Measuring HLA-B allele expression across differential cell types

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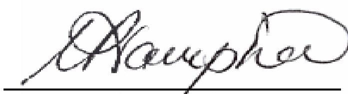
Preface

The practical component of this project described in this dissertation was carried out at the following laboratories: KwaZulu-Natal Research Innovation and Sequencing Platform (Krisp), HIV Pathogenesis Programme (HPP), Centre for the AIDS Programme of Research in South Africa (CAPRISA), located within the Nelson R Mandela School of Medicine at University of Kwa-Zulu of Natal in Durban. This research was carried out from April 2017 to December 2018, under the supervision and mentorship of Dr. Veron Ramsuran.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.



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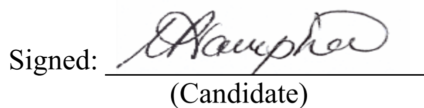
Signed:  Date: 14 December 2018
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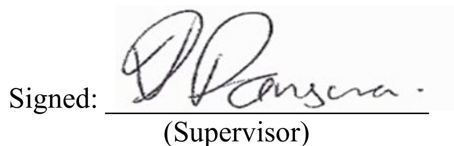
Signed:  Date: 14 December 2018
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Dedication

To my Mum & Da

My Sisters

My niece

In loving memory of my perfect baby boy,

Shenzi!

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Table 3: Summary of selected HLA-B alleles identified in corresponding donors, based on a 10% variation across affinity values of antibody REA274 obtained from the LSA assay used in this study **28**

List of Abbreviations

Ab	-	antibody
Ag	-	Antigen
APC	-	Antigen Presenting Cells
CTLs	-	Cytotoxic T Lymphocytes
CAPRISA	-	Centre for the Aids Programme of Research in South Africa
ddPCR	-	Droplet Digital Polymerase Chain Reaction
FACS	-	Fluorescence-activated cell sorting
FITC	-	fluorescein isothiocyanate
GOI	-	Gene of interest
GVHD	-	Graft-versus-host disease
GWAS	-	Genome Wide Association Studies
HBV	-	Hepatitis B Virus
HLA	-	Human Leukocyte Antigen
HLA-I	-	Human Leukocyte Antigen Class-I
HIV	-	Human Immunodeficiency Virus
HIV-	-	Human Immunodeficiency Virus Negative
HIV+	-	Human Immunodeficiency Virus Positive
HPP	-	HIV Pathogenesis Program
kDa	-	Kilo Daltons
Krisp	-	KwaZulu-Natal Research Innovation and Sequencing Platform
MHC	-	Major Histocompatibility Complex
mRNA	-	Messenger ribonucleic acid
Nef	-	Negative Regulatory Factor
PBMC	-	Peripheral blood mononuclear cells

Abstract

Background: The human leukocyte antigen (HLA) region has shown to have the strongest disease associations and recent studies have shown that expression levels of these HLA molecules play a major role in the clinical course of diseases. Differences in the expression levels of these molecules have been found to have a major effect on their ability to present specific peptide antigens. HLA molecules are critical to the interaction between diseases and components of the immune system. Expression of such molecules, namely HLA-C and *HLA-A*, have been shown to associate with HIV disease outcomes. An increase in expression of HLA-C leads to protection against HIV whereas an increase in *HLA-A* expression leads to rapid HIV progression. Furthermore, studies have shown the region with the strongest genetic effect falls within the *HLA-B* gene, as determined by genome wide association studies. However, limited information is available for *HLA-B* allelic expression levels and the variation across differential cell types.

Materials and Methods: Allelic expression levels of HLA-B were measured using cryopreserved PBMC samples from HIV negative and positive cohorts with HLA typing. Antibodies specific to the HLA-B protein were identified. The affinity of the antibodies relative to class-I alleles were determined. Based on these affinities, donors with specific alleles were selected for HLA-B cell surface measurement using the flow cytometer. mRNA levels were measured across HLA-A, -B, -C and -E genes within the following cell types T-cells, B-cells, Monocytes and NK cells. These levels and a comparison of HIV infected and uninfected mRNA levels from the same donor were measured using droplet digital PCR (ddPCR).

Conclusions: Contrary to HLA-B mRNA expression levels, we find cell surface expression levels vary in an allele-specific manner. We further observed differential mRNA expression patterns for HLA-A, HLA-B, HLA-C and HLA-E across cell types. We also observed no mRNA expression variation across pre- and post- HIV samples. When comparing HLA-B mRNA and surface expression across alleles and donors no significant correlation was found. However, at an donor level, some alleles may be differentially regulated at the cell surface. This study built existing

knowledge and fills in some of the gaps in knowledge surrounding HLA-B expression. We also report, for the first-time, variation in allele specific expression, variation in expression across differential cell types and lack of expression variation across pre- and post- HIV infection at the mRNA level.

Chapter one: Introduction

"Human leukocyte antigen (HLA) region, located within the Major histocompatibility complex (MHC), is the most polymorphic region of the human genome and comprises of just over 14,800 HLA-I alleles[1]. Since the discovery of the HLA molecules over five decades ago, the HLA research platform has explosively developed into a remarkable field making the HLA complex fundamental in basic and clinical immunology. HLA's have been recognized as a major predictor in the extremes of disease outcome and have been reported as being one of the two loci responsible for modifying Human Immunodeficiency Virus (HIV) disease[2].

The precise mechanism of HLA molecules governing protection against diseases is still a major gap in research. However, it has been speculated that the precise mechanism underlying the protective effects of HLA-I molecules may vary from one to another[3] yet the underlying effects of these class-I molecules are not completely understood[4] and is continuously being investigated. Possible mechanisms governing the transcriptional regulation of HLA-I gene expression have been discussed, however emphasis that a better comprehension of this highly regulated expression is required in order to consider therapeutic approaches aimed at modulating the level of HLA expression level[5]. Expression of these molecules are critical in transforming our understanding of host genetics, its application in disease modification and control and evolution[2,5].

Several genome wide association studies (GWAS) have been conducted with the aim of identifying host factors that contributed to HIV and other disease outcomes. The results of these studies indicate the human leukocyte antigen class-I genes exhibit the strongest associations supporting previous studies that have targeted the HLA genes[6–8]. The influence of variability in peptide binding region of HLA molecules on immune responses and HIV disease outcomes is

well appreciated. The most clearly defined escape mutations arise within CD8 T cell epitopes recognized by HLA-B alleles that are linked with successful control of HIV[9]. Allelic polymorphisms that influence differential antigen presentation and HIV disease outcomes have been extensively studied for HLA-B alleles[10,11]. However, despite HLA-B being widely studied not much is known about the allelic-specific expression levels.

This study, therefore, focused on measuring the allelic expression levels of HLA-I molecules, particularly HLA-B, and bridging the gaps in knowledge surrounding HLA-B expression and its potential impact on disease outcomes."

Chapter Two: Literature Review

2.1 The Human Leukocyte Antigen Region

2.1.1 Location, Structure and Function of HLA-I

HLA is the human version of the Major Histocompatibility Complex and is located on chromosome six of the genome (figure 1). The HLA region is comprised of three classes with the class-I region consisting of the three classic human leukocyte antigen (HLA) gene loci: HLA-A, HLA-B, and HLA-C; three non-classic HLA-E, HLA-F, and HLA-G, which show limited polymorphism compared to the classic class-I; and other related non-coding genes and pseudogenes. These molecules are fundamental to the development of innate and adaptive immune responses and are the most polymorphic loci in the human genome.

HLA COMPLEX

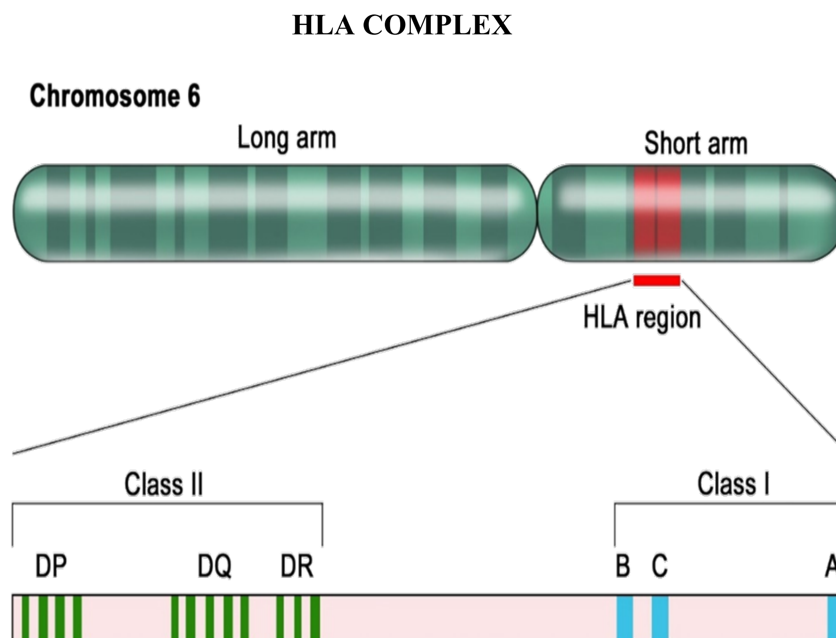

Figure 1: HLA Complex. Location of the HLA region on chromosome 6. Human chromosome 6 with amplification of the HLA region. Specific HLA-I B, C, and A alleles and the class-II DP, DQ, and DR alleles are shown[12].

These complexes are located on the short arm (p) of chromosome 6 and are composed of 252 genes in close approximation of each other[13]. The HLA class-I genes are a heterodimer, consisting of a heavy alpha chain and a light beta chain (beta-2 micro-globulin) of approximately 12 kDa in size. The heavy alpha chain is anchored in the membrane and gives rise to its transmembrane characteristic.

The heavy alpha chain is approximately 45 kDa in size and its gene contains 8 exons[5,14]. Exon 1 encodes the leader peptide, exon 2 and 3 encode the alpha1 and alpha2 domains, which both bind the peptide, exon 4 encodes the alpha3 domain, exon 5 encodes the transmembrane region and exons 6 and 7 encode the cytoplasmic tail. Polymorphisms within exon 2 and exon 3 are responsible for the peptide binding specificity of each class one molecule (figure 2).

These classical transmembrane HLA-I molecules are genetically inherited making them important host genetic risk factor in infectious disease that are expressed on the surfaces of most of the human nucleated cells[15]. Molecules encoded by the HLA region are involved in a multitude of functionalities such as: antigen presentation, inflammation regulation, the complement system, and the innate and adaptive immune responses, indicating the HLA's importance in immune-mediated, autoimmune, and infectious diseases[16]. In antigen presentation, the HLA molecules to bind to antigens derived from pathogens and display them on the cell surface for recognition by the appropriate cells CD8+ T-cells, which then kill the antigen-presenting cells (APCs)[17].

A defining feature of the HLA-I molecules are their tremendous polymorphism which is concentrated in the regions of the HLA molecule involved in peptide binding[14]. These polymorphisms influence the specificities of the peptide binding in the assembled HLA-I proteins to allow for the presentation of a distinct and diverse pool of antigenic peptides by each HLA-I molecule[18]. In principle, a single HLA molecule may bind to more than a million different peptides[19,20]. It was proposed that infectious diseases drive HLA-I polymorphism due to the phenomenon of HLA-I restriction[21].

(a)

(b)

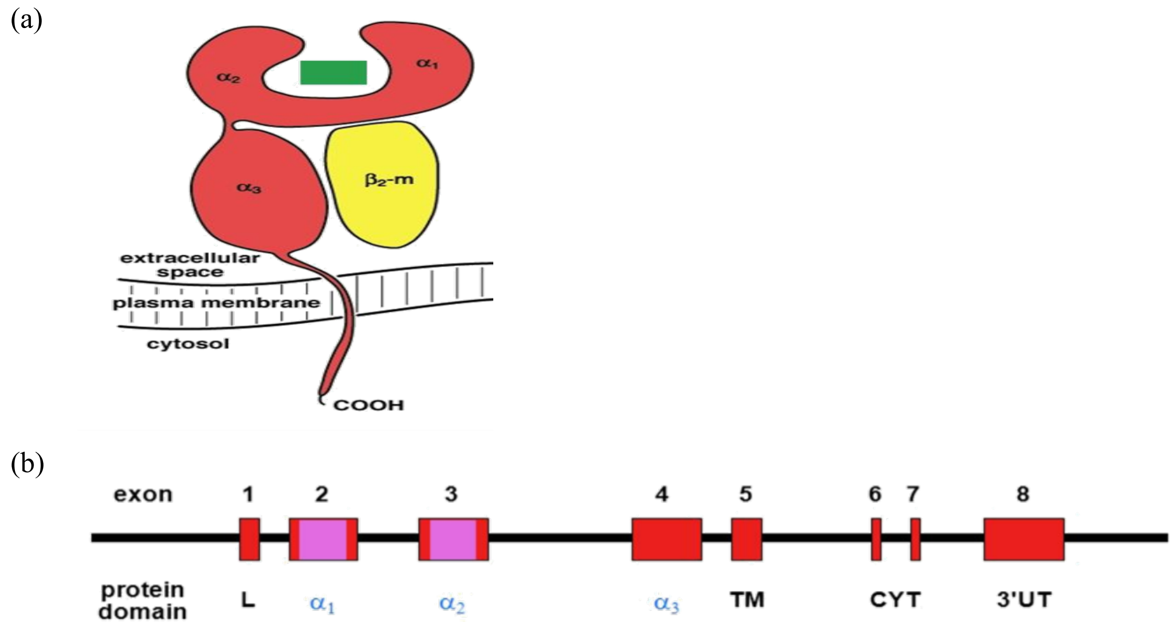

Figure 2: a) Typical transmembrane HLA-I molecular structure highlighting the heavy alpha chain and light beta-2-microglobulin complex. b) Coding region of the heavy alpha chain exons 1 to 8[22].

2.1.2 Disease Association

Being the richest segment in the human genome, the HLA region is associated with several diseases of various sources, such as bacterial, viral, inflammatory, cancer, neurologic disorders, and drug hypersensitivity[23]. MHC and autoimmune diseases have been associated since the early 1970s and were some of the earliest described genetic associations that have remained as the strongest risk factors for autoimmune diseases[24,25]. Allelic variations in the HLA-I molecules are known to have implications in susceptibility to infectious diseases, autoimmune conditions and malignancies[5].

It was reported that associations within the HLA-I locus suggest that CD8+ T-cell responses play a critical role in major viral infections such as HIV, dengue, and Hepatitis C (HCV)[26]. Slower HIV disease progression was attributed to the increase of CD8+ T-cell responses in infected donors, specific to conserved HIV proteins such as Gap p24 antigens presented by the protective alleles[24]. Another study reported an association of increased risk of

severe dengue disease with HLA-I due to weaker CD8+ T cells responses[27]. In HCV, HLA-B*27 presents the most conserved epitopes of HCV and elicits strong cytotoxic T-cell responses, thus reducing the ability of HCV to escape from host immune responses[28]. Table 1 highlights some of these and other major HLA disease associations that have been discovered over the decades.

