4.10.7 Autoantibodies

HLA autoantibodies, analyzed against self HLA genotypes at two-digit resolution, were not uncommon. At the first timepoint, 27% of highly exposed persistently seronegative controls expressed autoantibodies to at least one locus, 6.7% against at least one class 1 locus and 18.2% against at least one class 2 locus. At early timepoints, there was a trend to a higher frequency of class 2 HLA autoantibodies in highly exposed persistently seronegative controls (18.2% compared with 0% of HIV-seroconverters, p=0.07, Table 3), in keeping with the finding of more class 2 HLA antibodies generally in highly exposed persistently seronegative controls at the early timepoint.

At later timepoints, HIV-seroconverters, following seroconversion, tended to have a higher frequency than highly exposed persistently seronegative controls of HLA-B autoantibodies (16.7% in HIV-seroconverters compared with 5.1% in controls, p=0.07, Table 3) with no significant differences from highly exposed persistently seronegative controls in HLA autoantibody frequency at other loci.

Table 4.4. HLA autoantibodies in highly exposed persistently negative controls compared with HIV-seroconverters, at first and second timepoints

(n=115 highly exposed persistently seronegative controls, 28 HIV-seroconverters and 19 linked HIV-seroconverters)

AUTOANTIBODIES ONLY	controls			seroconverters			
Locus	% controls with autoAbs	# individuals with autoAbs present	# individuals with autoAbs absent	% seroconverters with autoAbs	# individuals with autoAbs present	# individuals with autoAbs absent	p value compared with controls
Time point 1							
HLA-A autoantibodies	2.4	2	82	0.0	0	24	1.00
HLA-B autoantibodies	3.3	3	87	8.0	2	23	0.30
HLA-C autoantibodies	0.0	0	99	0.0	0	27	1.00
Any HLA class 1 autoantibodies	6.7	5	70	8.3	2	22	0.68
HLA-DRB1 autoantibodies	5.8	6	98	0.0	0	24	0.59
HLA-DRB3/4/5 autoantibodies	2.8	3	103	0.0	0	27	1.00
HLA-DQB1 autoantibodies	7.3	7	89	0.0	0	25	0.34
HLA-DPA1 autoantibodies	1.0	1	95	0.0	0	27	1.00
HLA-DPB1 autoantibodies	0.0	0	89	0.0	0	26	1.00
HLA-DQA1 autoantibodies	0.0	0	83	0.0	0	26	1.00
Any class 2 autoantibodies	18.2	14	63	0.0	0	20	**0.07**
Any class 1 or 2 autoantibodies	27	18	42	10.5	2	17	0.13
Time point 2							
HLA-A autoantibodies	0.0	0	85	0.0	0	24	1.00
HLA-B autoantibodies	5.1	5	94	16.7	4	20	**0.07**
HLA-C autoantibodies	0.0	0	106	0.0	0	27	1.00
Any HLA class 1 autoantibodies	6.5	5	72	17.4	4	19	0.21
HLA-DRB1 autoantibodies	2.8	3	105	0.0	0	24	1.00
HLA-DRB3/4/5 autoantibodies	1.8	2	111	3.7	1	26	0.48
HLA-DQB1 autoantibodies	7.7	8	96	0.0	0	25	0.35
HLA-DPA1 autoantibodies	0.0	0	102	0.0	0	28	1.00
HLA-DPB1 autoantibodies	0.0	0	97	0.0	0	26	1.00
HLA-DQA1 autoantibodies	0.0	0	87	0.0	0	25	1.00
Any class 2 autoantibodies	15.6	12	65	5.3	1	18	0.45
Any class 1 or 2 autoantibodies	27.4	17	45	27.8	5	13	1.00

The proportion of highly exposed persistently seronegative controls and HIV-seroconverters with autoantibodies against their own genotype at each specific locus are indicated as percentages. The number of positive and negative individuals analyzed at each locus are also shown. p values were calculated using Fisher's exact test and apply to within-row comparisons; Abs: antibodies; bold figures indicate trend to significance approaching p=0.05. Vertical numbers are not cumulative as one individual may express antibodies to more than one HLA locus, and number of interpretable results differed per locus.

4.10.8 Complement-fixing antibodies

In order to expand our search to antibodies of isotypes other than IgG, we analyzed complement-fixing class 2 HLA antibodies. We considered only the first timepoint as we were particularly interested in whether IgM antibodies could differ between HIV-seroconverters and highly exposed persistently seronegative individuals, potentially conferring protection against HIV acquisition. We found that 7.5% (8/107) of controls and 4.0% of seroconverters (1/25) expressed complement-fixing class 2 HLA antibodies at the early timepoint. The specificities against which the complement-fixing antibodies were directed are shown in Supplementary Table 3. There was no significant difference between highly exposed persistently seronegative controls and HIV-seroconverters regarding the proportion of individuals with complement-fixing class 2 HLA antibodies.

4.10.9 Epidemiological factors in partners who were HIV-uninfected at enrolment associated with HIV-acquisition risk

Regarding demographic and clinical parameters in partners who were HIV-uninfected at enrolment, there was no significant difference in age, gender, cohort or ethnicity between HIV-seroconverters and highly exposed persistently seronegative individuals (Table 5). Amongst HIV-seroconverters, 50% came from Johannesburg and 50% from Cape Town, while amongst highly exposed persistently seronegative individuals, 76.1% were recruited from Johannesburg sites, showing significant difference in proportion between cities (p=0.01) (Table 5).

Given that pregnancy is known to be a trigger event for development of HLA antibodies[158][172], we analyzed numbers of pregnancies in the females originally HIV-uninfected at enrolment, of whom there were 31 highly exposed persistently seronegative females and 7 HIV-seroconverter females. At enrolment, the mean number of children in highly exposed persistently seronegative controls was 1.75 (SD 1.40) compared with 1.32 (SD 1.12) in HIV-seroconverters, with no significant difference between the groups (Table 5). Regarding pregnancies occurring during the study, pregnancy was reported by 6 of 31 highly exposed persistently seronegative females (19.4%) and 3 of 7 (42.9%) HIV-seroconverter females with no significant difference between the groups (data not shown, too few events for inclusion in regression analysis).

Regarding factors in the HIV-uninfected partner at enrollment that may be associated with HIV acquisition, the history of number of visits and proportion of visits in which unprotected sexual episodes were reported was similar in both groups (Table 5). Similarly the number of reported unprotected sex acts was similar in both groups (Table 5). There was no difference in proportion of HIV-seroconverters and highly exposed persistently seronegative controls using injectable contraception. *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria ghonorrhoeae* results at enrollment showed no difference between groups. Similarly, genital ulcer frequency was similar in both groups. HLA antibody variables summarized in Table 5 were investigated in univariate and multivariate regression.

In univariate analysis of risk factors in the partner who was HIV-uninfected at enrolment and incorporating viral load of the HIV-infected index partner, factors significantly associated with HIV acquisition included recruitment city, index partner viral load and any class 2 HLA antibodies at the early timepoint (Table 6). The Cape Town site was associated with a higher odds of HIV acquisition (OR 3.185, CI 1.351-7.510, p=0.01). Class 2 HLA antibodies at the early timepoint were associated with significantly lower odds of HIV acquisition (OR 0.393, CI 0.159-0.969, P=0.04) and each log viral load in the index

partner showed odds ratio of 2.192 (CI 1.137-4.225, P=0.02) for HIV acquisition by the partner who was HIV-uninfected at enrolment (table 6). In multivariate analysis, the only factors significantly associated with HIV acquisition was city of recruitment (OR 2.971 CI 1.029-8.580 p=0.04) and any class 2 HLA antibodies at the first timepoint (OR 0.330 CI 0.112-0.976, p=0.05).

We repeated univariate and multivariate regressions on the partner HIV-uninfected at enrolment, split by gender (Supplementary Table 6 for males and Supplementary Table 7 for females). For males, in univariate analysis, city of recruitment, maximum viral load and matching class 1 antibodies at the first timepoint were significantly associated with increased odds of HIV acquisition. Matching class 1 HLA antibodies at the early timepoint gave an odds ratio of 6.333 (CI 1.357-29.55, P=0.02) for HIV acquisition. Interpretation is limited by the low numbers (84 male highly exposed persistently seronegative controls, 21 male HIV-seroconverters) and resultant wide confidence interval. Any class 2 HLA antibodies at the first timepoint showed a protective trend (OR 0.346 CI 0.115-1.038, p=0.06), as did matching class 2 antibodies at the early timepoint (OR 0.149 CI 0.018-1.196, p=0.07), earliest viral load and any STI (Supplementary Table 6). The only variable significant in multivariate analysis for male partners who were HIV-uninfected at enrolment was city of recruitment (Supplementary Table 6).

In female partners, HIV-uninfected at enrolment, analysed alone (31 female highly exposed persistently seronegative controls and 7 female HIV-seroconverters), no variables were significantly associated with odds of HIV acquisition in univariate analysis, likely due to low sample numbers, therefore multivariable analysis was not performed.

Table 4.5: Characteristics of individuals who were HIV-uninfected at enrolment
(n=115 highly exposed persistently seronegative controls and 28 HIV-seroconverters)

Characteristics of the partner who was HIV-uninfected at enrolment			
Variable	**Did not acquire HIV**	**Acquired HIV**	**p value**
Mean (SD) age at enrolment (years)	35.81 (9.12)	35.20 (9.94)	0.7557
Gender n(%)			
Female	31 (27.19)	7 (25.00)	1.0000
Male	83 (72.81)	21 (75.00)	
Cohort n(%)			
Cos	6 (5.26)	3 (10.71)	0.3800
Hsv	108 (94.74)	25 (89.29)	
Ethnicity n(%)			
Other	29 (25.22)	4 (14.29)	0.2985
Sotho	27 (23.48)	5 (17.86)	
Zulu	59 (51.30)	19 (67.86)	
Site n(%)			
CT	27 (23.89)	14 (50.00)	**0.0100***
JHB	86 (76.11)	14 (50.00)	
Mean (SD) number of children at enrolment	1.75 (1.40)	1.32 (1.12)	0.1390

Ever unprotected sex n(%)			
No	52 (45.61)	12 (42.86)	0.8349
Yes	62 (54.39)	16 (57.14)	
Mean (SD) Proportion unprotected sex	0.20 (0.27)	0.17 (0.19)	0.6201
Mean (SD) Number unprotected sex	1.27 (1.93)	1.11 (1.29)	0.6689
Mean (SD) No. of no condom sex acts	9.77 (22.64)	6.39 (11.65)	0.4463
Contraception injection n(%)			
0	102 (89.47)	24 (85.71)	0.5210
1	12 (10.53)	4 (14.29)	
Mean (SD) Interval between 2 timepoints (mo)	14.29 (5.08)	15.46 (6.80)	0.3194
Chlamydia n(%)			
Negative	99 (93.40)	24 (96.00)	1.0000
Positive	7 (6.60)	1 (4.00)	
Trichomonas n(%)			
Negative	95 (89.62)	20 (80.00)	0.1883
Positive	11 (10.38)	5 (20.00)	
Genital ulcers n(%)			
No	85 (74.56)	21 (75.00)	1.0000
Yes	29 (25.44)	7 (25.00)	
Any STI n(%)			
Negative	93 (87.74)	19 (76.00)	0.2017
Positive	13 (12.26)	6 (24.00)	

STI sexually transmitted infection, VL viral load

4.10.10 Epidemiological factors in index partners who were HIV-infected at enrolment associated with HIV-transmission risk

Factors in index partners who were HIV-infected at enrolment that may be associated with transmission risk were analysed in univariate and multivariate regression (Supplementary Table 8). Regarding pregnancy in HIV-infected index partners, there were 11 pregnancies in 84 female index partners to highly exposed persistently seronegative controls and 2 pregnancies in 21 female index partners to HIV-seroconverters, yielding no difference between the groups and too few events for inclusion in regression analysis. For the regression analysis, we excluded unlinked couples as their index partners did not transmit HIV. In univariate regression, factors associated with increased transmission from HIV-infected index partners included reported unprotected sex and viral load measurements. There was a trend to reduced HIV transmission with increased number of children at enrollment in the HIV-infected index partner (OR 0.663 CI 0.418-1.051, p=0.0805), but no association with enrollment age. In multivariate analysis, median viral load and history of unprotect ted sex remained significant and there was a trend to reduced transmission with increased number of children at enrollment in the HIV-infected index partner (OR 0.576 CI 0.329-1.007, p=0.0528).

We then stratified HIV-infected index partners by gender (Supplementary Table 9 for males and Supplementary Table 10 for females). In males, no variable was significant on univariate regression, with only median viral load showing a trend to increased transmission risk. In females, in univariate analysis, history of unprotected sex and viral load indicators were associated with increased transmission risk. In multivariate analysis, higher number of children at enrollment in female HIV-infected index partners showed a trend to reduced odds of HIV transmission to their sexual partner (OR 0.558, CI 0.302-1.030, p=0.06).

Table 4.6: Risk factors for HIV acquisition by individuals who were HIV-uninfected at enrolment, by univariate and multivariate regression

(n=115 highly exposed persistently seronegative controls and 28 HIV-seroconverters)

HIV-seroconverters versus highly exposed persistently seronegative controls				
	Univariate		**Multivariate**	
Variable	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Gender: Females vs Males	0.892 (0.345-2.307)	0.8144		
Enrolment age (years)	0.993 (0.949-1.039)	0.7537		
Cohort: Cos vs Partners	2.160 (0.505-9.232)	0.2988		
Ethnicity: Sotho vs Other Xhosa vs Other Zulu vs Other	1.343 (0.326-5.529) 2.335 (0.727-7.494)	0.6833 0.1542		
Site: Cape Town vs Johannesburg	3.185 (1.351-7.510)	**0.0081***	2.971 (1.029-8.580)	**0.0441***
Number of children at enrolment	0.765 (0.536-1.092)	0.1395	0.777 (0.497-1.215)	0.2681
Ever unprotected sex: No vs Yes	0.894 (0.388-2.060)	0.7928		
Proportion unprotected sex	0.646 (0.116-3.592)	0.6180		
Number unprotected sex	0.948 (0.743-1.210)	0.6670		
No. of no condom sex acts	0.990 (0.964-1.017)	0.4510		
Contraception injection	1.417 (0.420-4.779)	0.5742		
Interval between 2 timepoints (mo)	1.040 (0.963-1.122)	0.3178		

Chlamydia: Positive vs Negative	0.589 (0.069-5.020)	0.6285		
Trichomonas: Positive vs Negative	2.159 (0.676-6.901)	0.1942		
Genital ulcers: Yes vs No	0.977 (0.377-2.536)	0.9620		
Any STI: Positive vs Negative	2.259 (0.763-6.692)	0.1413	2.878 (0.772-10.72)	0.1152
Earliest Log VL	2.192 (1.137-4.225)	**0.0191***	1.740 (0.831-3.644)	0.1416
Max Log VL	1.995 (1.119-3.557)	**0.0192***		
Median Log VL	1.920 (1.058-3.484)	**0.0320***		
Any class 1 HLA Abs first timepoint: yes vs no	0.904 (0.392-2.083)	0.8124		
Number of class 1 specificities first timepoint	0.993 (0.938-1.051)	0.8076		
Any class 2 HLA abs first timepoint: yes vs no	0.393 (0.159-0.969)	**0.0425***	0.330 (0.112-0.976)	**0.0452***
Number of DRB1 and DRB345 specificities first timepoint	0.750 (0.423-1.328)	0.3239		
Matching class 1 first timepoint: yes vs no	2.100 (0.680-6.485)	0.1972		
Matching class 2 first timepoint: yes vs no	**0.305 (0.084-1.107)**	**0.0710**		

STI sexually transmitted infection, VL viral load

4.11 Discussion

To explore the role of HLA antibodies in risk of HIV acquisition, we retrospectively studied samples from the Partners and COS studies, longitudinal analyses of HIV serodiscordant couples with both genetic material and sera available for study, including timepoints prior to HIV seroconversion. The cohorts were well characterized, including viral analysis of the seroconverting couples which could identify those who acquired HIV from their index partner (linked transmissions) and those who acquired HIV from a third party (unlinked transmissions). The HIV-uninfected partners at enrolment who did not seroconvert represent healthy individuals with no known comorbidities and can be considered highly exposed persistently seronegative individuals, as most reported episodes of unprotected sexual intercourse despite HIV risk counselling (Table 1). We analyzed the presence of any HLA antibody at each locus in HIV-seroconverters and highly exposed persistently seronegative controls to ascertain whether particular specificities may play a prominent role. We then analyzed a subset of HLA antibodies, namely partner-directed HLA antibodies ("matching antibodies"), which could potentially react with the HIV donor's HLA proteins. We performed these analyses twice – firstly incorporating all HIV-seroconverters,

to look for general factors in persistently seronegative individuals which may differ from individuals who acquired HIV. Secondly, we repeated the analysis using only linked seroconverters, to look for associations directly related to interaction between the sexual partners between whom HIV was transmitted.

At the first timepoint, prior to seroconversion, we found that **significantly fewer HIV-seroconverters had class 2 HLA antibodies compared to highly exposed persistently seronegative controls.** There was no particular class 2 locus in which the proportion of highly exposed persistently seronegative controls and HIV-seroconverters differed significantly, but for each class 2 locus there were fewer HIV-seroconverters than highly exposed persistently seronegative controls with antibodies. These findings suggest that class 2 HLA antibodies are protective against HIV acquisition.

We went further to analyze the matching antibodies directed particularly against HLA types of the index partner who was HIV-infected at enrolment. Prior to HIV-seroconversion there was a similar trend noted of fewer matching HLA class 2 antibodies in HIV-seroconverters than in highly exposed persistently seronegative controls. Thus, prior to HIV-seroconversion, HIV-seroconverters showed less alloimmunity against their partners than the highly exposed seronegative control group.

There are two schemas for interaction of HLA antibodies with HIV. The first is relevant to any enveloped virus. Enveloped viruses, such as HIV and measles, bud from the host cell membrane and incorporate host proteins, including HLA, into the envelope [176][239][240]. HLA proteins are present in the HIV envelope in higher quantities than gp120 trimers [42][152]. Incorporation of host HLA within the viral envelope of an HIV "donor" suggests that antibodies against HLA in the HIV "recipient" may play a role in protection from acquisition of HIV, similar to the role of HLA antibodies in humoral rejection of a solid organ transplant.

The second schema, of relevance only for HIV, is that HIV proteins, in particular gp120, show particular homology to HLA proteins, likely due to shared affinity for binding to the host CD4 protein. Notable homologies between HIV and host proteins has been summarized in Supplementary Table 1 [193][194][196][187][203][195][197][204][189][205] including additional homologies reported between class 2 HLA and other HIV proteins including gp41 and Nef [199][192][196][188]. HIV gp120 has even been shown to be able to present peptide as antigen, analogous to peptide presentation by class 2 HLA [42][186]. Some authors have speculated that HIV gp120 may have evolved from class 2 HLA or by mimicry of class 2 HLA [185][192].

Due to homologies between HIV proteins and HLA proteins, antibodies reactive against HLA may cross-react with gp120 or other HIV antigens as reported by others using *in vitro* models [211]. HLA-DR antiserum interfered with HIV replication in culture [213] and in some cases HLA class 1 and class 2 antibodies were able to neutralize strains of HIV [212]. Other antibody-mediated functions such as antibody-dependent cellular cytotoxicity or antibody-dependent cell mediated viral inhibition may play a role *in vivo* [54]. Complement fixation has been shown to be important in HIV transmission [151], however we found no difference between highly exposed persistently seronegative controls and HIV-seroconverters in proportion expressing complement-fixing antibodies against class 2 HLA. Alternatively, HLA antibodies may be a surrogate or correlate of protective T cell or innate immune responses.

Interestingly, while we found statistical difference between highly exposed persistently seronegative controls and HIV-seroconverters when including all HIV-seroconverters, we did not note any difference in class 2 HLA antibody frequencies comparing only linked HIV-seroconverters with highly exposed

persistently seronegative controls. Thus, presence of class 2 HLA antibodies seemed protective, even against HIV acquisition from a third partner who was not enrolled in the trial. While lack of significant associations in the linked group may be an artefact of small numbers of linked transmissions, these findings may also infer that protection derived from HLA class 2 antibodies is directed at HIV gp120 itself, which would apply to any HIV acquisition from any partner, rather than specifically directed towards donor HLA proteins incorporated into the viral envelope which would apply only between linked partners. That is, the protection afforded by class 2 HLA antibodies does not seem to derive from matching antibodies directed at the HIV-infected index partner's HLA type only, but rather from any class 2 antibodies. However, donor-directed antibodies (matching antibodies) also showed a trend towards expression in more highly exposed persistently seronegative controls than HIV-seroconverters (32% versus 17%, p=0.07). While such partner-directed antibodies may be of relevance in binding to partner HLA incorporated into viral envelope, they may be a result of stimulation by exposure to the partner which then play a role in binding to HIV gp120 regardless of partner HLA incorporation into the viral envelope.

There are important implications from our work, including absence of certain findings. A relevant finding was the lack of any one particular HLA antibody allele or locus specificity common to the majority of highly exposed persistently seronegative controls. Further, we did *not* find that *only* partner-directed antibodies were associated with lower odds of HIV acquisition. Similarly, we did not demonstrate any protection from complement-fixing antibodies against class 2 HLA, thus there is no suggestion that it is antibodies of IgM isotype or newly developed antibodies that explain any protective effect.

Using multivariable regression to control for well described risk factors for HIV acquisition, in partners originally HIV-uninfected at enrolment, presence of any class 2 HLA antibodies at the early timepoint were significantly associated with lower odds of HIV acquisition in both univariate (OR 0.393 CI 0.159-0.969, p=0.04) and multivariate analysis (OR 0.330, CI 0.112-0.976, P=0.05). This association was stronger for originally HIV-uninfected partners than traditional risk factors such as history of unprotected sex, sexually transmitted infection or partner viral load. The association of the Cape Town site with HIV acquisition is an artefact of our sample selection, as we intentionally recruited additional HIV-seroconverters from the Cape Town and Orange Farm sites to boost seroconverter numbers. Although ethnicity of Cape Town and Johannesburg participants differed, there was no association of ethnicity with odds of HIV acquisition. The reason for the association of number of children at enrollment in the originally HIV-infected index participants with increased HIV-transmission risk to their partners is unclear, but does not relate to pregnancy during the study. Whether some female index partners may have been in the peripartum period at enrolment is unknown.

Stratification by gender was limited by sample size. In male partners who were HIV-uninfected at enrolment, presence of HLA antibodies at the early timepoint showed a trend to reduced odds of HIV acquisition, as did presence of partner-directed class 2 HLA antibodies. Surprisingly, the presence of partner-directed matching class 1 HLA antibodies in males who were HIV-uninfected at enrollment was significantly associated with *increased* odds of HIV acquisition on univariate analysis, albeit with a wide confidence interval, not significant in the multivariable model. In females who were HIV-uninfected at enrolment analyzed alone, no variables were significantly associated with odds of HIV acquisition, possibly due to the low number of HIV-uninfected females at enrollment in each group. Only the index partner viral load showed a trend to increased HIV acquisition risk by the originally HIV-uninfected female partner.

Following seroconversion, there were significantly more HIV-seroconverters than highly exposed persistently seronegative controls with HLA class 1 antibodies. In particular, significantly more HIV-seroconverters than highly exposed persistently seronegative controls expressed HLA-A and HLA-B antibodies. Similarly, HIV-seroconverters expressed significantly more matching class 1 HLA antibodies at the second timepoint compared to highly exposed persistently seronegative controls, particularly at the HLA-A locus. The same pattern was seen with linked transmissions, where there were significantly more linked HIV-seroconverters with class 1 HLA antibodies than highly exposed persistently seronegative controls. At the second timepoint, HIV-seroconverters also expressed more class 1 specificities *per individual* than highly exposed persistently seronegative controls. The change from timepoint 1 to timepoint 2 illustrates that HIV acquisition is a sensitizing event stimulating production of antibodies against HLA-A and HLA-B loci. This is an expected finding and is in keeping with other reports that HIV-infected individuals make anti-lymphocyte antibodies and HLA antibodies [241][194][199]. HIV-infected patients often show polyclonal increases in gamma globulins and many viral infections result in polyclonal increases in IgG titres, often resulting in cross-reactive serological diagnostic assays [242][243]. We did not have data related to acute HIV seroconversion illness, but it may be that a feature of acute seroconversion illness includes *de novo* development of class 1 HLA antibodies in some patients which persist afterwards.

Regarding HIV acquisition as a sensitizing event for *de novo* induction of HLA antibodies, it is interesting to note, however, that the sensitization seems to be limited to class 1 rather than class 2 antibodies. Despite multiple homologies between HIV proteins and human proteins described, why HIV infection does not stimulate new class 2 antibodies in HIV-seroconverters is an intriguing question. It may be that homologies between HIV proteins and human class 2 MHC proteins result in some form of immune tolerance that limits *de novo* development of class 2 HLA antibodies. Alternatively repeated stimulation or exposure to certain HIV proteins homologous to class 2 antigens may result in blunting of the humoral immune response to class 2 antigens, such as that seen with pneumococcal polysaccharide vaccination [244].

Our data have relevance to alloimmunization strategies. Potential advantages of alloimmunization as an HIV prevention strategy includes the strong immunogenicity of HLA and avoidance of the high variability and mutability of HIV [216][214]. Potential disadvantages include the risk of induction of autoimmunity and limitations to future transplantation options in vaccinated individuals [216]. Also the HLA type of the potential HIV "donor" could not be predicted, which may require incorporation of a group of multiple HLA specificities into a vector [149] if "matching" antibodies were required for protection. Our findings however suggest that there may be some benefit even from "non-matching" class 2 HLA antibodies. The benefit:risk ratio of a successful HIV vaccine would likely outweigh concerns around organ transplantation for HIV endemic areas. Multiparous women and patients who have received multiple transfusions represent natural examples of people with alloimmunity without obvious autoimmune side effects. Our data lend weight to this opinion, showing that 79% of healthy highly exposed persistently seronegative individuals express HLA antibodies with no known deleterious consequences. Additionally, 27% of healthy highly exposed persistently seronegative individuals expressed potential HLA *autoantibodies* in at least one locus, tested to two-digit resolution, without apparent consequences. It may be these antibodies are non-reactive against the individual's HLA type tested at higher resolution, although others have also reported HLA autoantibodies in healthy individuals [171,245], including when using high resolution HLA typing [171].

Strengths of this work include inclusion of a rare sample group, namely HIV-seroconverters followed longitudinally from prior to the date of seroconversion and afterwards. Another strength was inclusion

of multiple loci for both HLA class 1 and HLA class 2 analysis, allowing a comprehensive overview. Many earlier studies have disregarded the potential influence of class 2 HLA antigens and focused solely on HLA class 1 antigens. We used highly sensitive Luminex single antigen methodology which detects even low-level antibody. We also looked specifically for complement-fixing class 2 HLA antibodies.

Weaknesses of the study include the relatively low number of HIV-seroconverters enrolled, leading to findings of borderline statistical significance that would benefit from replication in larger sample sets. Regarding complement-fixing antibodies, due to sample volume constraints, we focused on class 2 antibodies only and did not analyze complement-fixing class 1 HLA antibodies. We did not compare frequency of HLA alleles or haplotypes between groups. While HLA type and HLA antibodies may be interrelated variables, it is equally valid to interrogate either as the primary variable. We did not include antibody titre in our analysis, as each individual can have many HLA antibody specificities, each with its own titre. Also, the kits vary in the number of beads per low resolution antigen, for example kits may contain HLA-A*01:01 and HLA-A*01:02 beads, each of which may give a positive result with a different titre. Antibodies of a low titre may be no less relevant than antibodies of a high titre and information may be lost by summarizing an average or maximum titre antibody per locus. Hence we chose to express antibodies in a binary fashion as present or absent at the 2-digit resolution rather than add the complexity of multiple titres per individual per locus. While potential concerns of Luminex based methods include that of being overly sensitive or containing a mixture of intact and denatured HLA proteins, our single antigen kits have been shown to contain only intact HLA trimers, at least for class 1, [246], with clinical relevance of such antibodies best assessed through correlation with clinical outcome, unusually feasible in our rare cohort.

As most of our partners who were HIV-uninfected at enrolment were male, our reported frequencies of HLA antibodies may be lower than that of the general population, or of multiparous females in particular. It is interesting that we found such high frequencies of HLA antibodies in a predominantly male study cohort. Future analyses of HLA antibodies or alloimmunization strategies should be specifically powered to analyze males and females separately. Multiple host factors may influence HIV susceptibility (reviewed in[247]) and we could not control for all potential confounders. Our study was an epidemiological analysis and further mechanistic studies are warranted to probe interaction between HLA antibodies and HIV infection. Such mechanistic studies have already shown proof of concept that antibodies against heterologous proteins in the viral envelope do affect viral neutralization in vitro [248].

4.12 Conclusion

In summary, we have shown that more highly exposed persistently seronegative individuals had class 2 HLA antibodies than individuals who later acquired HIV infection. The presence of class 2 HLA antibodies was significantly associated with reduced odds of HIV acquisition, even after controlling for HIV-infected index partner viral load and other risk factors. These epidemiological data support the hypothesis that class 2 HLA antibodies may be partially protective against HIV acquisition; and re-invigorate the debate regarding potential merits of an alloimmunization approach against HIV, incorporating HLA class 2 alleles as vaccine antigens. A potential approach to explore the effect of class 2 HLA antibodies on HIV acquisition could include passive vaccination approaches which would be unlikely to harbour long-term risk of autoimmunity.

In terms of antibody prevalence, 79% percent of highly exposed, persistently seronegative healthy individuals harbored any HLA antibodies and 27% harbored HLA autoantibodies to at least one locus. HLA antibodies, including HLA autoantibodies, thus occur naturally without deleterious consequences. HLA antibodies are present in pharmaceutical preparations of intravenous immune globulins prepared from pooled blood donors (discussed in [171]) with no documented harm; while active immunization of pregnant women with partner white blood cells has previously been used as infertility treatment [249,250]. Fear of induction of autoantibodies remains the primary concern of an alloimmunization strategy and whether such antibodies are indeed harmful warrants directed investigation.

4.13 Author Contributions

MSS: lead author, conceptualization, sample retrieval, data retrieval, data analysis, wrote and revised original draft
NM: sample acquisition, critical review of manuscript
SM: laboratory work, critical review of manuscript
DdR: formative discussions, critical review of manuscript
RM: sample acquisition and retrieval, data retrieval, critical review of manuscript
JL: sample acquisition and retrieval, data retrieval, critical review of manuscript
XH: data retrieval, critical review of manuscript
HR: laboratory work, critical review of manuscript
SDM: sample acquisition, critical review of manuscript
DC: sample acquisition, critical review of manuscript
KO: data analysis, critical review of manuscript
LM: study planning, critical review of manuscript
CTT: study planning, critical review of manuscript
DMS: laboratory work, sample retrieval, data retrieval, study planning, critical review of manuscript

All authors attest they meet the ICMJE criteria for authorship. All authors have approved the final manuscript.

