Use of SAB in cross-match prediction

EQUIVALENECE OF ANTIBODY BINDING TO HLA ON BEADS AND CELLS: CRITICAL TESTS IN TRANSPLANATION

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ABSTRACT

AMR as a cause of graft rejection has been long recognized and the presence of pre formed antibodies against donor HLA is a risk factor for increased graft rejection. FlowXM is the current clinical gold standard for detecting harmful DSA in the recipients and a positive FlowXM is considered a strong contraindication to transplantation. However, newer techniques such as SAB provide with a highly sensitive and specific method for DSA detection that is unattainable by FlowXM. But due to the intrinsic limitations associated with SAB assays, the clinical relevance of DSA detected on SAB has been highly disputable. Therefore, the overall aim of this study was to investigate the utility of SAB in predicting harmful DSA levels, by establishing a fluorescence range on SAB that correlated to positive FlowXM. This was done by retrospectively testing the highest serum dilutions on FlowPRA SAB that produced positive B or T cell FlowXM from 15 variably sensitized patients. Thus, a very narrow MFI range on SAB was established, for B and T cells separately, that correlated to positive FlowXM. On B cells this correlate ranged from 2780-7772 MFI (Mean MFI =5641), whereas T cell range was 1089-6731 (Mean MFI= 3226). In order to test these ranges for prediction of positive FlowXM, B and T cell FlowXM tests were carried out using various serum/cell combinations. DSA MFI of >3000 on SAB resulted in a significantly higher T cell positive FlowXM ($p<0.05$); however, similar significant cutoff could not be achieved for B cells. DSA below FlowXM positive levels were also measured by carrying out serial dilutions on serum samples. A standard curve was obtained so that sub-clinical DSA levels could be measured in relation to positive FlowXM. Thus, this study provides with novel means of 1) establishing a DSA cutoff on SAB that predicts positive XM, 2) measuring DSA below XM positive levels. This could be beneficial in performing ‘quantitative virtual XM’ to preclude high risk transplants pre-transplant and monitoring patients post-transplant.

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ABBREVIATIONS USED

1x- 1:1 dilution

10x- 10:1 dilution

ab. - antibodies

ADCC- antibody dependent cell cytotoxicity

AHG- anti-human globulin

AMR- antibody mediated rejection

AT1R - angiotensin 1 receptor

APCs- antigen presenting cells

C1q- complement component 1q

c4d- proteolytic by-product of activated complement component 4b

CD3- cluster of differentiation 3 (T cell marker)

CD19 - cluster of differentiation 19 (B cell marker)

CDCXM- complement dependent cytotoxicity crossmatch

CIHI- Canadian Institute of Health Information

CKD- chronic kidney disease

DSA- donor specific antibodies

ESRD- end-stage renal disease

FlowXM- flow cytometry crossmatch

Fc region- fragment crystallizable region

FITC- fluorescein isothiocyanate

FSC- forward scatter

HLA- human leucocyte antigens

HRLMP- Hamilton Region Lab Medicine Program

Ig- immunoglobulin

IMDM-10 - Iscove's Modified Dulbecco's Medium-10

MBCs- memory B cells

MESF- molecules of equivalent soluble fluorophores

MFI- median fluorescence intensity

MHC- major Histocompatibility complex

MICA- MHC Class- I-polypeptide-related sequence A

MiHA- minor Histocompatibility antigens

ml- milliliters

NK cells- natural killer cells

NPV- negative predictive value

PPV- positive predictive value

PBS- phosphate buffered saline

PBMC- peripheral blood mononuclear cells

PCs- plasma cells

PE- phycoerythrin

PRA- panel reactive antibodies

REB- Research Ethics Board

rpm- revolutions per minute

RPMI- Roswell Park Memorial Institute medium

SAB- single antigen beads

S.D.- standard deviation

SOP- standard operating procedures

SSC- side scatter

TCAD- transplant related coronary artery disease

ul- microliter

XM- crossmatch/cross-match

CHAPTER 1- INTRODUCTION

1.1 Overall Introduction

Chronic kidney disease (CKD) includes a wide array of medical conditions that affect kidney structure and function (Levey & Coresh 2012**)**. It is becoming one of the major public health crises in Canada as increasing numbers of Canadians are being diagnosed with CKD every year. According to one statistic published by The Kidney foundation of Canada, every day an average of fifteen new people are diagnosed with kidney failure; and the rate of Canadians being treated for CKD has tripled over the last 20 years ("Facing the Facts" 2013). This is in part due to high prevalence of diabetes and high blood pressure, two major risk factors associated with CKD worldwide (Haroun et. Al. 2003). Patients suffering from CKD are also at increased risk for progression to end-stage renal disease (ESRD), an end result of prolonged insult to kidneys in CKD patients. As a result, nearly complete loss of kidney function occurs in ESRD and the body loses its ability to maintain metabolic and electrolyte balance causing conditions such as uremia or azotemia (Meyer & Hostetter 2007). Thus, renal replacement therapy in the form of dialysis or kidney transplantation is required in order to prolong patient survival.