Table 1: Categorized Summation of Major HLA Disease Associations

Disease Category	Disease Association
Autoimmune[13,26]	Ankylosing spondylitis
	Graves' disease
	Hashimoto's thyroiditis
	Myasthenia Gravis
	Addison's disease
	Rheumatoid arthritis
	Celiac disease
	Multiple sclerosis
	Type 1 diabetes
	Systemic lupus erythematosus
Viral[13,14,23,24,27–36]	Human immunodeficiency Virus (HIV)
	Hepatitis B Virus (HBV)
	Dengue hemorrhagic fever (DHF)/Dengue shock syndrome (DSS
	Hepatitis C Virus
	Adenovirus
	Coxsackie Virus Type II
	Parvovirus
	Herpes Simplex Virus
Bacterial[37–39]	Reactive arthritis (*Klebsiella pneumonia*, *Proteus mirabilis*)
	Ankylosing spondylitis
	Leprosy
	Reiter disease
	Acute Anterior uveitis
Parasitic[38]	Malaria
	Schistosomiasis
Others[5,23]	Graft versus host disease
	Cancers

We have also found that several HLA molecules that have shown to associate with diseases in conferring protection, have found to increase the risk or susceptibility to another disease. Due to our continuously evolving immune system, in relation to exposure to various infectious diseases, HLA molecules have thus adapted and evolved resulting in a variety of peptides being presented on the cell surface. The continuous evolution of HLA molecules gives rise to the number of alleles / HLA forms increasing at a rapid rate. However, having said this, infectious disease continues evolving and adapting as well. Rock *et al*., 2016 illustrated that some alleles present highly specific peptides at the cell surface and this specificity results in the reason as to why some alleles illicit a greater immune response to a particular disease than others[40]. This association directly links HLA molecules with diseases and their outcomes. The peptides that are presented by HLA-I molecules and the polymorphisms that exist have been shown to contribute to differences in disease outcome[41]. In addition to presenting peptides on the cell surfaces, HLA expression was found to also associate with diseases outcomes.

2.2 HLA Expression

2.2.1 HLA Expression and disease association

Over the past few years, a few studies have explored the expression of HLA and the association with several diseases[34,35,42,43]. It was observed that expression levels have a direct association with disease outcomes and vary at the allele level, that is most HLA genes show allele specific expression variation[4–6,14,18,23,34–36,42–48]. This variability in allelic-expression has shown to be important in HIV infection, Crohn's disease, Hepatitis B Virus (HBV) clearance, graft-versus-host disease (GVHD) after unrelated hematopoietic cell transplantation, Parkinson's disease, systemic lupus erythematosus and certain cancers[23,36,43,49]. Therefore, varied expression levels for a single gene within the HLA region could affect multiple disease outcomes. This was illustrated within the HLA-C locus where variation in expression was shown to associate with diseases HIV, Crohn's disease and Graft-Versus Host Disease (GVHD) as previously mentioned[5,23,49].

Expression levels of HLA molecules have an important influence on their function. It has been implicated that the nature of the HLA–viral peptide interaction is a major factor modulating durable control of the HIV infection[42]. The expression levels, however, of HLA-A and HLA-C were shown to associate with HIV disease in opposing ways[14,34,35,42]. Increased expression of HLA-C associated with decreased HIV disease progression, however, elevated expression of HLA-A associated with loss of HIV control. These genes were shown to work independently of each other. Higher expression of HLA-C correlated with increased cytotoxic responses and are also conferred greater immune pressure on HIV[35,42]. Elevated expression of HLA-A contributes significantly to level of HLA-E expression, which promotes inhibition of Natural Killer (NK) cells and subsequently leads to diminished NK cell killing of HIV infected target cells[35]. Currently, there is no supporting literature that is available for HLA-B expression in association with HIV disease outcomes.

In addition to disease associations, Ramsuran *et al*., 2017 observed that irrespective of the similarity between the promoter sequence within the HLA-I loci, there was poor correlation in expression levels and promoter regions as well as in expression across *HLA-A, HLA-B* and *HLA-C*. This was indicative that the classical class-I genes are regulated independently of each other, consequently by mechanisms not completely understood yet[23].

2.2.2 HLA-B

2.2.2.1 HLA-B Expression

Allelic variation of gene expression is common in humans and is of interest because of its potential contribution to variation in heritable traits. A decrease in expression may sometimes result in an increase in risk of disease susceptibility; and the extent of the risk is co-dependent on the variation in expression across donors.

The HLA-B gene has many different normal variations called alleles, allowing donor immune systems to react to a wide range of foreign invaders. Hundreds of alleles of HLA-B are

known, and closely related alleles are categorized together. Several variations of HLA-B genes appear to play a role in the progression of HIV infection to acquired immunodeficiency syndrome (AIDS). HLA-B*5701 has been associated with an extreme sensitivity to abacavir – drug used in the treatment of HIV-1 and slows he spread of HIV within the body. Previous studies have suggested that HIV positive donors who possess the HLA-B27 or HLA-B57 genes, tend to progress at a slower rate to AIDS. Whereas other researchers have observed the converse in HIV positive donors who have the HLA-B35 gene[45]. The mechanism by which HLA influences disease progression is not completely understood. It has been suggested that specific HLA molecules maybe directly involved in restricting HIV replication however this process is not fully understood.

Genetic makeup of a person's HLA affects the rate of HIV disease progression. Sites in HLA-B within the MHC have been reported to be highly associated with HIV control[45]. A recent study had explored this variation across alleles and found that the variation in expression across HLA-A and HLA-C alleles vary in an allele specific manner, whereas the expression across HLA-B alleles does not display a similar trend in allele specific variation (figure 3)[23,36,42]. In fact, the expression variation across various alleles were found to be relatively low and remained consistent throughout (figure 3b). Unlike *HLA-A* and *HLA-C*, *HLA-B* shows a much higher nucleotide diversity upstream of the transcription start site (TSS), therefore it is surprising that the mRNA variation in expression across alleles were low[23].

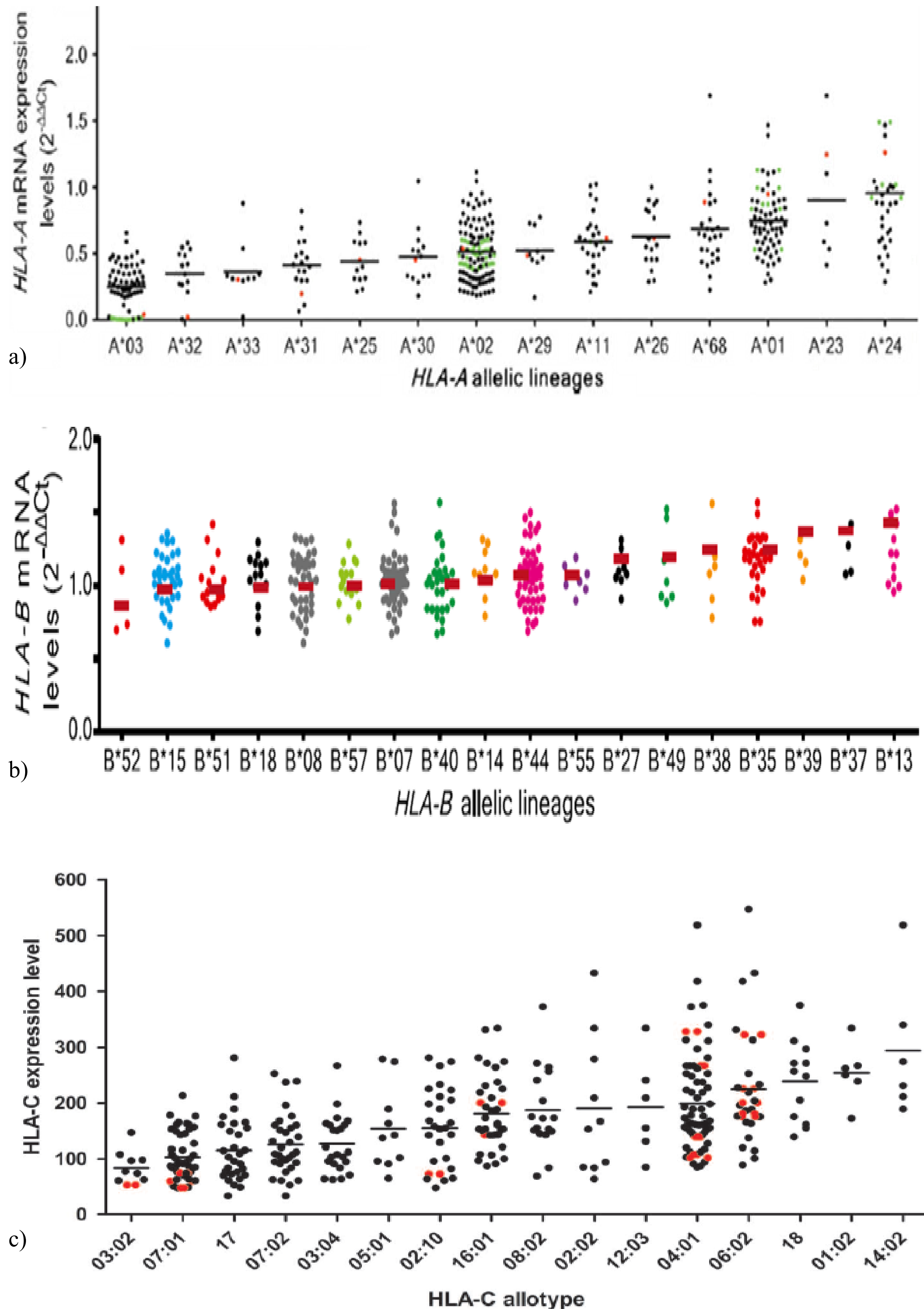

Figure 3: Differential expression of a) HLA-A[36], b) HLA-B[23] and c) HLA-C[42] across alleles – plotted in ascending order. Relative expression is plotted twice for each donor for their respective allele combination (black dots). a) HLA-A and b) HLA-B graphs are plotted for

relative mRNA allelic expression whilst c) denotes cell surface expression of HLA-C alleles. In a) red dots denote linear regression estimates, green dots denote homozygous donors and horizontal lines denote average allelic expression; whereas in b) horizontal line denotes the estimated HLA-B expression levels for each lineage as calculated using linear regression; c) red dot denotes the homozygous donors.

These findings substantiate that there is no correlation between promoter sequence diversity and expression at the mRNA level and further differentiates *HLA-B* from *HLA-A* and *HLA-C*. Interestingly, this was the only study that reported HLA-B allele specific mRNA expression using peripheral blood mononuclear cells (PBMC)[23]. These cells are composed collectively of lymphocytes (approximately 70 – 85 %) and monocytes (approximately 10 to 30 %) and current literature does not report the expression of HLA across cell types. It would be interesting if some cell types had higher expression than others. Such data would further validate the presence of alternate mechanisms that may be responsible for the variation in expression or with the nucleotide diversity observed in the promoter region of *HLA-B* as previously described[23].

Kiepiela, et al., 2004 stated that the variation in viral set-point was strongly associated with HLA-B, but not HLA-A, allele expression. It was also observed that greater selection pressure is imposed on HIV-1 by HLA-B alleles than by HLA-A[3]. This substantiates that the principal focus of HIV-specific activity is at the HLA-B locus. Furthermore, HLA-B gene frequencies in the population are those likely to be most influenced by HIV disease, consistent with the observation that B-alleles evolve more rapidly than A alleles. The dominant involvement of HLA-B in influencing HIV disease outcome is of specific relevance to the direction of HIV research[3]. HLA-B alleles have also been reported to pose better protective activity against HIV-1 than HLA-A alleles possibly due to differences in in HLA-restricted HIV-1-specific CD8+ cytotoxic T lymphocyte function, however the exact mechanism is unknown[3,50]. Expression levels of HLA molecules have an important influence on their function[14,45]. It is therefore pivotal to gain insight into the regulation of the HLA-B class-I allele expression as this area is yet to be further explored.

Gene expression levels are believed to be crucial in the involvement of the HLA complex in the pathogenesis of particular diseases[5]. Studies have also revealed that HIV protein, Negative Regulatory Factor (Nef), downregulates HLA-A and HLA-B types at the cell surface however with different efficiencies[50,51]. HLA-B is known to be more protective against HIV-1 and was also observed to exhibit a greater resistance to downregulation by Nef in comparison to HLA-A types[3,50–52]. This exploitation by HIV highlights the importance of HLA-I expression in modulating HIV disease progression.

Apps *et al*., 2015 showed that in HIV infected cells, HLA-A expression was 1 to 3 times higher and HLA-B was 2 to 5 times higher than HLA-C. HLA-C expression was not modified by Nef. HLA-A and HLA-B downregulation was approximately 4 times lower in HIV infected cells post infection[14]. To date, there is no comparative information on the difference in expression at the mRNA level pre and post HIV infection. It would be of interest to explore these sectors and build fundamental insight into the most polymorphic region within the human genome. Extensively studied HLA alleles, HLA-B*57 and B*27, have been proven to illicit protective properties against HIV-1 disease whilst HLA-B*35 is predisposing to the disease. To date, HLA-B has been known to have the strongest disease association as well as being the most polymorphic loci in the human genome, yet limited information is available with regard to the allelic expression of HLA-B at the mRNA and cell surface levels[6–8,53].

2.2.2.2 Measurement of HLA-B expression

Some alleles offer a better immune response than others and this may be attributed to the varying levels of expression observed[40]. However, as reported in previous studies, the variable expression may offer contrasting responses. We have observed that high surface expression of HLA-C confers protection against HIV as well as increases the risk of Crohn’s disease[42].

At the cell surface using specific haplotypes (A*02:01, B*44:02, C*05:01), HLA-B protein expression level were found to be similar to HLA-A, however 13 to 18 times higher than HLA-C using flowcytometry as a quantification method and 4 to 5 times higher than HLA-C when mass spectrometry was used as a quantification method[14]. Further to this, HLA-E expression was found to be 25 times lower than HLA-C surface expression using mass spectrometry[14]. This discrepancy using the two technologies may be attributed to the variation in protein conformation or perhaps the location of the protein measured[14].

While expression level has significant consequences for HLA function, HLA-B allele specific expression is not well established at the cell surface. Every donor can express six HLA alleles, two for each of the classical class-I HLAs, that is, HLA-A, HLA-B and HLA-C, with exception of HLA-E. Due to the high level of polymorphism that exists in the HLA loci and therefore the HLA-B loci, precise quantification is very challenging. Currently, the two methods in use are flowcytometry and mass spectrometry, as discussed above. However, due to availability or lack thereof, it is difficult to identify a suitable antibody that can be used across all alleles with equal affinity.

Unlike HLA-C, that uses the DT9 antibody to measure all HLA-C alleles with equal affinity, within the HLA-B locus there is no single monoclonal antibody with the ability to measure expression levels across all common HLA-B alleles[34]. It is therefore difficult to determine the expression across several HLA-B alleles for comparison. challenging. Commercially, there are several antibodies that are specific to molecules from each locus, however their binding cannot merely be compared because of the differences in affinity for their respective antigens. This illustrates that a single antibody cannot be used to measure a broad range of alleles for HLA-B as the affinity variation, which is defined as the strength to which an antibody binds to an antigen, is too broad and therefore expression levels measured thereafter are not comparable across alleles. Also, some antibodies can bind to a broad range of alleles across the HLA-I loci and these affinities skew the expression data if a donor expressed two out of the six

alleles from HLA-I. These were common hindsight's in several expression experiments previously performed and discussed[42,54,55].