4.14 Conflict of interest statement

The authors declare no potential conflicts of interest.

4.15 Acknowledgements

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4.17 Supplementary Data

Please see Supplementary Tables 4.1-4.10 and Supplementary Figures 4.1-4.14

4.18 Location of References

Included within reference list of thesis.

5. Chapter 5 - Natural History of HLA antibodies

5.1 Overview of work on mothers and infants

After investigation of HLA antibodies in the context of HIV infection risk, I wanted to characterize development of HLA antibodies using Luminex single antigen methodology in the physiological situation of pregnancy and early life. Development of HLA antibodies may shed light on their potential role in health of newborn infants. Such antibodies have unknown relevance to infectious risk in neonates. Published work discussed in the introduction explored association of HLA antibodies with risk of vertical HIV transmission from mother to child, however there are no studies to my knowledge exploring association of HLA antibodies with risk of other infections in young infants. A first step to understanding the role of HLA antibodies in infants is to explore whether maternal HLA antibodies are directed at antigens present in the foetus, whether such antibodies cross the placenta, and at what age infants produce their own HLA antibodies.

HLA antibodies present in maternal circulation raise the intellectual conundrum that antibodies are known to pass from mother to infant, yet it is well known that mothers make HLA antibodies against foetal HLA antigens derived from the infant's father. Thus HLA antibodies directed against infant HLA genotypes should likely cross the placenta to the infant's circulation. In the infant, such antibodies would be autoantibodies, directed against the infant's own HLA genotype. This question is an intriguing avenue by which to explore development of the neonatal immune system and raises questions of how immune tolerance to neonatal antigens comes about. Either there must be selective antibody transfer to the infant such that antibodies directed against neonatal antigens do not cross the placenta, or the presence of antibodies against the infants HLA may be harmless and present in healthy infants (perhaps even beneficial to the infant), or there may be active immunological mechanisms by which HLA antibodies are removed or suppressed by the infant immune system. Despite the obvious clash with the dogma that autoantibodies are associated with autoimmune disease, this question has not been thoroughly explored. I am aware of one previous study in which Ravindranath and colleagues showed mismatched HLA antibodies in the sera of mothers and cord blood of infants, including instances of HLA antibodies present in the mother not seen in the infant, HLA antibodies present in the infant undetected in the mother, and maternal HLA autoantibodies [171].

In this chapter, I explored the natural history of HLA antibody development by analyzing class 1 and class 2 HLA antibodies in pregnant women and their six week old infants. I analysed whether antibody specificities in the mother were identical, termed "shared antibodies" to that in their infants. Unfortunately, as I used residual samples from a bigger study, I did not have infant DNA for HLA genotyping to assess whether HLA antibodies present in the mothers and infants were directed against HLA genotypes of the infant (matching antibodies). In a small group of six infants from a second cohort we had DNA available from the infants and have presented these mother-infant dyads as case-studies. This work has been published in the American Journal of Reproductive Immunology [238] and is included here.

5.2 Published article on mother to child transmission of HLA antibodies

HLA ANTIBODY REPERTOIRE IN INFANTS SUGGESTS SELECTIVITY IN TRANSPLACENTAL CROSSING

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ABSTRACT

Problem: Late in pregnancy, women produce and transfer high amounts of antibodies to the foetus. During gestation, women produce antibodies against human leukocyte antigens (HLA), including antibodies directed at foetal HLA. There is paucity of data on transplacental crossing, specificity, and role of HLA antibodies in pregnancy and new-borns.

Methods: Using highly sensitive Luminex technology, we measured prevalence of IgG HLA antibodies in 30 mother-infant pairs six weeks post-partum. Additionally, in six pregnant women, we measured HLA antibodies longitudinally and HLA-typed infant DNA to assess whether maternal HLA antibodies were directed at infant specificities.

Results: Overall, 68% of mothers and 44% of infants expressed HLA-I antibodies and 56% of mothers and 52% of infants expressed HLA-II antibodies. Infants shared up to 78% of antibodies with their mothers, suggesting that the remaining antibodies were self-made. Less than 25% of maternal HLA antibodies were detected in infants, possibly due to selection in transplacental crossing. We detected complement fixing HLA antibodies in mothers and at low levels in infants. In a third of our pregnant subjects, we detected infant-directed HLA antibodies.

Conclusions: Our findings raise the possibility of selection in transplacental crossing of HLA antibodies. As HLA antibodies may act as autoantibodies in the neonate, the mechanism of a selective transfer may give important insights into immune tolerance. Findings also suggest that infants start producing their own HLA antibodies in the first weeks of life, which, together with maternally derived antibodies may impact the infant's immune reaction to HLA proteins.

KEYWORDS

HLA antibodies; HLA; pregnancy; alloimmunity; immune tolerance; placenta; transplacental crossing

INTRODUCTION

Human leukocyte antigens (HLA) are proteins responsible for presentation of short peptides to responding T lymphocytes[1-3]. By binding to T cell receptors on the surface of T lymphocytes, HLA proteins lead to T cell activation, as well as to recruitment of additional immune players[2,3]. HLA class I proteins, encoded by the loci HLA-A, HLA-B and HLA-C, are expressed on the surface of all nucleated cells, whereas HLA class II proteins, encoded by the loci DRB1, DQA/B, DRB3,4,5 and DPA/B, are expressed only on the surface of professional antigen presenting cells. Together, the seven HLA-encoding loci are the most polymorphic genes in the human genome, with thousands of alleles expressed in the population[1-3]. This high polymorphism may result in HLA-induced alloimmunity mediated by antibodies targeted against HLA, referred to as HLA antibodies.

For a long time, HLA antibodies were believed to be expressed only following sensitizing events such as blood transfusions, multiple pregnancies or intravenous drug use. However, modern bead-based technology has led to the discovery of previously undetectable low levels of HLA antibodies in up to 77 percent of healthy individuals with no history of sensitizing events[4-10]. Such antibodies may be against one or more HLA specificities[6,11], although whether they are produced in response to these HLA antigens or just cross-react with them is not clear at this point.

During pregnancy, women produce and transfer a large quantity of antibodies to the foetus[12,13], which is well described for antibodies against pathogens such as measles or pneumococcus[14-16]. This process begins at around 13 weeks of gestation and increases with gestational age, with the majority of antibodies crossing the placenta during the third trimester[12,17-20]. Transplacental crossing is mediated by neonatal FcRn receptors, and most antibodies transferred are IgG[12,20-24]. It has been found that infants born preterm have markedly lower IgG levels at birth compared to term infants[12,25]. IgA antibodies have also been shown to cross the placenta[13,26,27]; however, this occurs at low levels, as the main mode of transferring IgA antibodies to the baby is through breastfeeding[28].

Transplacental crossing of antibodies has recently been shown to involve some degree of selection based on post-translational modifications in the fragment crystalline (Fc) region[29,30]. In these studies, IgG molecules with digalactosylated glycans in the Fc region were shown to have higher binding affinity to FcRn, leading to more efficient transplacental crossing. Several studies have suggested that post-translational modifications in Fc regions of antibodies may be antigen-dependent[31,32]. However, since no evidence has been found so far for an antigen-dependent selection in transplacental crossing, we hypothesize that antibodies produced during pregnancy cross the placenta without depending on their target antigen. Intriguingly, in the case of HLA antibodies, placental transfer may result in the presence of maternally-derived, paternally directed antibodies in the foetus, potentially acting as autoantibodies. The impact of such antibodies on the foetus and later, infant, is not known.

The production of HLA antibodies during pregnancy was first observed by Van Rood et al. in 1958[33]. Since then, efforts to measure their prevalence and uncover their role have been relatively scarce. It has been shown that HLA antibodies – both non-specific, and those targeting paternal HLA proteins – develop in many, but not all healthy pregnancies after 20-28 weeks of gestation[19,34-37]. The frequency of these antibodies increases with parity, reaching 74% in women who have had more than two deliveries[38]. Interestingly, HLA antibodies that appear during pregnancy are not always detected in women several years after delivery but may persist in immunological memory[39].

The impact of HLA antibodies on pregnancy outcome is not understood. Some studies have found an association between high levels of HLA antibodies in pregnancy, both in early[35,40] and late[41-44] gestational age, and a poor pregnancy outcome. However, other studies suggest that high levels of HLA antibodies in pregnancy have a positive impact on pregnancy outcome[36,45]. In line with the latter, immunotherapy with paternal lymphocytes resulting in HLA antibody production may be a therapeutic option in women with recurrent miscarriages[46,47]. Yet a third line of studies, however, has found no substantial correlation between HLA antibodies and pregnancy outcome[48,49]. Supporting this, is the observation that pregnancy following oocyte donation – associated with a higher risk of developing child-specific HLA antibodies – is not associated with immunological complications[50].

The inconsistency arising from existing studies on HLA antibodies in pregnancy demonstrates gaps in our understanding. Similarly, we know little about the development of HLA antibodies in the foetus and after birth. In humans, the foetus has been shown to develop IgM and IgA antibodies[20,51,52]. IgM is usually the first class of antibody to be produced by B lymphocytes, having high avidity and agglutinating activity, but reduced specificity and antigen affinity[36]. When the human body further encounters antigen, B lymphocytes switch their isotype from IgM to IgG, which, in addition to being more specific, also possesses the widest range of effector functions[36]. Based on evidence so far[12,51,53], foetuses do not produce their own IgG antibodies, suggesting that IgG – including potential HLA antibodies – present at birth originate in the mothers. The specific timepoint and triggers for production of IgG in new-borns are not known.

In the current study, we investigated the HLA antibody profile in women and their infants six weeks post-partum, as well as in pregnant women. We used two cohorts; a first cohort of six-week-old infants and their mothers (referred to as the “mother-infant cohort”), and a second cohort of pregnant women at different timepoints in pregnancy and post-partum (referred to as the “pregnancy cohort”). We detected IgG HLA antibodies and characterized their specificities, sub-class distribution and expression levels; additionally, we tested the mother-infant cohort samples for the presence of complement-fixing HLA antibodies.

METHODS

Study design and sample collection

Mother-infant study

Thirty healthy mother-infant pairs available from a previously completed study on rotavirus vaccine immunogenicity (NCT02215226[54]) were used. The study, conducted in Soweto, South Africa in 2009-2010, explored the effect of at least an hour abstention from breast feeding at immunization visits on immune responses of infants to rotavirus vaccine. The study enrolled black South African mother-infant pairs who were human immunodeficiency virus (HIV)-negative, at their six weeks post-partum visit. Blood samples were drawn from all participants at enrolment, immediately before administration of the rotavirus vaccine to the infants. The serum fraction was separated by centrifugation and stored at -70^{0}C until further use. Infant DNA was not available from this cohort.

Longitudinal Pregnancy study

Twenty-nine women in their first trimester of pregnancy were recruited from collaborating gynaecologist practices (the Charlotte Maxeke Johannesburg Academic Hospital and Netcare private clinics in South Africa). Recruited patients were of both black and white South African ethnicity. All patients were HIV-negative, multigravida and over 18 years of age at enrolment. Blood samples were drawn from participants at routine visits to the clinic, including during the first (up to 13 weeks of pregnancy), second (14-26 weeks of pregnancy), and third (27-40 weeks of pregnancy) trimesters, and/or 8-11 weeks post-partum. Buccal swabs were collected from infants at the post-partum visit and later processed for DNA extraction. Mothers' serum was stored at -70^{0}C until further use. Samples of six participants with healthy and uncomplicated pregnancies, who had at least two visits to the clinic and for whom buccal swabs were successfully collected from their babies, were analysed and their results presented here as case studies. The remaining participants are not presented here due to missing data such as incomplete infant HLA typing results or maternal serum timepoints. We did not collect serum from infants in this study.

Measurement of HLA antibody levels

The prevalence of IgG HLA antibodies was measured in serum of mothers and infants from the mother-infant cohort, as well as that of mothers from the pregnancy cohort. Presence, strength (expression levels) and specificity of antibodies were measured using Luminex technology. Commercial kits for detection of IgG antibodies against HLA class I and HLA class II (LSA class I/II single antigen kits; Immucor, Norcross, Georgia, USA) were used according to manufacturer's instructions. Antibodies detected included anti-HLA-A, HLA-B, and HLA-C for class I, and anti-DRB1, DRB3,4,5, DQA/B and DPA/B for class II. The data obtained from these experiments was subsequently analysed using Match-It software (Immucor, Norcross, Georgia, USA), according to manufacturer's instructions. The HLA antibody strength was recorded as adjusted mean fluorescent intensity (AD-MFI), with additional interpretive comments: positive, weak, possible, or absent. Positive and weak were considered positive results, while possible and absent were considered negative results. Highly reactive sera samples were purified by adsorption of non-specific antibodies using commercial "Sera clean" kit (Immucor, Norcross, Georgia, USA), followed by a second analysis of HLA antibodies. For the mother-infant study, antibody specificities were also labelled as shared (detected in both mother and infant) or mother-only/infant-only. For the pregnancy study, HLA antibodies were categorized as baby-directed (targeting HLA types of the baby) or non-specific. Additionally, antibodies were categorized as recurring (expressed at more than one timepoint) or single timepoint, in order to describe development of new antibodies at various timepoints.

Complement-fixing HLA antibodies were detected in serum of 20 mothers and infants from the mother-infant study by running HLA-I/II antibody assays as described above but using complement component C3d as a detection agent to bind to complement fixing antibodies in serum. After a wash step, bound C3d was detected with fluorescently labelled antibody against C3d (C3d kit, Immucor, Norcross, Georgia, USA). The data obtained was subsequently analysed using Match-It software (Immucor, Norcross, Georgia, USA), as described above.

DNA extraction

Buccal swabs of infants in the pregnancy study were soaked in DNA extracting solution (QuickExtract™ DNA Extraction Solution, Epicentre, Madison, WI, USA), followed by DNA extraction using a commercial kit (QuickExtract™ DNA Extraction kit, Epicentre, Madison, WI, USA). Next, DNA was precipitated as follows. Briefly, samples were mixed with sodium acetate in a 1:10 volume, followed by addition of 2.5 volumes of absolute ethanol and incubation at -70^{o}C. Following incubation, the samples were centrifuged, the pellet was washed with 70% ethanol and then eluted with a commercial elution buffer (QIAamp DNA Mini kit, Qiagen, Germany), quantified, and stored at -20^{0}C. DNA was not available in the mother-infant cohort and therefore no DNA analysis was carried out on these samples.

HLA typing

Class I and II HLA genotyping was performed for DNA prepared from infant samples collected in the pregnancy cohort. Commercial kits for HLA-A, HLA-B, HLA-C, DRB1, DRB3,4,5, DQB1 and DPA/B loci (Immucor, Norcross, Georgia, USA) were used for locus-specific PCR amplification according to manufacturer's instructions, followed by detection via allele-specific probes using a Luminex instrument (Bioplex, Biorad, Hercules, California, USA) and Match-It DNA software (Immucor, Norcross, Georgia, USA). The typing was reported at the 2/4 - digit resolution.

Statistical analysis

Data analysis was performed using version 8.2.1 (441) of the GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A p value of 0.05 or lower was considered statistically significant. Variables were compared using χ^2 test (for number of specificities per individual) or Mann Whitney test (for antibody strength).

Ethics

Our mother-infant study, conducted on samples from the previously published NCT02215226 study, was approved by the Human Ethics Research Committee of the University of the Witwatersrand with consent for storage and testing (M1611144). The pregnancy study was approved by the Human Ethics Research Committee of the University of the Witwatersrand (M130230).

RESULTS

Mother-infant study

HLA antibody specificities in mother and infants

We first characterized the prevalence of IgG HLA antibodies in mothers and infants. HLA antibodies detected in each mother-infant pair are listed in Supplementary Tables 1 (class I) and 2 (class II), including those detected only in mother, those detected only in infant, and those that were shared between the two. Our data reveals great diversity in number and type of specificities between the pairs. We found that of 25 mothers, 20 (80%) expressed HLA antibodies of at least one class (Figure 1A), 17 (68%) expressed HLA class I antibodies (Figure 1B) and 14 (56%) expressed HLA class II antibodies (Figure 1C). In infants, of 25, 18 (72%) expressed HLA antibodies of either class, 11 (44%) expressed HLA class I antibodies, and 13 (52%) expressed HLA class II antibodies (Figure 1A, B and C, respectively).

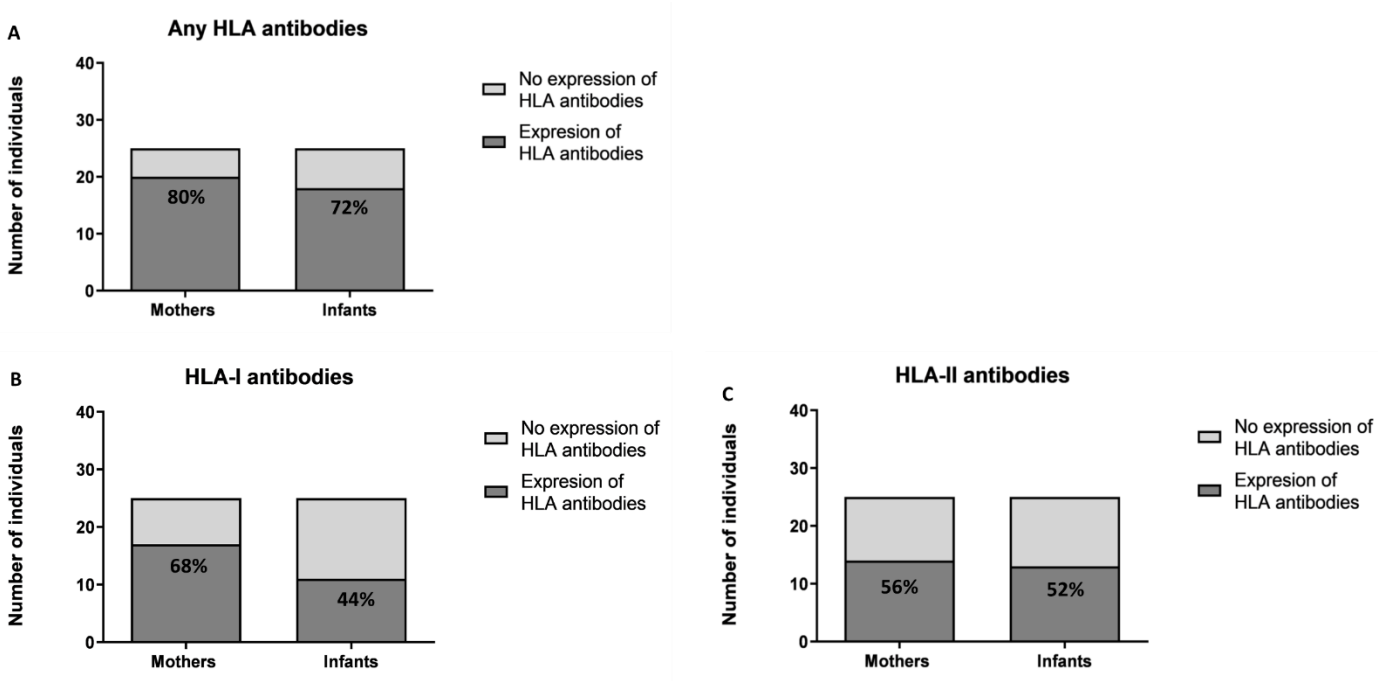


Figure 5.1: Number (and percentage) of mothers six weeks post-partum and their six-week-old infants, who express HLA antibodies of at least one class (**A**), HLA class I antibodies (**B**), and HLA class II antibodies (**C**) out of total number of individuals (n = 25; all antibodies are IgG)

HLA antibody subclasses

Each HLA-I antibody subclass, namely HLA-A, HLA-B and HLA-C, showed a similar pattern, with a higher proportion of specificities apparent in the mother only, and a low proportion of specificities apparent in infants only and in the group shared between mothers and infants (Figure 2A-C). More specifically, out of 29 mothers, 18 (62%), 18 (62%) and five (17%) expressed HLA-A, HLA-B and HLA-C antibodies that were not detected in their infants, respectively. However, only five (17%) infants expressed anti-HLA-A antibodies, five (17%) expressed anti-HLA-B, and only one (3%), expressed anti-HLA-C. Similarly, low proportions of antibodies were shared between mothers and infants, with six pairs (21%) expressing HLA-A antibodies and seven pairs (24%) expressing HLA-B antibodies; notably no HLA-C antibodies were shared between mothers and infants.

Similarly, for HLA class II, a high proportion of mothers (9/25, 12/25 , 4/25 and 4/25, which represented 36%, 48%, 16% and 16% out of all mothers) expressed DRB1, DQA/B, DRB3,4,5 and DPA/B antibodies,

respectively, that were not detected in their infants. There were few infant-only antibodies (2/25, 8/25, 1/25 and 2/25, equal to 8%, 32%, 4% and 8%, respectively) as well as few shared antibodies (1/25, 3/25, 2/25 and 2/25, equal to 4%, 12%, 8% and 8%, respectively). DQA/B antibodies was the predominant HLA-II antibody sub-class in mothers and babies (Figure 2D-G).

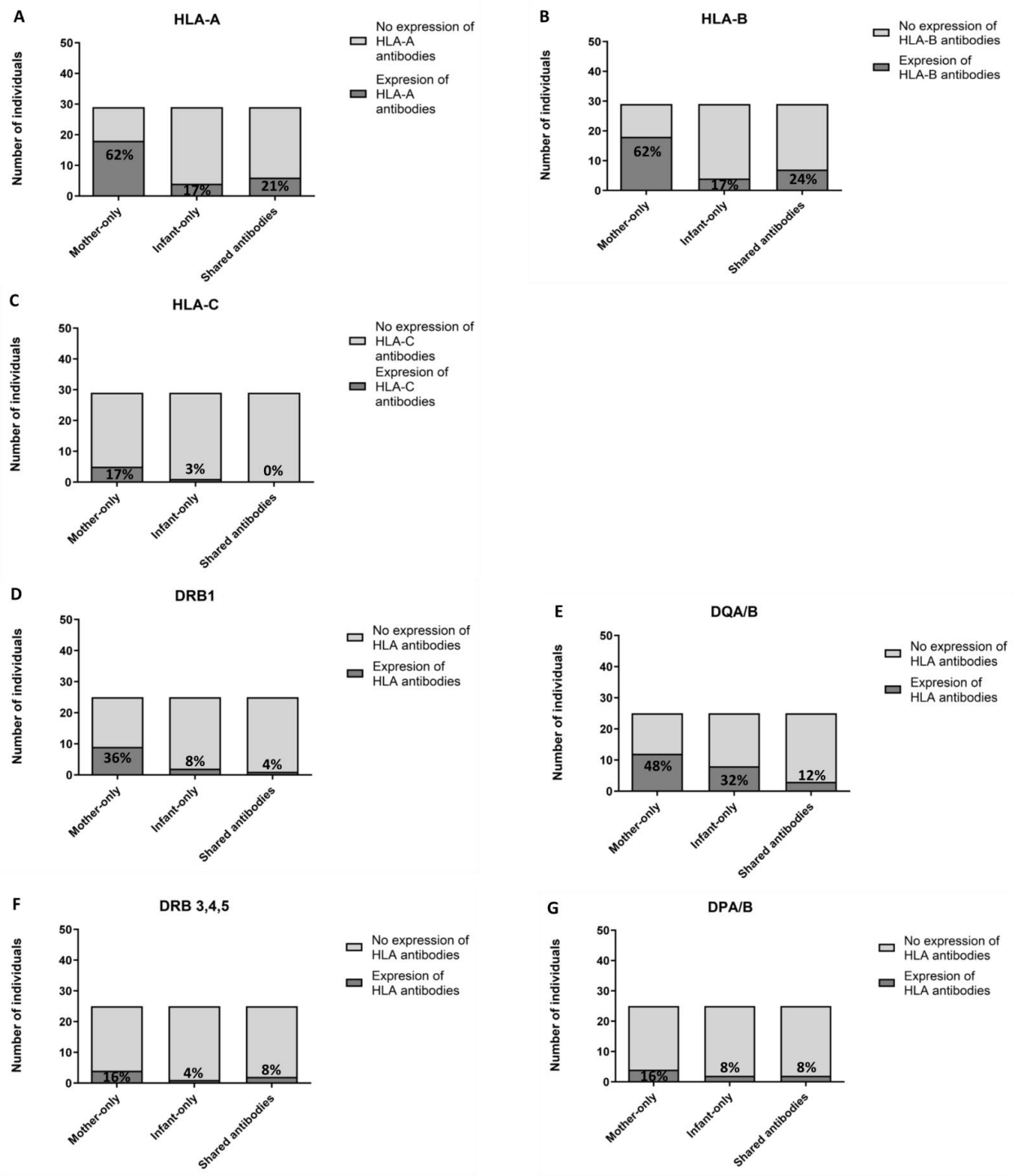


Figure 5.2: Number (and percentage) of mothers six weeks post-partum and their six-week-old infants, who express HLA class I antibodies (**A**, **B**, **C** for HLA-A, HLA-B and HLA-C, respectively; n = 29) and HLA class II antibodies (**D**, **E**, **F**, **G** for DRB1, DQA/B, DRB3,4,5 and DPA/B, respectively; n = 25) that were detected only in mothers (Mother-only antibodies), only in infants (Infant-only antibodies) and in both mother in infant (Shared antibodies); all antibodies are IgG.

Number of target HLA specificities per individual

Next, we measured the number of different HLA specificities targeted by mothers and infants (Figure 3). We sought to determine whether infants expressed the same repertoire of HLA antibodies as their mothers, with the assumption that such antibodies would be of maternal origin. In contrast, we postulated that antibodies detected in infants but not in their mothers were either self-made, or, less likely – of maternal origin but undetectable in the mothers; and that antibodies detected in mothers but not in their infants were either produced during pregnancy yet not transferred to the foetus, or produced post-partum.

We found that overall, infants targeted significantly fewer HLA specificities than mothers (p value < 0.05 for both HLA classes). Interestingly, only some of these specificities were shared with their mothers (a mean of 78% for HLA-I and 50% for HLA-II, Figure 3A and 3B, respectively). Surprisingly, we also found that most specificities detected in mothers were not detected in their babies (a mean of 84% for HLA-I and 76% for HLA-II, Figure 3A and 3B, accordingly).

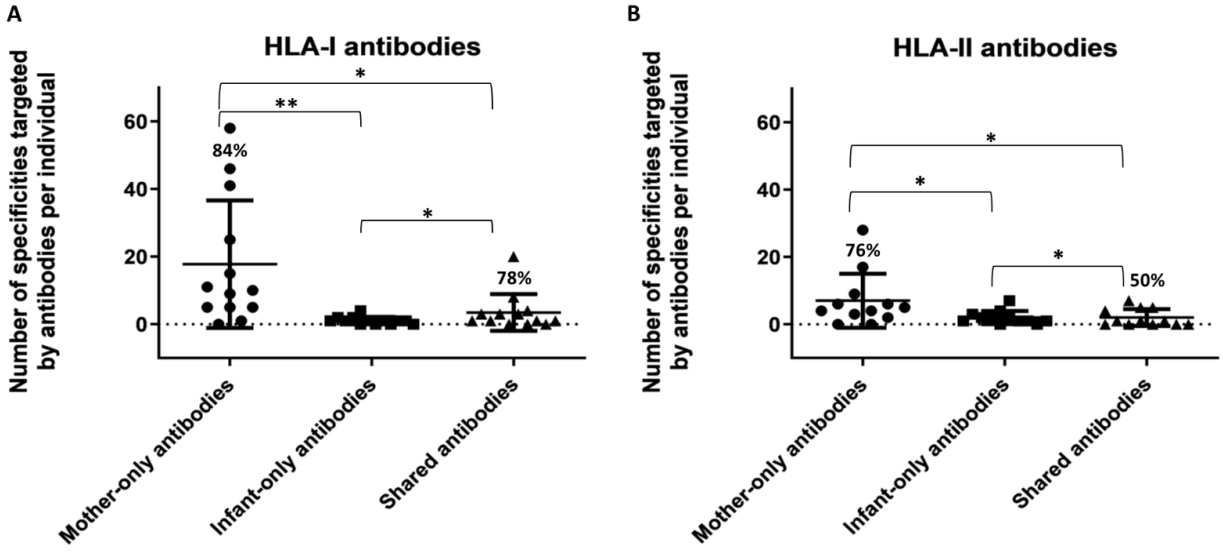


Figure 5.3: Number of different specificities targeted by HLA antibodies (class I, **A** and class II, **B**; all antibodies are IgG) in mothers six weeks post-partum and their six-week-old infants. Number of antibody specificities detected only in mother (Mother-only antibodies), only in infant (Infant-only antibodies), or those detected in both mother and infant (Shared antibodies) are shown (n = 29; * represents 0.05 > p value > 0.001; ** represents a p value < 0.001; p

value was determined by Student's T test; median, 25th and 75th percentiles are shown). On top of the Mother-only antibodies column – percentage out of total antibodies detected in mothers. On top of the Shared antibodies column – percentage out of total antibodies detected in infants.

Expression intensity of HLA antibodies

The expression intensity was significantly lower for antibodies detected in infants compared to antibodies detected in mothers (p value < 0.001 and < 0.05, presented in Figure 4A and 4B, for HLA-I and HLA-II antibodies, respectively). Interestingly, this included antibodies shared between mother and infant, possibly indicating that only a fraction of the amount of antibody produced in mothers crossed the placenta.