Kidney transplantation is a favorable option for patients suffering from ESRD and the only treatment shown to improve survival by providing maximum replacement of kidney function (Pesavanto 2009). In addition to significantly improving patient's quality of life, it is also an economically feasible option over long term dialysis. As a result, increased numbers of patient as well as physician are opting for kidney transplantation over dialysis as a preferred mode of treatment when possible. However, the number of

kidneys needed for transplantation surpasses there availability. In addition, not all kidney transplantations performed are successful over long period. Even though improved immunosuppressive protocols, detailed genotyping and better methods to detect donor specific antibodies (DSA) have successfully improved short term graft survival, attaining a superior long term graft function still remains a challenge (Haiyan & Xiaozhou 2011). This is mainly due to host immunologic reaction against the graft which could occur in the form of acute or chronic rejection episodes; this negatively affects the graft function and graft survival post-transplant. These rejection episodes could be the result of an antibody mediated rejection (AMR) due to DSA reacting against the graft, or due to host T cells causing a cellular rejection. As a result, patients with failed grafts end up back on dialysis or on the waiting list for new kidneys. Thus, it becomes important that transplant patients are monitored regularly post-transplant to detect for early development of DSA. Early detection of DSA can provide opportunity to intervene clinically before significant damage to the graft occurs due to AMR. This will help save patient lives lost from complications arising due to graft failure as well as help save valuable grafts by prolonging graft function in the transplanted patients.

Therefore, the main objective of this study was to assess the use of single antigen beads (SAB) in detecting low levels of DSA and its implications in monitoring patients post-transplant. Since SAB are very sensitive at detecting DSA (Gebel & Bray 2010), these assays were used to measure the lowest levels of DSA that were harmful to the graft; and a correlate was established on SAB that represented these harmful DSA levels. In addition, standard curve for DSA detection below the harmful levels were developed

on SAB. This could allow comparison of patient DSA to the level required for graft damage or AMR. Thus, patients that are at increased risk of graft failure due to presence of harmful levels of DSA could be readily identified post-transplant so that appropriate immunologic intervention could be used. Furthermore, using SAB to measure DSA pre-transplant could be helpful in performing “virtual crossmatch” in order to evaluate the compatibility of a donor-recipient pair, allowing better allocation of organs.

1.2 Antibody mediated rejection- a major barrier in transplantation

AMR remains one of the major barriers to successful solid organ transplantation and prolonging graft survival (Stegall & Gloor 2010). AMR is defined as graft rejection occurring due to the action of antibodies against mismatched donor human leukocyte antigens (HLA), ABO blood group antigens or antigens on the surface of endothelial cells (Lucas et. Al. 2011). AMR as one of the major barriers to successful solid organ transplantation was first shown by Patel and Teraski in 1969. They showed that presence of cytotoxic alloantibodies in the patient serum was a major risk factor for immediate graft loss in kidney transplant recipients. Immediate graft loss occurred in patients who had higher risk of having alloantibodies such as multiparous women or patients receiving secondary transplants (Patel & Terasaki 1969). Since then numerous studies have confirmed these findings in kidney recipients (Kerman et. Al. 1997, O'Rourke et. Al. 2000, Lefaucheur et. Al. 2008, Amico et. Al. 2009, Gloor et. Al. 2010). These studies looked at the level of antibodies against donor HLA antigens in the recipient serum as a predictor of graft survival post-transplant, with higher serum DSA associated to poor graft outcomes. More direct evidence on the role of alloantibodies in graft rejection comes from studies using various mouse models. In a severe combined immunodeficiency (SCID) mouse model of cardiac allograft, it was shown that SCID mice remained rejection free post-transplant whereas injection of anti-donor antibodies in SCID mice post-transplant resulted in lesions consistent with graft rejection (Russell et. Al. 1994). As a result, it is now a well-recognized fact that the presence of DSA is a

major risk factor for graft rejection and graft loss in transplant recipients can occur due to the action of DSA.