In order to overcome such observations, specific antibodies were required to be screened such that each antibody will only detect a subset of alleles with equal affinity within a large cohort. Apps *et al*., 2009 used a fluorescence bead based method that incorporated commercially available beads each coated with donor coated HLA purified from transfected 721 221 cells[56]. The LABScreen single antigen-bead (LSA) based assay, from Onelambda, allows for a precise determination of antibody profiles against HLA's (figure 4). The LSA is designed to screen HLA-I antibodies that are reactive to one or more dominant epitopes providing a comprehensive assay to evaluate antigens found frequently in the population. Using the LSA beads and the Luminex assay, a range of anti-Bw4 antibodies can be used screened in order to determine the affinity against 100 class-I HLA alleles, inclusive of 4 control beads. The affinities for specific alleles are obtained using a Luminex reader and used to selected specific alleles for further analysis[57].

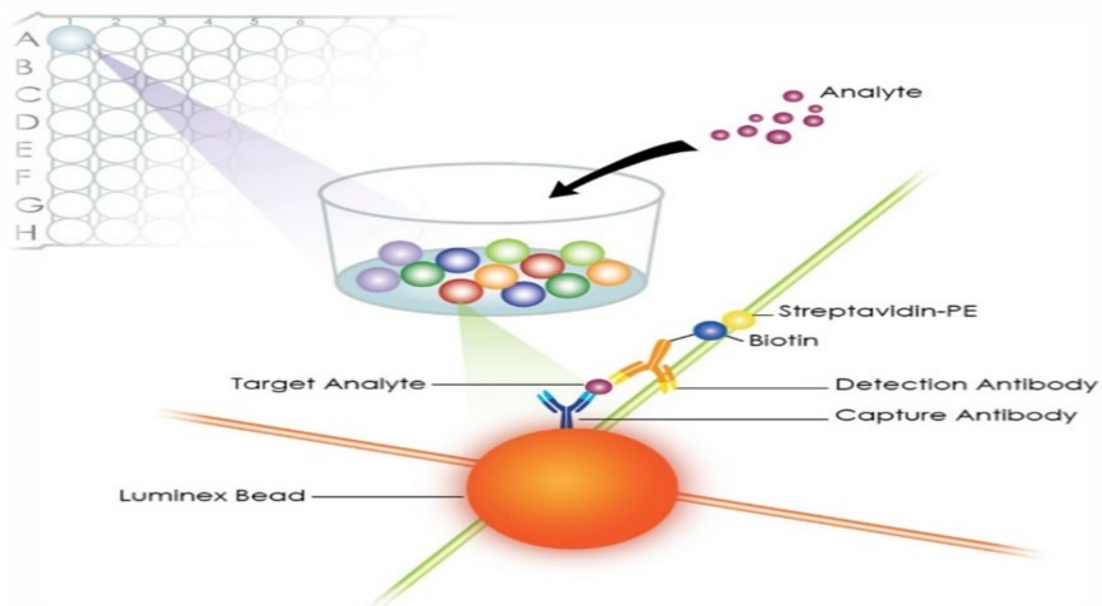

Figure 4: LABScreen Single Antigen assays leverage Luminex® bead-based multiplexing technology for monitoring Donor Specific Antibodies[57]

mRNA expression allows researchers to determine if a gene of interest (GOI) is expression at high or low levels, if expressed at all. This expression results the functional capability of the protein synthesised. The most sensitive method used for detection and quantification of mRNA expression exists in PCR (RT-qPCR and ddPCR). Digital PCR (dPCR) enables precise, highly sensitive quantification of nucleic acids. Traditional PCR is an end-point analysis that is semi-quantitative because the amplified product is detected by agarose gel electrophoresis. Real-time PCR (or qPCR) uses fluorescence-based detection to allow the measurement of accumulated amplified product as the reaction progresses. qPCR requires normalization to controls (either to a reference or to a standard curve), allowing only relative quantification. Furthermore, variations in amplification efficiency may affect qPCR results.

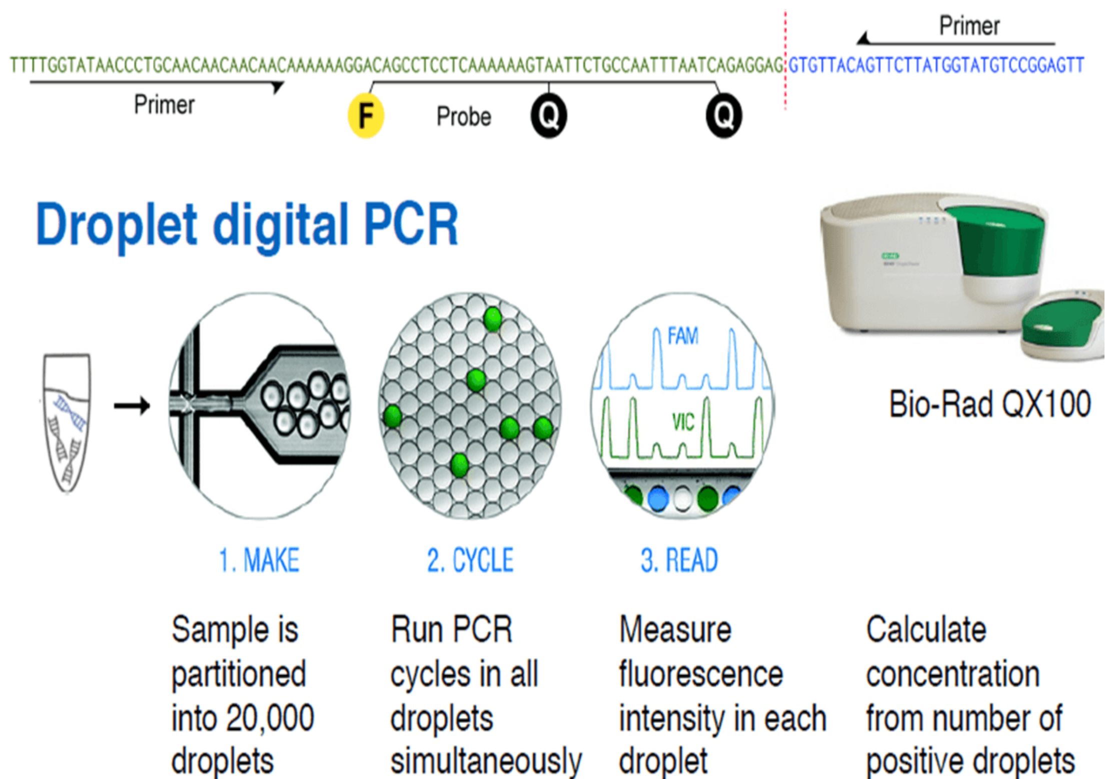

Figure 5: Schematic illustration of Droplet digital PCR (ddPCR) reaction. Sample is partitioned into ~20,000 droplets, followed by amplification. Thereafter droplets containing target sequence are detected by fluorescence and scored as positive, and droplets without fluorescence are scored as negative. Poisson statistical analysis of the numbers of positive and negative droplets yields absolute quantitation of the target sequence[58]

Digital PCR is a technique that combines traditional PCR amplification and fluorescent-probe–based detection methods to provide highly sensitive absolute quantification of nucleic acids without the need for standard curves[58,59]. Due to the similarity across the classical class-I genes, and the extreme of polymorphisms that exist within exons 2 and 3, primer efficiency and specificity is crucial in any qPCR mRNA allele lineage assay.

In this study, we use droplet digital PCR (ddPCR) which, as described above, is a method for performing absolute quantification of a product. This procedure is based on water-oil emulsion droplet technology where the sample was fractionated into 20 000 droplets, and PCR amplification of the specified molecules occurred in each donor droplet (figure 5). Samples are amplified using a thermocycler, and droplets from each sample are analysed using the QX100 Droplet Reader, purchased from Biorad. Using florescent detection, PCR-positive and PCR-negative droplets are counted to provide absolute quantification of the target DNA in digital form.

A study published in human molecular genetics looked at *HLA-A* and measured the allelic expression in healthy European donors using qPCR[36]. Each donor was plotted twice for their respective *HLA-A* allele and the illustrated findings revealed a gradient in mRNA allelic expression. The variation in expression was attributed to epigenetic factors, namely DNA methylation, that resulted in the gradient of expression across *HLA-A* alleles[36]. This observation was quite distinct in *HLA-A* for transcriptional regulation in comparison *HLA-B* and *HLA-C*, which was surprising since the CpG target sites across *HLA-A, HLA-B* and *HLA-C* are similar yet *HLA-B* and *-C* remain unmethylated[36].

mRNA expression provides valuable information from a pre-transcriptional level and provides insight on regulatory pathways that may contribute to disease outcomes. Several such studies explored different contributory mechanisms, regulatory factors and single nucleotide polymorphisms that have been linked to a variety of disease associations and outcomes[34–36]. The evolutionary pathway of the *HLA* loci is continuous and the regulatory mechanism behind *HLA-*

A, HLA-B and *HLA-C* are not completely understood. This is an area that receiving much attention in disease associations.

2.3 Research Project Overview

2.3.1 Rationale for the study:

Genome wide association studies (GWAS) have shown the HLA-I genes exhibit the strongest associations with HIV disease outcomes. The region showing the strongest genome-wide association falls within the HLA-B gene region. Recent studies have shown expression levels of HLA-A and HLA-C are associated with HIV disease outcomes. An increase in expression of HLA-C leads to delayed progression against HIV whereas an increase in HLA-A leads to rapid HIV disease progression.

Limited information is available for the allelic expression levels for HLA-B gene. Analysis of HLA-B expression level is likely to be important not only in HIV infection, but also other infectious diseases and immune-related disorders in which HLA-B has previously been shown to play a role. Measuring HLA expression levels adds another dimension to the extreme diversity of HLA-I molecules that accounts for variability in immune responses across donors and may suggest possible drug targets to modulate host responses to infections. Despite HLA-B being widely studied, not much was known about the allelic expression levels, variation of expression across differential cell types and HIV downregulation of HLA-I at the mRNA level.

2.3.2 Study Aims and Objectives

This study explores the HLA-B allelic surface expression variation and to further determine HLA-B expression variation across several cell types such as T cells, B cells, Monocytes, and NK cells.

The objectives were as follows:

- Objective 1: Determine HLA-B differential allele-specific expression levels on the cell surface using flow cytometry
- Objective 2: Assess *HLA-A, -B, -C* and *-E* mRNA expression levels across T-cells, B-cells, monocytes and natural killer (NK) cells using digital droplet PCR (ddPCR)
- Objective 3: Assess the effect of HIV on HLA-class-I types at the mRNA level

This study aimed to examine these gaps in knowledge and reports for the first-time variation in allele specific expression, variation in expression across differential cell types as well as a lack of expression variation in pre- and post- HIV infection at the mRNA level.

Chapter three: Materials and Methods

3.1 Study Ethics Consideration

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee (BREC). The BREC approval number was BE539/17. Ethics approval for use of samples previously obtained from the HIV negative and positive study cohorts that have had HLA typing successfully concluded, were referenced on the BREC application. This study was conducted within good clinical practice ethical guidelines. (See Appendix A)

3.2 Study Samples

HIV infected, and uninfected samples used in this study were obtained from the Centre for AIDS Program of Research (CAPRISA). All samples that were considered for analysis have been previously HLA typed. All samples were stored at -80 °C until required for analysis.

3.2.1 CAPRISA 002 cohort

The CAPRISA 002 cohort is an Acute Infection Study that was established between August 2004 and May 2005. The cohort consists of high-risk HIV-uninfected women, working as female sex workers in and around a large urban area. CAPRISA 002 is the first acute longitudinal study in South Africa that reports acute infection in a prospective cohort of HIV-1 subtype C infection[60]. Details of the establishment of this study cohort was published by Loggerenberg et al., 2008[60]. The aim of the CAPRISA 002 study was to identify host and viral factors during the acute and early phases of HIV-1 infection that may have a significant impact on the subsequent course of the disease.

3.3 HLA Expression

3.3.1 RNA extraction

RNA extraction was performed on PBMC's and sorted cell types followed by cDNA synthesis using the iScript cDNA synthesis kit (BioRad). HIV negative (HIV-) samples were

used to measure cell surface expression of HLA-B and mRNA expression of *HLA-A, HLA-B, HLA-C* and *HLA-E*. HIV positive (HIV+) samples were used in conjunction with the corresponding HIV- samples for pre- and post-HIV infection mRNA expression comparison study.

3.3.2 Cell surface expression

3.3.2.1 Antibody affinity screening

Using the Luminex assay with LABScreen single antigen-beads (LSA) purchased from one lambda, we screened two commercially available antibodies, REA274 and 22E1, against 96 HLA-I alleles, as per previously described[34,56]. Each of the one hundred alleles tested were attached to different size polystyrene bead. Briefly, the beads were incubated with unlabelled primary or isotype control mAb for 30 min at room temperature, washed five times and labelled with monoclonal FITC-conjugated antibody to murine IgG or IgM (Sigma-Aldrich). The FITC-conjugated mAb labelling were analysed using a Luminex 100 reader. Results shown are median fluorescence intensities (MFI) of a minimum of 100 events for each HLA-I-coated bead. (Miltenyi Biotec).

3.3.2.2 Allele Selection

HLA-B alleles were selected based on the affinities against each antibody. Allele within a 10% affinity variability were selected for comparison. Therefore, each antibody will only detect a subset of alleles with similar affinity. Donors with a selected allele, based on the affinities, were used to compare HLA-B allelic expression levels. Donors were carefully examined to ensure other HLA-I alleles were not detected by the antibody.

3.3.2.3 Flowcytometry

CD3+ T-cells were centrifuged at 300 *x*g for 10 min and washed with wash buffer. Samples were then stained using *FITC* conjugated monoclonal Antibodies (22E1 or REA274)

and incubated for 10 minutes. Sampled were washed using wash buffer and centrifuged at 300 xg for 10 min. Cell pellet was resuspend in a suitable amount of buffer for analysis by flow cytometry using the BD LSRFortessa. A combination of gating strategies was applied to distinguish our cells of interest – CD3+ T-cells. A Bw6 allele was selected from the cohort of samples and used as negative control with each set of antibody specific allele subset. All data generated from flow cytometry were analysed using flowjo software (version 10.5.2). All plotted graphs and statistical analysis were performed using GraphPad Prism version 5.0. HLA-B allelic expression levels was reported as (MFI).

3.3.3 mRNA Expression

3.3.3.1 Cell sorting

Seven homozygous donor samples were selected and sorted using Fluorescence-activated cell sorting (FACS), PBMC samples were sorted into the following cell types: T-cells, B-cells, Monocytes and NK cells. All sorted cells were stored at -80 °C for further analysis.

3.3.3.2 RNA extraction and cDNA synthesis

As mentioned in section 3.3.1, RNA was extracted for the sorted cells types: T-cell, B-cells, Monocytes and NK cells. cDNA synthesis was performed using the cDNA two step synthesis kit purchased from Bio-Rad. Synthesised samples were stored at -80 °C for further analysis.

3.3.3.3 Effect of HIV on HLA-I mRNA Expression

Two donors were selected to assess the effect of HIV pre- and post- infection on HLA-I expression. HIV- and HIV+ PBMC samples were used to extract RNA, thereafter synthesising cDNA for analysis by ddPCR as described in 3.3.1 and 3.3.3.4, respectively. ddPCR was used to measure the expression of *HLA-A, HLA-B, HLA-C* and *HLA-E* pre- and post- HIV infection. Results were reported as copies per microliter (µL).