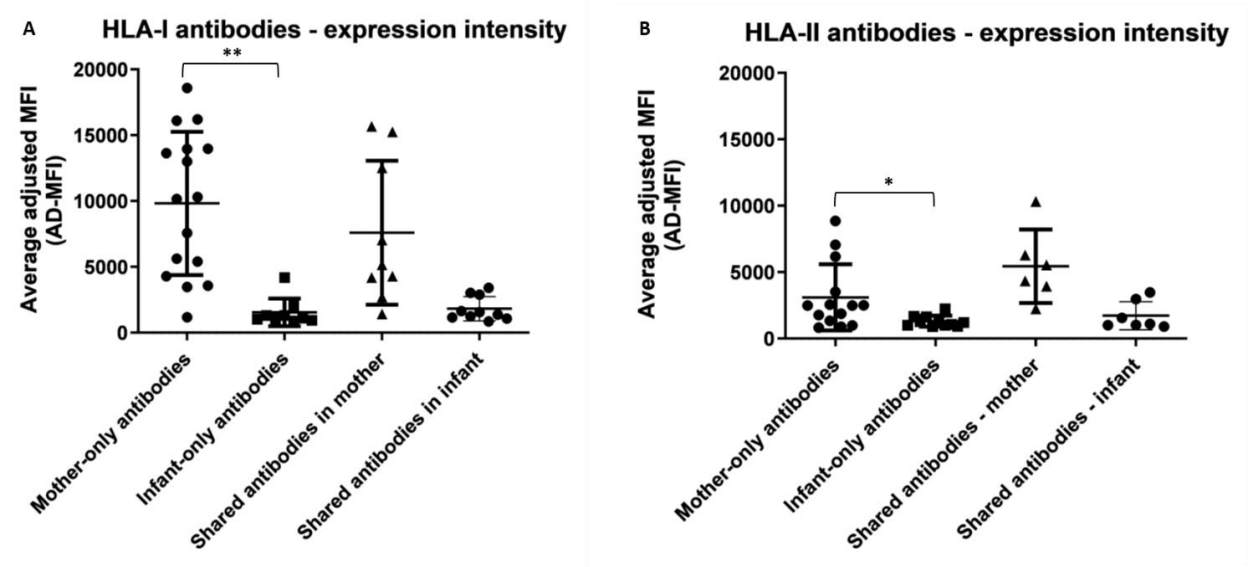


Figure 5.4: Intensity of antibody expression. Mean AD-MFI (adjusted MFI as calculated by the MatchIt analysis software) of antibodies detected only in mothers (Mother-only), only in infants (Infant-only), and those detected in both mother and infant (Shared antibodies) are shown for HLA-I (**A**, n = 17; ** represents p value < 0.001) and HLA-II (**B**, n = 15; * represents 0.05 > p value > 0.001) antibodies (all antibodies are IgG; p value was determined by Mann Whitney test; median, 25th and 75th percentiles are shown).

Complement-fixing ability of detected HLA antibodies

As expected, we detected complement-fixing HLA antibodies in mothers, with 55% and 40% expressing HLA-I and HLA-II antibodies, respectively (Figure 5A). The number of HLA specificities varied between mothers (Figure 5B) but was in the same range as the maternal HLA IgG antibodies (Figure 3). For HLA-I, the expression intensity of these complement fixing antibodies was generally lower than that observed for maternal IgG; however, for HLA-II the intensity was overall higher than that of maternal IgG (Figure 5C). In infants, we could not detect complement-fixing HLA class I antibodies (Figure 5A), indicating that whether self-made or of maternal origin, HLA class I antibodies do not activate complement at six weeks of age. In contrast, we did detect complement-fixing HLA class II antibodies in 35 percent of the infants (Figure 5A). However, HLA class II antibody numbers per individual (Figure 5B) and expression levels (Figure 5C) were extremely low (p value < 0.001 for mean antibody intensity of mothers versus infants),

possibly because their production might only begin at six weeks of age. We detected infant complement fixing HLA-II antibodies shared with the mother in only one infant (not shown).

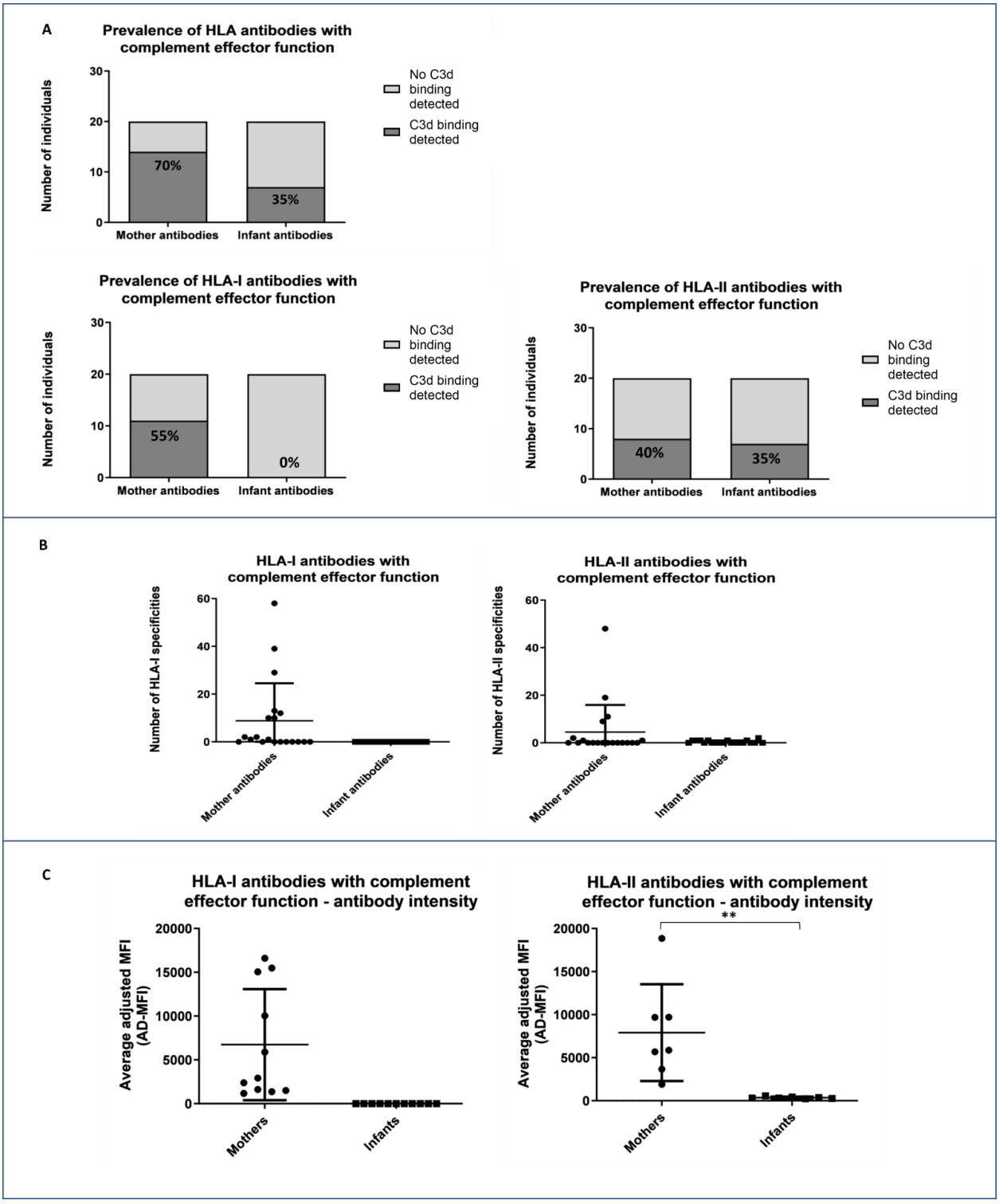


Figure 5.5: HLA antibodies with complement effector function (as detected by C3d binding) in mothers six weeks post-partum and their six-week-old infants: (**A**) Number (and percentage) of mothers and infants who express complement-activating HLA antibodies out of total number of individuals (n = 20); (**B**) Number of different HLA specificities per individual of complement-activating antibodies (n = 20; median, 25th and 75th percentiles are shown); (**C**) Antibody expression intensity (average AD-MFI as calculated by the MatchIt analysis software) of complement-activating HLA antibodies (n = 20; ** represents p value < 0.001; p value was calculated using Mann-Whitney test; median, 25th and 75th percentiles are shown).

Pregnancy study

In six women, referred to as Mother A-F, we measured the number and specificities targeted by maternal IgG HLA antibodies (Figure 6, class I and Figure 7, class II) at different stages of gestation and/or post-partum. All women expressed HLA class I (Figure 6), as well as HLA class II (Figure 7) antibodies in the pregnancy timepoints tested; however, there were noticeable variations between women in number of specificities. In the post-partum timepoint three out of four women expressed HLA class I antibodies (Figure 6) and two out of four women expressed HLA class II antibodies (Figure 7). We further assessed whether maternal antibodies targeted HLA specificities of the infant by genotyping the infant HLA-B, HLA-C, DRB1, DQA/B, DRB3,4,5, DPA/B, and classifying the maternal antibodies into baby-directed or non-specific. For both classes, two out of six women (mothers D and E for HLA-I, and E and F for HLA-II) expressed baby-directed HLA antibodies.

Next, we classified the maternal antibodies into single timepoint (detected only at one timepoint) or recurrent (detected in at least two timepoints), in an attempt to assess the appearance of new antibodies. Overall, once antibodies were present in the mother, they recurred at subsequent timepoints. However, we did find antibody specificities that were detected once but not subsequently, as seen in mothers A, C and F (Figure 6) for HLA-I and mothers A, C, D and F (Figure 7) for HLA-II. Development of new HLA antibodies in the second timepoint compared to the first, was apparent in four mothers for HLA-I (A, D, E and F, Figure 6), and four mothers for HLA-II (B, D, E and F, Figure 7).

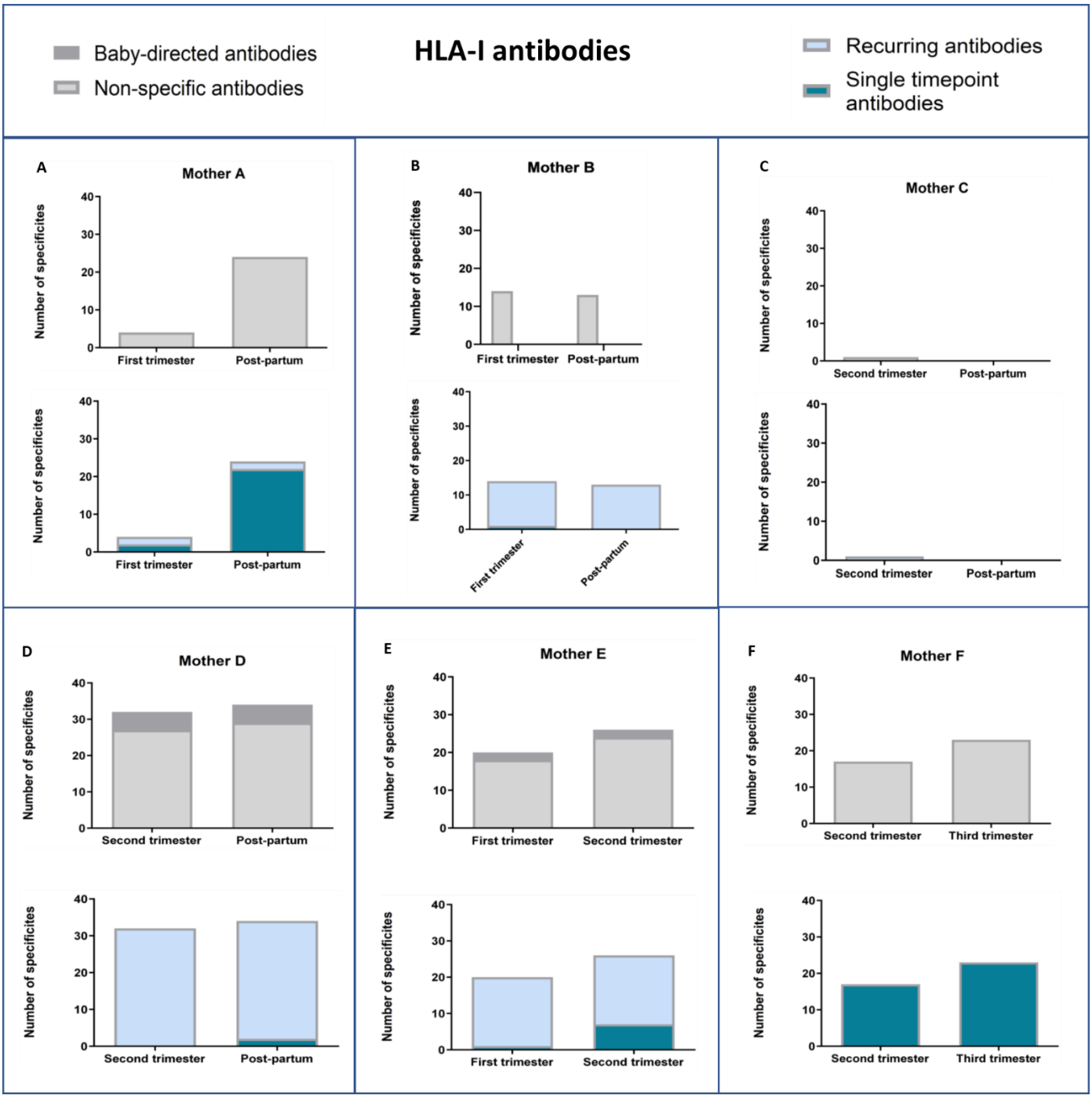


Figure 5.6: Number of different HLA class I antibodies in six pregnant women (**A-F**) as detected at different timepoints in pregnancy (all antibodies are IgG). Two graphs are shown for each woman: the top graph presents baby-directed and nonspecific antibodies (shown in dark and pale grey, respectively); the lower graph presents recurring and single timepoint antibodies (shown in pale and dark blue, respectively). Baby-directed HLA-I antibodies were detected in two out of six mothers (mothers D and E). Development of new HLA-I antibodies is apparent in mothers A, D, E and F. Certain antibody specificities in mothers A, C and F were detected once but not subsequently.

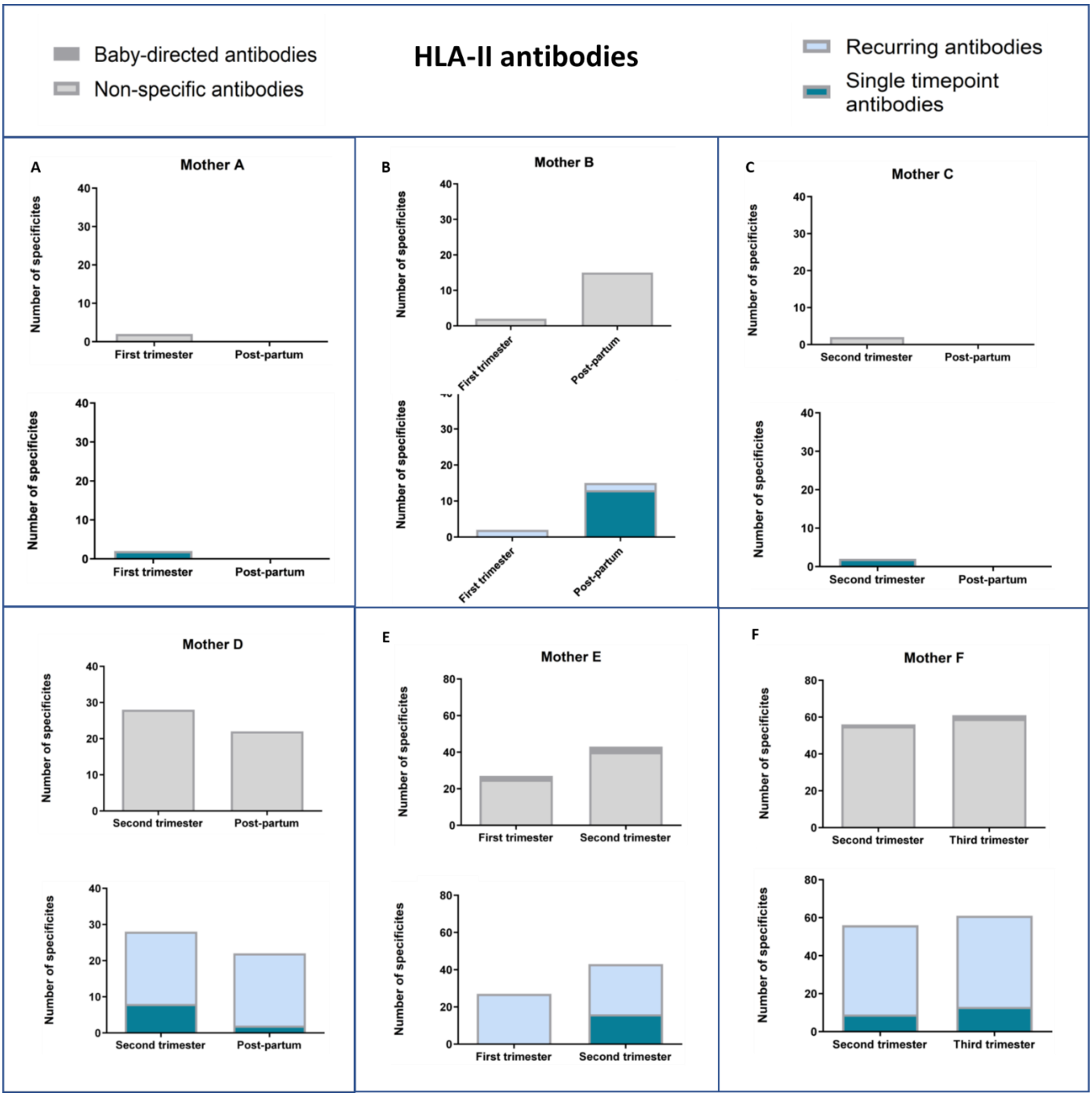


Figure 5.7: Number of different HLA class II antibodies in six pregnant women (**A-F**) as detected at different timepoints in pregnancy (all antibodies are IgG). Two graphs are shown for each woman: the top graph presents baby-directed and nonspecific antibodies (shown in dark and pale grey, respectively); the lower graph presents recurring and single timepoint antibodies (shown in pale and dark blue, respectively). Baby-directed HLA-II antibodies were detected in two out of six mothers (mothers E and F). Development of new HLA-II antibodies is apparent in mothers B, E and F. Certain antibody specificities in mothers A, C, D and F were detected once but not subsequently.

DISCUSSION

Currently, it is not known whether HLA antibodies play a role in pregnant women or if they are essential for maintaining a healthy pregnancy. However, many studies suggest that high levels of HLA antibodies, at least (and perhaps only) in late stages of pregnancy, are important for a successful outcome. We found that most women in our two cohorts expressed HLA antibodies in their pregnancy and/or post-partum (Figures 1, 6, 7). To our surprise, in the mother-infant cohort, a relatively small fraction of IgG HLA antibodies detected in mothers post-partum was found in the infants (these were termed "shared antibodies"). If we assume that the mothers produced the HLA antibodies detected post-partum during pregnancy, the low levels of shared antibodies in infants suggests selection in transplacental crossing. So far, the efficiency of placental crossing has not been shown to depend on antibody specificity, and hence, the mechanism behind such selection remains to be explored.

It is also possible, however, that pregnant women produce and transfer HLA antibodies to the foetus, but rather than being selected against at placental crossing, some of the transferred antibodies might be degraded in the infants by the time they are six weeks of age, thus resulting in lower prevalence and intensity of shared antibodies in infants compared to mothers.

Alternatively, some of the maternal HLA antibodies we detect in mothers post-partum may have been produced during the first six weeks after delivery rather than during pregnancy. Theoretically, HLA antibodies produced post-partum may still be passed on to the infants through breastfeeding. However, this would likely be at low levels as transfer of IgG antibodies via breastmilk is limited[55,56].

In the future, it would be interesting to explore the possibility of selection in transplacental crossing of HLA antibodies, including possible post-translational modifications that would increase their FcRn binding affinity, and a potential link between such modifications and antigen specificity. One interesting aspect of a theoretical antibody selection would be the fate of antibodies that may become autoantibodies in the foetus, directed at paternal HLA proteins. In our longitudinal pregnancy study, half of the women expressed a small proportion of baby-directed HLA antibodies of at least one class. However, we do not know whether these antibodies were transferred to the foetuses, and if so, what impact they might have had on the foetal and later, infant immune system.

Another interesting aspect of a potential selection is the transfer of the HLA-C antibodies. In the mother-infant cohort we found that the shared antibodies did not include any HLA-C antibodies, which may be due to our small sample size, especially in light of the small proportion of maternal HLA-C antibodies within the class-I HLA antibodies. However, it is also possible that HLA-C antibodies are less preferentially transferred to the foetus. Interestingly, at the beginning of pregnancy, placental trophoblast cells express HLA-C proteins on their membranes, later internalizing them. The levels of HLA-C in placental trophoblast cells are then highly increased at birth[57]. Hypothetically, HLA-C antibodies may be selected against for transplacental crossing, possibly to lower the chances of transferring autoantibodies to the foetus. However, at this point, this scenario remains speculative.

Unlike antibodies targeting pathogenic antigens, whose role is well understood, the role of HLA antibodies in the foetus, and later in the child, is not known. A study by Gros and colleagues[58] suggested that maternal antibodies transferred to the foetus during pregnancy or to the infant via breast milk may act as immunomodulators, leading to the development of specific and long-lasting immune responses in the infant. However, whether this is the case for HLA antibodies is not known. It has been shown that antibodies against a variety of HLA alleles may be produced after exposure to a specific HLA antigen[59]. The

significance of HLA alloimmunity and whether humans need to develop immunological tolerance or alloimmunity against HLA proteins, remain to be established.

HLA antibodies may play a crucial role in infection with enveloped viruses, such as the Human Immunodeficiency Virus (HIV), Epstein-Barr Virus (EBV), influenza or measles. While budding out of host cells, enveloped viruses incorporate different host proteins, including HLA, which they then introduce to new hosts[60]. Therefore, individuals who express HLA antibodies may have pre-existing immunity to pathogens. However, whether this is beneficial, harmful or simply irrelevant, still needs to be determined. If pre-existing antibodies increase protection, we expect that the more HLA antibodies present, the higher their protection. However, it is also possible that HLA antibodies may, in fact, increase susceptibility to certain viruses. This may be due to the HLA antibody-induced recruitment of immune cells that some viruses may easily infect. To note, previous studies in macaques and non-human primates have suggested a protective role for HLA antibodies against infection with the enveloped simian immunodeficiency virus (SIV)[61-63]. Interestingly, it was found that administration of vaccines against infectious agents such as the influenza and measles viruses may induce *de-novo* production of HLA antibodies[64-66]; the impact of these antibodies on future infections remains to be determined. It is tempting to envision the use of HLA antibody-based alloimmunity for vaccine strategies in pathogenic diseases caused by enveloped viruses that incorporate host-derived HLA proteins or those like HIV that express viral proteins with high structural homology to HLA proteins[62,67-70]. Interestingly, antibody transfer from mother to foetus is compromised in various conditions affecting placental integrity, including placental malaria and HIV infection[15,16,30,71,72]. It would be interesting to see whether these conditions also impact the transfer of HLA antibodies, and if so, what effect impaired transfer has on susceptibility to infection in the foetus and after birth.

It has recently been shown that in the first weeks of life, the infant's immune system undergoes drastic changes that comprise maturation of most immune cells, including B lymphocytes[73,74]. This rapid maturation results in differences between the immune profile at birth (as seen in cord blood) and that of infants several weeks of age. Maturation may include the ability of B cells to switch from IgM (which is already produced in the foetus) to other types of antibodies, presumably in response to specific antigens. In our study, we find that at six weeks of age, infants express IgG HLA antibodies, including a considerable number of antibodies that are not found in their mothers, which may be self-made antibodies. Whether these antibodies are produced as "natural antibodies" or in response to specific antigens warrants further investigation. To note, we cannot exclude the possibility that at least some of the HLA antibodies found in infants but not in their mothers, are in fact of maternal origin, having been present antenatally yet becoming undetectable in the mother post-partum.

In our study we show that amongst HLA antibodies produced by the mothers, some antibodies have complement effector functions, likely in keeping with IgM isotype. The complement system may be activated by IgM or IgG antibodies; however, unlike IgG, which has multiple effector functions, complement activation is almost the sole effector function of IgM antibodies[75]. Thus, in infants, the absence of complement-fixing HLA class I (but not class II) antibodies suggests that their HLA-I antibodies are unlikely to be IgM.

Limitations and Strengths

Strengths of this study include the use of highly sensitive single antigen technology which can detect low intensity antibodies. The study is unusual in that it includes healthy Black African women and infants with no underlying infectious diseases. Additionally, we explored multiple HLA loci including class II loci, rarely analysed in previous studies. A main limitation of this study is that we could not test any of the women

prior to their pregnancy due to lack of available samples, and therefore our findings in pregnancy and post-partum could not be compared to a basal, non-pregnant state. In the mother-infant cohort, we did not have cord blood samples available, and therefore could not compare the infant samples at six weeks to those at birth. Additionally, we had no DNA available to explore the presence of antibodies directed at the infants' HLA. In the pregnancy cohort, we had HLA type of the infants but there was no infant serum available to establish whether baby-directed antibodies crossed to the baby. Additionally, we did not HLA type the mothers so could not confirm whether antibodies were anti-paternal. Gravidity and parity data was not collected in the mother-infant study, and thus, the possible effect of these on HLA antibody repertoire could not be tested.

Summary

In this study we show that up to 80 percent of women after birth express IgG HLA antibodies. Surprisingly, we detect less than 25 percent of these antibodies (16 percent for HLA-I and 24 percent for HLA-II) in the neonate, raising the possibility of HLA antibody selection in placental transfer. Our study also suggests that infants start producing their own IgG HLA antibodies in the first weeks of life. Further investigation is required to confirm these two hypotheses and the mechanisms behind them. The presence of HLA antibodies in neonates may impact their immune reaction to HLA proteins, including those incorporated in enveloped viruses. Whether and how HLA antibodies affect susceptibility to enveloped viruses needs to be further explored using prospective studies. Overall, our findings may provide a preliminary baseline for future studies focusing on pregnancy complications that are not fully understood yet, such as preeclampsia, as well as infections with (and possibly vaccines against) enveloped viruses in new-borns, in which HLA antibodies may play a role.

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References

Please see pdf of published paper attached in appendix

6. Chapter 6 - Discussion

6.1 Overall rationale of this thesis

The major hurdle hindering exploration of an alloimmunization strategy against HIV is the fear of induction of autoimmune disease through induction of HLA antibodies. There remain large gaps in our knowledge, however, regarding the natural history of HLA antibodies in healthy individuals, including various life stages such as infancy, adulthood and pregnancy. My controls from the Partners and COS studies represent healthy HIV-uninfected adults, in whom I explored presence and repertoire of antibody specificities. In addition, to better understand the physiological role of HLA antibodies I characterized the HLA antibody repertoire in a cohort of healthy pregnant women and their six week old infants. This group had no known complications of pregnancy nor auto-immune disease. My characterization of antibody specificities in mothers and infants adds to our understanding of HLA antibodies and draws attention to the perplexing question of whether HLA antibodies in the mother, likely directed at foetal (paternal) HLA antigens, cross to the baby during pregnancy. If such antibodies cross the placenta, they would be autoantibodies in the baby. If the antibodies do not cross the placenta, the mechanism of why they would behave in a manner different to antibodies against infectious pathogens which do cross to the infant, would warrant exploration. Protection against pathogens may be an evolutionary advantageous role of HLA antibodies in infants, pregnant women or healthy adults. An infectious agent of particular relevance is HIV.

6.2 Relevance of HLA antibodies to HIV acquisition

Enveloped viruses, such as HIV and measles, bud from the host cell membrane and incorporate host proteins, including HLA, into the envelope (156)[239][240]. HLA proteins are present in the HIV envelope in higher quantities than gp120 trimers [42][152]. Incorporation of host HLA within the viral envelope of an HIV “donor” opens the possibility that antibodies against HLA in the HIV “recipient” may play a role in protection from acquisition of HIV similar to the role of HLA antibodies in humoral rejection of a solid organ transplant. Evidence for this hypothesis first arose from studies in macaques immunized with cellular antigens that were protected from viral challenge by HLA antibodies [147].

Although any enveloped virus can incorporate HLA antigens from its host, HIV also shows particular homologies between its proteins and HLA proteins, likely due to shared affinity of gp120 and class 2 HLA for binding to for host CD4 protein as an entry receptor. The notable homologies between HIV gp120 and host proteins has been summarized in Table 1 [187,189,193–197,203–205] including additional homologies reported between class 2 HLA and other HIV proteins including gp41 and Nef [188,192,196,199]. HIV gp120 has even been shown to be able to present peptide as antigen, analogous to peptide presentation by class 2 HLA [42,186], leading some authors to speculate that HIV gp120 may have evolved from class 2 HLA or by mimicry of class 2 HLA [185,192].

Thus two plausible biological explanations exist for potential interaction of HLA antibodies and HIV virions. The first is interaction of HLA antibodies in the viral recipient with HLA proteins incorporated in the viral envelope, analogous to the role of HLA antibodies in solid organ transplantation. This mechanism could be envisioned for any enveloped virus, such as measles or influenza, budding from a viral donor to a viral recipient, perhaps with particular relevance to HIV as a blood-borne pathogen. The second plausible biological interaction is of HLA antibodies in the viral recipient interacting with HIV gp120 protein itself in the viral envelope. Certain HLA antibodies may have conformations that can interact with gp120, while other HLA antibody specificities may be unable to bind HIV gp120, leading to heterogeneity in protective effect at the population level. Much *in vitro* and animal work has been performed to interrogate these concepts, but no prior human cohorts of highly HIV-exposed seronegative individuals have been compared to individuals who acquired HIV in terms of their HLA antibody repertoires.

Macaque vaccination studies using simian immunodeficiency virus (SIV) or simian/human immunodeficiency virus (SHIV) reported protection against SIV challenge using SIV grown in human cells until Stott noted that *immunization with human cells alone* conferred protection [147,232]. This was a model of xenoimmunization, whereby *macaques* were immunised by *human* cells. Further work showed that macaques immunised *with purified HLA-DR protein* were protected against subsequent SIV challenge with SIV grown in human, but not macaque, cells [148]. Additional relevant examples include immunization of macaques with recombinant HLA class 1 or 2 proteins, either alone or in combination with HIV and SIV proteins and adjuvant [149]. Complete or partial protection was achieved in macaques vaccinated with all three components and was transferrable by serum. Shearer and colleagues have

suggested that alloimmunization with lymphocytes would be preferable than immunization with peptide antigens, in order to stimulate allogeneic T cell responses [59].

Despite intense interest in allo- and xenoimmunity for HIV vaccine development in the early 2000's [215] there remains no coordinated effort to explore whether intentional induction of HLA antibodies may indeed reduce HIV acquisition. Potential advantages of alloimmunization as an HIV prevention strategy includes the strong immunogenicity of HLA and avoidance of the high variability and mutability of HIV [214,216]. Potential disadvantages include the risk of induction of autoimmunity and limitations to future transplantation options in vaccinated individuals [216]. Also, if the mechanism of protection was found to be due to HLA antibodies in the individual reacting against human HLA incorporated into the viral envelope, a disadvantage would be that "matching" antibodies would be required for protection. As the HLA type of the potential HIV "donor" could not be predicted, an allo-vaccine would then require incorporation of multiple HLA specificities into a vector [149]. Future pregnancy would debatably not be a concern, as HLA antibodies are prevalent and incident during pregnancy and have not been convincingly shown to affect pregnancy outcome [249].

6.3 HLA frequencies and haplotypes in the Partners and COS studies

The allele frequencies in my cohort were similar to recent reported frequencies in the South African black population. HLA-A*30 was predominant, in agreement with prior reports in the same population group [223,227,229,230]. HLA-A*02 and HLA-A*68 also agree with previously reported studies as amongst the commonest HLA-A specificities in South African black individuals [224,229,230]. Using the online database allelefrequencies.net (www.allelefrequencies.net, accessed 03/03/2020), my results are in agreement with that for South African black individuals. The database reports HLA-A*30 alleles at a combined two-digit allele frequency of 0.25, HLA-A*02 at 0.16 and HLA-A*68 at 0.11.