In addition, different types of graft rejection can occur due to presence of antibodies against donor antigens. These include hyperacute rejection, acute rejection and chronic rejection. These rejection episodes differ in their severity and the period of onset following transplantation. Hyperacute rejection manifests within minutes to a few days of graft implantation and occurs due to the presence of pre-formed high titre DSA in the donor. The histopathologic features of this type of rejection include severe endothelial injury, arteritis, interstitial edema, and severe cortical necrosis (Puttarajappa et. Al. 2012). As a result of severe damage arising to the graft, this type of graft rejection almost always requires graft nephrectomy following transplantation. Whereas this type of rejection has mainly been eliminated due to advancement in various methods to detect pre-formed DSA, acute rejection and chronic rejection still remain an issue. Acute rejection is classified as graft dysfunction occurring over a period of few days to few weeks. This type of rejection is typically diagnosed by the presence of C4d deposition in peritubular or glomerular capillary endothelium and presents pathologic features similar to hyperacute rejection (Colvin & Smith 2005). Antibodies can also cause chronic graft rejection which can occur years after transplantation in many cases. This could be due to the action of de-novo antibodies formed against the graft or up regulation of DSA due to immunologic memory. Various studies have shown presence of circulating antibodies in the patients prior to occurrence of chronic graft rejection (Lee et. Al. 2002, Worthington et. Al. 2003). It is hypothesized that antibody mediated injury to the graft might be slow

during chronic rejection, which might take months or years before any pathologic features appear (Colvin & Smith 2005, Terasaki 2003).

1.3 Human Leucocyte antigens (HLA) - the main target for DSA reaction.

Graft rejection is mainly attributed to DSA reacting against the mismatched antigens of Major histocompatibility complex (MHC) present on the surfaces of donor cells. In humans, MHC antigens are divided in to two main classes: HLA Class 1 (HLA-A, B and C) and HLA Class II antigens (HLA-DR, DQ, DP, DM and DO). HLA Class 1 antigens are found on all nucleated cells, whereas HLA Class II antigens are primarily found on antigen presenting cells (APCs) as well as endothelial cells and renal tubular epithelial cells (Haiyan & Xiaozhou 2011). The main role of MHC Class I and Class II is presentation of antigens from intracellular and extracellular pathogens to the immune cells. This allows an appropriate immune response against the invading pathogen to be mounted. The presence of HLA molecules on cell surfaces also allows the immune system to distinguish self from non-self when a foreign pathogen invades. Thus, a high degree of MHC polymorphism exists in human population (Figure 1.1). This allows unique combinations of alleles to be expressed on the cell surfaces. Even though this is evolutionarily advantageous (Jin & Wang 2003), this can cause potential problems during solid organ transplantation as it is nearly impossible to find an exact HLA match between the donor and the recipient, other than monozygotic twins or some dizygotic twins. Thus, if there is a HLA mismatch between the graft and the transplanted recipient, it could cause the host immune system to mount an immune attack against the graft which could potentially result in graft rejection.