3.3.3.4 Droplet Digital PCR (ddPCR)

In this study, ddPCR was used to quantify the expression levels of *HLA-A*, *HLA-B*, *HLA-C* and *HLA-E* at the mRNA level for all seven donor samples analysed. We also measured the expression of the above HLA types on the differential cell sorts to assess the variability in expression across cell types. ddPCR was performed using the cDNA from PBMC's and the sorted cell types: T-cells (CD3), B-cells (CD19), Monocytes (CD14) and NK cells (CD56). Primers previously designed specifically for each *HLA* type with high efficiency and specificity were used in measuring expression[23]. Concentrations were reported in copies per microliter (μL).

Chapter Four: Results

4.1 Cell Surface Expression

The Luminex-LSA bead-based assay was used to screen two Bw4 antibodies against 96 HLA-I alleles[56,57]. The affinities were detected based on the binding of antibody to the respective allele and the fluorescence was measured accordingly. The antibodies, 22E1 (figure 6) and REA274 (figure 7), exhibited a broad affinity range for HLA-I alleles and were used to identify donors with specific alleles of interest. Common allotypes found within South African populations were comprised of 31 HLA-A, 49 HLA-B and 16 HLA-C molecules. Alleles which showed high binding affinities were bound to the antibody with greater strength than those which showed lower affinities. Affinities (blue) are represented on the x-axis and alleles on the y-axis in figures 6 and 7.

Using our sample cohort, we screened for donors from a pool of 300 samples to identify specific donors with alleles of interest that were within a 10% variation in antibody affinities. A total of 37 donors were selected to assess the variability in HLA-B allele specific expression using the anti-Bw4 antibodies. Using monoclonal antibody 22E1 (table 2), we obtained a total of 23 healthy donors which were separated into 2 groups, were each group contained 4 alleles each. Alleles selected in 22E1 group 1 were: HLA-B*57:01, HLA-B*49:01, HLA-B*44:02 and HLA-B*58:01; and group 2: HLA-B*27:05, HLA-B*37:01, HLA-B*38:01 and HLA-B*51:01. Grouping of alleles detected using ab 22E1 were represented by purple and green lines in figure 6, respectively. Using the REA274 antibody (table 3), we had a total of 5 alleles from 14 donors with 3 alleles in one group and 2 alleles in the other. Alleles selected in REA274 group 1 were: group 1: HLA-B*51:01, HLA-B*13:02, and HLA-B*44:03; and group 2: HLA-B*57:01, and HLA-B*58:01. Grouping of alleles detected using ab REA274 were represented by yellow and red lines in figure 7, respectively.

22E1 (anti-Bw4)

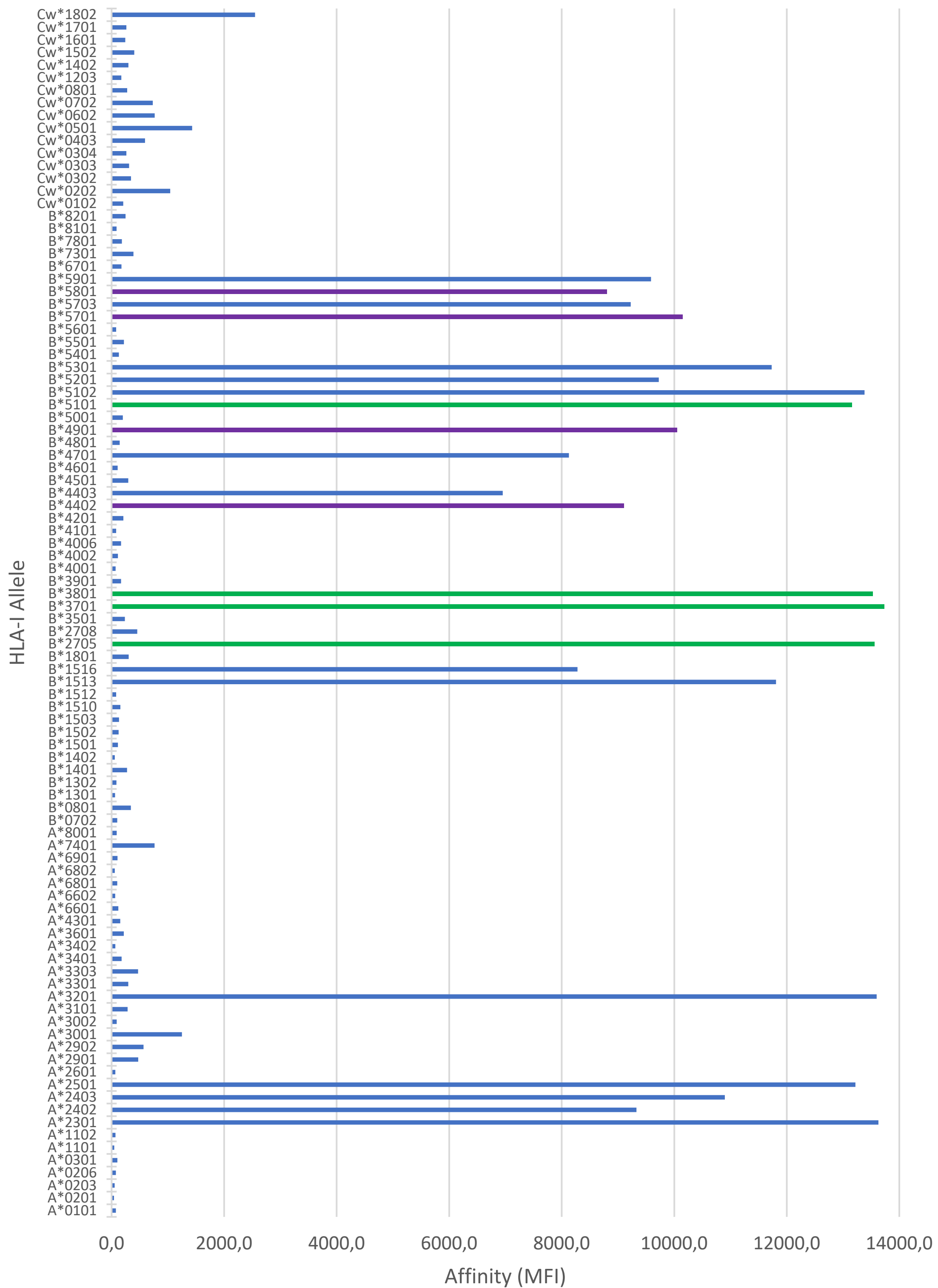

Figure 6: Classification of antibody 22E1 affinity against HLA-I alleles. The affinity of anti-Bw4 22E1 used in this study was tested experimentally against 96 of the common HLA-I alleles, using LABScreen single antigen-beads coated with donor HLA-I molecules. Grouping of alleles detected using ab 22E1 were highlighted in purple (group 1) and green (group 2).

REA274 (anti-Bw4)

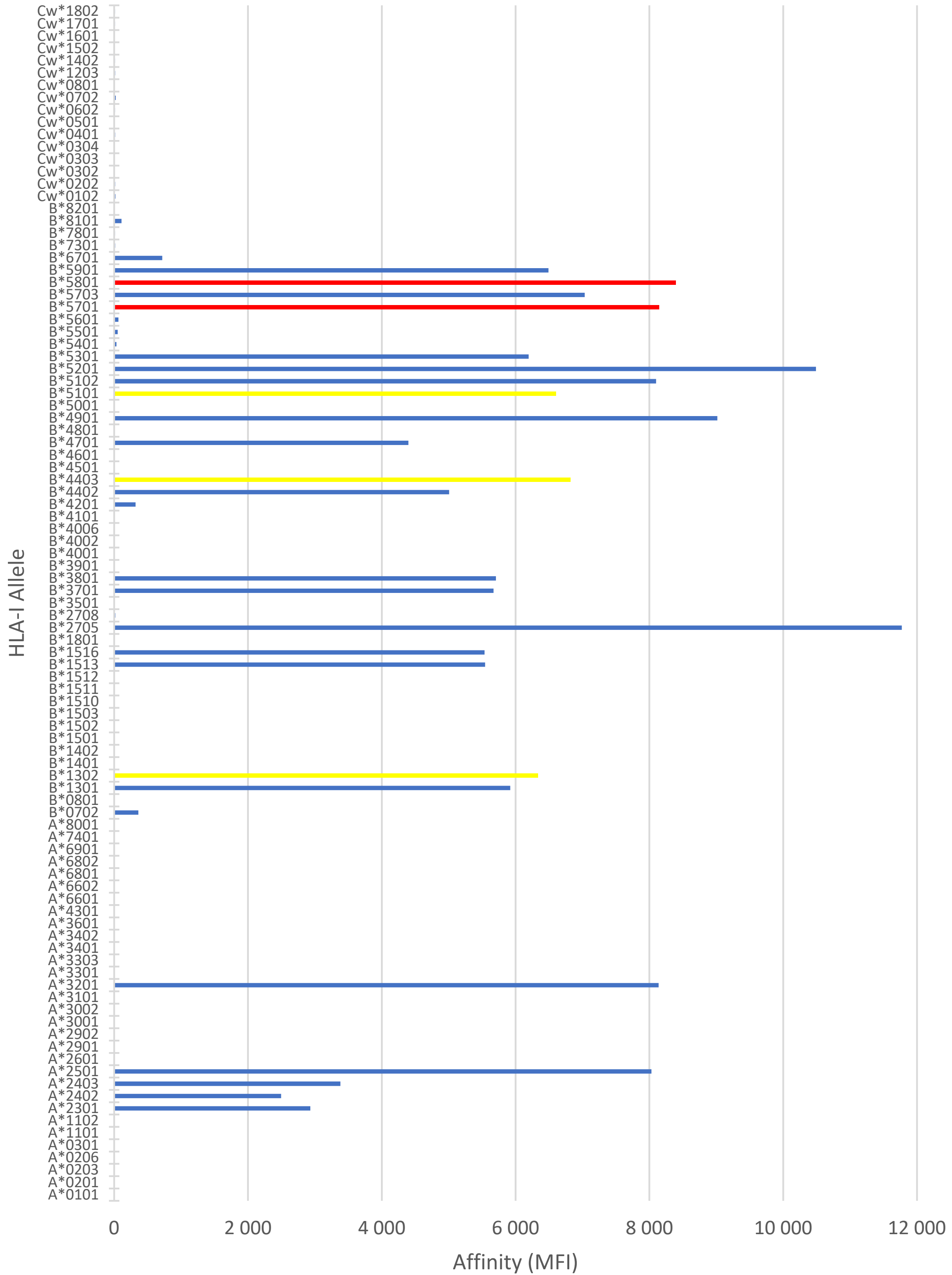

Figure 7: Classification of antibody REA274 affinity against HLA-I alleles. The affinity of anti-Bw4 REA274 used in this study was tested experimentally against 96 of the common HLA-I alleles, using LABScreen single antigen-beads coated with donor HLA-I molecules. Grouping of alleles detected using ab REA274 were highlighted in yellow (group 1) and red (group 2).

A single Bw6 allele, obtained from 7 donors, was used as a negative control (data not shown) for Ab 22E1 and REA274. Selected alleles were highlighted in bold print.

Selection of donors is a critical parameter to consider as if another allele is detected by the antibody with a high affinity, the expression of the selected allele is skewed as the other allele contributes towards the measured expression. For example: if a donor had the following HLA-I alleles, with corresponding Ab 22E1 affinity values in brackets – HLA-A*34:02 (66.3), A*23:01 (13629.7), B*37:01 (13734.8), B*14:02 (55.7), C*01:02 (206.0) and C*12:03 (171.8) – and we were interested in determining the expression of B*3701. Using the affinity graph for antibody 22E1 (figure 6), this donor would not be appropriate and therefore would not be selected primarily due to the affinity of A*23:01 being similar to B*37:01 and therefore, reflecting a combined expression of these two alleles. If A*29:01 was substituted for A*23:01, this donor would be selected. This approached for selection of specific alleles eliminated error for contributing expression from another specific allele that may be detected by the respective antibody.

Samples selected as per tables 2 and 3, were prepared and analysed using flowcytometry to establish the corresponding HLA-B cell surface allelic expression. As previously described, alleles were grouped together based on a 10% variation in antibody affinities for Bw4 antibodies 22E1 and REA274, and expression was quantified for comparison. Tables 2 and 3 tabulates the grouping of alleles based on affinities for antibodies, 22E1 and REA274, respectively. The corresponding HLA-B allele from the selected donors were used to quantify expression, as graphically represented in figures 8 and 9, respectively.

Table 2: Summary of selected HLA-B alleles identified in corresponding donors, based on a 10% variation across affinity values of antibody 22E1 obtained from the LSA assay used in this study

Group 1				
PID[1]	**HLA-B**		**Antibody 22E1 Affinity**	
	Allele 1	**Allele 2**	**Allele 1**	**Allele 2**
ID0001	*50:01	***57:01**[a]	5	8148
ID0002	*07:02	***57:01**[a]	362	8148
ID0003	*07:02	***58:01**[a]	362	8394
ID0004	*14:02	***44:02**[a]	56	9110
ID0005	*35:01	***44:02**[a]	235	9110
ID0006	*13:02	***44:02**[a]	85	9110
ID0007	*07:02	***44:02**[a]	101	9110
ID0008	*08:01	***44:02**[a]	343	9110
ID0009	*07:02	***44:02**[a]	101	9110
ID0010	*08:01	***44:02**[a]	343	9110
ID0011	*14:02	***44:02**[a]	56	9110
ID0012	*08:01	***44:02**[a]	343	9110
ID0013	*15:01	***49:01**[a]	112	10053
ID0014	*07:02	***49:01**[a]	101	10053
Group 2				
PID	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
ID0015	*15:01	***51:01**[a]	112	13161
ID0016	*08:01	***51:01**[a]	343	13161
ID0017	*41:01	***51:01**[a]	80	13161
ID0018	*13:02	***27:05**[a]	85	13561
ID0019	*15:01	***27:05**[a]	112	13561
ID0020	*08:01	***27:05**[a]	343	13561
ID0021	*07:02	***37:01**[a]	101	13735
ID0022	*35:01	***37:01**[a]	235	13735
ID0023	*14:01	***38:01**[a]	275	13533

[1] Participant Identity number;
[a] Selected allele

Table 3: Summary of selected HLA-B alleles identified in corresponding donors, based on a 10% variation across affinity values of antibody REA274 obtained from the LSA assay used in this study

Group 1				
PID[1]	HLA-B		Antibody REA274 Affinity	
	Allele 1	Allele 2	Allele 1	Allele 2
ID0024	*40:02	***44:03**[a]	5	6819
ID0025	*08:01	***44:03**[a]	3	6819
ID0026	*08:01	***44:03**[a]	3	6819
ID0027	*15:01	***44:03**[a]	4	6819
ID0028	***13:02**[a]	*35:01	6337	5
ID0029	*08:01	***13:02**[a]	3	6337
ID0030	*07:02	***13:02**[a]	362	6337
ID0031	*07:02	***13:02**[a]	362	6337
ID0015	*15:01	***51:01**[a]	4	6600
ID0016	*08:01	***51:01**[a]	3	6600
ID0017	*41:01	***51:01**[a]	5	6600
Group 2				
PID[1]	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
ID0001	*50:01	***57:01**[a]	5	8148
ID0002	*07:02	***57:01**[a]	362	8148
ID0003	*07:02	***58:01**[a]	362	8394

Participant Identity number;
[a] Selected Allele

Cell surface expression of specific HLA-B alleles (tabulated in Table 2 and 3, respectively) were measured using FITC-conjugated antibodies 22E1 and REA274, respectively. Selected Bw6 alleles were used as a negative control in the presence of anti-Bw4 antibodies. HLA-B cell surface expression is plotted on the y-axis and the HLA-B alleles for each 22E1/REA274 group is plotted on the x-axis. HLA-B allelic expression is compared across alleles per group. Variation in HLA-B expression is represented per Ab group in figures 8 and 9, for 22E1 and REA274 respectively. Alleles in group 1 of Ab 22E1 show a variability in expression across alleles, with HLA-B*57:01 being the highest expressed allele followed by B*49:01, B*44:02 and B*58:01, plotted in descending order. In 22E1 group 2, a similar trend in expression was observed with HLA-B*27:05 showing the highest cell surface expression followed by B*37:01, B*38:01 and B*5101 in descending order.