My commonest HLA-B alleles, HLA-B*15, HLA-B*58 and HLA-B*07 are not notably different from prior reports. Paximadis *et al* [230] and Tshabalala *et al* [227] found HLA-B*58:02 as the commonest four-digit HLA-B allele. Williams *et al* [229], Loubser *et al* [223] and Mayne *et al* [224] found HLA-B*15 allele frequencies highest, with HLA-B*42 also common. HLA-B*07 is reported by Loubser et al [223] and Allele frequencies.net at a frequency of 0.06 and Tshabalala *et al* [227] as 0.08. My commonest HLA-C alleles,

HLA-C*06, HLA-C*07 and HLA-C*04 were similarly common in prior reports [223,224,227]. Thus my class 1 allele frequency appears comparable with that previously reported and does not suggest any particular skewing of allele frequencies within my sample (assessed at two-digit resolution).

My commonest HLA-DRB1 alleles, HLA-DRB1*13, HLA-DRB1*11 and HLA-DRB1*15 were similarly common in prior reports [223,224,227]. Allelefreq.net reports combined HLA-DRB1*13 (HLA-DRB1*13:01, HLA-DRB1*13:02 and HLA-DRB1*13:03) frequencies at 0.1796. Allelefrequencies.net reports HLA-DRB1*03 (comprising HLA-DRB1*03:01 and HLA-DRB1*03:02) at 0.1691 and DRB1*15 (comprising HLA-DRB1*15:01 and HLA-DRB1*15:02) at 0.1268.

HLA-DPA1 and HLA-DPB1 alleles are much less commonly reported, and none are available on allelefrequencies.net for the South African black population. For HLA-DPB1, my three commonest alleles, HLA-DPB1*01, HLA-DPB*105 and HLA-DPB*02 are in agreement with Loubser [223], while Tshabalala *et al* [227]found HLA-DPB1*13 commonest. None of the cited papers reported HLA-DPA1 specificities. For HLA-DQB1, my commonest allele, HLA-DQB1*06 was most the most prevalent HAL-DQB1 allele in prior reports [223,224,227]. Thus my class 2 allele frequencies are in keeping with prior reports and suggest that my sample was drawn from South African black individuals with HLA types representative of the wider community. There is no suggestion that presence of rare HLA types could be a reason for my findings related to HLA antibodies.

I have reported my commonest class 1 (3 locus) and class 2 (5 locus) haplotypes, which add information to that already published for South African black haplotypes [223,251] and other African studies [100]. I detected some notably unusual DRB1 –DRB3/4/5 two-locus haplotypes, not typically described in Caucasian populations. Unusual combinations may occur, particularly in the black African population with its high degree of HLA diversity. Using next-generation sequencing, Thorstenson et al also reported unusal DRB1 - DRB345 two-locus haplotypes [251], not identical to my unusual haplotypes. Similarly Lacap *et al* commented on an unusual HLA-DRB1*08 linked to HLA-DRB3*02 [100]. In order to confirm that these unusual haplotypes are not artefacts of my typing method, confirmation using sequencing is recommended, but has not been performed to date. In general, the HLA-DRB*3/4/5 Luminex-based single-stranded oligonucleotide typing method did differentiate the presence of homozygous alleles from an absent allele, but further work will include confirmation of these haplotypes and phasing via a second typing method.

I did not directly compare allele or haplotype frequencies between HIV seroprevalent/seroincident individuals and control individuals, and this aspect may still be explored. Instead I grouped my participants to give a more comprehensive perspective on South African allele frequencies and haplotypes. The Partners study had already looked in the larger multi-country study for associations between class 1 HLA and HIV setpoint [102] and had performed genome wide analysis for single nucleotide polymorphisms associated with transmission[220]. The Partners study had not analysed influence of class 2 HLA alleles. Class 2 HLA alleles have been studies in other cohorts, although to a far less extent than class 1. In a Zambian study of 292 HIV -discordant couples, Tang *et al* [96] showed that the allele HLA-DRB1*13:01 in initially seropositive partners led to delayed transmission of HIV to their spouses. In the same study, the haplotypes HLA-DRB1*03:01 – HLA DQB1*02:01 and HLA-DRB1*15:03 - HLA-DQB1*06:02 in initially seronegative partners showed accelerated seroconversion. There were no overt differences in HLA-DRB1 or HLA-DQB1 loci when analysed at the 2-digit resolution however. Tang *et al* analysed HLA-DRB1 further as the 5 "known lineages" termed DR1, DR51, DR52, DR53 and DR8 and found no association with HIV transmission or acquisition. This "lineage" terminology obscures allelic differences that may exist even at two-digit resolution of the HLA-DRB3, HLA-DRB4 or HLA-DRB5 loci, for example a chromosome with the DR52 lineage may harbour a HLA-DRB3*01 or a HLA-DRB3*02 allele in addition to its HLA-DRB1 allele. Tang *et al* did not type HLA-DRB3/4/5 loci.

6.4 HLA antibodies and odds of HIV transmission

To explore the role of HLA antibodies in HIV acquisition, I performed a nested case-control study using samples from the Partners and COS studies. Partners and COS were longitudinal analyses of heterosexual HIV serodiscordant couples with both genetic material and sera available for study, including timepoints prior to HIV seroconversion. The cohorts were well characterized including viral analyses classifying HIV transmissions as linked (HIV acquired from the index partner) or unlinked (HIV acquired from third party). The couples who did not seroconvert represent healthy individuals with no known comorbidities. Most couples reported episodes of unprotected sexual intercourse despite HIV risk counselling and therefore at least some represent highly exposed persistently seronegative individuals. Despite my low numbers of 28 seroconverters, the group of interest was the group I have termed controls, of whom 115 represents a relatively high number.

I proceeded to analyse HLA antibodies in seroconverting and control couples. At the first timepoint, prior to seroconversion, I found that **significantly fewer seroconverters had class 2 HLA antibodies compared to controls.** There was no particular class 2 locus in which the proportion of controls and seroconverters differed significantly, but for each class 2 locus there were fewer seroconverters than controls with antibodies. These findings show an association between presence of class 2 HLA antibodies and lower odds of HIV acquisition, confirmed on multivariate logistic regression.

I further analyzed the "matching" antibodies directed particularly against HLA types of the index partner. Prior to seroconversion there was a similar trend noted of fewer matching HLA class 2 antibodies in seroconverters than controls. Thus, prior to seroconversion, seroconverters showed less alloimmunity against their partners than the highly exposed seronegative control group.

Importantly, my statistical associations were weaker when limiting analysis to partners with linked HIV transmissions. Thus, presence of class 2 HLA antibodies seemed protective, even against HIV acquisition from a third partner who was not enrolled in the trial. While lack of significant associations in the linked group may be an artefact of small numbers of linked transmissions, these findings may also infer that protection derived from HLA class 2 antibodies is generic, rather than specifically directed towards donor HLA proteins incorporated into the viral envelope. That is, the protection afforded by class 2 HLA antibodies does not seem to derive from *matching antibodies* directed at the index partner HLA type, but rather from any class 2 antibodies. Should this finding be confirmed it could be highly advantageous for an allo-vaccination strategy.

Following seroconversion, there were significantly more seroconverters than controls with HLA class 1 antibodies. In particular, significantly more seroconverters than controls expressed HLA-A and HLA-B antibodies. Similarly, seroconverters expressed significantly more matching class 1 HLA antibodies at the second timepoint compared to controls, particularly at the HLA-A locus. The same pattern was seen with linked transmissions, where there were significantly more linked seroconverters with class 1 HLA antibodies than controls. At the second timepoint, seroconverters also expressed more class 1 specificities *per individual* than controls. The change from timepoint 1 to timepoint 2 illustrates that HIV acquisition is a sensitizing event stimulating production of antibodies against HLA-A and HLA-B loci. This is an expected finding and is in keeping with other reports that HIV-infected individuals make anti-lymphocyte antibodies and HLA antibodies [194,199,241] .

HIV-infected patients often show polyclonal increases in gamma globulins and many viral infections result in polyclonal increases in IgG titres, often resulting in cross-reactive serological diagnostic assays [242,243]. Regarding HIV acquisition as a sensitizing event for *de novo* induction of HLA antibodies, it is interesting to note, however, that the sensitization seems to be limited to class 1 rather than class 2 antibodies. Despite multiple homologies between HIV proteins and human proteins described, why HIV infection does not stimulate new class 2 antibodies is an intriguing question. It may be that homologies between HIV proteins and human class 2 HLA proteins result in some form of immune tolerance that limits *de novo* development of class 2 HLA antibodies. Alternatively, repeated stimulation or exposure to certain HIV proteins homologous to class 2 antigens may result in blunting of the humoral immune response to class 2 antigens, such as that seen with pneumococcal polysaccharide vaccination [244].

My findings suggest that there may be some benefit even from "non-matching" class 2 HLA antibodies. Multiparous women and patients who have received multiple transfusions represent natural examples of people with alloimmunity without obvious autoimmune side effects. My data lend weight to this opinion, showing that 79% of healthy individuals express HLA antibodies with no known deleterious consequences.

While it is also usually assumed that individuals do not harbour antibodies directed against their own HLA type (potentially autoreactive HLA antibody), I found that a low proportion of individuals do express such autoantibodies. Interestingly, at the early timepoint, there was a trend to higher frequencies of class 2 autoantibodies in controls than in those who later seroconverted. There were in fact no class 2 autoantibodies at all in the seroconverter group at this early time. At the second timepoint however, after seroconversion, seroconverters tended to express higher frequencies of class 1 antibodies than controls, particularly at the HLA-B locus. Thus autoantibodies in controls at the early timepoint may also be in some way protective against HIV acquisition, in a similar fashion to more general alloantibodies. HIV acquisition may increase the frequency of HLA-B autoantibodies, which may or may not relate to HIV pathogenesis.

There are multiple potential mechanisms by which HLA antibodies may affect HIV acquisition. Hypotheses that span the diverse functions of non-neutralising antibodies. Although the prime objective of HIV vaccine design has been to develop vaccine candidates that stimulate broadly neutralising

antibodies, recognition has been given to the potential role of non-neutralising antibodies. Non-neutralising antibodies, termed HIV-inhibitory antibodies, may function in a multitude of ways, such as reducing movement of virions across mucosal surfaces or epithelia, influencing cytokine production or influencing F_c receptor function. Influencing F_c receptor function may result in alteration of antibody dependent cellular cytotoxicity or antibody dependent cellular viral inhibition [252–254]. Non-neutralising antibodies against HIV may opsonize virus and enhance phagocytosis of viral particles [254,255].

After attachment to the cell surface but before entry, retroviruses, including HIV, may "surf" along the cell membrane to a more favourable location for entry [21] or closer to the nucleus where viral nucleic acid can integrate. It is conceivable that host HLA protein incorporated into the virion envelope may alter such trafficking of the virus along the cell surface prior to entry, either enhancing or reducing likelihood of fusion. Recipient HLA antibody bound in turn to the incorporated donor-derived HLA may therefore interfere with virion "surfing" prior to CD4 binding to gp120.

The class, or isotype of IgG, may also impact the function of non-neutralising or neutralising antibodies, such as impacting the ability of the antibody to mediate opsonisation and result in phagocytosis of virus. Both neutralising and non-neutralising antibodies could mediate uptake of virus into monocytes. Different isotypes of antibody (IgG1 versus IgG3; IgA1 versus IgA2) of the same specificity (targeting the same envelope region) differed in their ability to mediate viral uptake by monocytes. IgG3 was more efficient than IgG1 at mediating viral uptake, and IgA was less efficient than IgG1 [255].

It has been proposed that a successful vaccine may need to induce other functions such as antibody dependent cellular cytotoxicity or antibody dependent cell mediated virus inhibition [70]. The functionality of non-neutralising antibodies *in vivo* is however difficult to predict. In certain diseases such as dengue, antibodies to dengue serotypes other than the infecting serotype can worsen disease severity [232][256]. Complement dependent non-neutralising antibodies may even enhance HIV infectivity [256].

James Stott, author of the original observation that immunization with human cells alone could protect macaques against SIV challenge, co-authored a 2014 paper investigating correlates of protection with Luminex based anti-HLA methodology in macaque sera from the earlier trials. Using only class

identification kits rather than single antigen beads, protection against challenge correlated with anti-HLA antibody that could fix complement. Protection appeared mediated by antibody against framework determinants of HLA (both class 1 and 2) rather than particular specificities [257]. Incorporation of host complement regulatory molecules such as CD55 and CD59 into the viral envelope inhibited the action of complement [258,259].

I therefore included analysis of class 2 complement-fixing HLA-antibodies at the early time point, in order to exclude the presence of complement-fixing IgM antibodies that I may have missed with my analysis of alloantibodies of IgG isotype. A very low frequency of individuals (7.5% of controls and 4.0% of seroconverters) expressed complement-fixing class 2 HLA antibodies at the early timepoint expressed complement-fixing HLA antibodies, with no difference in frequency noted between controls and those who went on to acquire HIV.

6.5 HLA antibodies in mother-infant pairs

In healthy mother-infant pairs, we used the same highly sensitive Luminex technology to measure prevalence of HLA antibodies. In six additional pregnant women, we measured HLA antibodies longitudinally and HLA-typed infant DNA to assess whether maternal HLA antibodies were directed at infant specificities. We found that 68% of mothers and 44% of infants expressed class 1 HLA antibodies and 56% of mothers and 52% of infants expressed class 2 HLA antibodies. These are important findings, as they form a baseline estimate of what proportion of mothers and infants are expected to harbour HLA antibodies. Such information is of relevance to future studies of vaccination during pregnancy, where fear of induction of alloimmunity may be considered an adverse event following immunization. Background rates of such alloimmunity will be important with which to compare future rates during clinical trials.

Further, Infants shared only 78% of antibodies with their mothers, suggesting that the remaining antibodies were self-made. Self-made antibodies in such young infants was unanticipated and a novel finding that requires repeat confirmation. While detection of antibodies against pathogens in infants such as antibodies against measles or rubella is often thought to derive from maternal serum, even when present at higher concentration in cord blood than maternal blood, the unique “fingerprint” of antibody repertoires in mothers and infants suggest that infants synthesize their own IgG antibodies even at the age of six months.

Less than 25% of maternal HLA antibodies were detected in infants. The reasons for this finding are uncertain, as mothers are thought to transfer their antibody repertoire, in the case of antibodies against pathogens like measles or rubella, to their infants. The absence of certain HLA antibodies from infants raises the possibility of selectivity in which antibodies cross the placenta. In infants, certain maternal HLA antibodies detected at paternal antigens would be "autoantibodies" in the infant. In a third of our pregnant subjects studied longitudinally, we detected infant-directed HLA antibodies. We can hypothesize that there may be an active selection process to select certain antibody specificities to cross the placenta or to determine which antibodies, after crossing the placenta, are maintained in the foetus until six weeks of post-natal life. We did not have a full sample set of longitudinal maternal and infant DNA and matching sera with which to fully interrogate this question. Further work will be required, but our data shows that the HLA antibody repertoire in mothers is not directly correlated with that of their infants.

6.6 Weaknesses and Strengths of this work

I used a two-digit resolution for HLA-typing and therefore classified antibodies as allo-antibodies or auto-antibodies on the basis of a two-digit resolution. There may be some cases of over-estimation of auto or allo-antibodies, for example an antibody against HLA-B*15 may not react with all HLA-B*15 genotypes. However autoantibodies have been reported by others even using 4-digit resolution HLA-typing [171,245].

I studied serum only and did not analyse fluids from mucosal sites, such as cervico-vaginal lavage fluid. Authors have reported differential findings from serum and mucosae. Lund *et al* reported IgA in cervico-vaginal lavage fluid in women taking pre-exposure prophylaxis for HIV which could neutralize HIV in vitro [260]. I cannot comment on the effect of HLA-antibodies in mucosal secretions. I did not study T cell or innate immune cell responses to allo-antigens. Alloimmunity may not be limited to antibody responses but may also have a T cell, natural-killer cell or other immune mediated components.

This work has a number of important strengths. Strengths of the work on HIV serodiscordant couples include inclusion of a rare sample group, namely HIV-infected patients followed longitudinally from prior to the date of seroconversion. Strengths of the work on pregnant women include that the participants

were healthy and not drawn from cohorts of HIV-infected women nor women with pregnancy disorders such as preeclampsia or miscarriage, who have been more frequently studies.

I studied HLA class 2 extensively, often overlooked in preference of HLA class 1 in other work. Within each HLA class, I included multiple loci, allowing a comprehensive overview not limited solely to HLA-A, B and DRB1, the most frequently studied histocompatibility loci. I used highly sensitive Luminex single-antigen methodology which detects even low-level antibody. We compared the strength of our findings in HIV serodiscordant couples with that of more traditional HIV risk factors including HIV viral load, presence of sexually transmitted infections.

A perceived weakness of this work can be use of only one methodology for detection of HLA antibodies, with a concern that the method may be overly sensitive. Luminex based detection of HLA antibodies using single antigen beads is now considered the gold standard in renal transplantation laboratories, and low level HLA antibodies have been shown to be important in outcomes of renal tansplants [156,161]. Whether Luminex detectable HLA antibodies are of relevance in the context of infectious diseases requires similar epidemiological studies correlating clinical infectious endpoints with presence or absence of antibodies. Investigating whether HLA antibodies detectable through Luminex based assays were clinically relevant was a major aim of this work. Our finding in multivariable regression of a significant association of class 2 HLA antibodies with reduced HIV acquisition risk suggests that the antibodies detected are not “overly sensitive” but physiologically relevant.

6.7 Relationship between HLA antibodies and autoimmunity

The major concern hampering further exploration of an alloimmune vaccine strategy for HIV has been fear of induction of autoimmunity. It is prudent to review some perceptions and misperceptions regarding the role of autoantibodies in autoimmune disease.

Interestingly, with many auto-, allo- and xenoantibodies, antibodies are often present without any identified triggers nor symptoms. Natural antibodies are known to be present at birth, including IgM antibodies against blood group antigens. IgG auto-antibodies are found at low frequencies in healthy individuals, and increase in frequency with age, even in asymptomatic individuals. For example, anti-parietal cell antibodies, associated with the autoimmune condition pernicious anemia, are found at

frequencies of 20% in the *healthy* elderly population [261]. Similarly antibodies against thyroid peroxidase, associated with Hashimoto's disease, can be found in more than 40% of *healthy* populations [262,263]. Occasionally autoantibodies are discovered incidentally because of interference with laboratory assays, such as antibodies against prolactin, termed "macroprolactin" [264,265]. For multiple autoimmune illnesses, intravenous immune globulin (presumably containing multiple HLA antibodies from its donors from) are used as therapeutics [266].

Xenoantibodies are also common. Termed heterophile antibodies, they can be found incidentally interfering with laboratory assays [267–269]. Heterophile antibodies increase in titre during certain diseases and can be used diagnostically. For diagnosis of infectious mononucleosis, an acute EBV infection, heterophile antibodies against sheep or horse red blood cells are commonly assayed (the Paul Bunnell test and Monospot test, respectively) [270,271]. Additionally antibodies against lecithin, cardiolipin and cholesterol ("reagin") are used to indentify syphilis infections [272].

Additional counterintuitive features of antibodies in disease amelioration are coming to light. Vacccination with one antigen may increase protection against other antigens [273–275] and polyclonal B cell activation may be relevant to protection [195]. The presence of anti-gp120 antibodies, as well as unrelated antibodies, has been described in HIV-uninfected patients with various autoimmune diseases and in mice treated with lipopolysaccharide [195]. A high proportion of patients recovering from measles infection have antibodies that are cross-reactive with HIV antigens, causing indeterminate HIV immunoblots, as well as with antigens on lymphoid cells, likely HLA antigens [276]. Influenza vaccination can also result in development of *de novo* HLA-antibodies and alloreactive T cell responses [277,278]. Certain lipopolysaccharides from gram negative bacteria such as *E.coli* and Salmonella species were shown to cross react with anti-HLA allosera [279].

Thus our understanding of why certain antibodies are present in individuals is more complex than mere previous exposure to the same antigen. The presence of a pre-existing network of antibodies, including autoantibodies, existing regardless of foreign antigen exposure has been proposed as the immunological homunculus or "immunculus"[280]. The patterns of natural autoreactive antibodies are remarkably similar in different individuals. In addition to the presence of naturally occurring, low affinity IgM antibodies (such as those used towards blood group antigens of the ABO system) at least half of naturally occurring antibodies are of IgG isotype [280]. The naturally occurring antibodies seem to be

formed as early as the intrauterine period. Characterising the spectrum of IgM, IgG and IgA in paired maternal and cord blood by antigen microarray, babies were shown to develop relatively uniform patterns of IgM and IgA auto-antibodies in utero, which differed from maternal patterns [281]. The authors did not discuss HLA antibodies, and no HLA proteins were included in their array format. HLA antibody development may occur from early infancy, with HLA antibodies found in infants as early as 6 weeks of age, in some cases differing from specificities in their mothers [238] .

The natural history of HLA antibodies in pregnancy also leaves many questions unanswered. The comparison between maternal tolerance of a foetus at the placenta with allograft tolerance was first made by Sir Peter Medawar in 1953 [282,283]. HLA antibodies during pregnancy are not thought to harm the foetus. In fact, there has been suggestion that they are a sign of a good immunological reaction between the mother and foetus. Lymphocyte immunotherapy has been used since the 1980s with the intention of inducing HLA antibodies to assist pregnancy in couples with poor obstetric histories thought due to immunological reasons. Intradermal lymphocyte infusions from the male partner injected intradermally into the female partner were safe for mother and baby and had variable efficacy in increasing rates of successful pregnancy [284–286]. In some instances, even third party lymphocytes (rather than paternal lymphocytes) were used, with comparable safety and similar induction of regulatory cell subsets.

Hoffman *et al* suggested an MHC-image-anti MHC-image (MIAMI) model of HIV pathogenesis in 1991 [200]. In this paper, the authors proposed that an alternative approach for an HIV vaccine would be to attempt to induce tolerance to HIV in order to lessen severity of AIDS symptoms and slow disease progression. They note that induction of tolerance to HIV is precisely the opposite approach of most HIV vaccine strategies. The authors concede that a less radical approach may be to attempt induction of tolerance to parts of HIV that mimic MHC, while inducing immunity to other HIV components. The hypothesis of tolerance induction to MHC mimicking parts of HIV has not been comprehensively explored, although a few approaches have been reported. Lu has proposed that a tolerogenic vaccine against HIV is the “paradigm shift” required [287] to deal with the unique features of HIV, a host-mimicking virus. Whether such tolerogenic approach would correlate with HLA antibodies is unclear.

7. Chapter 7 - Concluding Remarks

In this thesis I have presented two novel studies of HLA antibodies, with the aim of understanding more about the natural history of such antibodies and their physiological significance, particularly in prevention of HIV sexual transmission.

in HIV serodiscordant couples, I have found epidemiological evidence that a higher proportion of control couples than seroconverter couples harboured class 2 HLA antibodies at early timepoints. Even controlling for other HIV transmission risk factors in logistic regression, presence of class 2 HLA antibodies was associated with an odds ratio of 0.33 (CI 0.112-0.976, p=0.045) for HIV acquisition. This data is in keeping with the concept that an alloimmunisation approach may be of value as a prophylactic HIV vaccine strategy, although I cannot exclude that mine is a chance finding. Unexpectedly, I found no difference in proportion of controls compared to seroconverters who expressed HLA antibodies matching the HLA genotype of their sexual partner. Thus a protective benefit of HLA antibodies, if any, appears to derive from generic non-matching class 2 HLA antibodies present in healthy individuals, rather than by specific antibodies matching the HIV-transmitting sexual partner. My study is observational in nature and therefore can never prove causation, but the association of a high proportion of healthy individuals expressing class 2 HLA antibodies is supportive of earlier *in vitro* and animal work which required corroboration in humans. Additional human cohorts should be investigated for reproducibility of my results. Further work should also explore class 2 HLA antibodies in cell culture or humanized mice models, but true validation would only be possible through human trials, either using passive antibody administration or a class 2 HLA immunogen.

While a natural concern around alloimmunisation is that autoimmune disease may be induced by autoantibodies against HLA, I have added to the body of data challenging this concept. Healthy participants in the Partners and COS studies showed class 1 and class 2 autoantibodies, with class 2 autoantibodies present in significantly more controls than seroconverters, with no obvious health defects. Certainly, natural exposure to HLA antigens identical to self, or foreign to self, is a natural occurrence in sexual partners and pregnant women with no known deleterious consequences. Herein I have found a potential positive effect of HLA antibodies at the population level associated with reduced odds of HIV acquisition, even when controlling for other variables.

I have also shown in my study of mother-infant pairs that healthy neonates often harbour HLA antibodies, in some cases directed against their own HLA genotypes. Such infants showed no adverse health effects of such antibodies.

Mothers and infants shared most specificities of HLA antibodies, showing that HLA antibodies present in mothers do transfer to fetuses, but the antibody repertoires were not identical, with certain specificities present only in either the mother or the infant. A more thorough understanding of triggers of antibody development in infants, or mechanisms of selective antibody transfer to infants, are avenues for future research. A focus on reproductive immunology is likely to lend insights into mechanisms of immune tolerance, a field which has never been fully integrated into our understanding of infectious disease immunology [288].

My results support calls to evaluate and deliberate options for an alloimmunization approach against HIV, using either passive antibody administration or incorporating HLA class 2 alleles as vaccine antigens. It is my hope that this work raises pertinent questions regarding the intersection of alloimmunity and immune tolerance with infectious disease immunology. Vaccines have been the most successful public health intervention in the last century. Harnessing improved understanding of immune tolerance to develop improved vaccines is our challenge for the twenty-second century.

8. CHAPTER 8. References

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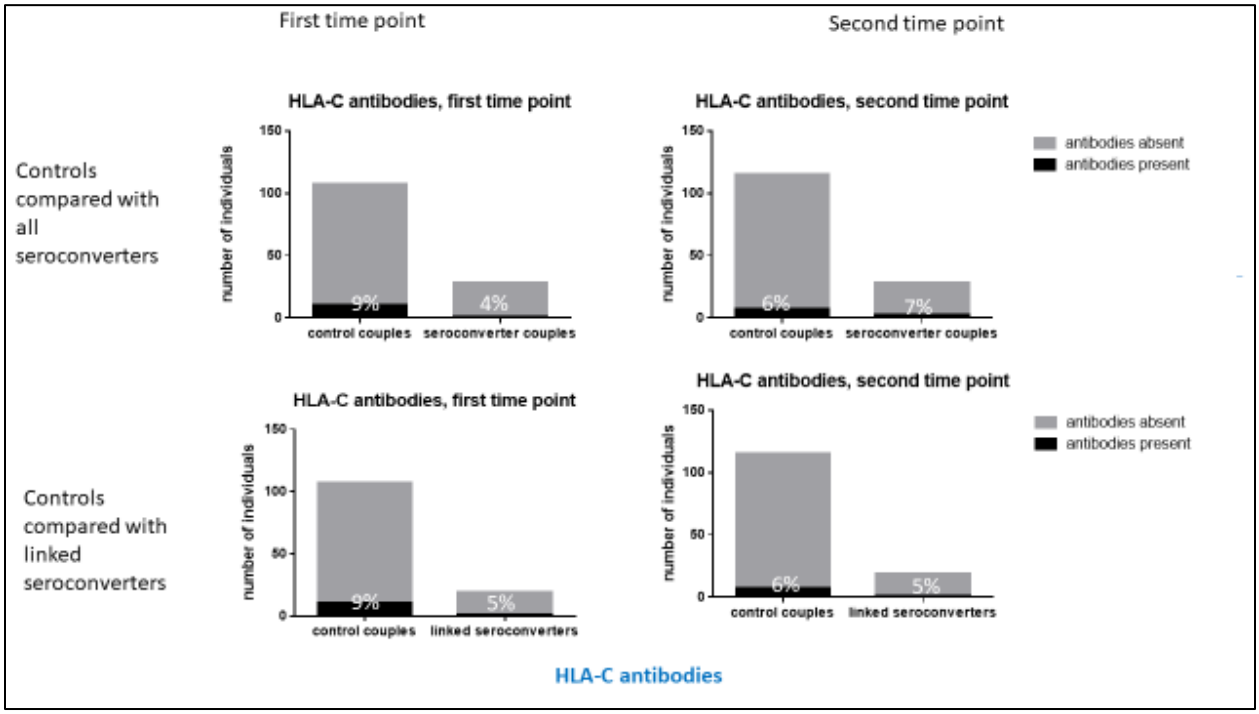
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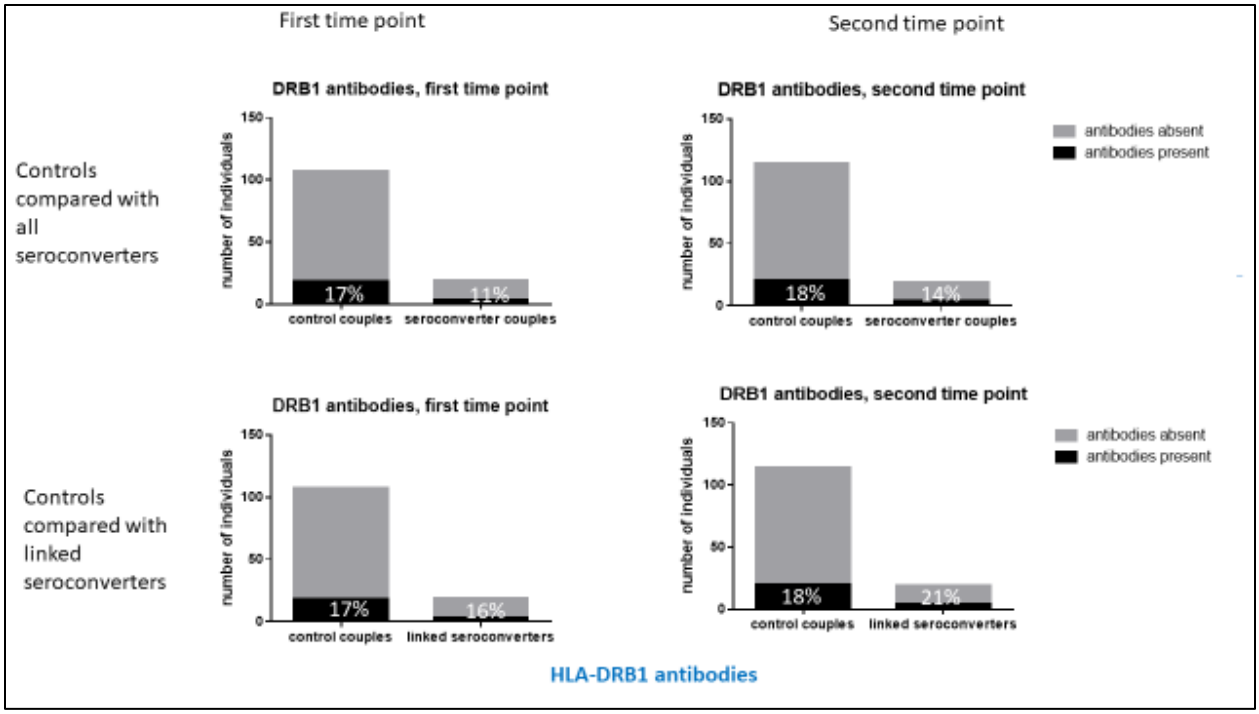
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Appendix