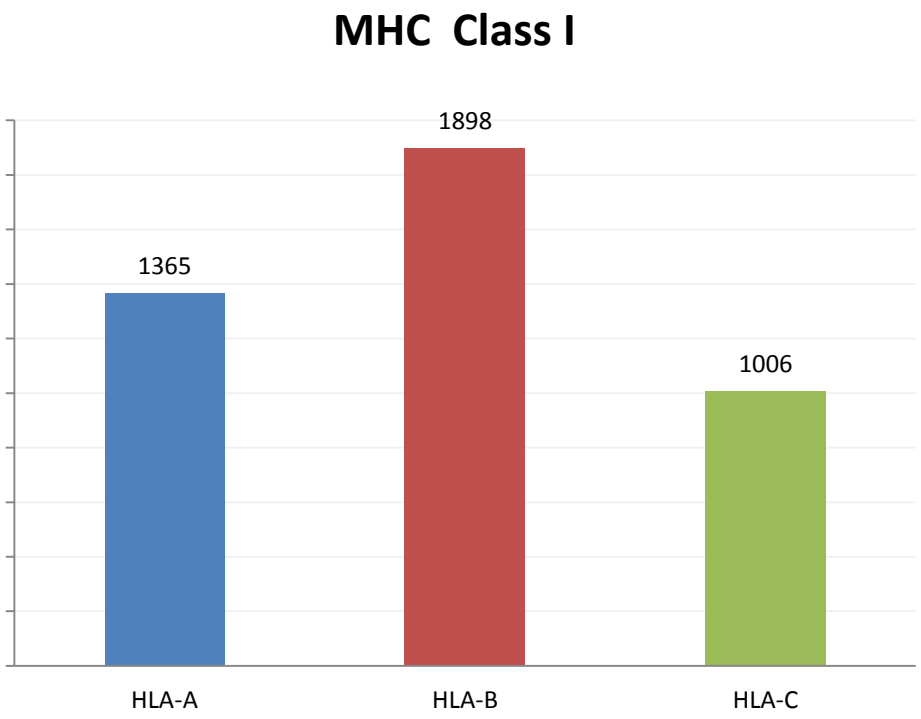
In addition, preformed antibodies against HLA Class I and Class II antigens can also be found in patients that have been previously sensitized to HLA. Studies show that approximately 30% of the patients awaiting transplant are sensitized to HLA antigens (Lucas et. Al. 2011). Patients can be sensitized when they are exposed to foreign HLA during blood transfusions, previous transplants or during pregnancies in women (Colvin & Smith 2005). Once introduced in to the host, these HLA antigens can be presented to the host immune cells in secondary lymphoid tissues via three main pathways. Indirect pathway results in HLA sensitization when the host antigen presenting cells (APCs) take up donor antigens (including peptides from donor MHC molecules) and present these in context of self MHC molecules to T cells. Second pathway known as the direct pathway involves the donor APCs. Donor APCs are also capable of presenting intact donor MHC molecules present on their surface when these cells migrate from the graft into the secondary lymphoid tissues of the host. The "novel" third pathway involves host APCs taking up membrane fragments from the donor cells and presenting intact donor MHC molecules from these membrane fragments (Herrera et. Al. 2004). This presentation of donor HLA antigens to the host immune cells causes activation of host B and T cells against HLA in the secondary lymphoid tissues. As a result, alloreactive memory B cells (MBCs) and Plasma cells (PCs) are formed from naïve B cells through antibody production pathways. PCs are responsible for producing and secreting high levels of anti-HLA antibody which can be detected in the sera of sensitized patients. On the other hand, MBCs are responsible for long term immunologic memory against HLA antigens. It is hypothesized that post-transplant when sensitized individuals undergo secondary

exposure to HLA antigens, it reactivates alloreactive MBCs and causes them to differentiate into antibody secreting PCs. Thus, preexisting sensitization can also result in production of high levels of anti-HLA antibodies post-transplant.

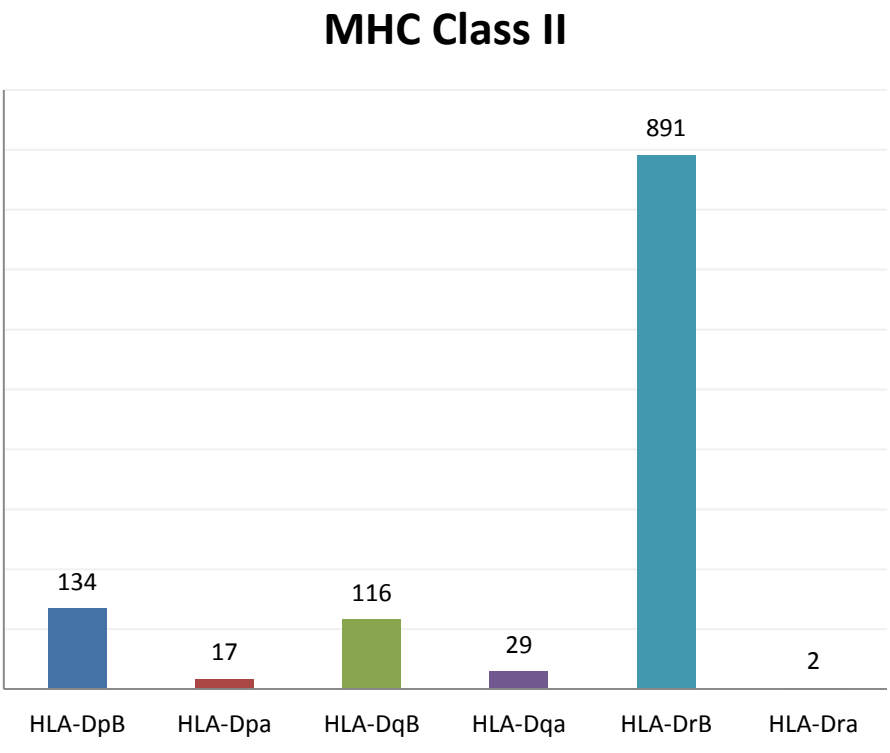
In addition to antigens of MHC as primary targets for antibody reaction, antibodies against minor histocompatibility antigens (MiHA) can also be involved in graft damage and AMR. MHC Class- I-polypeptide-related sequence A (MICA) expressed on endothelial cell surface has been shown as a potential target for antibody reaction in transplant patients (Zou et. Al. 2007, Terasaki et. Al. 2007). Studies have also shown presence of non-HLA antibodies against angiotensin 1 receptor (AT1R-Ab) in patients with vascular rejection episodes (Dragun et. Al. 2005). Thus, in some cases a mismatch in minor histocompatibility antigens can also cause problems during organ transplantation.