Alleles in group 1 of Ab REA274 also show a variability in expression across HLA-B alleles, with HLA-B*51:01 being the highest expressed allele followed by B*13:02, and B*44:03, plotted in descending order. In REA274 group 2, HLA-B*57:01 showed the highest cell surface expression followed by B*58:01 in descending order. A Bw6 alleles was found to have an affinity to Ab 22E1 that was below threshold and was therefore used as negative control in group 1 and group 2 for antibodies 22E1 and REA274.

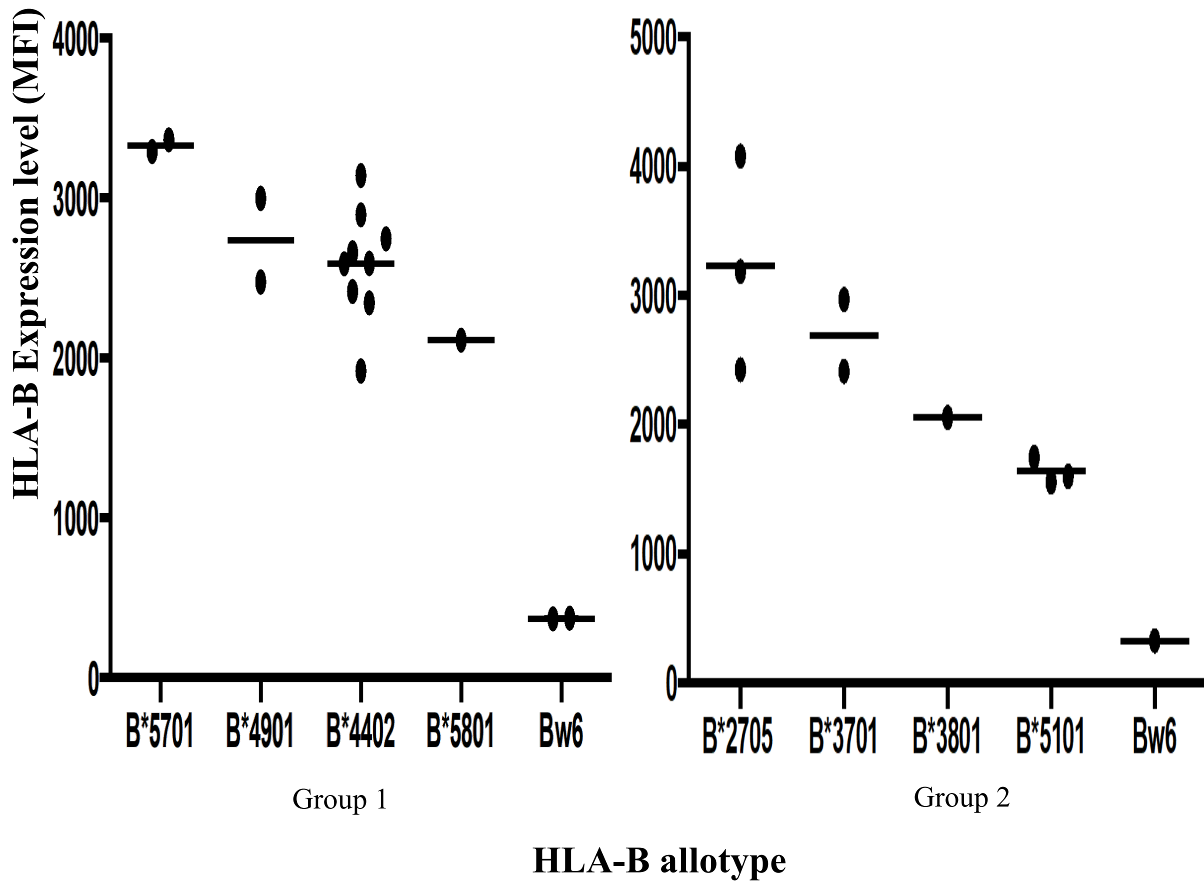

Figure 8: The distribution in expression levels (*y-axis*) of HLA-B allotypes (*x-axis*) present in healthy HIV- donors. Peripheral blood CD3+ cells from 23 healthy donors were analysed by flow cytometry for HLA-B expression level using the monoclonal antibody 22E1. An additional 3 donors with Bw6 alleles were used as a negative control. MFI of HLA-B staining is plotted once for each donor based on the selected allele. Average expression is expressed by a horizontal line. Each group is plotted separately for comparison of data based on affinity variation.

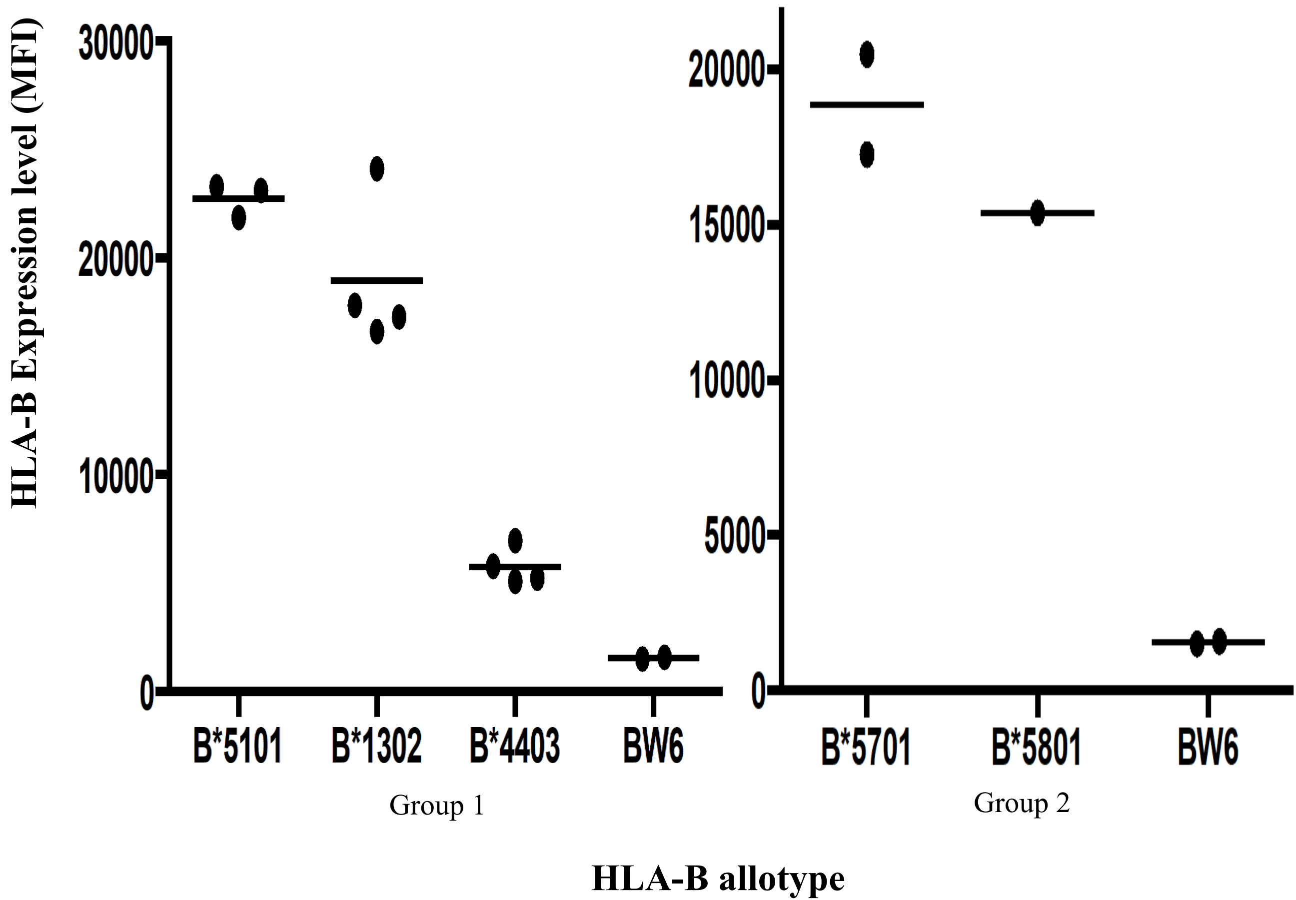

Figure 9: The distribution in expression levels (*y-axis*) of HLA-B allotypes (*x-axis*) present in healthy HIV- donors. Peripheral blood CD3+ cells from 14 healthy donors were analysed by flowcytometry for HLA-B expression level using the monoclonal antibody REA274. An additional 4 donors with Bw6 alleles were used as a negative control. MFI of HLA-B staining is plotted once for each donor based on the selected allele. Average expression is expressed by a horizontal line. Each group is plotted separately for comparison of data based on affinity variation.

4.2 mRNA Expression

4.2.1 HLA Class-I

Digital droplet PCR was used to measure the mRNA expression of HLA-I types. Seven healthy donors were selected based on sample availability. RNA extraction and cDNA synthesis were performed as previously mentioned. Samples were prepared for analysis and primers used for each HLA type were reported previously in Ramsuran *et al*., 2017. The expression of *HLA-A, HLA-B, HLA-C* and *HLA-E* were measured for each donor using PBMC samples and graphically illustrated in figure 10 with average *HLA-I* expression detailed in figure 11. Each donor reports a combination of their allelic expression for each HLA-I type. Donors 089, 174, 127, 196, 134, 158, and 187 were each plotted with their corresponding mRNA expression (measured in copies per microliter) for each HLA-I type (figure 10). The concentration was plotted on the y-axis and the donors were plotted on the x-axis. Based on the data demonstrated in figure 10, we observed a fluctuation in mRNA expression across donors as well as across HLA types. *HLA-B* shows a higher concentration in copy numbers across the other HLA-I types. The average mRNA expression (figure 11) displayed a similar trend for HLA-I types, which showed that *HLA-B* had a greater average mRNA expression followed by *HLA-A*, *HLA-C* and *HLA-E,* listed in descending order of mRNA expression.

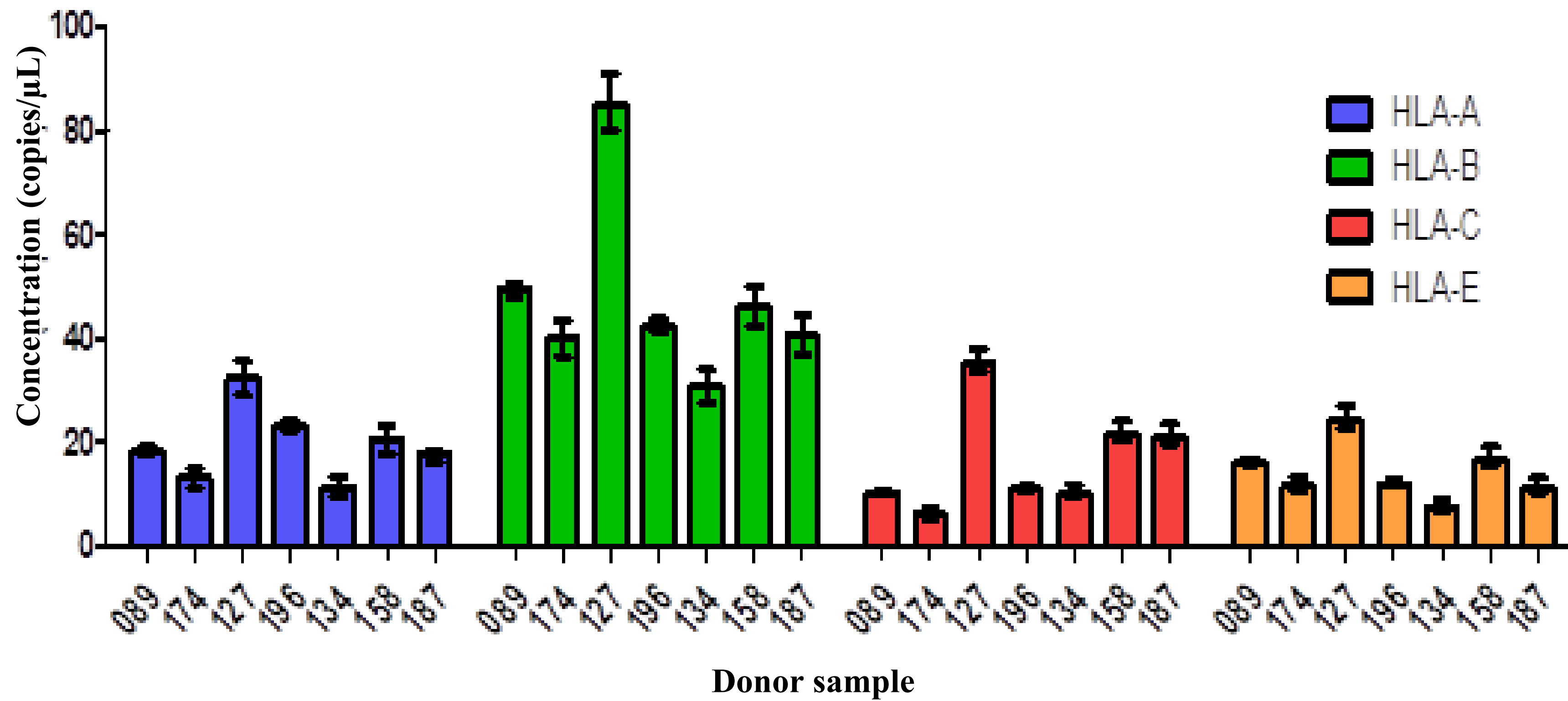

Figure 10: mRNA expression for *HLA-I* types across donor samples. The variation in expression across 7 healthy donor samples (*x-axis*) for overall expression of *HLA-A, HLA-B, HLA-C* and *HLA-E*. mRNA expression is reported as the concentration in copies per microliter (µL) (*y-axis)*

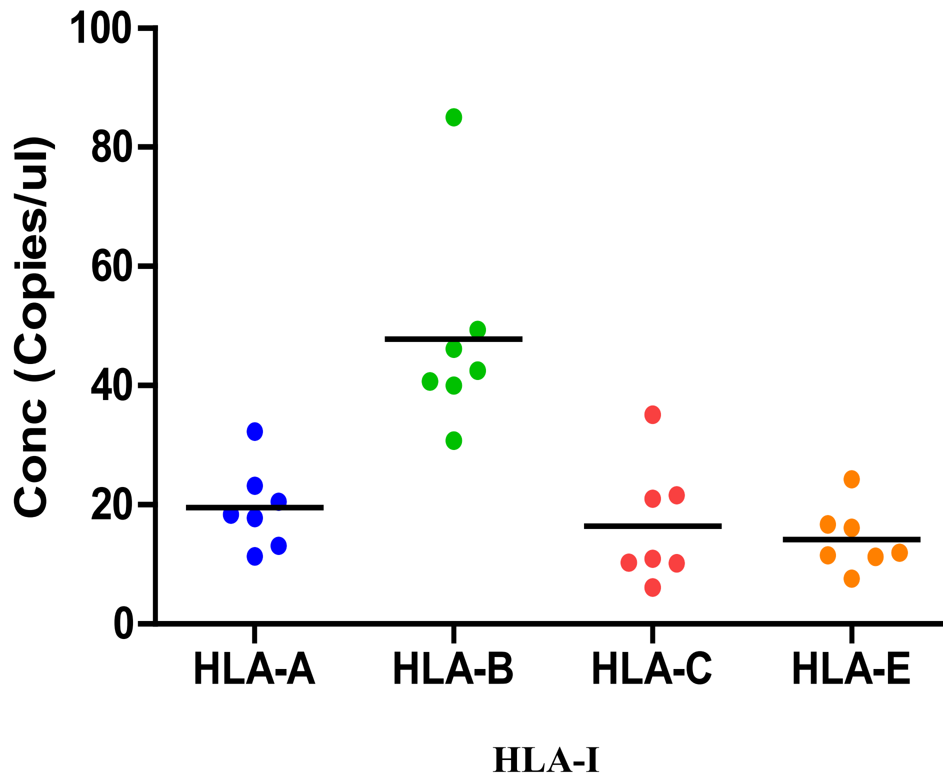

Figure 11: Average mRNA expression levels for *HLA-I* types across 7 healthy donor samples for *HLA-A, HLA-B, HLA-C* and *HLA-E,* respectively (*x-axis*). mRNA expression is reported as the concentration in copies per microliter (µL) (*y-axis*). Each donor reports a combined expression for each allele across the HLA-I type. Each donor is plotted 4 times, that is, each dot represents the combined mRNA expression per donor for each HLA-I type. The average expression per HLA-I type is denoted by a horizontal black line.