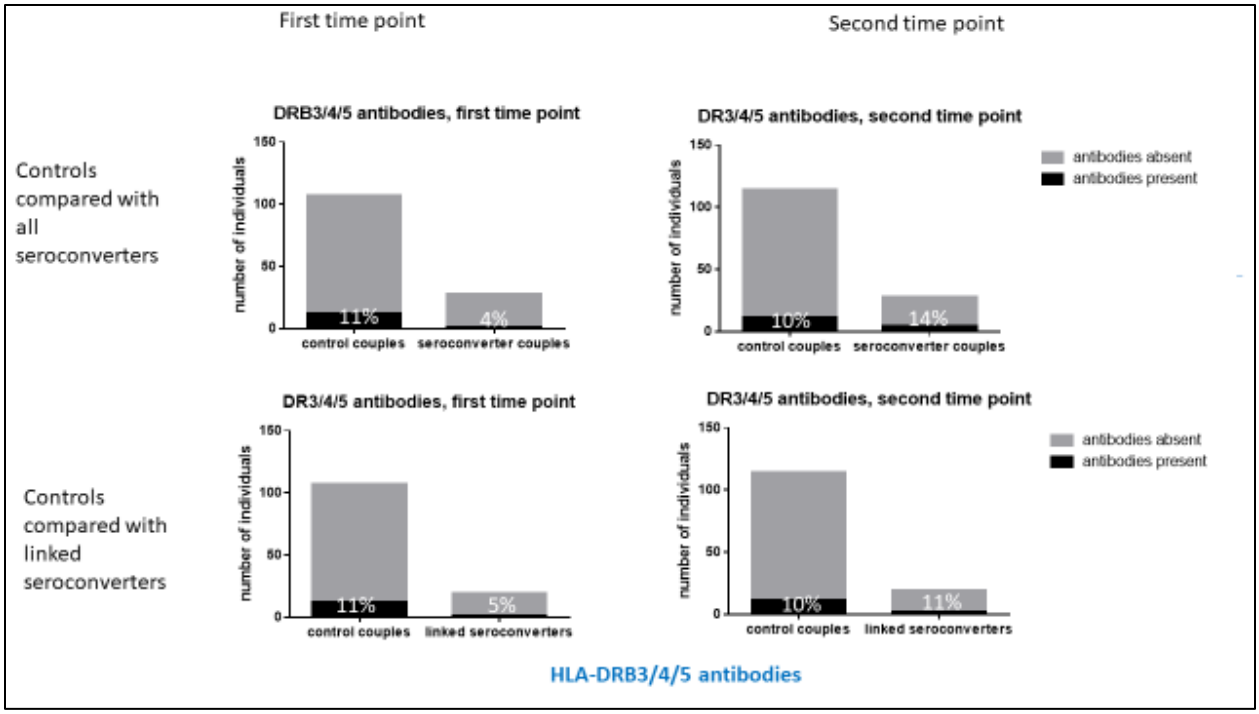
Supplementary Figures for HIV-serodiscordant couples (for Chapter 4)



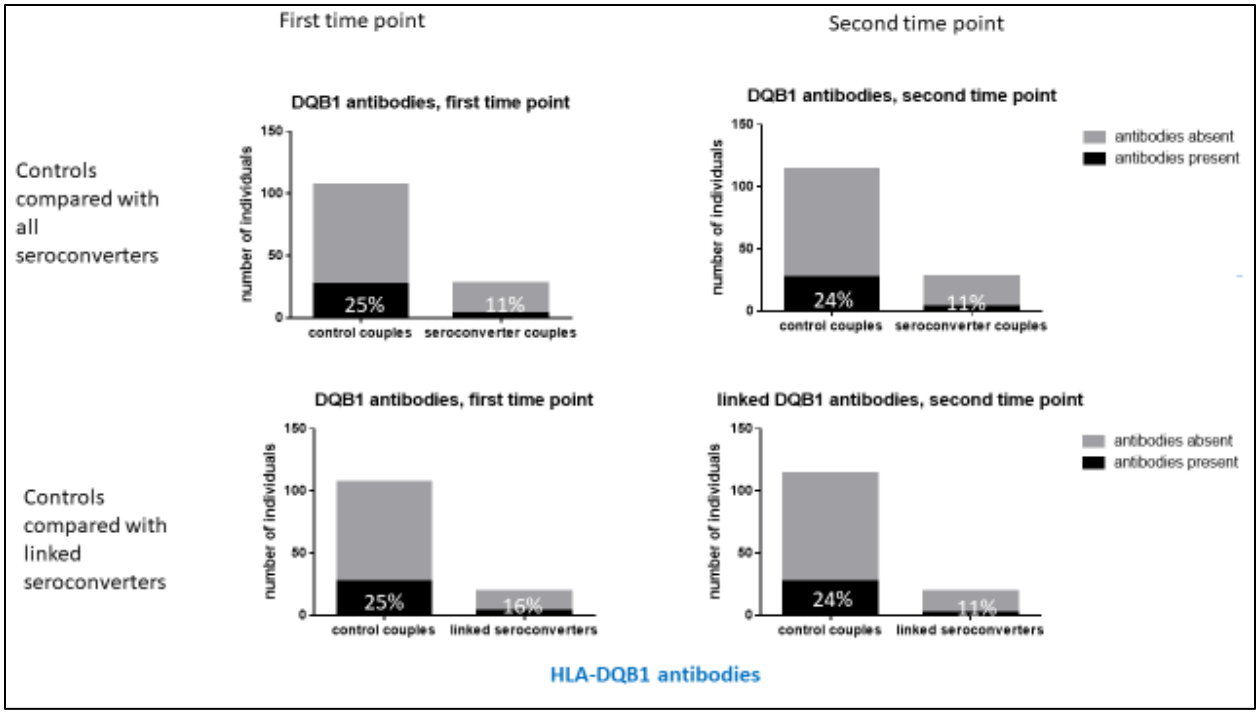
Supplementary figure 4.1 HLA-C antibodies in seroconverter couples compared with control couples at the first and second timepoints.



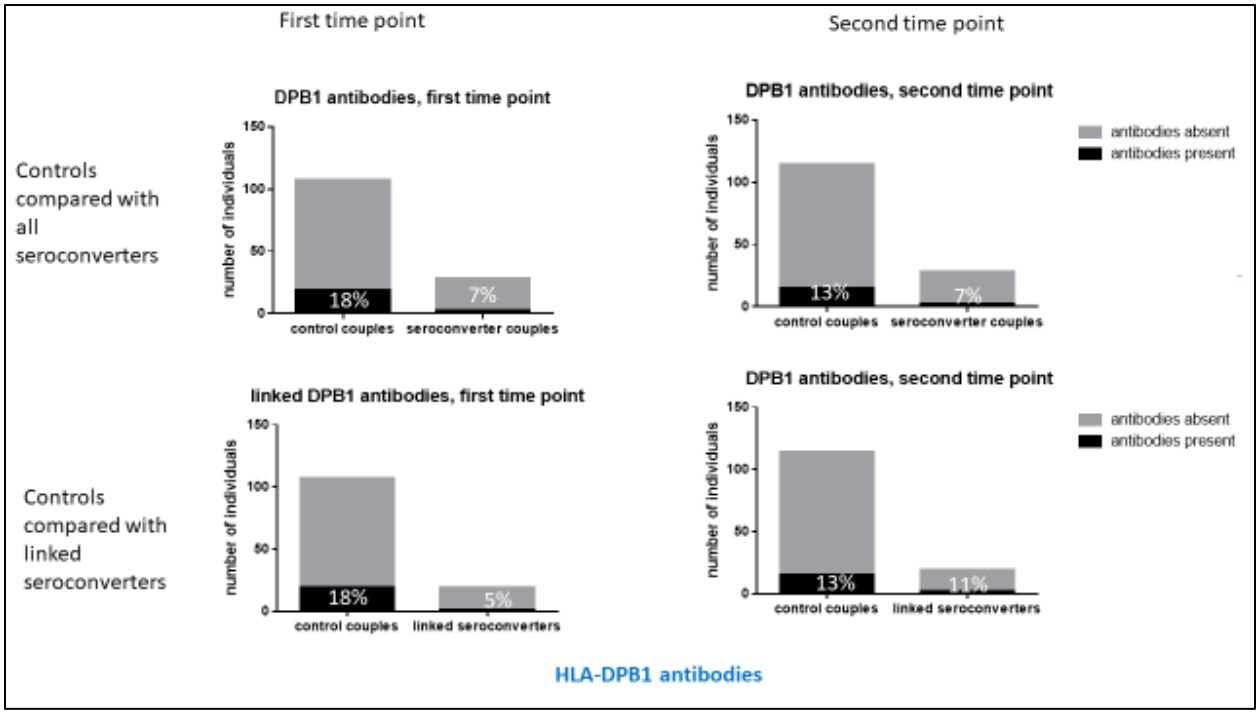
Supplementary figure 4.2. HLA-DRB1 antibodies in seroconverter couples compared with control couples at the first and second timepoints



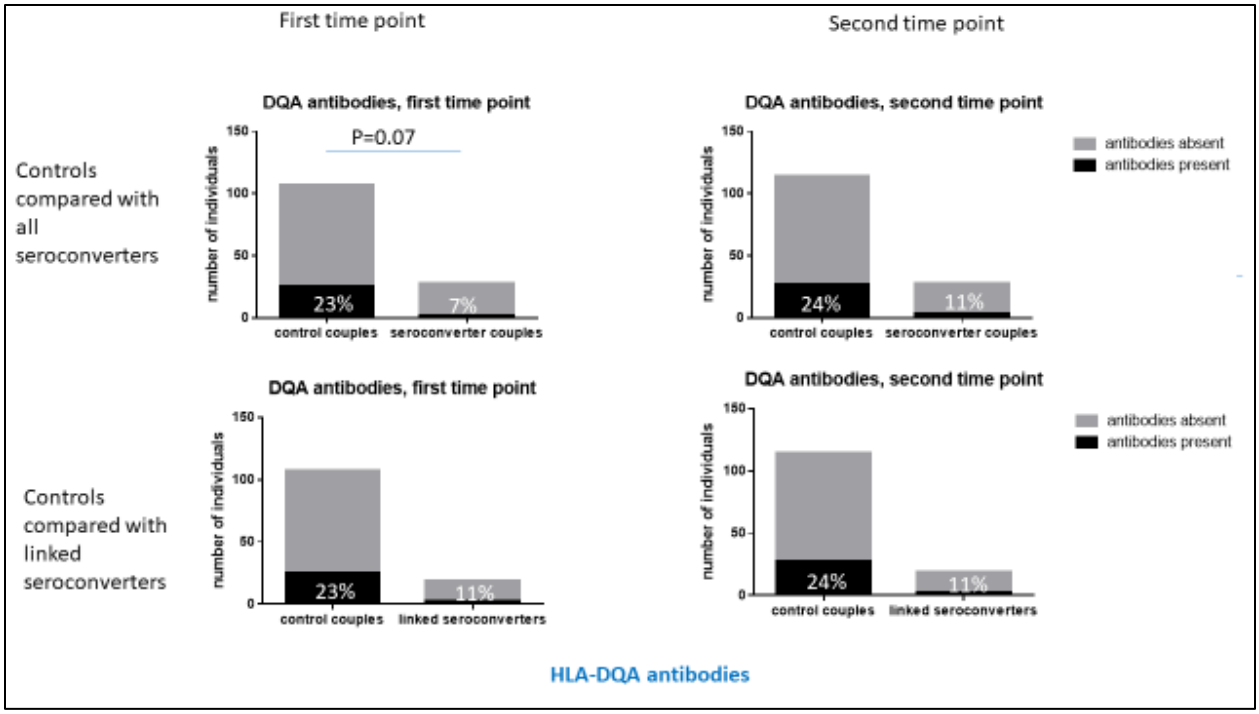
Supplementary figure 4.3. HLA-DRB3,4,5 antibodies in seroconverter couples compared with control couples at the first and second timepoints



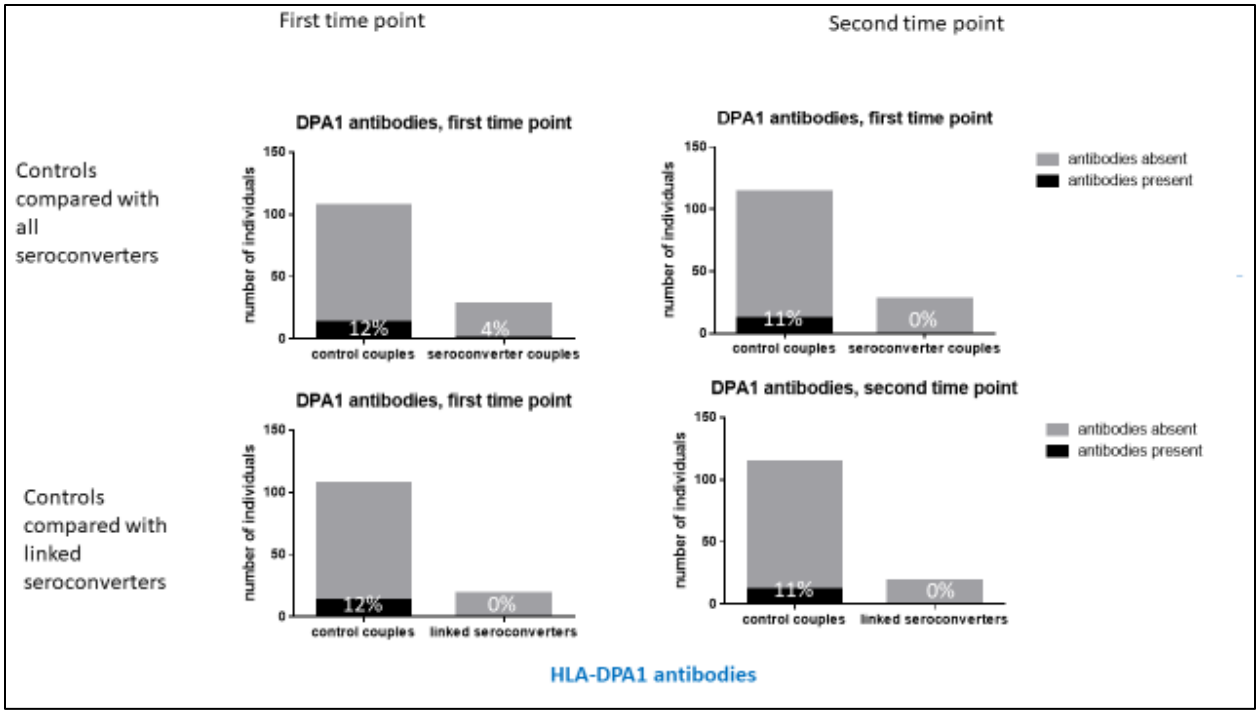
Supplementary figure 4.4. HLA-DQB1 antibodies in seroconverter couples compared with control couples at the first and second timepoints



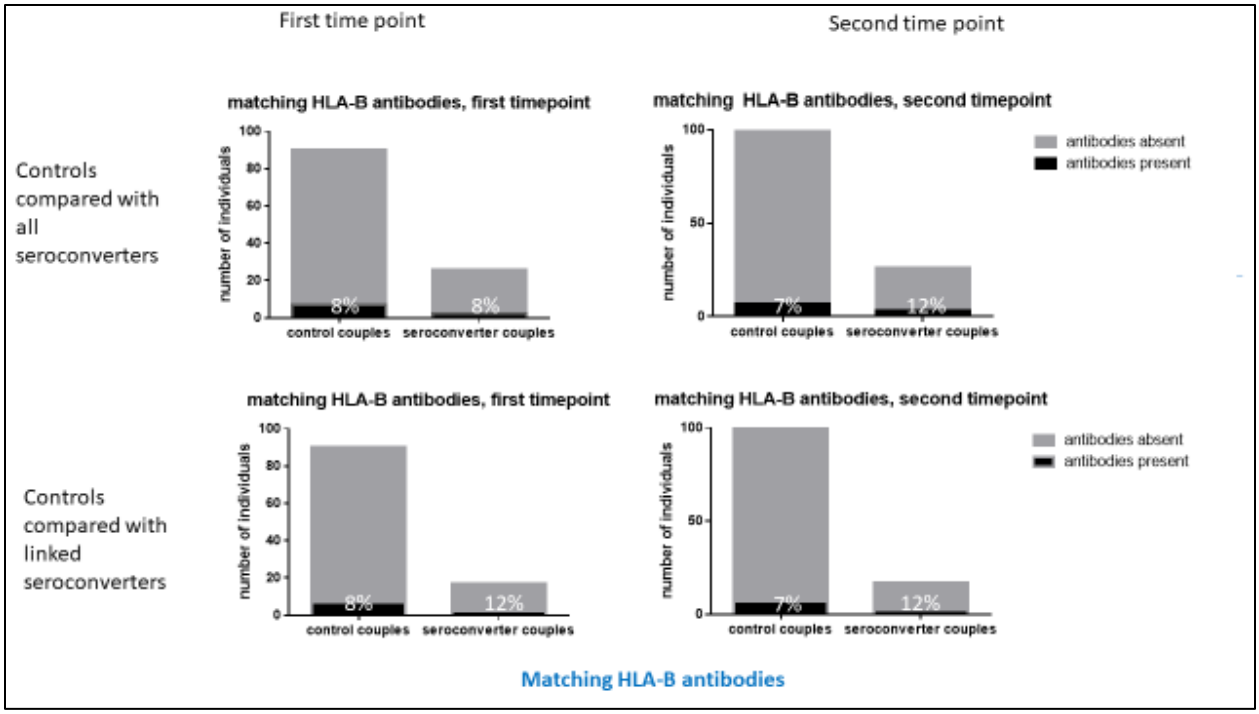
Supplementary figure 4.5. HLA-DPB1 antibodies in seroconverter couples compared with control couples at the first and second timepoints



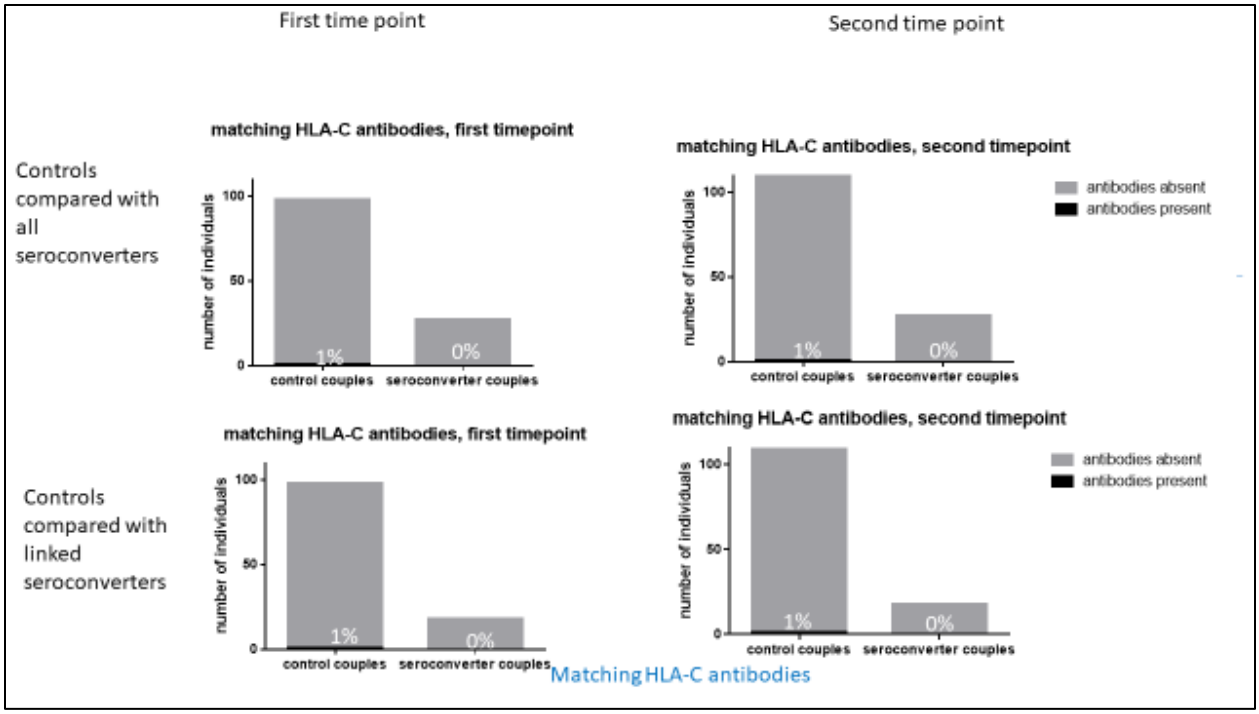
Supplementary figure 4.6. HLA-DQA antibodies in seroconverter couples compared with control couples at the first and second timepoints



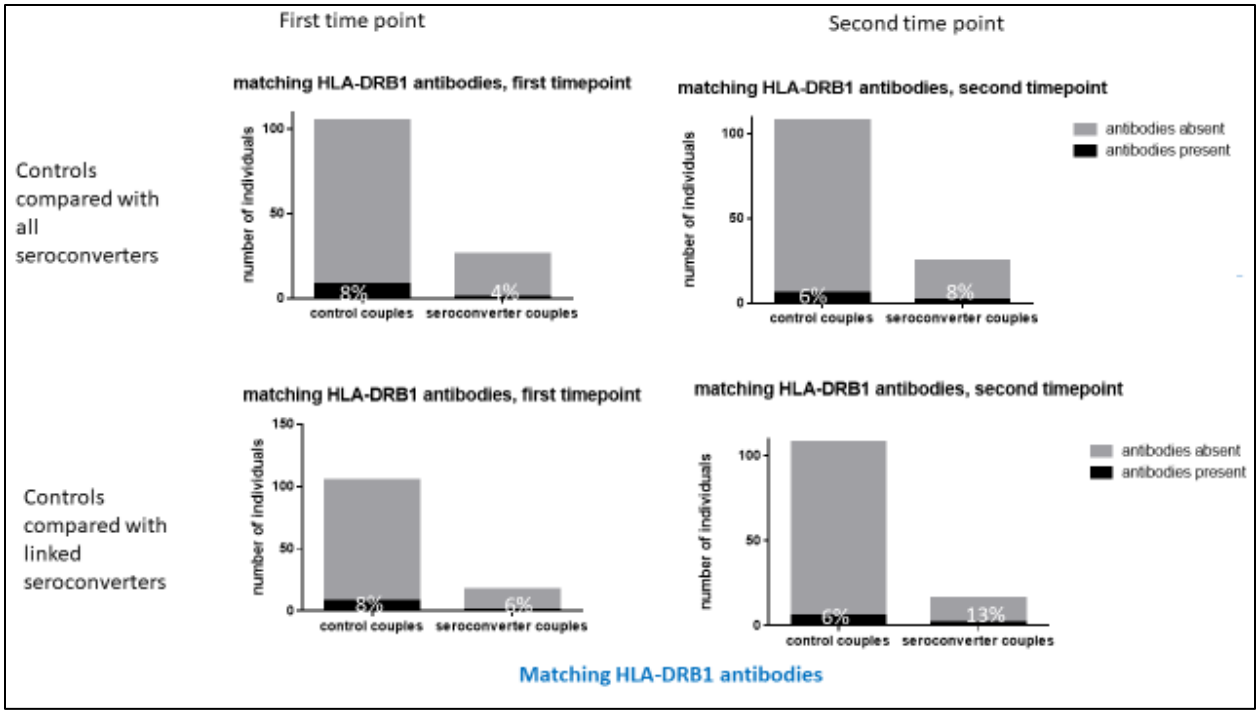
Supplementary figure 4.7. HLA-DPA1 antibodies in seroconverter couples compared with control couples at the first and second timepoints



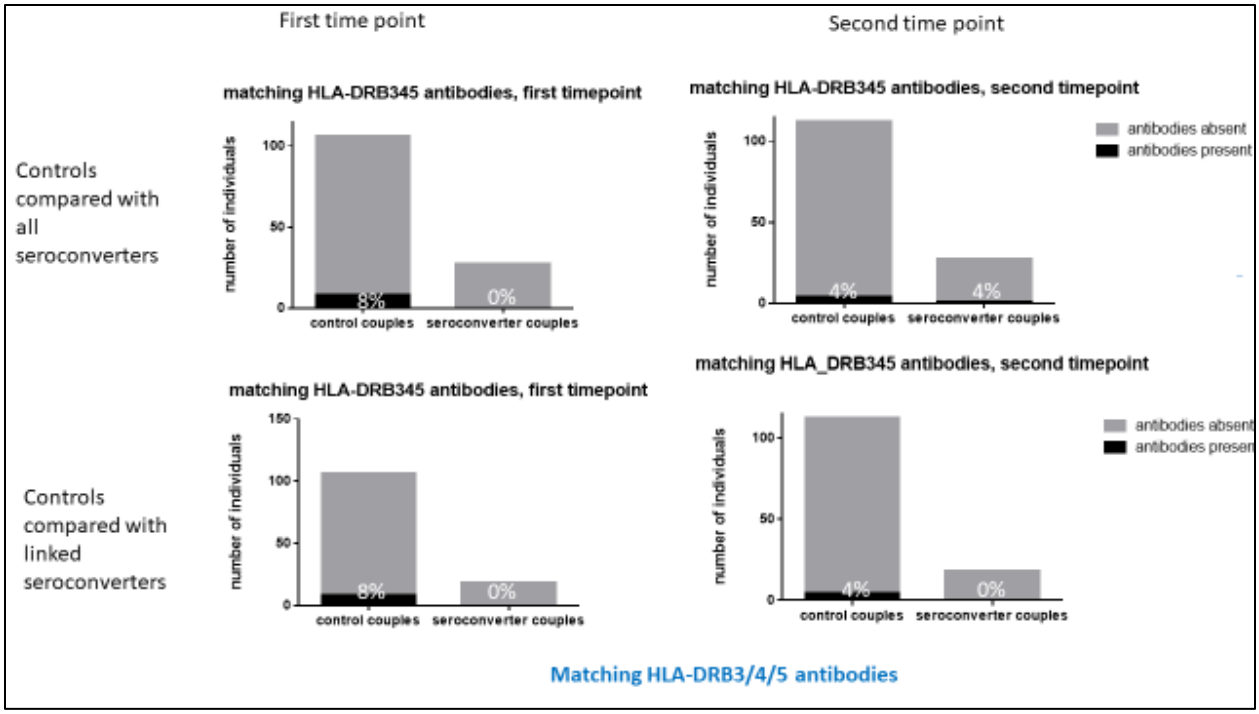
Supplementary figure 4.8. Matching HLA-B antibodies in seroconverter couples compared with control couples at the first and second timepoints



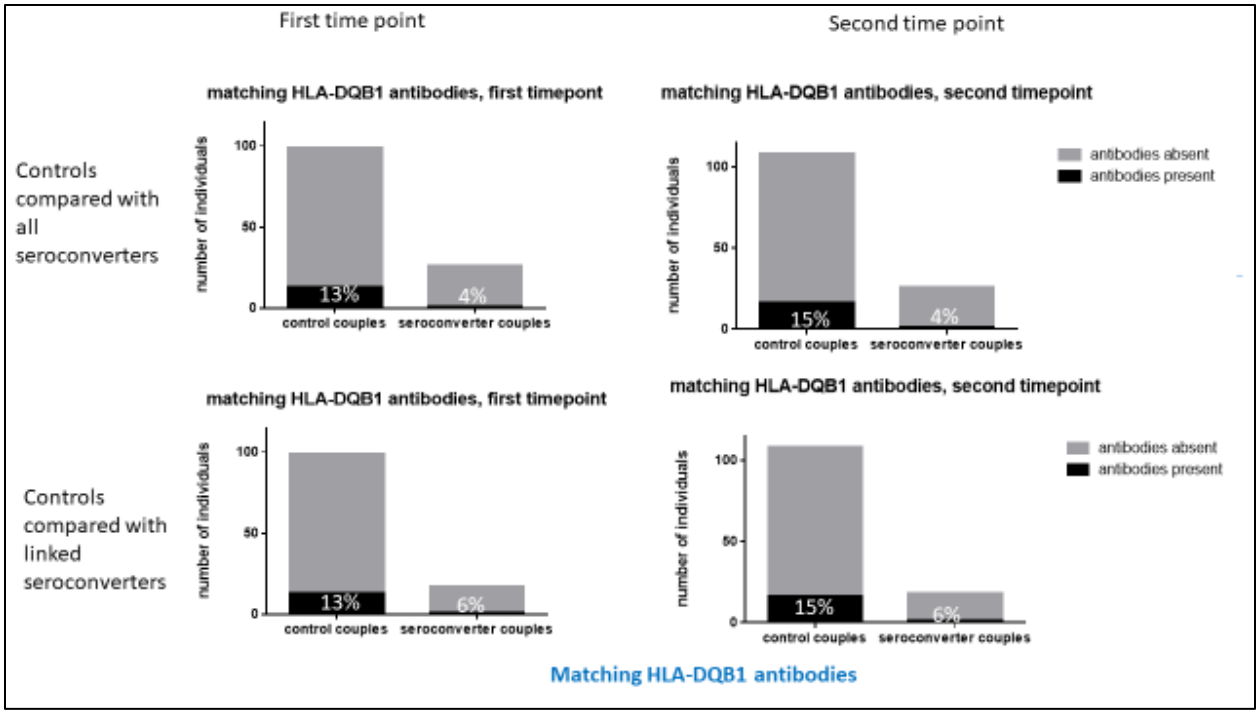
Supplementary figure 4.9. Matching HLA-C antibodies in seroconverter couples compared with control couples at the first and second timepoints