Figure 1.1: Number of different HLA alleles identified in human population a) Class 1 HLA. b) Class II HLA. (Figure adapted from Laperrousaz et. al. 2012)

a)


MHC Class I
1898
1365
1006
HLA-A
HLA-B
HLA-C

b)


MHC Class II
891
134
116
17
29
2
HLA-DpB
HLA-Dpa
HLA-DqB
HLA-Dqa
HLA-DrB
HLA-Dra

1.4 Humoral theory of graft rejection and different types of antibodies involved

The presence of anti-HLA antibodies pre transplant as well as post-transplant are a major risk factor for graft rejection. According to the humoral theory of transplantation, AMR occurs when recipient DSA bind to donor antigens present on the graft surfaces and cause endothelium damage via complement dependent and complement independent manner (Terasaki 2003). Complement dependent damage involves activation of the classical complement pathway when antibodies react against antigens present on endothelial cells and form antigen-antibody complexes. This activation of complement pathways results in the formation of membrane attack complexes which causes cellular injury and vascular damage to the graft. Various by-products of complement activation such as C3a and C5a fragments can further amplify the immune response targeted at the injury site. Following this initial damage, a series of inflammatory and pathological changes in the endothelium occur (Cai & Terasaki 2005), which eventually results in graft rejection. Anti-HLA antibodies can also cause graft damage via complement independent mechanism such as antibody dependent cell cytotoxicity (ADCC). ADCC involves activation of macrophages and cytotoxic cells such as Natural Killer cells (NK cells) when Fc receptors on these cells bind to Fc region of the antibody (Puttarajappa et. Al. 2012). This activation of innate immune cells cause lysis of the antibody bound cells, which also results in vascular injury and graft damage. However, not all antibody isotypes directed against HLA antigens might be involved in AMR. AMR has been primarily attributed to the presence of anti HLA IgG antibodies. Furthermore, different subtypes of IgG exist (IgG1, IgG2,

IgG3 and IgG4), of which only IgG1 and IgG3 are known to be complement fixing. In accordance, studies have shown successful kidney transplantation in patients with highly reactive DSA of non-complement binding IgG2 and IgG4 subtypes (Lobashevsky et. Al. 2010, Honger et. Al. 2011). These studies showed that recipients with IgG2 and IgG4 anti HLA class II DSA had similar graft survival times as recipients without DSA, indicating that only subtypes IgG1 and IgG3 might be involved in AMR.

The role of IgM antibodies in AMR is not as clear. Even though IgM is able to fix complement, IgM positive crossmatch (XM) is often not seen as a contraindication to transplant as IgM antibodies detected are mostly harmless autoantibodies. In addition, studies have shown that presence of IgM DSA do not pose any risk of hyper acute rejection post-transplant (McCalmon et. Al. 1997); in fact presence of IgM might be beneficial to the graft survival in some cases (Kerman et. Al. 1999, Melero et. Al. 1997). Contrary, a recent study by Stastny et. Al. showed that preformed donor specific IgM antibodies predicted AMR in kidney recipients and transplant related coronary artery disease (TCAD) in heart transplants (2009). Furthermore, preformed IgA antibodies in transplant recipients have also been shown to induce a protective effect against graft damage (Koka et. Al. 1993, Lim et. Al. 1993).

1.5 Different methods for detecting anti-HLA antibodies.

Given the imperative role of different types of anti-HLA antibodies in mediating AMR, it is crucial that transplant recipients are tested for the presence of preformed DSA. It is also important that these antibodies are detected with precise specificity in order to check compatibility between a donor-recipient pair. The significance of testing for preformed anti-HLA antibodies in kidney recipients was first shown by Patel and Terasaki in 1969. Their findings showed that the recipients with a positive complement dependent cytotoxicity cross-match (CDCXM) were at a significant higher risk of having a graft rejection post-transplant (Patel & Terasaki 1969). As a result, CDCXM became a gold standard for testing for anti-HLA antibody prior to a transplant. However, since then more sensitive and specific techniques have emerged in the field of cross-match testing. These techniques allow transplant physicians to better evaluate immunologic risks pre-transplant as well as post-transplant so that successful organ transplantation can be carried out.