4.2.2 mRNA expression across differential cell types

PBMC's of the healthy donors were further sorted into differential cell types using FACS. RNA was extracted followed by cDNA synthesis for each cell type: T-cells, B-cells, Monocytes and NK cells. The expression of *HLA-A, HLA-B, HLA-C* and *HLA-E* were measured across each cell type using ddPCR as previously described. Figure 12 graphically represents the average expression of HLA-I types across the differential cell types. The mRNA expression is plotted on

the y-axis and the differential cell types are plotted on the x-axis for each HLA-I type. In descending order of HLA-I mRNA expression: B-cells, followed by monocytes, and T-cells and NK cells at similar levels. Across each HLA-I type, B-cells showed the highest mRNA expression and HLA-B showed the highest expression across cell types. A similar trend in hierarchy of expression across HLA-I types were observed. Differential expression was observed across cell types.

4.2.3 Effect of HIV on HLA-I mRNA expression

Two donors were selected based on previous data available for pre and post infection using PBMC samples. These donors with identity numbers 284 and 326, were each plotted before and after HIV infection to establish an alteration in expression across *HLA-A, HLA-B, HLA-C* and *HLA-E,* respectively. mRNA expression for pre- and post- HIV infection is plotted on the y-axis and the respective donor samples for each HLA-I type is plotted on the x-axis. Post- HIV infection data show a very slight decrease in mRNA expression in comparison to pre- infection data. The minor decrease in expression is not significant and therefore it can be hypothesised that HIV may not downregulate HLA-I at the mRNA level (figure 13) as compared to the cell surface expression. The lack in downregulation of HLA-I by HIV can be further investigated using a larger sample number of matched donors for statistical power to verify and conclude the hypothesis.

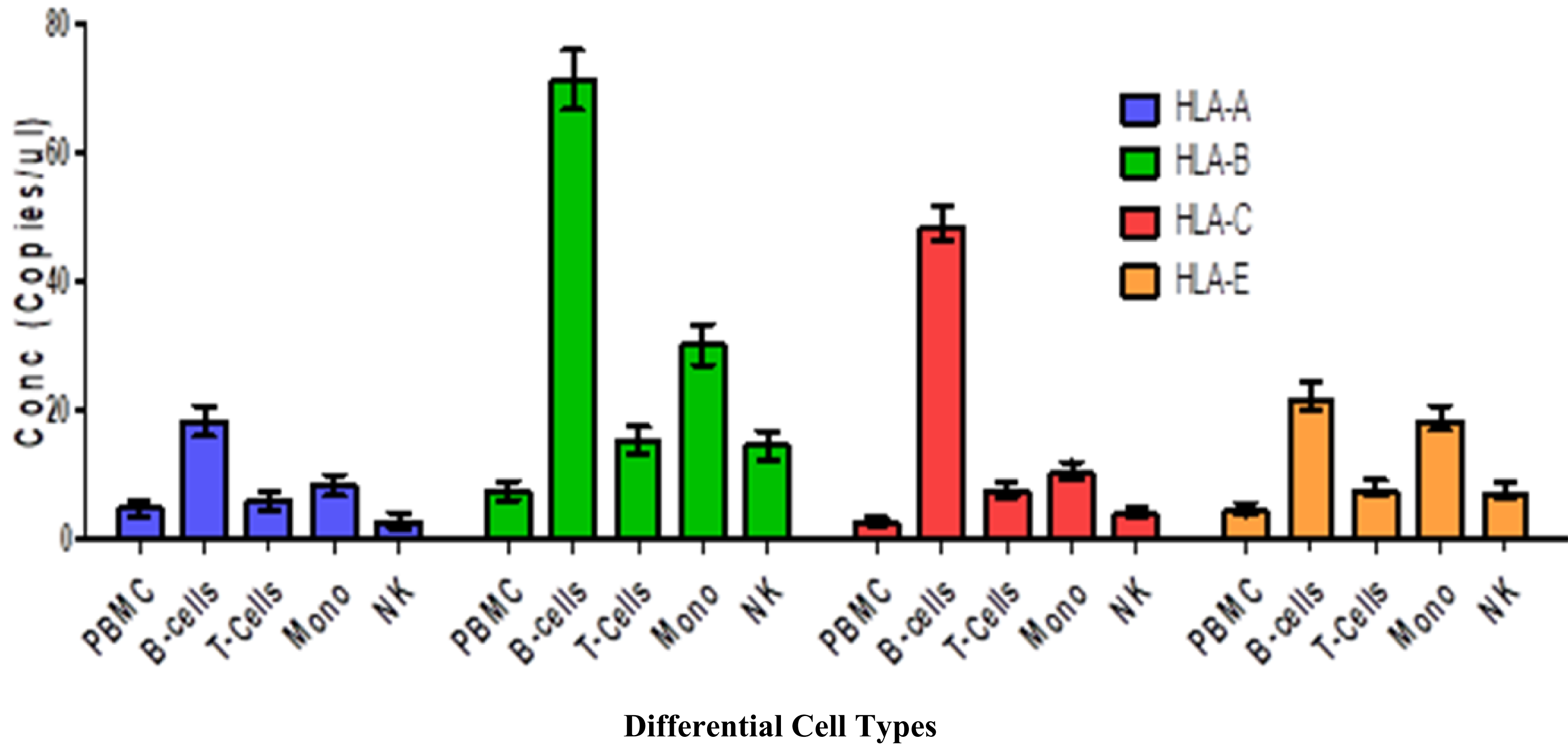

Figure 12: Variability in HLA-I mRNA expression across differential cell types. *HLA-A, HLA-B, HLA-C* and *HLA-E* expression (*y-axis*) measured across cell types: PBMC's, B-cells, T-cells, Monocytes and NK cells (*x-axis*). Variability in expression across cell types with B-cells showing the overall highest level of mRNA expression.

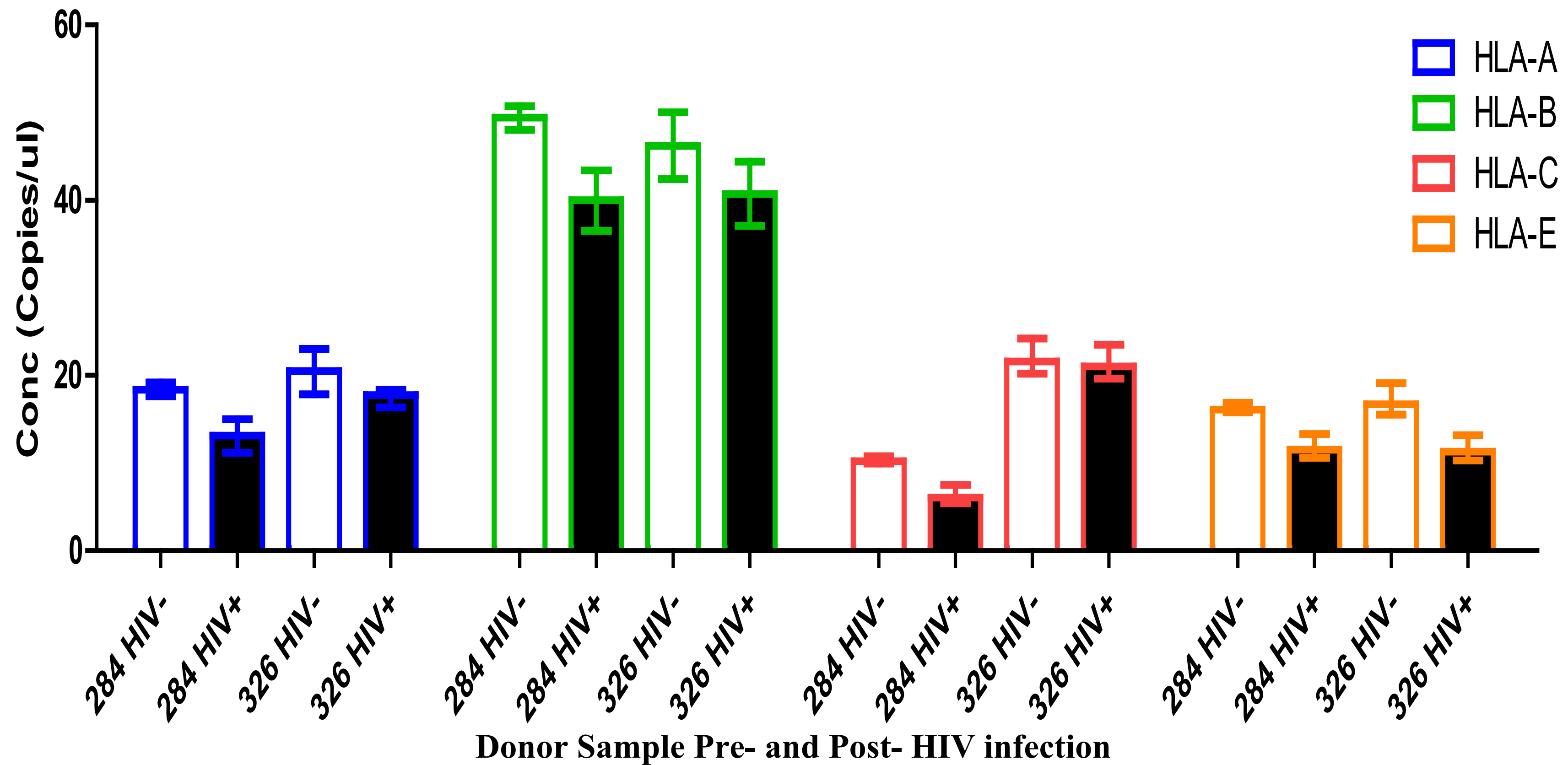

Figure 13: Effect of HIV Infection on HLA-I expression levels. mRNA expression of HLA-I is represented on the *y-axis* with the corresponding donor sample pre- and post- HIV infection on the *x-axis*. HIV infection appears to have no significant impact on the regulation of HLA-I types based on the illustrated trend.

4.3 Expression comparison

The alleles measured for cell surface expression, were categorised based on their relative antibody affinities, and used to extrapolate the corresponding mRNA expression using expression estimates. The mRNA expression estimates were obtained by averaging the mRNA expression data available for a selected allele from previous data and therefore estimating the mRNA expression to obtain an average expression estimate. Expression estimates were thereafter used to determine if there was a correlation in mRNA and cell surface expression for a given allele. After analysing mRNA and cell surface expression estimates, we observed a trend that demonstrated a lack in correlation of estimated mRNA expression and estimated cell surface expression at the allelic level per Ab group (figure 14).

We further attempted to explore the correlation between mRNA expression and cell surface expression for each donor per antibody affinity group for each respective antibody. The HLA-B mRNA expression for each donor selected was an estimated average of their respective allele and a total average HLA-B mRNA expression estimate was reported. The mRNA expression estimate was compared to the corresponding measured cell surface expression for that particular donor. Correlation data was reported per group per antibody and are illustrated in figures 15, 16, 17 and 18, respectively. There was a lack in correlation of mRNA and cell Surface expression levels per donor as selected by monoclonal antibody 22E1 in group 1 (figure 15), REA274 group 1 (figure 17) and REA274 group2 (Figure 18). Presence of a Correlation in mRNA and cell Surface expression levels per donor as selected by monoclonal antibody 22E1 in group 2. The correlation observed in this instance was suggestive that expression may be regulated at the cell surface for some alleles, however this correlation was only observed at a donor level. The average mRNA expression estimates were tested for a correlation in expression to the measured cell surface expression obtained from previous analysis using flowcytometry data. The mRNA expression estimates are plotted on the y-axis and the cell surface expression

estimates are plotted on the x-axis. Each dot represents the HLA-B expression estimates calculated for each donor participant.