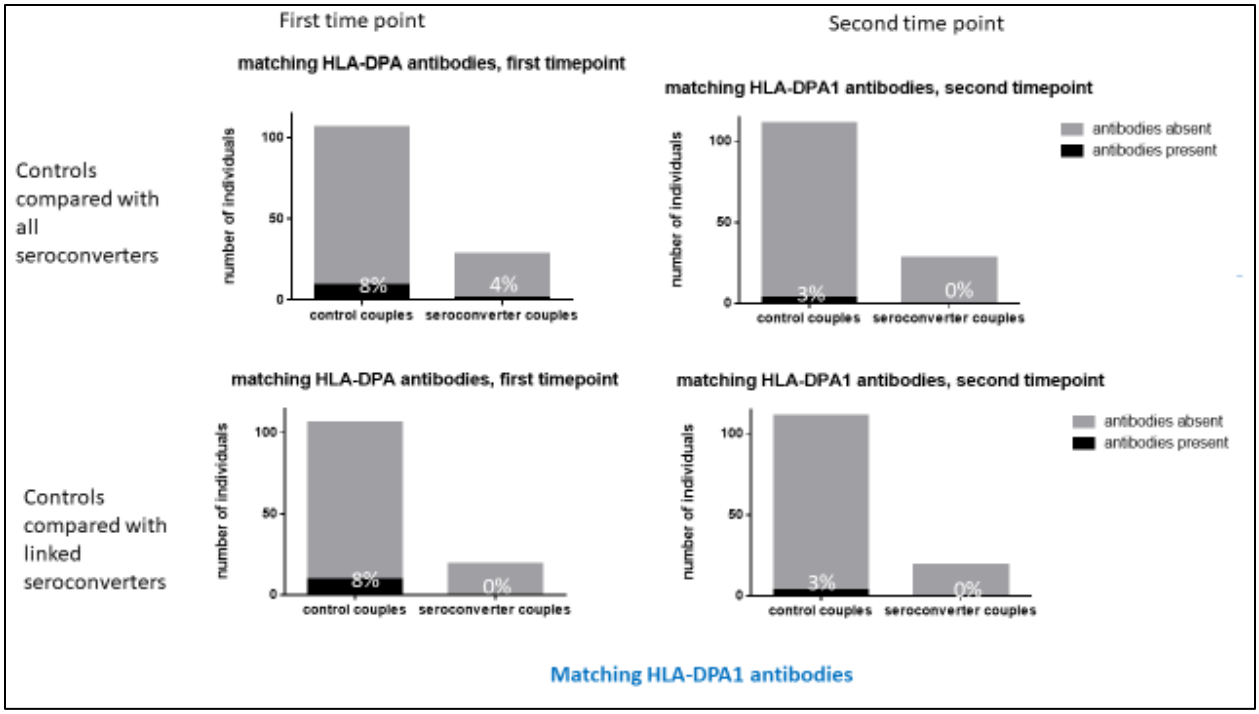
Supplementary figure 4.10. Matching HLA-DRB1 antibodies in seroconverter couples compared with control couples at the first and second timepoints



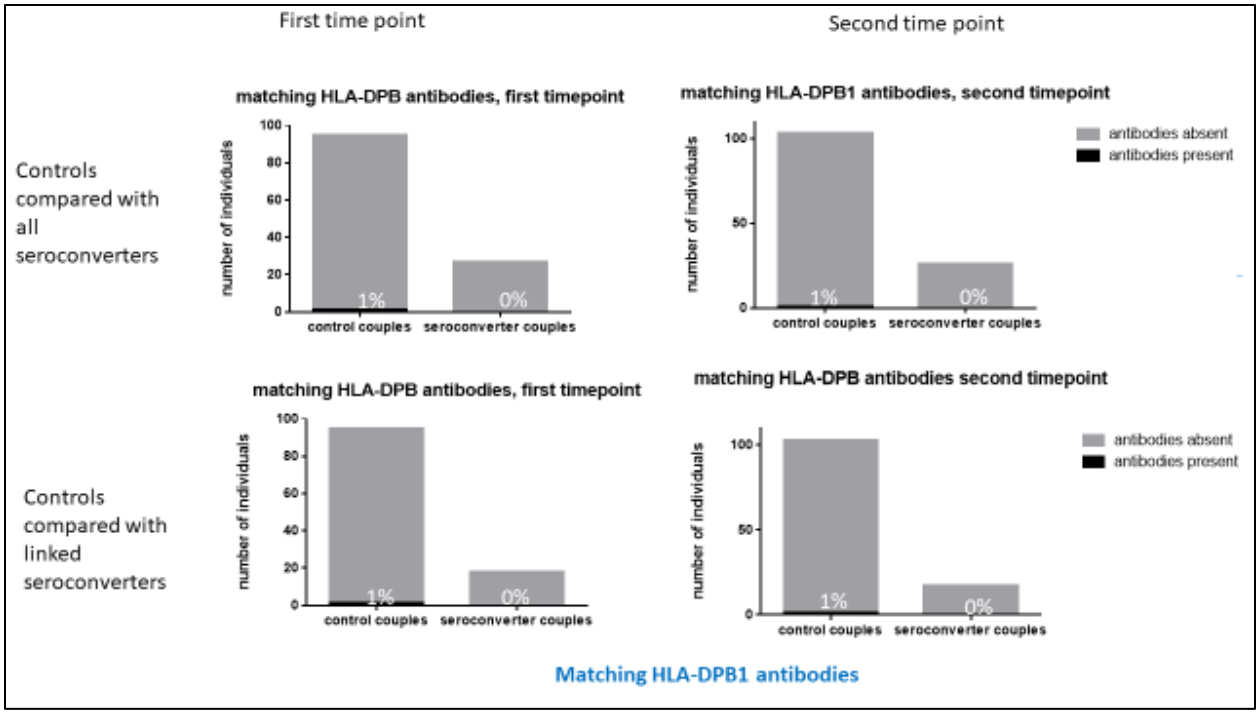
Supplementary figure 4.11. Matching HLA-DRB3,4,5 antibodies in seroconverter couples compared with control couples at the first and second timepoints



Supplementary figure 4.12. HLA-DQB antibodies in seroconverter couples compared with control couples at the first and second timepoints



Supplementary figure 4.13. HLA-DPA antibodies in seroconverter couples compared with control couples at the first and second timepoints



Supplementary figure 4.14. HLA-DPB1 antibodies in seroconverter couples compared with control couples at the first and second timepoints

Supplementary Tables for HIV-serodiscordant couples

Supplementary tables 4.1 appears in thesis as Table 1.1
Supplementary table 4.2 appears in thesis as Table 3.1

Supplementary Table 4.3. Example of antibody specificities in five highly exposed persistently seronegative controls

	CONTROLS			
	Class 1 antibodies (timepoint 1)	**Class 1 antibodies (timepoint 2)**	**Class 2 antibodies (timepoint 1)**	**Class 2 antibodies (timepoint 2)**
1	B*73:01 B*08:01	B*73:01 B*08:01	DRB1*14:04 DRB1*08:01 DRB1*08:02 DRB1*12:02 DRB1*12:01	DRB1*14:04 DRB1*08:01 DRB1*08:02 weak DRB1*12:02 weak DRB1*12:01
2	A*26:01	A*26:01 A*25:01 A*33:01 A*66:02	DQA1*01:01 DQA1*01:02 DQA1*01:03 DQA1*01:04 DQB1*05:01 DQB1*05:02 DQB1*05:03 DQB1*06:01 DQB1*06:02 DQB1*06:03 DQB1*06:04	DQA1*01:01 DQA1*01:02 DQA1*01:03 DQA1*01:04 DQB1*05:01 DQB1*05:02 DQB1*05:03 DQB1*06:01 DQB1*06:02 DQB1*06:03 weak DQB1*06:04
3	A*03:01	A*03:01 A*66:02	DRB1*08:01 DQA1*01:01 DQA1*01:02 DQA1*01:03 DQA1*01:04 DQB1*05:01 DQB1*05:02 DQB1*05:03 DQB1*06:01 DQB1*06:02 DQB1*06:03 DQB1*06:04	DRB1*08:01 DQA1*01:01 DQA1*01:02 DQA1*01:03 DQA1*01:04 DQA1*06:01 DQB1*05:01 DQB1*05:02 DQB1*05:03 DQB1*06:02 DQB1*06:03 DQB1*06:04
4	A*11:02 A*25:01 B*37:01 weak	A*11:02 A*25:01 B*55:01	-	-
5	-	.	DRB1*15:01	DRB1*15:01 DRB1*15:02 weak

*In these highly exposed persistently seronegative controls, individual 1 show reproducible class 1 and class 2 specificities, with a decrease in mean fluorescent intensity of HLA-DRB1*12:01 and HLA-DRB1*12:02 antibodies from strong at the first timepoint to weak at the second timepoint. Individual 2 shows a consistent HLA-A*26:01 antibody, with development of new HLA-A*25:01, HLA-A*33:01 and HLA-A*66:02 antibodies at the second timepoint. Individual 2's class 2 specificities were highly reproducible over time, with only a weakened intensity of HLA-DQB1*06:04 antibody from strong to weak. For individual 3, the HLA-A*03:01 antibody was reproducible, with new development of an HLA-A*66:02 antibody at the second timepoint. The class 2 antibodies were highly comparable at the two timepoints, with new development of a HLA-DQA1*06:01 specificity and loss of a HLA-DQB1*06:01 specificity. In individual 4, HLA-A*11:02 and HLA-A*25:01 recurred at both timepoints, while a weak HLA-B*37:01 antibody present at the first timepoint did not recur, and a new HLA-B*55:01 antibody was noted at the second timepoint. Individual 4 had no class 2 antibodies. Individual 5 had no class 1 antibodies. In individual 5, HLA-DRB1*15:01 antibody was present at both timepoints and weak HLA-DRB1*15:02 antibody only at the second timepoint. Individuals presented were chosen to show a diverse range of possible presentations, from no specificities to many specificities for class 1 or class 2 loci.*

Supplementary Table 4.4 Example of antibody specificities in five HIV-seroconverters

	SEROCONVERTERS			
	Class 1 antibodies (timepoint 1)	**Class 1 antibodies (timepoint 2)**	**Class 2 antibodies (timepoint 1)**	**Class 2 antibodies (timepoint 2)**
6	-	A*23:01 weak A*24:03 weak A*24:02 weak C*04:01 weak A*02:02 weak	-	DRB3*01:01
7	A*24:02 A*24:03	A*24:02 B*15:12 B*82:02	-	-
8	-	A*24:02 A*24:03 B*27:05 B*15:16 B*54:01 B*58:01 B*39:01	DRB1*13:05	DRB1*13:05
9	A*30:01 A*31:01 weak B*46:01	A*30:01 A*31:01 A*43:01 B*44:02 weak B*45:01	-	-
10	-	A*03:01 A*11:01 possible A*30:01 possbile A*36:01 possible A*11:02 possible A*01:01	-	DRB1*07:01

*In HIV-seroconverters, individual 6 had no HLA-A antibodies at the first timepoint (prior to HIV seroconversion) but developed weak HLA-A*02:02, HLA-A*23:01, HLA-A*24:02, HLA-A*24:03 and HLA-C*04:01 antibodies at the second timepoint (after HIV-seroconversion). There were no class 2 antibodies at either timepoint. Individual 7 showed a reproducible HLA-A*24:02 antibody but loss of a HLA-A*24:03 antibody and development of HLA-B*15:12 and HLA-B*82:02 antibodies. Individual 7 had no class 2 antibodies at either timepoint. Individual 8 had no HLA antibodies at the first timepoint but developed HLA-A*24:02, HLA-A*24:03, HLA-B*27:05, HLA-B*15;16, HLA-B*54:01, HLA-B*58:01 and HLA-B*39:01 antibodies at the second timepoint. There was a reproducible class 2 HLA-DRB1*13:05 antibody at both timepoints. In individual 9, There was a reproducible HLA-A*30:01 antibody and a weak HLA-A*31:01 antibody that became stronger at the second timepoint. There was disappearance of a HLA-B*46:01 specificity and development of new HLA-A*43:01, HLA-B*45:01 and weak HLA-B*44:02 specificities. There were no class 2 antibodies at either timepoint. In couple 10 we noted development of new HLA-*

*A*02:01, HLA-A*11:01 and some other possible HLA-A specificities. "Possible" specificities were not considered further in the statistical analysis. Only "strong" and "weak" specificities were included in the chi square analyses. There was new development of a HLA-DRB1*07:01 specificity at the second timepoint. Individuals presented were chosen to show a diverse range of possible presentations, from no specificities to many specificities for class 1 or class 2 loci.*

Supplementary Table 4.5. Complete list of class 2 complement-fixing HLA antibodies in highly exposed persistently seronegative controls and HIV-seroconverters at the first timepoint

couple ID	partner 2	Complement-fixing antibody specificities
control couples		
10143	1014319	DRB1*03:03, DRB1*03:02, DRB1*03:01, DRB3*01:01, DRB1*11:04, DRB1*13:01, DRB1*13:03, DRB1*11:03, DRB3*03:01,DRB1*11:04, DRB1*11:01, DRB1*14:01
10139	1013917	DQB1*03:02
10161	1016116	DPB1*17:01
10005	1000510	DRB4*01:01
10025	1002511	DRB4*01:01, DPB1*17:01
10106	1010616	DQA1*01:04
20165	2016512	DRB4*01:01
19066	1906610	DRB4*01:01
Seroconverting couples		
20091	2009113	DRB4*01:01

"Control couples" represent highly exposed persistently seronegative individuals. "Seroconverting couples" represent individuals who acquired HIV. Table represents all individuals who had any complement-fixing class 2 HLA antibodies. Complement-fixing antibodies against HLA class 1 were not investigated.

Supplementary Table 4.6. Risk factors for HIV acquisition by males who were HIV-uninfected at enrolment

(n=84 highly exposed persistently seronegative male controls and 21 male HIV-seroconverters)

Male seroconverters versus male highly exposed persistently seronegative controls				
	Univariate		**Multivariate**	
Variable	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Cohort: Cos vs Partners	1.334 (0.132-13.51)	0.8075		
Ethnicity: Sotho vs Other	2.222 (0.363-13.61)	0.3881		
Zulu vs Other	3.333 (0.696-15.96)	0.1320		
Site: Cape Town vs Johannesburg	3.195 (1.188-8.596)	**0.0214***	3.218 (1.136-9.117)	**0.0278**
Number of children at enrolment	0.774 (0.518-1.159)	0.2135		
Ever unprotected sex: No vs Yes	0.977 (0.375-2.549)	0.9625		
Proportion unprotected sex	0.705 (0.106-4.684)	0.7177		
Number unprotected sex	0.929 (0.708-1.219)	0.5930		
No. of no condom sex acts	0.993 (0.967-1.020)	0.6225		
Interval between 2 timepoints (mo)	1.030 (0.942-1.126)	0.5202		
Chlamydia: Positive vs Negative	1.027 (0.108-9.718)	0.9818		
Trichomonas: Positive vs Negative	3.441 (0.704-16.81)	0.1268		
Genital ulcers: Yes vs No	1.128 (0.364-3.500)	0.8342		
Any STI: Positive vs Negative	3.850 (0.930-15.94)	**0.0629**		
Earliest Log VL	1.971 (0.944-4.119)	**0.0710**	1.731 (0.801-3.744)	0.1631

Max Log VL	1.949 (1.026-3.700)	**0.0414**		
Median Log VL	1.650 (0.870-3.128)	0.1254		
Any class 1 HLA Abs first timepoint: yes vs no	1.100 (0.419-2.886)	0.8464		
Number of class 1 specificities first timepoint	1.115 (0.910-1.367)	0.2934		
Any class 2 HLA abs first timepoint: yes vs no	0.346 (0.115-1.038)	**0.0584**		
Number of DRB1 and DRB345 specificities first timepoint	0.670 (0.280-1.602)	0.3676		
Matching class 1 first timepoint: yes vs no	6.333 (1.357-29.55)	**0.0188***		
Matching class 2 first timepoint: yes vs no	0.149 (0.018-1.196)	**0.0732**		

STI sexually transmitted infection, VL viral load

Supplementary Table 4.7. Risk factors for HIV acquisition by females who were HIV-uninfected at enrolment

(n=31 highly exposed persistently seronegative female controls and 7 female HIV-seroconverters)

Female HIV-seroconverters versus female highly exposed persistently seronegative controls		
	Univariate	
Variable	**OR (95% CI)**	**P value**
Cohort: COS vs Partners	3.733 (0.492-28.33)	0.2026
Ethnicity: Sotho vs Other	0.444 (0.034-5.880)	0.5383
Zulu vs Other	1.143 (0.170-7.693)	0.8908
Site: Cape Town vs Johannesburg	3.125 (0.547-17.84)	0.1999
Number of children at enrolment	0.696 (0.316-1.532)	0.3679
Ever unprotected sex: No vs Yes	0.634 (0.106-3.802)	0.6176
Proportion unprotected sex	0.436 (0.008-25.04)	0.6880
Number unprotected sex	1.039 (0.591-1.826)	0.8939
Number of no condom sex acts	0.967 (0.876-1.067)	0.5020
Number of class 1 specificities first timepoint	0.982 (0.912-1.058)	0.6337
Any class 1 HLA Abs first timepoint: yes vs no	0.533 (0.097-2.939)	0.4704
Number of DRB1 and DRB345 specificities first timepoint	0.837 (0.424-1.652)	0.6084
Any class 2 HLA abs first timepoint: yes vs no	0.563 (0.106-2.999)	0.5004
Matching class 1 first timepoint: yes vs no	0.371 (0.036-3.838)	0.4059
Matching class 2 first timepoint: yes vs no	0.778 (0.117-5.162)	0.7947
Interval between 2 timepoints (months)	1.067 (0.920-1.237)	0.3887

Trichomonas: Positive vs Negative	1.619 (0.220-11.89)	0.6358
Genital ulcers: Yes vs No	0.727 (0.121-4.388)	0.7284
Any STI: Positive vs Negative	1.333 (0.184-9.660)	0.7758
Earliest Log VL	3.494 (0.765-15.95)	0.1063
Max Log VL	2.188 (0.603-7.947)	0.2340
Median Log VL	4.331 (0.891-21.07)	**0.0693**

No multivariate analysis was performed as no factors had p values below 0.05 in univariate analysis. STI sexually transmitted infection. VL viral load.

Supplementary Table 4.8. Risk factors for HIV transmission from index partner who was HIV-infected at enrolment

(n=115 HIV-infected index partners to highly exposed persistently seronegative controls and 19 HIV-infected index partners to linked HIV-seroconverters)

HIV-infected Index Partners – HIV-transmitters versus HIV-non-transmitters				
	Univariate		**Multivariate**	
Variable	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Gender: Females vs Males	1.033 (0.344-3.107)	0.9537		
Enrolment age (years)	1.011 (0.951-1.074)	0.7333		
Cohort: Cos vs Hsv	3.406 (0.774-14.99)	0.1050		
Ethnicity: Sotho vs Other Xhosa vs Other Zulu vs Other	1.706 (0.220-13.24) 2.836 (0.572-14.06) 3.782 (0.697-20.52)	0.6096 0.2019 0.1232		
Site: Cape Town vs Johannesburg	2.260 (0.827-6.176)	0.1120		
Number of children at enrolment	0.663 (0.418-1.051)	**0.0805**	0.576 (0.329-1.007)	**0.0528**
Ever unprotected sex: No vs Yes	0.148 (0.033-0.669)	**0.0131***	0.088 (0.018-0.440)	**0.0031***
Proportion unprotected sex	0.828 (0.078-8.814)	0.8756		
Number unprotected sex	0.972 (0.850-1.111)	0.6776		
No. of no condom sex acts	0.992 (0.970-1.013)	0.4469		
Contraception injection	1.091 (0.408-2.919)	0.8624		
Genital ulcers: Yes vs No	1.653 (0.595-4.587)	0.3347		
Any STI: Positive vs Negative	1.152 (0.369-3.595)	0.8081		
Earliest Log VL	3.813 (1.563-9.305)	**0.0033***		
Max Log VL	2.098 (1.062-4.143)	**0.0328***		
Median Log VL	2.482 (1.182-5.212)	**0.0163***	3.038 (1.282-7.200)	**0.0116***

STI sexually transmitted infection, VL viral load

Supplementary Table 4.9. Risk factors for HIV transmission from male index partners who were HIV-infected at enrolment

(n=31 male index partners of highly exposed persistently seronegative individuals and 5 male index partners of linked HIV-seroconverters)

Male HIV-infected Index Partners – HIV transmitters versus HIV-non-transmitters		
	Univariate	
Variable	**OR (95% CI)**	**p value**
Enrolment age (years)	0.947 (0.817-1.098)	0.4688
Cohort: Cos vs Partners	6.222 (0.725-53.37)	**0.0955**
Site: Cape Town vs Johannesburg	2.778 (0.376-20.50)	0.3164
Number of children at enrolment	0.695 (0.278-1.738)	0.4367
Ever unprotected sex: No vs Yes	0.267 (0.027-2.665)	0.2604
Proportion unprotected sex	0.526 (0.004-72.73)	0.7986
Number unprotected sex	0.952 (0.693-1.307)	0.7597
No. of no condom sex acts	0.980 (0.906-1.059)	0.6053
Genital ulcers: Yes vs No	2.286 (0.316-16.51)	0.4126
Any STI: Positive vs Negative	1.278 (0.112-14.59)	0.8435
Earliest Log VL	7.945 (0.927-68.09)	**0.0586**
Max Log VL	2.925 (0.638-13.41)	0.1672
Median Log VL	6.810 (0.945-49.07)	**0.0569**

STI sexually transmitted infection, VL viral load

Supplementary Table 4.10. Univariate risk factors for HIV transmission from female index partners who were HIV-infected at enrolment

(n=84 female HIV-infected index partners of highly exposed persistently seronegative individuals and 14 female HIV-infected index partners of HIV-seroconverters)

Female HIV-infected index partners - HIV-transmitters versus HIV-non-transmitters				
	Univariate		**Multivariate**	
Variable	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Enrolment age (years)	1.026 (0.960-1.096)	0.4505		
Cohort: Cos vs Partners	2.077 (0.201-21.51)	0.5400		
Ethnicity: Sotho vs Other Xhosa vs Other Zulu vs Other	0.607 (0.050-7.415) 1.653 (0.310-8.815) 2.000 (0.322-12.41)	0.6960 0.5563 0.4568		
Site: Cape Town vs Johannesburg	2.114 (0.659-6.775)	0.2080		
Number of children at enrolment	0.644 (0.374-1.108)	0.1121	0.558 (0.302-1.030)	**0.0621**
Ever unprotected sex: No vs Yes	0.103 (0.013-0.820)	**0.0318***	0.088 (0.017-0.451)	**0.0036***
Proportion unprotected sex	0.960 (0.063-14.54)	0.9767		
Number unprotected sex	0.977 (0.841-1.134)	0.7556		
No. of no condom sex acts	0.993 (0.972-1.015)	0.5416		
Genital ulcers: Yes vs No	1.474 (0.447-4.862)	0.5243		
Any STI: Positive vs Negative	1.104 (0.303-4.025)	0.8806		
Earliest Log VL	3.164 (1.165-8.596)	**0.0239***	4.683 (1.677-13.08)	**0.0032***
Max Log VL	1.930 (0.909-4.096)	**0.0870**		
Median Log VL	2.007 (0.903-4.458)	**0.0873**		

STI sexually transmitted infection, VL viral load

Supplementary Data related to Mothers and Infants (for Chapter 5)

Supplementary Table 5.1. List of specificities targeted by HLA class I antibodies in mothers-only antibodies, infants-only antibodies and antibodies shared by mothers and their infants (six weeks after birth); hyphen – no antibodies detected.

Mother-infant pair number	HLA class I antibodies
1	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
2	-
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
3	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
4	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
5	
Shared HLA I Abs	B*15:12
Mother HLA I Abs	A*23:01
Baby HLA I Abs	-
6	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
7	
Shared HLA I Abs	-
Mother HLA I Abs	B*14:02 B*15:18 B*08:01 B*14:01 B*39:01 B*27:08 B*18:01 B*82:02 B*07:02 B*45:01 B*67:01 B*15:12 B*56:01 B*15:01 B*07:03 B*35:08 B*15:02 B*50:01 B*55:01 B*35:01 B*54:01 B*78:01 B*42:01 B*40:02 B*15:03 B*41:01 B*40:01 B*81:01 B*46:01 B*51:01 A*23:01 A*24:03 A*24:02 C*01:02
Baby HLA I Abs	-
8	
Shared HLA I Abs	-
Mother HLA I Abs	B*44:03 B*44:02 B*15:12 B*45:01 B*49:01 B*82:02 B*50:01 B*47:01 B*40:01 B*41:01 B*40:02 B*13:02 B*15:01 B*15:03 B*15:18 B*53:01 A*01:01 B*37:01 B*15:02 B*78:01 B*15:13 B*35:08 B*27:05 B*18:01 B*52:01 B*07:03 B*14:01 B*59:01 B*51:01 B*35:01 B*38:01 B*27:03 B*39:01 B*27:08
Baby HLA I Abs	-
9	
Shared HLA I Abs	-
Mother HLA I Abs	B*57:01 B*45:01 B*15:12 B*44:03 A*02:02 A*02:03 A*69:01 B*58:01 B*82:02 A*03:01 A*11:02 A*31:01 A*66:02 A*32:01 A*34:02 A*29:01 A*26:01 A*01:01 A*33:03 B*49:01 B*40:01 B*41:01 A*68:01 A*68:02 B*44:02 A*02:05 A*02:01 A*24:03 A*24:02 A*23:01 A*11:01 A*29:02 B*40:02 A*66:01 A*74:01 A*25:01 A*33:01 A*43:01 A*36:01 A*80:01 B*13:02 B*50:01 B*47:01
Baby HLA I Abs	-
10	
Shared HLA I Abs	A*24:02 A*24:03 B*15:12 A*23:01
Mother HLA I Abs	A*68:02 A*02:02 A*02:01 A*69:01 B*39:01 B*18:01 B*67:01 B*82:02 B*14:01 B*15:16 B*15:13 A*66:01 B*51:01 A*11:02 C*03:04 B*37:01 A*02:03 A*68:01 B*15:03 B*78:01 B*15:02 B*07:02 B*55:01 B*42:01 B*53:01 B*81:01 B*46:01 A*26:01 A*03:01 A*66:02 A*02:05 B*15:18 B*56:01 B*50:01 B*57:01 B*45:01 B*35:08 B*40:02 B*49:01 B*14:02 B*38:01 A34:02 A*11:01 A*33:01 B*27:08 B*54:01 B*35:01 B*15:01 B*08:01 B*58:01 B*41:01 B*07:03 B*40:01 A*25:01 B*48:01 B*52:01 A*33:03 C*03:03
Baby HLA I Abs	-
11	
Shared HLA I Abs	-
Mother HLA I Abs	A*23:01
Baby HLA I Abs	-
12	
Shared HLA I Abs	B*37:01
Mother HLA I Abs	B*27:08 B*27:05 B*40:02 B*47:01 B*73:01 B*40:01 B*07:02 B*81:01 B*13:02 A*66:02 B*07:03 C*18:01 C*05:01 C*03:04 A*66:01 A*29:02 A*29:01 C*04:03 B*27:03 C*02:02 C*06:02 C*15:02 B*48:01 A*43:01 A*26:01
Baby HLA I Abs	B*15:12 B*44:03 B*44:02 B*45:01
13	
Shared HLA I Abs	-
Mother HLA I Abs	B*67:01 B*42:01 B*55:01 B*82:02 B*56:01 B*08:01 B*07:02 B*81:01 B*27:08 B*37:01 B*54:01
Baby HLA I Abs	A*24:02
14	
Shared HLA I Abs	-
Mother HLA I Abs	A*32:01 A*25:01 A*66:02 A*43:01 A*33:01 A*68:02 A*68:01 A*02:01 A*31:01 A*02:02 A*66:01 A*34:02 A*26:01 A*29:01 A*74:01 A*02:03 A*33:03 A*02:05 A*69:01 A*29:02 B*46:01 B*73:01 B*55:01 B*42:01 B*37:01 C*12:02 C*08:01 C*07:01 C*07:02 C*03:04 C*03:03 C*01:02 C*14:02 C*08:02 C*16:01 C*15:02 C*06:02
Baby HLA I Abs	-

15	
Shared HLA I Abs	B*53:01 A*24:02 B*51:01
Mother HLA I Abs	A*25:01 A*23:01 B*35:01 B*35:08 B*78:01
Baby HLA I Abs	B*37:01
16	
Shared HLA I Abs	-
Mother HLA I Abs	B*27:08 B*07:02 A*02:05 A*02:02 A*02:03 B*27:05 A*68:01 A*24:03 A*68:02 A*02:01 A*25:01 A*69:01 A*66:02 A*66:01 A*34:02 A*24:02 B*40:01 A*26:01 A*33:03 B*81:01 A*23:01 B*07:03 A*33:01 B*40:02 B*15:16 B*47:01 B*73:01 B*13:02 B*54:01 B*39:01 B*27:03 B*48:01 B*38:01 A*11:01 A*11:02 B*15:02 B*15:13 B*53:01 A*29:02 B*15:18
Baby HLA I Abs	-
17	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
18	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	-
19	
Shared HLA I Abs	-
Mother HLA I Abs	A*29:02 A*29:01 A*43:01 B*13:02 B*15:12 B*15:16 B*15:01 B*15:13 B*57:01 B*15:02 A*24:02 A*80:01 A*23:01 A*68:01 A*30:01 A*34:02 A*03:01 A*33:03 A*24:03 A*33:01 A*31:01 A*02:05 A*25:01 A*02:03 A*02:02 B*27:05 A*02:01 A*26:01 A*68:02 A*36:01 A*66:02 B*27:08 A*11:01 A*69:01 A*66:01 A*11:02 A*01:01 B*44:02 B*47:01 B*44:03 B*49:01 B*46:01 B*07:02 B*40:02 B*40:01 B*45:01 B*50:01 A*32:01 B*81:01 B*07:03 B*41:01 B*58:01 B*27:03 B*59:01 B*67:01 B*53:01 B*38:01 B*37:01
Baby HLA I Abs	-
20	
Shared HLA I Abs	A*24:02 A*24:03 A*23:01
Mother HLA I Abs	B*15:02 B*15:13 B*18:01 A*36:01 A*01:01 B*07:03 B*48:01 B*81:01 A*26:01 A*25:01
Baby HLA I Abs	A*02:05
21	
Shared HLA I Abs	-
Mother HLA I Abs	B*15:03
Baby HLA I Abs	-
22	
Shared HLA I Abs	-
Mother HLA I Abs	B*15:12 B*44:03 B*45:01 B*82:02 B*44:02
Baby HLA I Abs	A*66:01
23	
Shared HLA I Abs	-
Mother HLA I Abs	A*33:01 A*31:03 A*33:03
Baby HLA I Abs	-
24	
Shared HLA I Abs	A*24:02
Mother HLA I Abs	B*39:01 B*67:01 B*38:01 A*24:03 B*82:02
Baby HLA I Abs	B*57:01 B*58:01
25	
Shared HLA I Abs	B*15:12 B*44:03 B*82:02 B*45:01 B*44:02 A*01:01 A*24:02 A*23:01
Mother HLA I Abs	B*49:01 B*15:16 B*59:01 B*57:01 B*51:01 B*38:01 B*52:01 B*53:01 B*58:01
Baby HLA I Abs	C*17:01
26	
Shared HLA I Abs	-
Mother HLA I Abs	-
Baby HLA I Abs	B*57:01 B*58:01
27	
Shared HLA I Abs	A*23:01 A*24:02 A*24:03
Mother HLA I Abs	A*02:02 A*02:05 A*02:01 A*02:03 A*01:01 B*45:01 B*44:03 B*44:02 B*15:12 B*50:01 B*82:02 B*49:01 B*41:01 B*15:03 B*15:01 B*15:18 B*35:01 B*39:01 B*40:01 B*38:01 B*78:01 B*15:02 B*40:02 B*53:01 B*18:01 B*35:08 B*47:01 B*15:13 B*13:02 B*37:01 B*14:01 B*07:03 B*52:01 B*08:01 A*69:01 B*59:01 B*51:01 B*48:01 B*56:01 B*54:01 B*14:02
Baby HLA I Abs	-
28	
Shared HLA I Abs	B*44:03 B*37:01 B*44:02 B*38:01 B*53:01 B*27:03 B*27:05 B*47:01 B*49:01 B*15:13 B*13:02 B*57:01 B*59:01 B*15:16 B*51:01 B*58:01 B*52:01 A*24:03 A*24:02 A*23:01
Mother HLA I Abs	A*02:01 A*02:02 A*02:03 A*01:01 A*02:05 A*68:02 A*68:01 A*69:01 B*15:12 B*45:01 B*82:02 B*50:01 B*40:02 B*15:01 B*15:03 B*40:01 B*15:18 B*41:01 B*15:02 B*39:01 B*35:01 B*78:01 B*18:01 B*35:08 B*14:01 B*48:01 B*46:01 B*07:03 B*14:02 B*73:01 B*08:01 B*27:08 B*56:01 B*55:01 B*67:01 B*54:01 C*12:02 C*03:04 C*03:03 C*08:01 C*14:02 C*01:02 C*16:01 C*08:02 C*07:02 C*07:01
Baby HLA I Abs	A*32:01
29	
Shared HLA I Abs	B*15:12
Mother HLA I Abs	B*08:01 B*59:01 A*33:01 B*18:01 B*14:01 B*14:02 B*78:01 A*31:01 A*33:03 B*54:01 B*46:01 B*51:01 A*29:01 A*29:02 B*39:01 B*48:01 B*81:01 A*26:01 A*25:01

Supplementary Table 5.2. List of specificities targeted by HLA class II antibodies in mothers-only antibodies, infants-only antibodies and antibodies shared by mothers and their infants (six weeks after birth); hyphen – no antibodies detected; n/a – not analysed.