1.5.1 Complement dependent cytotoxicity cross-match

First pioneered by Patel and Terasaki in 1969, CDCXM has been widely used to detect donor reactive alloantibodies in patient serum. CDCXM is performed by incubating patient serum with donor lymphocytes, B cells and T cells separately, followed by addition of complement factors. If donor reactive alloantibodies are present in the patient serum, these serum antibodies bind to antigens present on donor lymphocytes. This can cause activation of complement system via classical pathway resulting in donor cell lysis,

if DSA are present in sufficient titre. The results are interpreted as positive or negative based on the percentage of dead vs. live cells. Degree of patient sensitization and incompatibility is assessed based on CDCXM tests performed on a panel of lymphocytes from normal donors. Thus, the percentage of donors incompatible for a particular sensitized patient can be known, with highly sensitized patients having higher panel reactive antibodies (PRA).

However, this is the least sensitive method for detecting DSA in the patient serum. This is because activation of complement is dependent on the amount of antibodies bound to donor cell antigens. Thus, low amounts of DSA that are not sufficient to activate complement but might otherwise be harmful to the graft cannot be detected by CDCXM. As a result, many patients that are CDCXM negative can have graft rejection episodes post-transplant. In order to make CDCXM more sensitive to the presence of DSA, addition of antihuman globulin (AHG) antibody along with complement factors has been proposed. This causes better crosslinking of DSA that are bound to donor cells as well as improved activation of C1q complex (Tan et. Al. 2007). Thus, sensitivity of the CDCXM can be improved using AHG. Nevertheless, this method of XM testing is still limited in its ability to identify DSA specificities as it is not possible to identify HLA antigens that are targeted by these antibodies. Furthermore, CDCXM does not distinguish between different isotypes of anti-HLA antibodies present in the patient serum. Since IgM antibodies are also capable of fixing complement, it can cause false positive CDCXM; and thus preclude otherwise compatible donor-recipient pairs.

1.5.2 Flow cytometric cross match (FlowXM)

FlowXM, a complement independent technique, is a much more sensitive method of DSA detection than CDXXM or AHG enhanced CDCXM (Bray et. Al. 2004). This method of DSA detection uses fluorescin labeled secondary antibodies that are detected by a Flow cytometer on an individual cell basis. In order to carry out FlowXM, donor lymphocytes are incubated in patient serum for a specific period of time. This allows various anti-HLA antibodies to bind against donor antigens expressed on the cell surfaces. These cell bound alloantibodies are detected by a fluorochrome labeled anti-IgG or anti-IgM antibody, which produces a signal on Flow cytometer when XM samples are analyzed on the instrument. B cells and T cells are also distinguished based on a fluorochrome labeled anti-CD19 or anti-CD3 antibodies. Thus, depending on the amount of antibodies bound to the cell surfaces, varying intensity of signal on B and T cells is produced on the Flow cytometer. Thus, using FlowXM it is possible to detect very low levels of DSA that might not be sufficient to activate complement (Cecka 2011).

This method of DSA detection is currently the gold standard for testing potential kidney recipients for compatible donors in many transplant clinics around the world including Hamilton Region Lab Medicine Program (HRLMP) Histocompatibility lab. FlowXM testing is advantageous as it incorporates the cell based functional aspect of CDCXM without dependency on the activation of complement pathway as an antibody indicator. In addition, this method of XM testing is far more sensitive at detecting serum anti-HLA antibodies that might not be detected by cell based methods such as CDCXM

(Gebel & Bray 2000). Many studies have also shown a positive FlowXM test to be a better predictor of AMR post-transplant (O'Rourke et. Al. 2000, Ilham et. Al. 2008, Karpinsky et. Al. 2001). As a result, a positive FlowXM test pre-transplant is considered as a strong contraindication to transplant. However, one of the major limitations of this technique is its lack of specificity in identify HLA antibodies. FlowXM is an indirect measure of anti-HLA antibodies present in the recipient serum as this method detects all antibodies bound to the donor cells. This might also include auto-antibodies (Ting et. Al. 1977) or other clinically irrelevant DSA (Bray et. Al. 2004). This makes it harder to assign antibody specificity to a particular antigen in highly sensitized patients due to the masking effect of multiple antigens present on the donor cells. Therefore, its major limitation lies in its inability to specifically detect clinically relevant DSA, which might lead to false positives in many cases. In addition, serum factors might also impede with DSA binding to the cell surfaces. This can also result in weak positive or false negative FlowXM test.