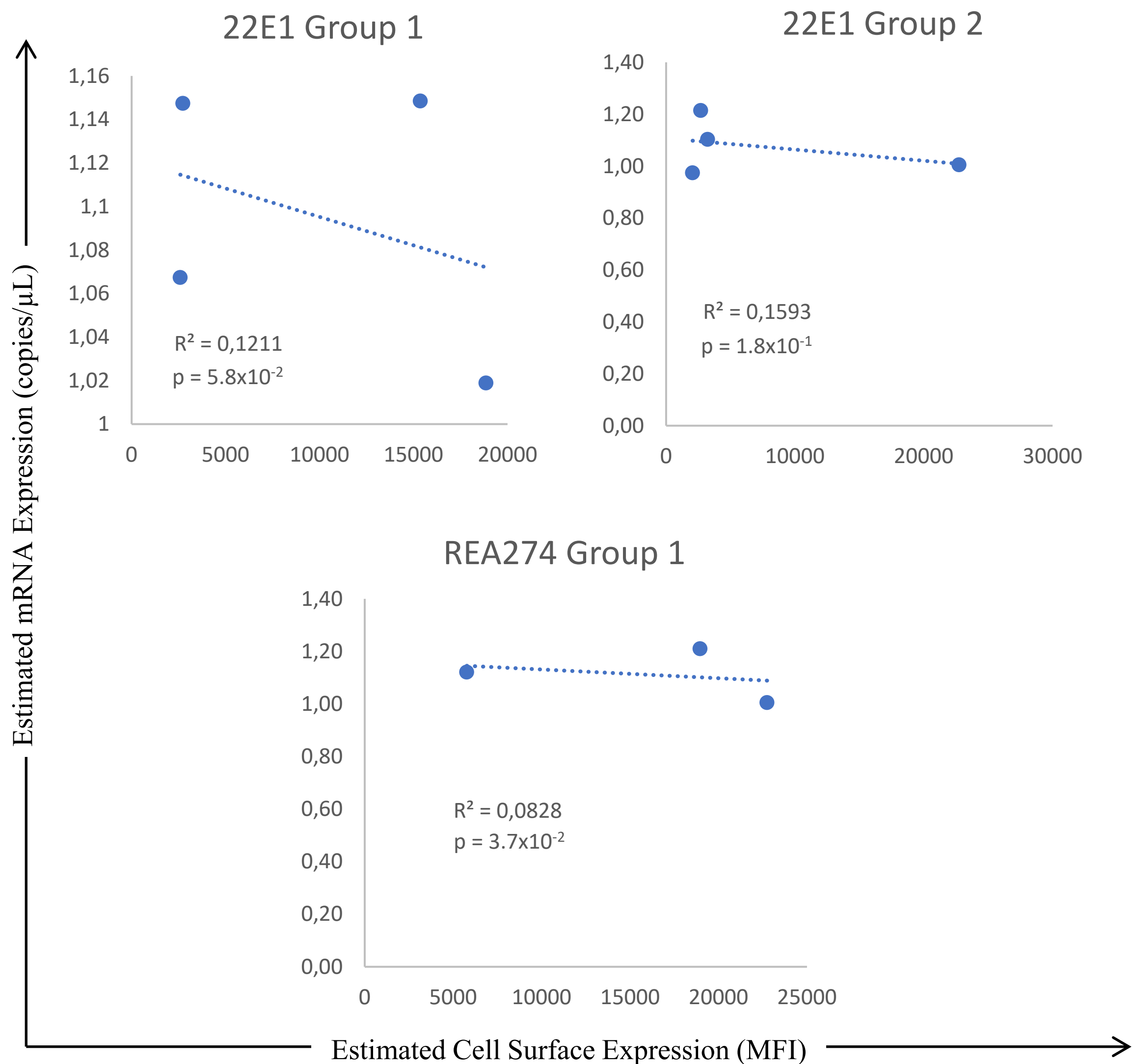

Figure 14: Lack of a correlation in mRNA and cell Surface expression levels at the allelic level for several common alleles. The mRNA expression estimates are plotted on the *y-axis* and the cell surface expression estimates are plotted on the *x-axis*. Each dot represents the allelic expression estimates calculated for each donor allele measured per antibody group. The average mRNA expression estimates were tested for a correlation in expression to the estimated cell surface expression obtained from previous analysis using flowcytometry data, per antibody groups for Ab 22E1 (group 1 & 2) and REA274 (group 1). Correlation in expression for Ab REA274 group 2 was not illustrated as only two alleles were measured resulting in insufficient data for comparison.

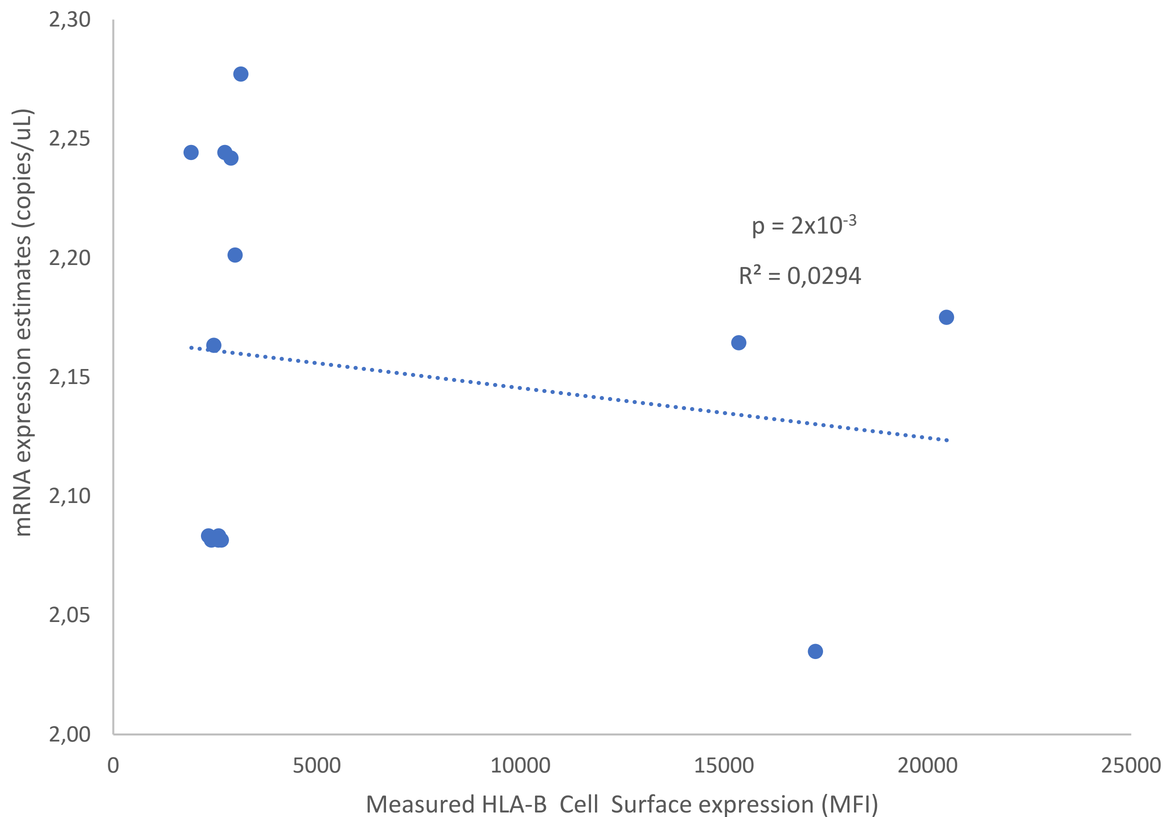

Figure 15: Lack of correlation in mRNA and cell Surface expression levels per donor as selected by monoclonal antibody 22E1 in group 1. The average mRNA expression estimates were tested for a correlation in expression to the measured cell surface expression obtained from previous analysis using flowcytometry data. The mRNA expression estimates are plotted on the *y-axis* and the cell surface expression estimates are plotted on the *x-axis*. Each dot represents the HLA-B expression estimates calculated for each donor participant.

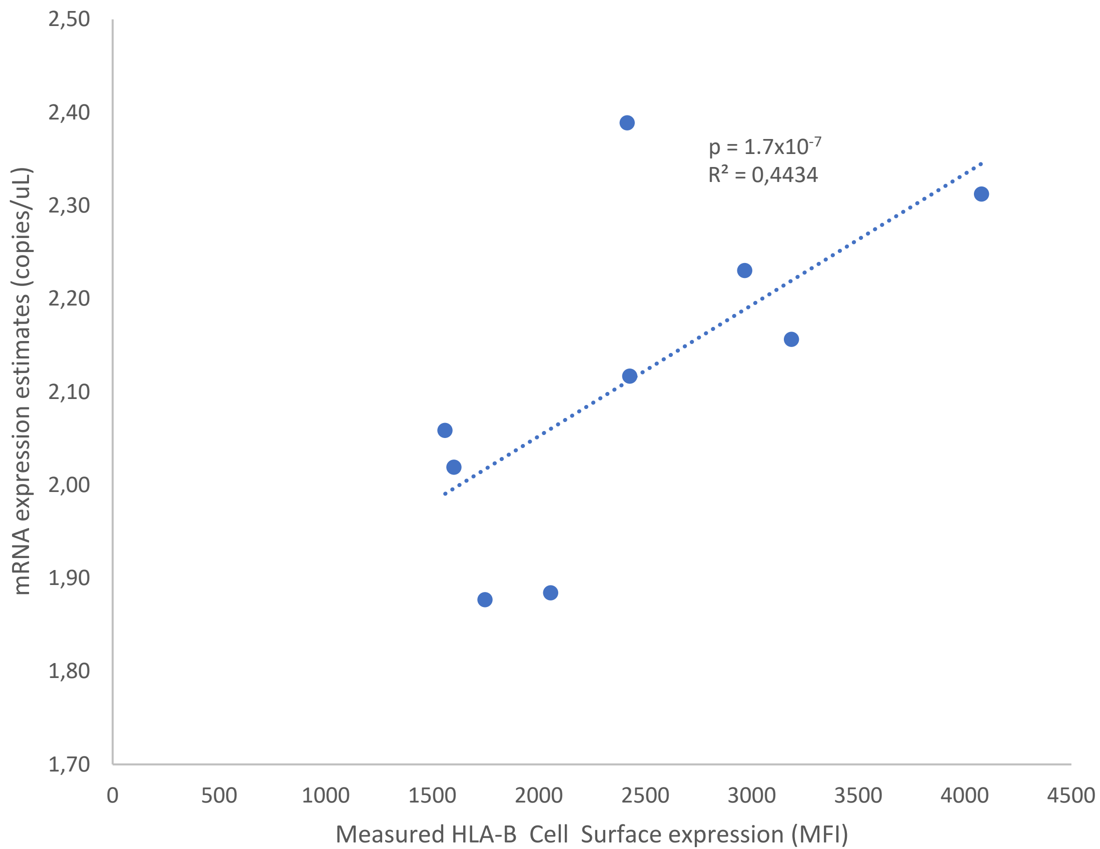

Figure 16: Presence of a Correlation in mRNA and cell Surface expression levels per donor as selected by monoclonal antibody 22E1 in group 2. The correlation observed in this instance was suggestive that expression may be regulated at the cell surface for some alleles, however this correlation was only observed at a donor level. The average mRNA expression estimates were tested for a correlation in expression to the measured cell surface expression obtained from previous analysis using flowcytometry data. The mRNA expression estimates are plotted on the *y-axis* and the cell surface expression estimates are plotted on the *x-axis*. Each dot represents the HLA-B expression estimates calculated for each donor participant.

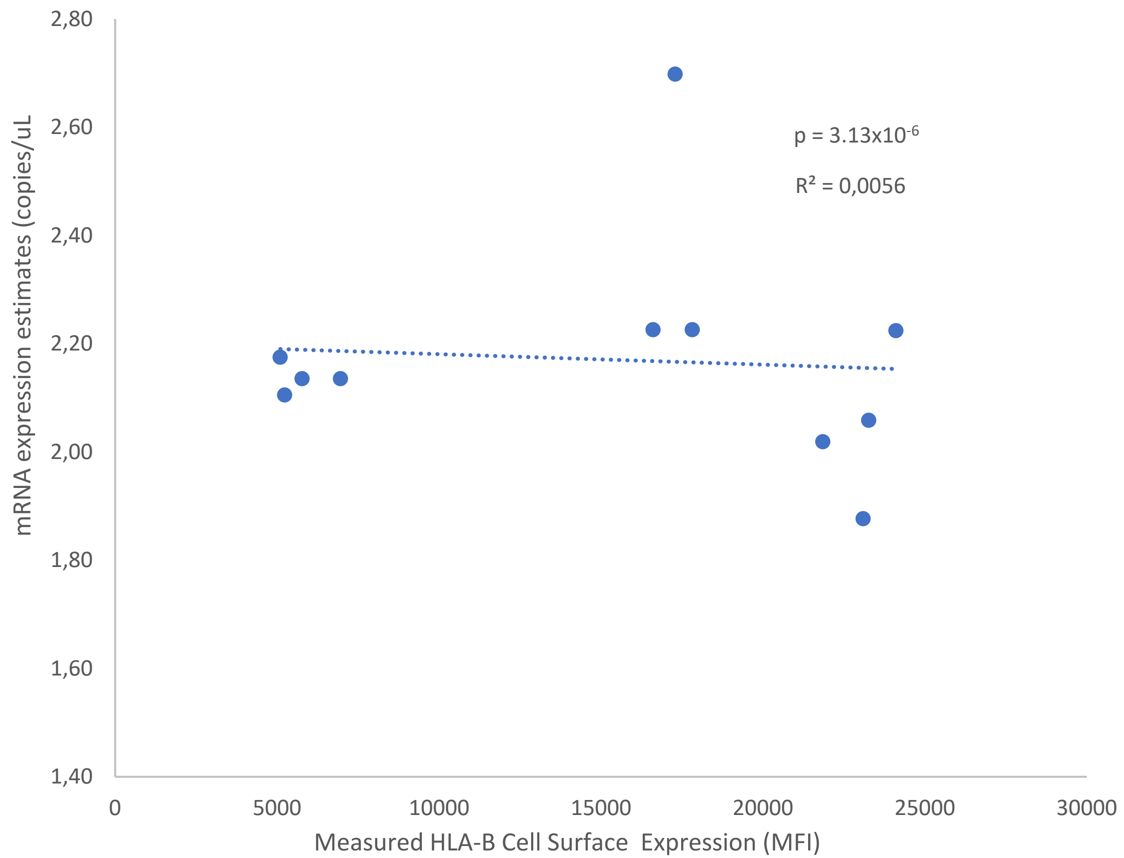

Figure 17: Lack of correlation in mRNA and cell Surface expression levels per donor as selected by monoclonal antibody REA274 in group 1. The average mRNA expression estimates were tested for a correlation in expression to the measured cell surface expression obtained from previous analysis using flowcytometry data. The mRNA expression estimates are plotted on the *y-axis* and the cell surface expression estimates are plotted on the *x-axis*. Each dot represents the HLA-B expression estimates calculated for each donor participant.

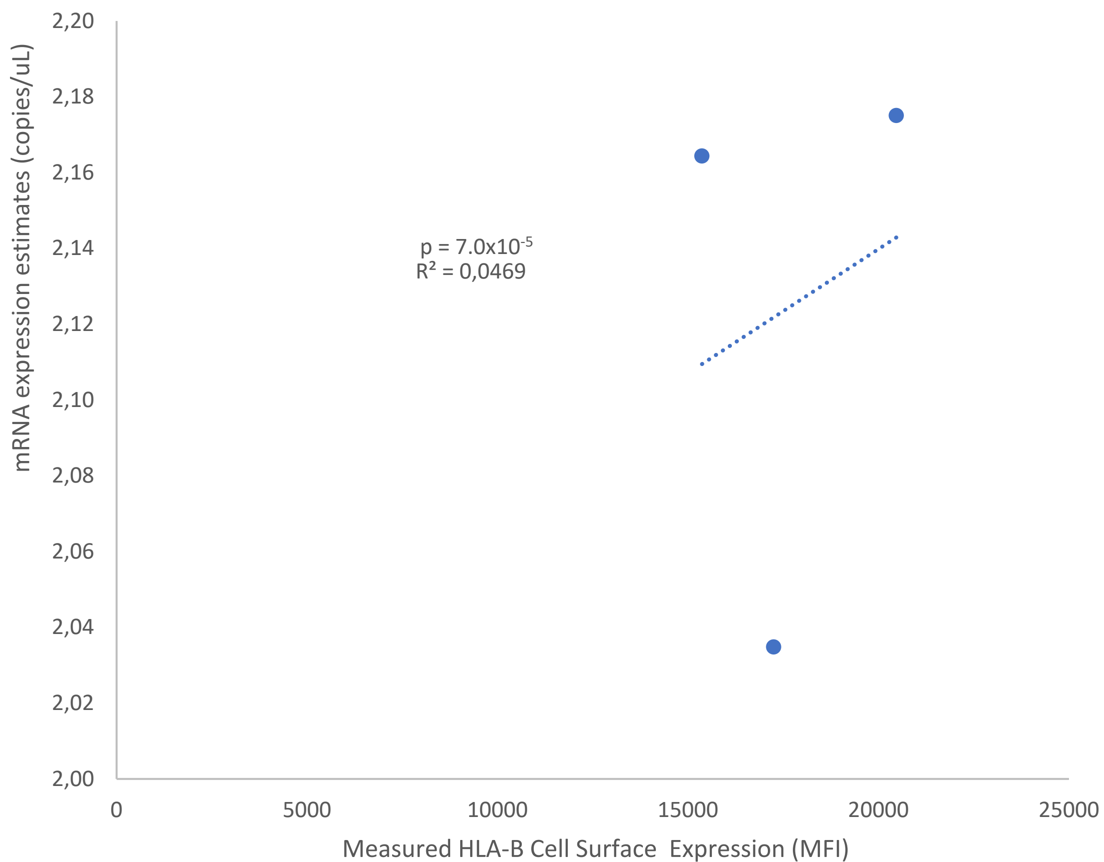

Figure 18: Lack of correlation in mRNA and cell Surface expression levels per donor as selected by monoclonal antibody REA274 in group 2. The average mRNA expression estimates were tested for a correlation in expression to the measured cell surface expression obtained from previous analysis using flowcytometry data. The mRNA expression estimates are plotted on the *y-axis* and the cell surface expression estimates are plotted on the *x-axis*. Each dot represents the HLA-B expression estimates calculated for each donor participant.