Mother-infant pair number	HLA class II antibodies
1	
Shared HLA II Abs	-
Mother HLA II Abs	DRB1*16:02 DRB1*12:02 DQA1*01:03 DQA1*02:01 DQB1*06:01 DQA1*04:01 DQB1*04:01
Baby HLA II Abs	-
2	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
3	
Shared HLA II Abs	DPA1*02:02 DPB1*28:01 DPB1*04:01 DPA1*03:01 DPB1*04:02
Mother HLA II Abs	DQA1*04:01 DQA1*05:01 DQB1*03:03 DPA1*02:01 DPA1*01:03 DPB1*05:01
Baby HLA II Abs	DRB3*01:01
4	
Shared HLA II Abs	DRB3*01:01
Mother HLA II Abs	DRB1*08:01 DQA1*01:02 DQB1*05:01
Baby HLA II Abs	DQA1*02:01 DQB1*02:01 DQA1*05:01 DQB1*02:02 DPA1*02:01 DPB1*01:01
5	
Shared HLA II Abs	-
Mother HLA II Abs	DRB1*03:01 DPA1*01:03 DPA1*02:02 DPA1*02:01
Baby HLA II Abs	DQA1*02:01 DQB1*03:02
6	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	DQA1*02:01 DQB1*03:02 DQA1*04:01 DQB1*04:02
7	
Shared HLA II Abs	-
Mother HLA II Abs	DQA1*04:01 DQB1*03:03
Baby HLA II Abs	-
8	
Baby HLA I Abs	-
Shared HLA II Abs	DPA1*01:03 DPB1*06:01 DPA1*02:01 DPB1*14:01
Mother HLA II Abs	DRB1*15:01 DRB1*15:02 DRB1*15:03 DRB5*02:02 DRB1*16:01 DRB1*16:02 DRB1*04:05 DRB1*01:01 DRB1*01:02 DRB1*04:04 DQA1*01:03 DQA1*01:02 DQB1*06:03 DQB1*06:02 DPB1*05:01 DPB1*13:01 DPA1*02:02 DPA1*03:01 DPA1*04:01 DPB1*01:01 DPB1*04:01 DPB1*11:01 DPB1*19:01 DPB1*15:01 DPB1*03:01 DPB1*09:01 DPB1*17:01 DPB1*28:01
Baby HLA II Abs	-
9	
Shared HLA II Abs	n/a
Mother HLA II Abs	DQA1*02:01 DQB1*03:02
Baby HLA II Abs	n/a
10	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
11	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
12	
Shared HLA II Abs	DQB1*06:04 DQA1*01:02 DQA1*01:03 DQB1*06:01 DQB1*06:02 DQB1*06:03 DQA1*01:04
Mother HLA II Abs	DRB1*15:01 DRB1*15:02 DRB1*15:03 DRB1*16:01 DRB1*16:02 DQA1*01:01 DQA1*02:01 DQA1*03:02 DQA1*03:01 DQA1*04:01 DQA1*05:01 DQB1*05:01 DQB1*05:02 DQB1*05:03 DQB1*03:02 DQB1*03:03 DQB1*03:01
Baby HLA II Abs	DQB1*05:01
13	
Shared HLA II Abs	DRB3*01:01 DQA1*05:01 DQB1*03:01 DQA1*06:01 DQA1*03:02
Mother HLA II Abs	DRB3*03:01 DQA1*04:01 DQB1*04:01 DQB1*03:03 DQB1*04:02
Baby HLA II Abs	DQA1*03:01
14	
Shared HLA II Abs	-
Mother HLA II Abs	DQA1*04:01 DQB1*03:03
Baby HLA II Abs	DRB1*07:01 DQA1*05:01 DQB1*02:02

15	
Shared HLA II Abs	DQA1*03:02
Mother HLA II Abs	DQA1*02:01 DQB1*04:01 DQA1*03:01 DQB1*06:01 DQA1*04:01 DQB1*04:02 DQB1*03:01 DQA1*06:01 DQB1*02:01
Baby HLA II Abs	DQB1*03:02
16	
Shared HLA II Abs	-
Mother HLA II Abs	n/a
Baby HLA II Abs	-
17	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	DRB1*04:01 DRB1*16:02
18	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
19	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
20	
Shared HLA II Abs	DRB1*10:01
Mother HLA II Abs	DRB4*01:01 DQA1*05:01 DQB1*02:02 DQB1*02:01
Baby HLA II Abs	-
21	
Shared HLA II Abs	-
Mother HLA II Abs	DRB1*01:01 DRB1*09:01 DRB1*01:02 DRB1*01:03 DRB5*02:02 DRB5*01:01 DRB1*10:01 DQA1*01:02 DQA1*01:01 DQA1*01:03 DQA1*01:04 DQB1*06:02 DQB1*06:04 DQB1*05:01 DQB1*05:02 DQB1*06:03 DQB1*05:03 DQB1*06:01
Baby HLA II Abs	-
22	
Shared HLA II Abs	-
Mother HLA II Abs	n/a
Baby HLA II Abs	-
23	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	DQA1*01:02 DQB1*06:02
24	
Shared HLA II Abs	-
Mother HLA II Abs	DRB1*11:04 DRB1*13:01 DRB1*01:03 DRB1*16:01 DRB1*16:02 DRB1*11:03 DRB1*04:02 DRB1*13:05 DRB1*13:03 DRB1*11:01 DRB1*12:01 DRB1*08:01 DRB1*12:02 DRB5*01:01 DRB1*14:03 DRB1*15:01 DRB5*02:02 DRB1*15:02 DRB1*08:02 DRB1*15:03 DRB1*14:01 DRB1*04:05 DRB1*04:04 DRB1*04:01 DRB1*01:01 DRB1*01:02 DRB1*07:01
Baby HLA II Abs	-
25	
Shared HLA II Abs	-
Mother HLA II Abs	DRB1*15:03 DRB1*15:01 DRB1*15:02 DRB1*16:01
Baby HLA II Abs	-
26	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
27	
Shared HLA II Abs	n/a
Mother HLA II Abs	n/a
Baby HLA II Abs	-
28	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	-
29	
Shared HLA II Abs	-
Mother HLA II Abs	-
Baby HLA II Abs	DRB3*02:02

R14/49 Dr Melinda Suchard et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1411118

NAME: (Principal Investigator) Dr Melinda Suchard et al

DEPARTMENT: Molecular Medicine and Haematology
National Institute for Communicable Disease

PROJECT TITLE: HLA Antibodies - Gearing for Novel Allo-Immunization Strategies against HIV

DATE CONSIDERED: Adhoc

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR:

APPROVED BY: Professor P Cleaton-Jones Chairperson, HREC (Medical)

DATE OF APPROVAL: 04/02/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee: (Medical)
FWA Registered No IRB 00001223

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa • Tel: +27-11-274-9200 • Fax: +27-11-274-9281

19 January 2007

COURIERED

Dr G de Bruyn,
Perinatal HIV Research Unit
Box 114
Diepkloof

2013

Fax: 011 986 9762

Dear Dr de Bruyn,

PROTOCOL: HOST GENETIC FACTORS - INVESTIGATIONS OF HOST GENETIC FACTORS AFFECTING HIV, HIV-2, OTHER STDS AND VIRUSES INTERACTING WITH HIV IN THE PARTNERS IN PREVENTION STUDY

ETHICS REFERENCE NO: 060808

RE : FINAL ETHICS APPROVAL

This is to certify that the above-mentioned trial was reviewed by the University of the Witwatersrand, Human Research Ethics Committee (HREC) and the Protocol Review Committee (PRC) on: 25 August 2006.

The University of the Witwatersrand, Human Research Ethics Committee Approval Granted for the above mentioned study is valid for five years. Where required by Sponsor to have approval on a more frequent basis it remains the responsibility of the Sponsor and Investigator to apply for continuing review and approval, or for the duration of the Tria .

1. THIS APPROVAL IS SUBJECT TO THE FOLLOWING PROVISOS:

* A copy of the MCC Approval and/or MCC Notification letter must be submitted to the Ethics Regulatory Office Secretariat before the study commences.

* The study is conducted according to the protocol submitted to the University of the Witwatersrand, Human Research Ethics Committee. Any amendments to the protocol must first be submitted to the Human Research Ethics Committee for approval.

* During the study, the University of the Witwatersrand Human Research Ethics Committee is informed immediately of :
  - Any Unexpected Serious Adverse Events or Unexpected Adverse Drug Reactions, which, in the Investigator and/or the Sponsor's opinion are suspected to be related to the study drug. (International and Local Reports).
  - Any data received during the trial which, may cast doubt on the validity of the continuation of the study

* The University of the Witwatersrand, Human Research Ethics Committee is notified of any decision to discontinue the study and the reason stated.

* The Investigators authorised by this approval participate in this study. Additional Investigators shall be submitted to the University of the Witwatersrand, Human Research Ethics Committee for approval prior to their participation in the study.

* In the event of an authorised Investigator ceasing to participate in the study, the University of the Witwatersrand, Human Research Ethics Committee must be informed and the reason for such cessation given.

2. PRINCIPLES OF INFORMED CONSENT:

R14/49 Miss Vania Duxbury

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130230

NAME: (Principal Investigator) Miss Vania Duxbury

DEPARTMENT: School of Physiology
Medical School

PROJECT TITLE: Natural History of HLA antibodies in Pregnancy

DATE CONSIDERED: 22/02/2013

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr K Scheuermaier

APPROVED BY: _____
Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 27/05/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

_____ _____
Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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ORIGINAL ARTICLE

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HLA antibody repertoire in infants suggests selectivity in transplacental crossing

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Funding information
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Abstract

Problem: Late in pregnancy, women produce and transfer high amounts of antibodies to the foetus. During gestation, women produce antibodies against human leukocyte antigens (HLA), including antibodies directed at foetal HLA. There is paucity of data on transplacental crossing, specificity and role of HLA antibodies in pregnancy and new-borns.

Method of study: Using highly sensitive Luminex technology, we measured prevalence of IgG HLA antibodies in 30 mother-infant pairs six weeks post-partum. Additionally, in six pregnant women, we measured HLA antibodies longitudinally and HLA-typed infant DNA to assess whether maternal HLA antibodies were directed at infant specificities.

Results: Overall, 68% of mothers and 44% of infants expressed HLA-I antibodies and 56% of mothers and 52% of infants expressed HLA-II antibodies. Infants shared up to 78% of antibodies with their mothers, suggesting that the remaining antibodies were self-made. Less than 25% of maternal HLA antibodies were detected in infants, possibly due to selection in transplacental crossing. We detected complement-fixing HLA antibodies in mothers and at low levels in infants. In a third of our pregnant subjects, we detected infant-directed HLA antibodies.

Conclusion: Our findings raise the possibility of selection in transplacental crossing of HLA antibodies. As HLA antibodies may act as autoantibodies in the neonate, the mechanism of a selective transfer may give important insights into immune tolerance. Findings also suggest that infants start producing their own HLA antibodies in the first weeks of life, which, together with maternally derived antibodies may impact the infant's immune reaction to HLA proteins.

KEYWORDS

alloimmunity, HLA, HLA antibodies, immune tolerance, placenta, pregnancy, transplacental crossing

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1 | INTRODUCTION

Human leukocyte antigens (HLA) are proteins responsible for presentation of short peptides to responding T lymphocytes.[1-3] By binding to T-cell receptors on the surface of T lymphocytes, HLA proteins lead to T cell activation, as well as to recruitment of additional immune players.[2,3] HLA class I proteins, encoded by the loci HLA-A, HLA-B and HLA-C, are expressed on the surface of all nucleated cells, whereas HLA class II proteins, encoded by the loci DRB1, DQA/B, DRB3,4,5 and DPA/B, are expressed only on the surface of professional antigen-presenting cells. Together, the seven HLA-encoding loci are the most polymorphic genes in the human genome, with thousands of alleles expressed in the population.[1-3] This high polymorphism may result in HLA-induced alloimmunity mediated by antibodies targeted against HLA, referred to as HLA antibodies.

For a long time, HLA antibodies were believed to be expressed only following sensitizing events such as blood transfusions, multiple pregnancies or intravenous drug use. However, modern bead-based technology has led to the discovery of previously undetectable low levels of HLA antibodies in up to 77 per cent of healthy individuals with no history of sensitizing events.[4-10] Such antibodies may be against one or more HLA specificities,[6,11] although whether they are produced in response to these HLA antigens or just cross-react with them is not clear at this point.

During pregnancy, women produce and transfer a large quantity of antibodies to the foetus,[12,13] which is well described for antibodies against pathogens such as measles or pneumococcus.[14-16] This process begins at around 13 weeks of gestation and increases with gestational age, with the majority of antibodies crossing the placenta during the third trimester.[12,17-20] Transplacental crossing is mediated by neonatal FcRn receptors, and most antibodies transferred are IgG.[12,20-24] It has been found that infants born preterm have markedly lower IgG levels at birth compared with term infants.[12,25] IgA antibodies have also been shown to cross the placenta[13,26,27]; however, this occurs at low levels, as the main mode of transferring IgA antibodies to the baby is through breastfeeding.[28]

Transplacental crossing of antibodies has recently been shown to involve some degree of selection based on post-translational modifications in the fragment crystalline (Fc) region.[29,30] In these studies, IgG molecules with digalactosylated glycans in the Fc region were shown to have higher binding affinity to FcRn, leading to more efficient transplacental crossing. Several studies have suggested that post-translational modifications in Fc regions of antibodies may be antigen-dependent.[31,32] However, since no evidence has been found so far for an antigen-dependent selection in transplacental crossing, we hypothesize that antibodies produced during pregnancy cross the placenta without depending on their target antigen. Intriguingly, in the case of HLA antibodies, placental transfer may result in the presence of maternally derived, paternally directed antibodies in the foetus, potentially acting as autoantibodies. The impact of such antibodies on the foetus and later, infant, is not known.

The production of HLA antibodies during pregnancy was first observed by Van Rood et al in 1958.[33] Since then, efforts to measure their prevalence and uncover their role have been relatively scarce. It has been shown that HLA antibodies—both non-specific, and those targeting paternal HLA proteins—develop in many, but not all healthy pregnancies after 20-28 weeks of gestation.[19,34-37] The frequency of these antibodies increases with parity, reaching 74% in women who have had more than two deliveries.[38] Interestingly, HLA antibodies that appear during pregnancy are not always detected in women several years after delivery but may persist in immunological memory.[39]

The impact of HLA antibodies on pregnancy outcome is not understood. Some studies have found an association between high levels of HLA antibodies in pregnancy, both in early[35,40] and late[41-44] gestational age, and a poor pregnancy outcome. However, other studies suggest that high levels of HLA antibodies in pregnancy have a positive impact on pregnancy outcome.[36,45] In line with the latter, immunotherapy with paternal lymphocytes resulting in HLA antibody production may be a therapeutic option in women with recurrent miscarriages.[46,47] Yet a third line of studies, however, has found no substantial correlation between HLA antibodies and pregnancy outcome.[48,49] Supporting this, is the observation that pregnancy following oocyte donation—associated with a higher risk of developing child-specific HLA antibodies—is not associated with immunological complications.[50]

The inconsistency arising from existing studies on HLA antibodies in pregnancy demonstrates gaps in our understanding. Similarly, we know little about the development of HLA antibodies in the foetus and after birth. In humans, the foetus has been shown to develop IgM and IgA antibodies.[20,51,52] IgM is usually the first class of antibody to be produced by B lymphocytes, having high avidity and agglutinating activity, but reduced specificity and antigen affinity.[36] When the human body further encounters antigen, B lymphocytes switch their isotype from IgM to IgG, which, in addition to being more specific, also possesses the widest range of effector functions.[36] Based on evidence so far,[12,51,53] foetuses do not produce their own IgG antibodies, suggesting that IgG—including potential HLA antibodies—present at birth originate in the mothers. The specific time point and triggers for production of IgG in new-borns are not known.

In the current study, we investigated the HLA antibody profile in women and their infants six weeks post-partum, as well as in pregnant women. We used two cohorts; a first cohort of six-week-old infants and their mothers (referred to as the "mother-infant cohort"), and a second cohort of pregnant women at different time points in pregnancy and post-partum (referred to as the "pregnancy cohort"). We detected IgG HLA antibodies and characterized their specificities, sub-class distribution and expression levels; additionally, we tested the mother-infant cohort samples for the presence of complement-fixing HLA antibodies.

2 | METHODS

2.1 | Study design and sample collection

2.1.1 | Mother-infant study

Thirty healthy mother-infant pairs available from a previously completed study on rotavirus vaccine immunogenicity (NCT02215226[54]) were used. The study, conducted in Soweto, South Africa in 2009-2010, explored the effect of at least an hour abstention from breastfeeding at immunization visits on immune responses of infants to rotavirus vaccine. The study enrolled black South African mother-infant pairs who were human immunodeficiency virus (HIV)-negative, at their six weeks post-partum visit. Blood samples were drawn from all participants at enrolment, immediately before administration of the rotavirus vaccine to the infants. The serum fraction was separated by centrifugation and stored at −70°C until further use. Infant DNA was not available from this cohort.

2.1.2 | Longitudinal Pregnancy study

Twenty-nine women in their first trimester of pregnancy were recruited from collaborating gynaecologist practices (the Charlotte Maxeke Johannesburg Academic Hospital and Netcare private clinics in South Africa). Recruited patients were of both black and white South African ethnicity. All patients were HIV-negative, multigravida and over 18 years of age at enrolment. Blood samples were drawn from participants at routine visits to the clinic, including during the first (up to 13 weeks of pregnancy), second (14-26 weeks of pregnancy) and third (27-40 weeks of pregnancy) trimesters, and/or 8-11 weeks post-partum. Buccal swabs were collected from infants at the post-partum visit and later processed for DNA extraction. Mothers' serum was stored at −70°C until further use. Samples of six participants with healthy and uncomplicated pregnancies, who had at least two visits to the clinic and for whom buccal swabs were successfully collected from their babies, were analysed and their results presented here as case studies. The remaining participants are not presented here due to missing data such as incomplete infant HLA typing results or maternal serum time points. We did not collect serum from infants in this study.

2.2 | Measurement of HLA antibody levels

The prevalence of IgG HLA antibodies was measured in serum of mothers and infants from the mother-infant cohort, as well as that of mothers from the pregnancy cohort. Presence, strength (expression levels) and specificity of antibodies were measured using Luminex technology. Commercial kits for detection of IgG antibodies against HLA class I and HLA class II (LSA class I/II single antigen kits; Immucor, Norcross, Georgia, USA) were used according to manufacturer's instructions. Antibodies detected included anti-HLA-A, HLA-B and HLA-C for class I, and anti-DRB1, DRB3,4,5, DQA/B and DPA/B for class II. The data obtained from these experiments were subsequently analysed using Match-It software (Immucor, Norcross, Georgia, USA), according to manufacturer's instructions. The HLA antibody strength was recorded as adjusted mean fluorescent intensity (AD-MFI), with additional interpretive comments: positive, weak, possible or absent. Positive and weak were considered positive results, while possible and absent were considered negative results. Highly reactive sera samples were purified by adsorption of non-specific antibodies using commercial "Sera clean" kit (Immucor), followed by a second analysis of HLA antibodies. For the mother-infant study, antibody specificities were also labelled as shared (detected in both mother and infant) or mother-only/infant-only. For the pregnancy study, HLA antibodies were categorized as baby-directed (targeting HLA types of the baby) or non-specific. Additionally, antibodies were categorized as recurring (expressed at more than one time point) or single time point, in order to describe development of new antibodies at various time points.

Complement-fixing HLA antibodies were detected in serum of 20 mothers and infants from the mother-infant study by running HLA-I/II antibody assays as described above but using complement component C3d as a detection agent to bind to complement-fixing antibodies in serum. After a wash step, bound C3d was detected with fluorescently labelled antibody against C3d (C3d kit, Immucor). The data obtained were subsequently analysed using Match-It software (Immucor), as described above.

2.3 | DNA extraction

Buccal swabs of infants in the pregnancy study were soaked in DNA extracting solution (QuickExtract™ DNA Extraction Solution, Epicentre, Madison, WI, USA), followed by DNA extraction using a commercial kit (QuickExtract™ DNA Extraction kit, Epicentre, Madison, WI, USA). Next, DNA was precipitated as follows. Briefly, samples were mixed with sodium acetate in a 1:10 volume, followed by addition of 2.5 volumes of absolute ethanol and incubation at −70°C. Following incubation, the samples were centrifuged, the pellet was washed with 70% ethanol and then eluted with a commercial elution buffer (QIAamp DNA Mini kit, Qiagen, Germany), quantified and stored at −20°C. DNA was not available in the mother-infant cohort, and therefore no DNA analysis was carried out on these samples.

2.4 | HLA typing

Class I and II HLA genotyping was performed for DNA prepared from infant samples collected in the pregnancy cohort. Commercial kits for HLA-A, HLA-B, HLA-C, DRB1, DRB3,4,5, DQB1 and DPA/B loci (Immucor, Norcross, Georgia, USA) were used for locus-specific PCR amplification according to manufacturer's instructions, followed by detection via allele-specific probes using a Luminex instrument (Bioplex, Biorad) and Match-It DNA software (Immucor). The typing was reported at the 2/4 - digit resolution.

2.5 | Statistical analysis

Data analysis was performed using version 8.2.1 (441) of the GraphPad Prism (GraphPad software Inc, San Diego, CA, USA). A *P* value of .05 or lower was considered statistically significant. Variables were compared using □2 test (for number of specificities per individual) or Mann-Whitney test (for antibody strength).

2.6 | Ethics

Our mother-infant study, conducted on samples from the previously published NCT02215226 study, was approved by the Human Ethics Research Committee of the University of the Witwatersrand with consent for storage and testing (M1611144). The pregnancy study was approved by the Human Ethics Research Committee of the University of the Witwatersrand (M130230).

3 | RESULTS

3.1 | Mother-infant study

3.1.1 | HLA antibody specificities in mother and infants

We first characterized the prevalence of IgG HLA antibodies in mothers and infants. HLA antibodies detected in each mother-infant pair are listed in Supplementary Tables S1 (class I) and S2 (class II), including those detected only in mother, those detected only in infant, and those that were shared between the two. Our data reveals great diversity in number and type of specificities between the pairs. We found that of 25 mothers, 20 (80%) expressed HLA antibodies of at least one class (Figure 1A), 17 (68%) expressed HLA class I antibodies (Figure 1B) and 14 (56%) expressed HLA class II antibodies (Figure 1C). In infants, of 25, 18 (72%) expressed HLA antibodies of either class, 11 (44%) expressed HLA class I antibodies, and 13 (52%) expressed HLA class II antibodies (Figure 1A, B and C, respectively).

3.1.2 | HLA antibody subclasses

Each HLA-I antibody subclass, namely HLA-A, HLA-B and HLA-C, showed a similar pattern, with a higher proportion of specificities apparent in the mother only, and a low proportion of specificities apparent in infants only and in the group shared between mothers and infants (Figure 2A-C). More specifically, out of 29 mothers, 18 (62%), 18 (62%) and five (17%) expressed HLA-A, HLA-B and HLA-C antibodies that were not detected in their infants, respectively. However, only five (17%) infants expressed anti-HLA-A antibodies, five (17%) expressed anti-HLA-B, and only one (3%), expressed anti-HLA-C. Similarly, low proportions of antibodies were shared between mothers and infants, with six pairs (21%) expressing HLA-A antibodies and seven pairs (24%) expressing HLA-B antibodies; notably no HLA-C antibodies were shared between mothers and infants.

Similarly, for HLA class II, a high proportion of mothers (9/25, 12/25, 4/25 and 4/25, which represented 36%, 48%, 16% and 16% out of all mothers) expressed DRB1, DQA/B, DRB3,4,5 and DPA/B antibodies, respectively, that were not detected in their infants. There were few infant-only antibodies (2/25, 8/25, 1/25 and 2/25, equal to 8%, 32%, 4% and 8%, respectively) as well as few shared antibodies (1/25, 3/25, 2/25 and 2/25, equal to 4%, 12%, 8% and

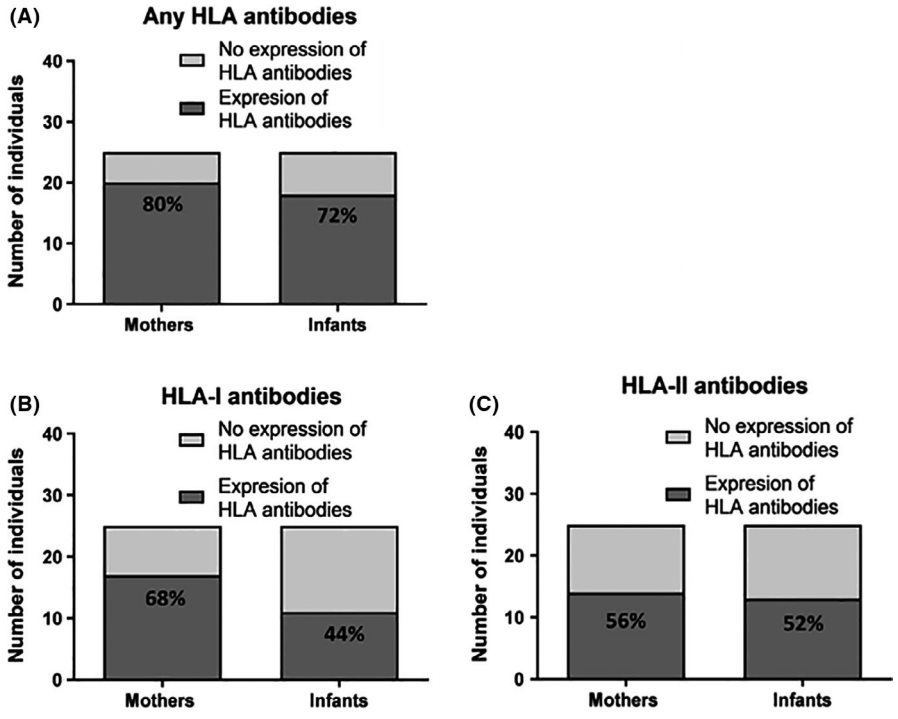


FIGURE 1 Number (and percentage) of mothers six weeks post-partum and their six-week-old infants, who express HLA antibodies of at least one class (A), HLA class I antibodies (B), and HLA class II antibodies (C) out of total number of individuals (n = 25; all antibodies are IgG)