1.5.3 SAB assays

SAB are polystyrene beads that contain purified Class I and Class II HLA antigens bound to their surfaces in very high density. HLA present on these microspheres are produced using recombinant technology and bound to their surface via hydrophobic interactions (Pei et. Al. 2003). Thus, when SAB are incubated in patient sera containing anti-HLA antibodies, these antibodies react and bind to HLA antigens present on the bead surfaces.

The bound anti-HLA antibodies are then detected using a fluorescin labeled anti-IgG secondary antibody which can produce varying amount of signal depending on the amount of serum anti-HLA antibodies binding to the beads. Thus, in addition to detecting for the presence of anti-HLA antibodies SAB also allow relative quantification of various anti-HLA antibody levels present in the patient depending on the fluorescence intensity produced by various beads. This is important as it has been shown that in 'desensitized' patients evaluation of DSA strength might be a better predictor of post-transplant outcomes then crossmatch test (Mujtaba et. Al. 2011). In addition, since SAB contain purified HLA antigens, only those antibodies that are specific to the HLA antigen on the bead will bind and produce a signal. This makes SAB very specific at detecting anti-HLA antibodies in the patients and discriminating between Class I and Class II antibodies, which is not possible using cell based assays.

Furthermore, SAB are coated with HLA antigens in very high density, far more than it is possible for the cells to express. Thus, these beads are highly sensitive at detecting even very low levels of DSA that might otherwise go undetected by CDCXM or FCXM. Two studies conducted by Ishida et. al. (2005) and Patel et. al. (2007) are worth mentioning in this regard. In these studies, detectable DSA could be found in serum samples by more sensitive SAB that were otherwise negative for CDCXM and FlowXM. In addition, these studies reported increased rate of AMR in DSA positive patients compared to patients without DSA detected by SAB (Ishida et. Al. 2005, Patel et. Al. 2007). Similarly, a retrospective study by Amico et. al. (2009) reported increased incidence of clinical and sub-clinical AMR 200 days post-transplant in patients with pre-

transplant HLA-DSA compared to patients without pre-transplant HLA DSA (71% vs. 35%). This indicates that low levels of DSA exist in some patients that are not detected by cell based assays, and these low levels of DSA detected on SAB might carry an increased risk of graft rejection or graft dysfunction post-transplant. Thus, using SAB it might be possible to predict AMR pre-transplant as well post-transplant with greater sensitivity that is unattainable by other cell based assays.

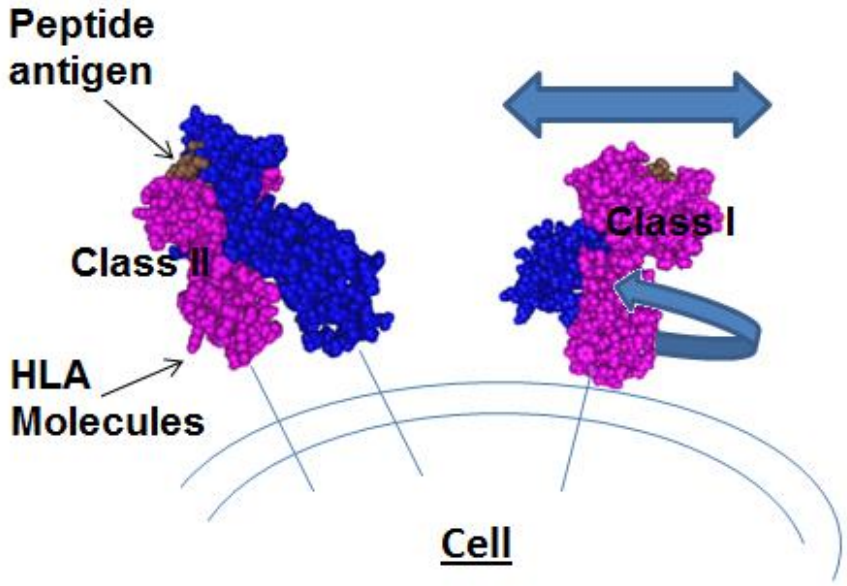
However, there are some intrinsic limitations associated with SAB assays as well. Since these assays contain recombinant HLA antigens bound by hydrophobic interactions, it has been argued that the conformation of HLA molecules present on the surface of beads could be different from their native conformation on the cell surfaces (Figure 1.2). This can expose 'cryptic epitopes' on HLA molecules which can result in non-specific antibody binding (El-Awar et. Al. 2009). In addition, due to their nature of production various peptide antigens are absent on the HLA molecules coating SAB, whereas peptides are loaded on to MHC before they are expressed on the cell surfaces. HLA molecules are also able to move and twist on cellular surfaces, allowing increased space for antibody binding; however, this is not possible on SAB. These factors could potentially result in difference in binding of anti-HLA antibodies to HLA molecules on beads and cells. Since many donors also have DSA against multiple HLA antigens, it is also not clear how to accurately interpret serum samples with multiple DSA specificities tested on SAB.