Chapter Five: Discussion

5.1 HLA-I diversity and Antibody Selection

The HLA region is the most polymorphic region in the human genome, listing HLA as being diversely associated with a broad range of diseases[5,23,24,26–28,36,49]. Some of the diversity in HLA's arises from their ability to present a wide array of peptides, similarity in nucleotide sequences yet variability in their promoter regions and complexity in mechanistic regulation of expression and variation in expression across alleles. HLA-I alleles have been shown to play a major role in immune mediated control of HIV infection[4], and several studies have associated HLA expression with HIV disease outcomes[14,34,35,42,43]. Elevated expression of HLA-C was found to reduce progression of HIV whereas elevated HLA-A expression resulted in the converse of HLA-C[35,42]. It was also observed that the expression was variable across alleles, with some alleles being expressed at higher levels than others. Several HLA-B alleles, such as HLA-B*57, HLA-B*27, HLA-B*5, have been extensively studied, due to their association with HIV. HLA-B is also the most polymorphic region within the human genome with over 5,500 known alleles, more than HLA-A and HLA-C[61]. However, not much is known about expression across HLA-B alleles or the variability across cell types.

This study bridged the lack in knowledge across HLA-B expression and discovered great insight into the expression of HLA-I at the cell surface and mRNA levels. Literature emphasizes that the production and characterization of monoclonal antibodies against HLA molecules makes it possible to analyse HLA expression in human cells[62]. Critical factors to consider when measuring allelic variations in HLA-I cell surface expression are the: (a) the specificities of the antibodies that are used for the quantification of expression, and (b) the differences in the binding affinities of detecting antibodies towards the HLA-I alleles that are selected for comparison[26].

Prior to measuring the cell surface expression, commercially available Bw4 antibodies, 22E1 and REA274, were required to be screened against a range of HLA alleles to assess affinity, that is, the strength to which an antibody binds to an allele. HLA-C allelic expression was easily measured in a previous study, using DT9 as the antibody of choice[34]. DT9 binds to HLA-C alleles with equal affinity and can therefore measure the expression across all HLA-C alleles for comparison. This was not the case with HLA-B alleles. Using the Luminex-LSA assay, two BW4 antibodies were selected and used in analysis of HLA-B variation in cell surface expression. Donors within a 10% variability in antibody affinity of each other were selected and used to assess the variability in allele specific expression at the cell surface.

5.2 HLA Expression

HLA-B alleles were selected based on antibody affinity and presence of alleles within the sample cohort. Selected alleles were tabulated in tables 2 and 3, respectively. HLA-B allele-specific expression was measured using flowcytometry. A FITC conjugated monoclonal antibody, that is, 22E1 or REA27, was used to quantify the surface expression of selected HLA-B alleles. Expression of alleles in group 1: HLA-B*5701, HLA-B*4901, HLA-B*4402 and HLA-B*5801; and group 2: HLA-B*2705, HLA-B*3701, HLA-B*3801 and HLA-B*5101 are shown in figure 8 and were within a 10% variation in antibody affinity of each other per group. In each group, we observed a variation in expression across HLA-B alleles measured with antibody 22E1. In figure 9, a similar trend across HLA-B alleles measured with antibody REA274 was observed. Expression of alleles in group 1: HLA-B*5101, HLA-B*1302, and HLA-B*4403; and group 2: HLA-B*5701, and HLA-B*5801 are shown in figure 9 and were within a 10% variation in antibody affinity of each other per group. Based on the alleles selected and antibodies used, we can conclude that some antibodies have a greater specificity for some alleles than others and hence antibody selection is a critical factor in quantifying cell surface expression. In contrast to other studies[63,64] and based on visual trends, we have demonstrated that irrespective of antibody affinity

and antibody used, HLA-B*57:01 has an overall higher cell surface expression in comparison to HLA-B*58:01 from an allele specific level. However, this observation can be further examined by increasing the number of alleles to assess hierarchy in allelic specific expression at the cell surface.

Interestingly, the only one other study that assessed HLA-B expression at the mRNA levels, observed a lack in variation across donors and no HLA-B allele-specific mRNA expression variation[23]. The trend observed for the dissimilarity of allelic expression was also supported by a previous study that attributed allele variations due to antigen acquisition pathways having an influence on HLA-B surface expression levels[65]. Other similar studies suggest that cell surface expression levels of HLA-B does vary in a lineage-specific manner to a degree, and this may be attributed to result from lineage-specific translational events[63,64]. The findings in this study further substantiates that other mechanisms drive the expression of HLA-B post transcriptional, as discussed by Yarzabek *et al*, 2018; and that there is no correlation in cell surface and mRNA expression levels[23,65]. Quantifying HLA-I cell surface expression variability is also antibody specific as well as on the cell type measured. As demonstrated in a study that used three selective antibodies (Tu149, B1.23.2, and 22E1), it was found that the expression of HLA-B alleles (in ascending order of expression): HLA-B*35, HLA-B*07, HLA-B*27, and HLA-B*57 were inversely correlated with the promiscuity of peptide binding[63]. However, in another study that examined the surface expression of HLA-B*57 and HLA-B27 using a different antibody, clone 0007, it was found that HLA-B*27 had higher expression than HLA-B*57[64]. Ramsuran *et al.*, 2017 attributed these differences in measurement to the use of different cell types in the respective studies, therefore supporting that some cell types express HLA-I at different expression levels[23,63,64]. Dependent on the HLA type, it can be observed that higher expressed alleles tend to have lower peptide promiscuity and lower expressed alleles have higher peptide promiscuity, and to some extent, HLA-B expression varies in a lineage specific manner[23,63]. However, the data that we have obtained during antibody affinity analysis, illustrated that some antibodies have a better

affinity for specific alleles and therefore antibody selection is a critical factor that must be considered when measuring HLA cell surface expression. It is also critical to perform a titration of the antibody selected for use in order to establish the correct concentration and quantity of antibody that is acceptable for use when measuring cell surface expression using the LSA assay. Previous studies that have measured expression of HLA-I without establishing an effective antibody concentration and based on the previously reported data, the expression measured for specific alleles using the selected antibody was not comparable to other reported data and therefore not accurately reproducible[63,66,67]. It is proposed to further explore a larger selection of alleles using a single antibody and group, as well as across different ethnicities, to establish a possible hierarchy in HLA-B allelic cell surface expression.

The mRNA expression across donors for each HLA-I type: *HLA-A, HLA-B, HLA-C* and *HLA-E* were found to be variable across types. The seven donors selected showed consistency in variability across donors as well as across HLA types. Using ddPCR, samples were partitioned in to ~20,000 droplets and amplified using highly specific primers used in a previous study[23]. Concentration of positive target sequences were reported in copies per microliter for all ddPCR analysis graphs. Data reported in this study observed a fluctuation in mRNA expression across the donors. Overall, we observed a higher concentration of *HLA-B* across the donors in comparison to the other HLA-I types. This trend in expression was also detected in the average mRNA expression reported for HLA-I types, which showed that *HLA-B* had a greater expression across class-I HLA genes, followed by *HLA-A*, *HLA-C* and *HLA-E* in descending order. This trend in mRNA expression follows that reported by Apps *et al*., 2013 for cell surface expression for HLA-I types[42]. However, it has also been reported that there is no correlation in mRNA and cell surface expression as observed with HLA-C[34,42,43].

Ramsuran *et al*., 2017 reported for the first time HLA-B allelic mRNA expression using healthy Caucasian PBMC samples[23]. Due to the large difference in the cell subtype population

comprising PBMC's, variation in expression observed in the lower population cell subtypes will not be detected. We therefore attempted to repeat that experiment across cell types to determine if HLA-I expression varies across differential cell types. Using FACS, a technique based on the principle of flowcytometry, we sorted the PBMC samples into T-cells, B-cells, Monocytes and Natural Killer Cells. Samples were prepared and analysed as previously described. The mRNA expression was graphically illustrated in figure 12. Expression was plotted for PBMC samples, T-Cells, B-cells, Monocytes and NK cells for each of the class-I types: *HLA-A*, *HLA-B, HLA-C* and *HLA-E*. As previously observed, expression levels measured using PBMC's shows a lack in variability across HLA-I types[23], however, across cell types, a distinct variation can be observed. Across the HLA-I types, a trend highlighting increased mRNA expression in B-cells, followed by monocytes, and similar expression observed in T-cells and NK cells. Interestingly, it was also observed that HLA-B mRNA expression is predominately higher than the other HLA-I types. This trend in HLA-I expression was also supported by figures 10 and 11, as previously mentioned.

It is known that HIV protein, Nef, downregulates HLA-A and B at the cell surface, however with different efficiencies[14,50,51]. However, it is unknown if this downregulation, if any, is also observed at the mRNA level. We attempted to further investigate the possible downregulation on pre- and post- HIV infected cells for the same donors. Two donors were identified from the CAPRISA 002 cohort and samples were obtained before and after HIV infection. mRNA expression of *HLA-A*, *HLA-B, HLA-C* and *HLA-E* was measured for each donor at the two time points. Figure 13 graphically illustrates the mRNA expression measured pre- and post- HIV infection. Post infection expression demonstrates a slight reduction in expression in comparison to pre- infection expression of *HLA-A, HLA-B, HLA-C* and *HLA-E*. Nevertheless, the reduction was insignificant and therefore, it can be assumed that in contrast to cell surface expression, the HIV protein, Nef, does not affect HLA-I mRNA expression levels.

5.3 Comparison of Expression in HLA-I

It was previously reported that there is no correlation in mRNA and cell surface expression however based on the similarity of trends observed in the hierarchy of HLA-I expression as observed in figure 11, and reports by Apps et al., 2015[14,65], we attempted to explore a possible correlation of mRNA and cell surface expression across donors as well as across alleles. Based on the average expression estimates obtained for specific alleles (figure 14), there was a lack of correlation in mRNA and cell Surface expression levels at the allelic level for each group of selected alleles. Alleles were grouped based on antibody affinity values as determined in 4.1. We further attempted to identify a correlation in expression at a donor level for each group of alleles selected per antibody. In figures 15, 17 and 18, no significant correlation in expression was observed at the donor level. Interestingly, a correlation in mRNA and cell surface expression level was observed in donors selected by monoclonal antibody 22E1 in group 2 (figure 16). The correlation observed in this instance was suggestive that expression may be differentially regulated at the cell surface for some alleles, however this correlation was only observed at a donor level and not allelic level and should therefore be analysed with careful consideration. It is possible, if explored with a larger selection of alleles using a single antibody and group, as well as across different ethnicities, that a stronger correlation may also be established for specific alleles at a donor level. This is an area that may provide some insight as to the variation across donors.

Chapter Six: Conclusion

Human Leukocyte Antigens are specialised molecules that directly links the hosts immune system with the internal environment within every nucleated cell. Aside from such linkage, these molecules play a pivotal role in diseases and up until recently have been associated with disease outcomes. Several HLA-B alleles have been extensively studied and associations have led to the discovery of protective and unfavourable alleles. Published literature has to date, reported the outcome of HIV disease in association with expression of HLA-A and HLA-C. Although several genome wide association studies have reported HLA-B to have the strongest disease associations, limited information exists for HLA-B expression. With HLA-B being the most polymorphic region within the human genome, we decided to fill some gaps in knowledge surrounding HLA-B expression. In this study, we have determined that HLA-B expression is variable across alleles at the cell surface, however antibody and allele selection prior to measuring cell surface expression are critical aspects that require a thorough understanding and attention to detail. As previously described, *HLA-B* expression at the mRNA level was found to be stable across alleles in comparison *HLA-A* and HLA-C. Since the study was conducted using PBMC's to quantify variability in expression across alleles, we were posed with the theory that expression varied across cell types. Interesting, across HLA-I types, we observed that B-cells have an overall higher expression across cell types and HLA-B had an overall higher expression across HLA-I types. We also found that the expression of HLA-I types was not downregulated at the mRNA level as observed on the cell surface level in the presence of HIV. Based on analysis of expression data, we observed that there was no correlation in mRNA and cell surface expression. In contrast, we also observed that some alleles may be differently regulated at the cell surface, however this correlation was found at the donor level and not across the alleles. Notable limitations of the study were that not all HLA-B alleles were assessed for variability in expression, and greater power can be achieved if the comparison was performed using multiple cohorts of various ethnicity.

Analysis of HLA-B expression level is likely to be important not only in HIV infection, but also other infectious diseases and immune-related disorders in which HLA-B is implicated. HLA expression levels adds another dimension to the extreme diversity of HLA-I molecules that accounts for variability in immune responses across donors and may suggest possible drug targets to modulate host responses to infections. Further insight into the factors that may be involved in the regulation of *HLA-B* at the genomic level could provide valuable information in understanding possible mechanisms that exist for HLA-B.

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Affiliations

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Appendices

Appendix A – BREC Certificates

04 October 2017

Dear Ms U Ramphal (200303380)
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Dear Ms Ramphal

Protocol: Measuring HLA-B allele expression levels on differential cell types
Degree: MMedSc
BREC Ref No: BE539/17

EXPEDITED APPROVAL

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 17 August 2017.

The conditions have now been met and the study is given full ethics approval and may begin as from 04 October 2017.

This approval is valid for one year from 04 October 2017. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be RATIFIED by a full Committee at its next meeting taking place on 14 November 2017.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator
cc supervisor: Dr V Ramsuran

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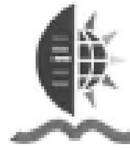

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20 November 2018

Dear Ms U Ramphal (200303380)
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Dear Ms Ramphal

Protocol: Measuring HLA-B allele expression levels on differential cell types
Degree: MMedSc
BREC Ref No: BE539/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 04 September 2018
Expiration of Ethical Approval: 03 September 2019

I wish to advise you that your application for Recertification received on 14 November 2018 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 December 2018.

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator
cc supervisor: Dr V Ramsuran