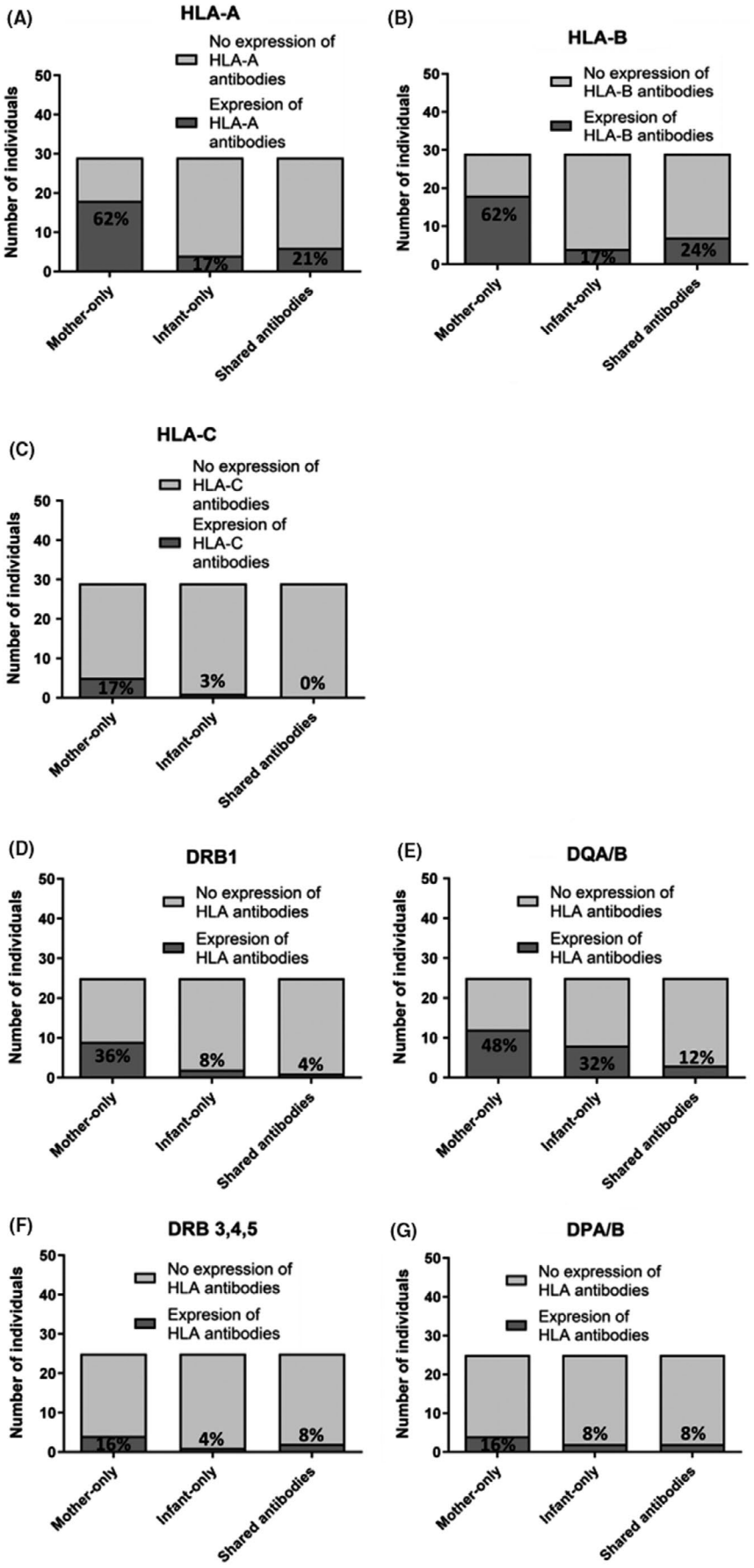


FIGURE 2 Number (and percentage) of mothers six weeks post-partum and their six-week-old infants, who express HLA class I antibodies (A, B, C for HLA-A, HLA-B and HLA-C, respectively; n = 29) and HLA class II antibodies (D, E, F, G for DRB1, DQA/B, DRB3,4,5 and DPA/B, respectively; n = 25) that were detected only in mothers (Mother-only antibodies), only in infants (Infant-only antibodies) and in both mother in infant (Shared antibodies); all antibodies are IgG

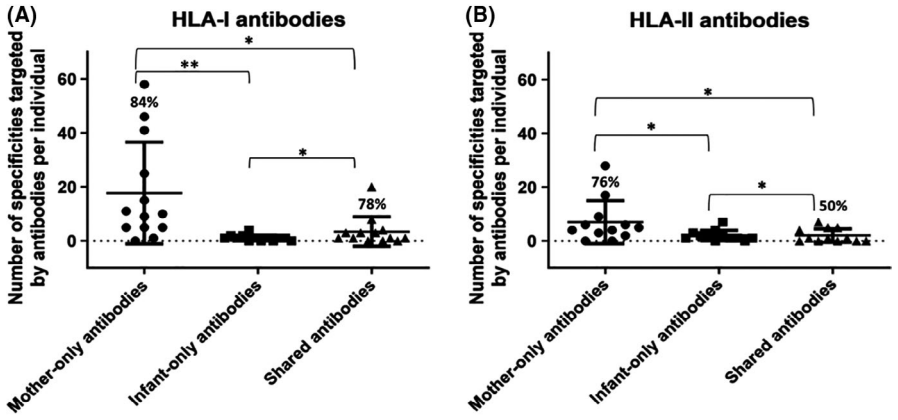


FIGURE 3 Number of different specificities targeted by HLA antibodies (class I, A and class II, B; all antibodies are IgG) in mothers six weeks post-partum and their six-week-old infants. Number of antibody specificities detected only in mother (Mother-only antibodies), only in infant (Infant-only antibodies), or those detected in both mother and infant (Shared antibodies) are shown (n = 29; * represents .05 > *P* value > .001; ** represents a *P* value < .001; *P* value was determined by Student's *t* test; median, 25th and 75th percentiles are shown). On top of the Mother-only antibodies column—percentage out of total antibodies detected in mothers. On top of the Shared antibodies column—percentage out of total antibodies detected in infants

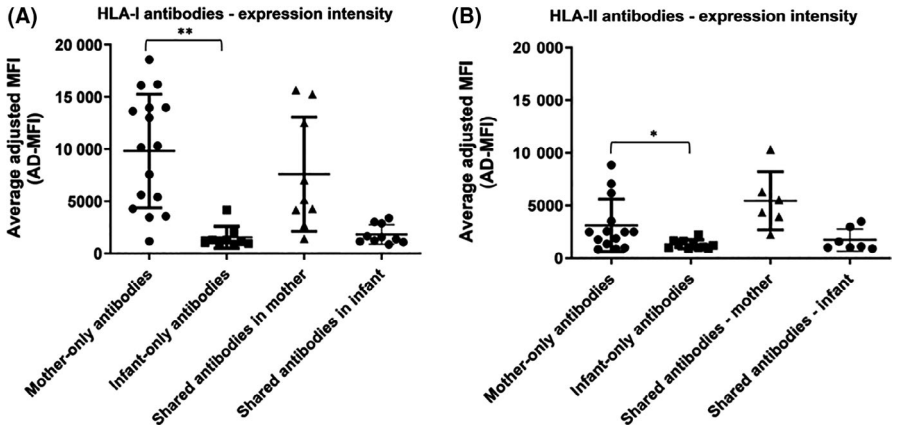


FIGURE 4 Intensity of antibody expression. Mean AD-MFI (adjusted MFI as calculated by the MatchIt analysis software) of antibodies detected only in mothers (Mother-only), only in infants (Infant-only), and those detected in both mother and infant (Shared antibodies) are shown for HLA-I (A, n = 17; ** represents *P* value < 0.001) and HLA-II (B, n = 15; * represents 0.05 > *P* value > 0.001) antibodies (all antibodies are IgG; *P* value was determined by Mann-Whitney test; median, 25th and 75th percentiles are shown)

8%, respectively). DQA/B antibodies were the predominant HLA-II antibody sub-class in mothers and babies (Figure 2D-G).

3.2 | Number of target HLA specificities per individual

Next, we measured the number of different HLA specificities targeted by mothers and infants (Figure 3). We sought to determine whether infants expressed the same repertoire of HLA antibodies as their mothers, with the assumption that such antibodies would be of maternal origin. In contrast, we postulated that antibodies detected in infants but not in their mothers were either self-made, or, less likely—of maternal origin but undetectable in the mothers; and that antibodies detected in mothers but not in their infants were either produced during pregnancy yet not transferred to the foetus, or produced post-partum.

We found that overall, infants targeted significantly fewer HLA specificities than mothers (*P* value <.05 for both HLA classes). Interestingly, only some of these specificities were shared with their mothers (a mean of 78% for HLA-I and 50% for HLA-II, Figure 3A and 3B, respectively). Surprisingly, we also found that most specificities detected in mothers were not detected in their babies (a mean of 84% for HLA-I and 76% for HLA-II, Figure 3A and 3B, accordingly).

3.3 | Expression intensity of HLA antibodies

The expression intensity was significantly lower for antibodies detected in infants compared with antibodies detected in mothers (p value < 0.001 and < 0.05, presented in Figure 4A and 4B, for HLA-I and HLA-II antibodies, respectively). Interestingly, this included antibodies shared between

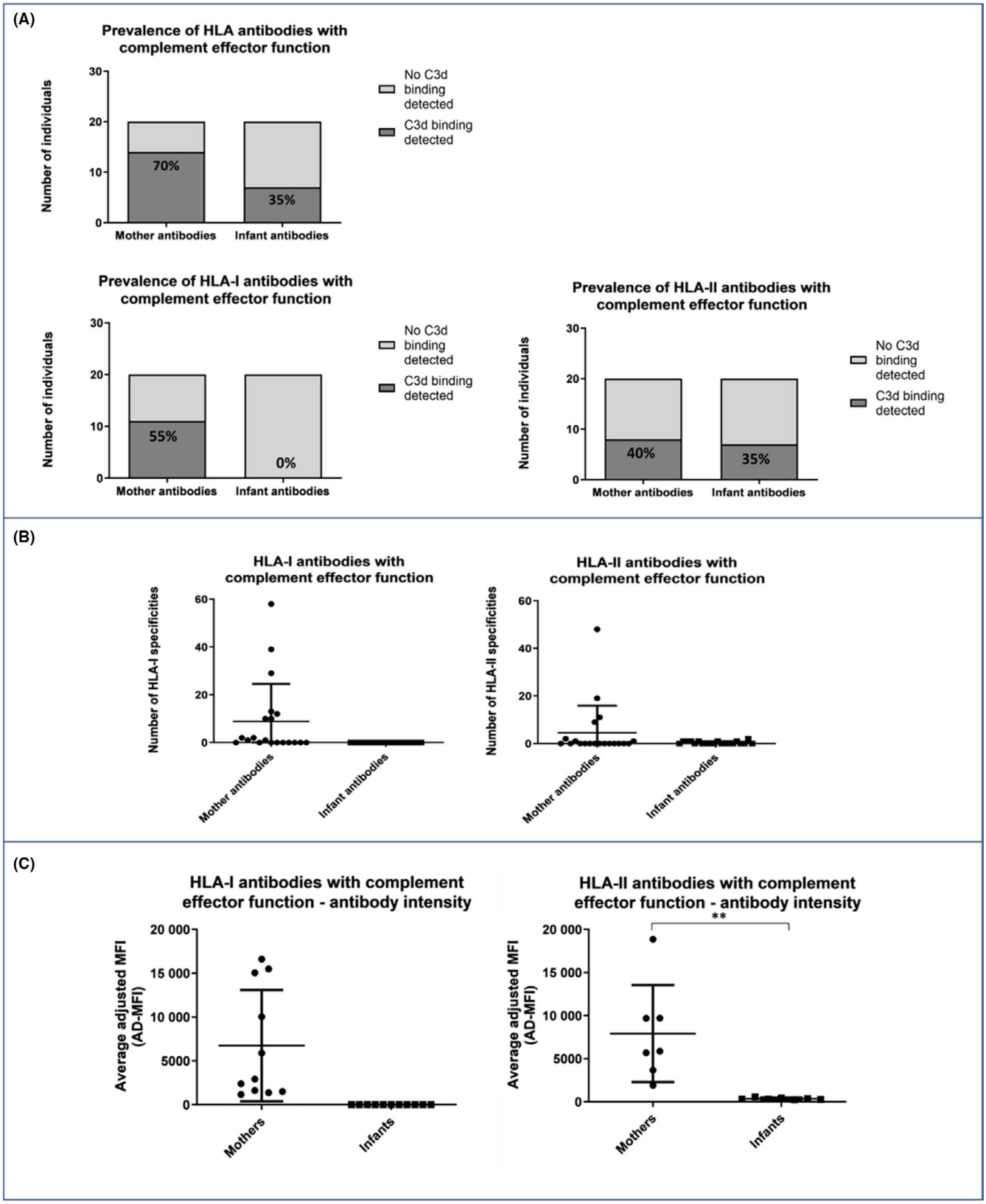


FIGURE 5 HLA antibodies with complement effector function (as detected by C3d binding) in mothers six weeks post-partum and their six-week-old infants: A. Number (and percentage) of mothers and infants who express complement-activating HLA antibodies out of total number of individuals (n = 20); B. Number of different HLA specificities per individual of complement-activating antibodies (n = 20; median, 25th and 75th percentiles are shown); C. Antibody expression intensity (average AD-MFI as calculated by the MatchIt analysis software) of complement-activating HLA antibodies (n = 20; ** represents *P* value < .001; *P* value was calculated using Mann-Whitney test; median, 25th and 75th percentiles are shown)

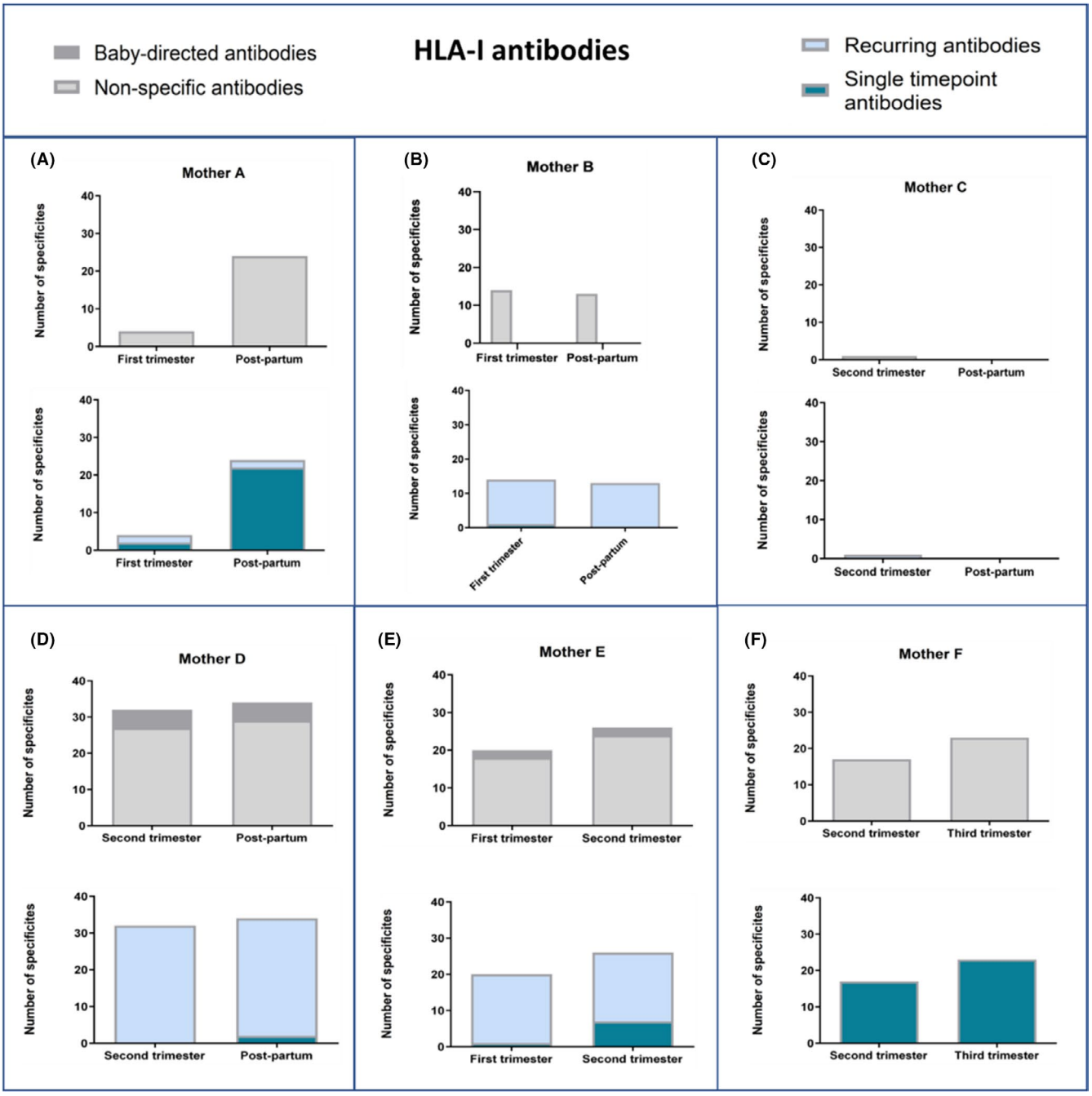


FIGURE 6 Number of different HLA class I antibodies in six pregnant women (A-F) as detected at different time points in pregnancy (all antibodies are IgG). Two graphs are shown for each woman: the top graph presents baby-directed and non-specific antibodies (shown in dark and pale grey, respectively); the lower graph presents recurring and single time point antibodies (shown in pale and dark blue, respectively). Baby-directed HLA-I antibodies were detected in two out of six mothers (mothers D and E). Development of new HLA-I antibodies is apparent in mothers A, D, E and F. Certain antibody specificities in mothers A, C and F were detected once but not subsequently

mother and infant, possibly indicating that only a fraction of the amount of antibody produced in mothers crossed the placenta.

3.4 | Complement-fixing ability of detected HLA antibodies

As expected, we detected complement-fixing HLA antibodies in mothers, with 55% and 40% expressing HLA-I and HLA-II antibodies, respectively (Figure 5A). The number of HLA specificities varied between mothers (Figure 5B) but was in the same range as the maternal HLA IgG antibodies (Figure 3). For HLA-I, the expression intensity of these complement-fixing antibodies was generally lower than that observed for maternal IgG; however, for HLA-II the intensity was overall higher than that of maternal IgG (Figure 5C). In infants, we could not detect complement-fixing HLA class I antibodies (Figure 5A), indicating that whether self-made or of maternal origin, HLA class I antibodies do not activate complement at six weeks

of age. In contrast, we did detect complement-fixing HLA class II antibodies in 35 per cent of the infants (Figure 5A). However, HLA class II antibody numbers per individual (Figure 5B) and expression levels (Figure 5C) were extremely low (*P* value <.001 for mean antibody intensity of mothers versus infants), possibly because their production might only begin at six weeks of age. We detected infant complement-fixing HLA-II antibodies shared with the mother in only one infant (not shown).

3.5 | Pregnancy study

In six women, referred to as Mother A-F, we measured the number and specificities targeted by maternal IgG HLA antibodies (Figure 6, class I and Figure 7, class II) at different stages of gestation and/or post-partum. All women expressed HLA class I (Figure 6), as well as HLA class II (Figure 7) antibodies in the pregnancy time points tested; however, there were noticeable variations between women

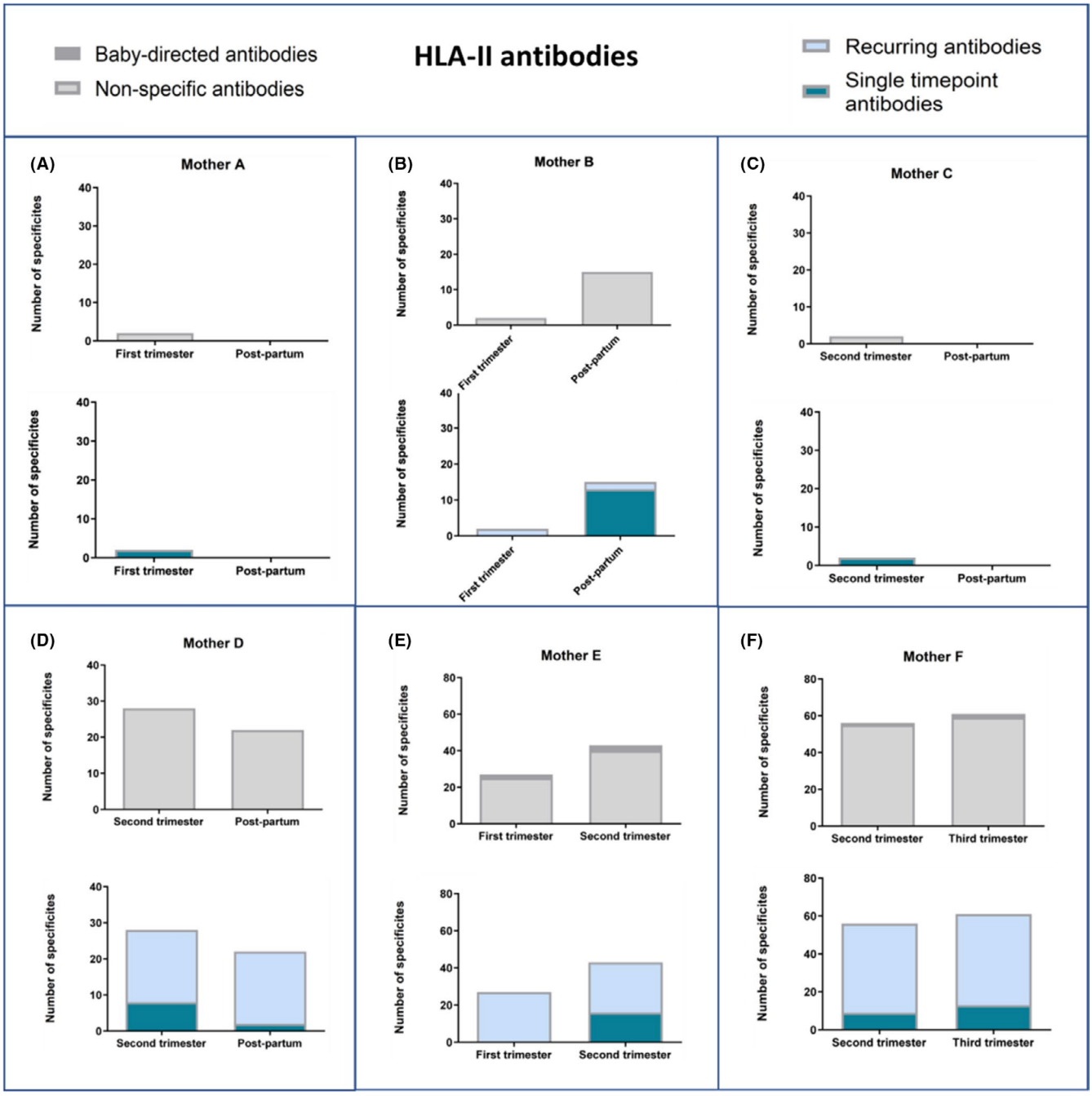


FIGURE 7 Number of different HLA class II antibodies in six pregnant women (A-F) as detected at different time points in pregnancy (all antibodies are IgG). Two graphs are shown for each woman: the top graph presents baby-directed and non-specific antibodies (shown in dark and pale grey, respectively); the lower graph presents recurring and single time point antibodies (shown in pale and dark blue, respectively). Baby-directed HLA-II antibodies were detected in two out of six mothers (mothers E and F). Development of new HLA-II antibodies is apparent in mothers B, E and F. Certain antibody specificities in mothers A, C, D and F were detected once but not subsequently

in number of specificities. In the post-partum time point, three out of four women expressed HLA class I antibodies (Figure 6) and two out of four women expressed HLA class II antibodies (Figure 7). We further assessed whether maternal antibodies targeted HLA specificities of the infant by genotyping the infant HLA-B, HLA-C, DRB1, DQA/B, DRB3,4,5, DPA/B, and classifying the maternal antibodies into baby-directed or non-specific. For both classes, two out of six women (mothers D and E for HLA-I, and E and F for HLA-II) expressed baby-directed HLA antibodies.

Next, we classified the maternal antibodies into single time point (detected only at one time point) or recurrent (detected in at least two time points), in an attempt to assess the appearance of new antibodies. Overall, once antibodies were present in the mother, they recurred at subsequent time points. However, we did find antibody specificities that were detected once but not subsequently, as seen in mothers A, C and F (Figure 6) for HLA-I and mothers A, C, D and F (Figure 7) for HLA-II. Development of new HLA antibodies in the second time point compared with the first, was apparent in four mothers for HLA-I (A, D, E and F, Figure 6), and four mothers for HLA-II (B, D, E and F, Figure 7).

4 | DISCUSSION

Currently, it is not known whether HLA antibodies play a role in pregnant women or if they are essential for maintaining a healthy pregnancy. However, many studies suggest that high levels of HLA antibodies, at least (and perhaps only) in late stages of pregnancy, are important for a successful outcome. We found that most women in our two cohorts expressed HLA antibodies in their pregnancy and/or post-partum (Figures 1, 6, 7). To our surprise, in the mother-infant cohort, a relatively small fraction of IgG HLA antibodies detected in mothers post-partum was found in the infants (these were termed "shared antibodies"). If we assume that the mothers produced the HLA antibodies detected post-partum during pregnancy, the low levels of shared antibodies in infants suggests selection in transplacental crossing. So far, the efficiency of placental crossing has not been shown to depend on antibody specificity, and hence, the mechanism behind such selection remains to be explored.

It is also possible, however, that pregnant women produce and transfer HLA antibodies to the foetus, but rather than being selected against at placental crossing, some of the transferred antibodies might be degraded in the infants by the time they are six weeks of age, thus resulting in lower prevalence and intensity of shared antibodies in infants compared with mothers.

Alternatively, some of the maternal HLA antibodies we detect in mothers post-partum may have been produced during the first six weeks after delivery rather than during pregnancy. Theoretically, HLA antibodies produced post-partum may still be passed on to the infants through breastfeeding. However, this would likely be at low levels as transfer of IgG antibodies via breastmilk is limited.[55,56]

In the future, it would be interesting to explore the possibility of selection in transplacental crossing of HLA antibodies, including possible post-translational modifications that would increase their FcRn binding affinity, and a potential link between such modifications and antigen specificity. One interesting aspect of a theoretical antibody selection would be the fate of antibodies that may become autoantibodies in the foetus, directed at paternal HLA proteins. In our longitudinal pregnancy study, half of the women expressed a small proportion of baby-directed HLA antibodies of at least one class. However, we do not know whether these antibodies were transferred to the foetuses, and if so, what impact they might have had on the foetal and later, infant immune system.

Another interesting aspect of a potential selection is the transfer of the HLA-C antibodies. In the mother-infant cohort, we found that the shared antibodies did not include any HLA-C antibodies, which may be due to our small sample size, especially in light of the small proportion of maternal HLA-C antibodies within the class-I HLA antibodies. However, it is also possible that HLA-C antibodies are less preferentially transferred to the foetus. Interestingly, at the beginning of pregnancy, placental trophoblast cells express HLA-C proteins on their membranes, later internalizing them. The levels of HLA-C in placental trophoblast cells are then highly increased at birth.[57] Hypothetically, HLA-C antibodies may be selected against for transplacental crossing, possibly to lower the chances of transferring autoantibodies to the foetus. However, at this point, this scenario remains speculative.

Unlike antibodies targeting pathogenic antigens, whose role is well understood, the role of HLA antibodies in the foetus, and later in the child, is not known. A study by Gros and colleagues[58] suggested that maternal antibodies transferred to the foetus during pregnancy or to the infant via breast milk may act as immunomodulators, leading to the development of specific and long-lasting immune responses in the infant. However, whether this is the case for HLA antibodies is not known. It has been shown that antibodies against a variety of HLA alleles may be produced after exposure to a specific HLA antigen.[59] The significance of HLA alloimmunity and whether humans need to develop immunological tolerance or alloimmunity against HLA proteins, remain to be established.

HLA antibodies may play a crucial role in infection with enveloped viruses, such as the Human Immunodeficiency Virus (HIV), Epstein-Barr Virus (EBV), influenza or measles. While budding out of host cells, enveloped viruses incorporate different host proteins, including HLA, which they then introduce to new hosts.[60] Therefore, individuals who express HLA antibodies may have pre-existing immunity to pathogens. However, whether this is beneficial, harmful or simply irrelevant, still needs to be determined. If pre-existing antibodies increase protection, we expect that the more HLA antibodies present, the higher their protection. However, it is also possible that HLA antibodies may, in fact, increase susceptibility to certain viruses. This may be due to the HLA antibody-induced recruitment of immune cells that some viruses may easily infect. To note, previous studies in macaques and non-human primates have suggested a protective role for HLA antibodies against infection with the enveloped simian immunodeficiency virus (SIV).[61-63] Interestingly, it was found that administration of vaccines against infectious agents such

as the influenza and measles viruses may induce de-novo production of HLA antibodies[64-66]; the impact of these antibodies on future infections remains to be determined. It is tempting to envision the use of HLA antibody-based alloimmunity for vaccine strategies in pathogenic diseases caused by enveloped viruses that incorporate host-derived HLA proteins or those like HIV that express viral proteins with high structural homology to HLA proteins.[62,67-70] Interestingly, antibody transfer from mother to foetus is compromised in various conditions affecting placental integrity, including placental malaria and HIV infection.[15,16,30,71,72] It would be interesting to see whether these conditions also impact the transfer of HLA antibodies, and if so, what effect impaired transfer has on susceptibility to infection in the foetus and after birth.

It has recently been shown that in the first weeks of life, the infant's immune system undergoes drastic changes that comprise maturation of most immune cells, including B lymphocytes.[73,74] This rapid maturation results in differences between the immune profile at birth (as seen in cord blood) and that of infants several weeks of age. Maturation may include the ability of B cells to switch from IgM (which is already produced in the foetus) to other types of antibodies, presumably in response to specific antigens. In our study, we find that at six weeks of age, infants express IgG HLA antibodies, including a considerable number of antibodies that are not found in their mothers, which may be self-made antibodies. Whether these antibodies are produced as "natural antibodies" or in response to specific antigens warrants further investigation. To note, we cannot exclude the possibility that at least some of the HLA antibodies found in infants but not in their mothers, are in fact of maternal origin, having been present antenatally yet becoming undetectable in the mother post-partum.

In our study, we show that amongst HLA antibodies produced by the mothers, some antibodies have complement effector functions, likely in keeping with IgM isotype. The complement system may be activated by IgM or IgG antibodies; however, unlike IgG, which has multiple effector functions, complement activation is almost the sole effector function of IgM antibodies.[1] Thus, in infants, the absence of complement-fixing HLA class I (but not class II) antibodies suggests that their HLA-I antibodies are unlikely to be IgM.

4.1 | Limitations and strengths

Strengths of this study include the use of highly sensitive single-antigen technology which can detect low intensity antibodies. The study is unusual in that it includes healthy Black African women and infants with no underlying infectious diseases. Additionally, we explored multiple HLA loci including class II loci, rarely analysed in previous studies. A main limitation of this study is that we could not test any of the women prior to their pregnancy due to lack of available samples, and therefore our findings in pregnancy and post-partum could not be compared with a basal, non-pregnant state. In the mother-infant cohort, we did not have cord blood samples available, and therefore could not compare the infant samples at six weeks to those at birth. Additionally, we had no DNA available to explore the presence of antibodies directed at the infants' HLA. In the pregnancy cohort, we had HLA type of the infants but there was no infant serum available to establish whether baby-directed antibodies crossed to the baby. Additionally, we did not HLA type the mothers so could not confirm whether antibodies were anti-paternal. Gravidity and parity data were not collected in the mother-infant study, and thus, the possible effect of these on HLA antibody repertoire could not be tested.

4.2 | Summary

In this study, we show that up to 80 per cent of women after birth express IgG HLA antibodies. Surprisingly, we detect less than 25 per cent of these antibodies (16 per cent for HLA-I and 24 per cent for HLA-II) in the neonate, raising the possibility of HLA antibody selection in placental transfer. Our study also suggests that infants start producing their own IgG HLA antibodies in the first weeks of life. Further investigation is required to confirm these two hypotheses and the mechanisms behind them. The presence of HLA antibodies in neonates may impact their immune reaction to HLA proteins, including those incorporated in enveloped viruses. Whether and how HLA antibodies affect susceptibility to enveloped viruses needs to be further explored using prospective studies. Overall, our findings may provide a preliminary baseline for future studies focusing on pregnancy complications that are not fully understood yet, such as preeclampsia, as well as infections with (and possibly vaccines against) enveloped viruses in new-borns, in which HLA antibodies may play a role.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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