Furthermore, since SAB assays contain polystyrene beads with very high HLA expression, their functional relevance has also been argued. This is because it is not yet

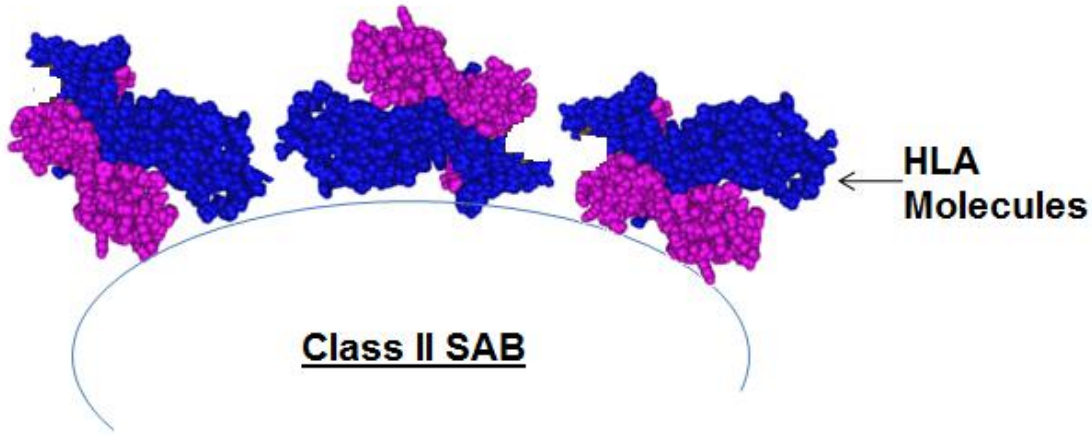
clear what level of DSA detected on SAB are harmful to the graft function and whether very low levels of DSA are even clinically relevant (Tinckam 2012). Thus, it is important to define a clinically relevant cutoff value for DSA detection using various serum samples that maximize the predictive value of SAB. If such a cutoff value can be successfully established on SAB, it will help detect DSA levels in patients that are harmful to the graft and can predict AMR. Multiple studies have taken various approaches to define such relevant cutoff thresholds (Zachary et. Al. 2009, Morris et. Al. 2010, Batal et. Al. 2010). These studies used patient serum samples that produced a positive B or T cell XM as a clinical correlate to establish a fluorescence range on SAB. However, results have been highly variable between studies. This is because these studies used neat serums samples from variably sensitized patients. Thus, highly sensitized patients with high DSA titre produced higher fluorescence whereas low fluorescence was produced by patients with lower serum DSA titre, resulting in a very broad range that corresponded to harmful DSA levels.

Figure 1.2: HLA molecules on **a)** cell surfaces and **b)** SAB. HLA are present on cell surfaces in their native conformation. These molecules are able to twist and move on the cell surface, allowing increased space for antibody binding. On SAB, HLA molecules are present in high density and bound by hydrophobic interactions to the beads. Since similar HLA movement is not possible on the beads, it can potentially hide some epitopes for antibody binding. Peptide antigens are also absent on SAB. This can result in expression of certain 'cryptic epitopes'.

a)


Peptide antigen
Class II
HLA Molecules
Class I
Cell

b)


HLA Molecules
Class II SAB

1.6 Hypothesis and Objectives

Due to their highly sensitive and specific nature, SAB assays are an attractive option that can prove useful in detecting harmful anti-HLA antibody levels in patients pre- as well as post-transplant. This could provide numerous benefits over cell based assays. However, this requires overcoming various limitations associated with these assays, as previously discussed. In order for these assays to be useful, it is important to show that DSA binding to HLA on SAB has a measurable quantitative correlate to cell based assays; and a clinical relevant cutoff can be successfully established that predicts harmful DSA levels (XM positive levels).

In order to establish a clinically relevant fluorescence range on SAB, it is important that serum samples being used have just enough DSA concentration required to produce a positive XM. This ensures that: 1) The SAB fluorescence range established is very narrow, even when serum samples from variably sensitized patients are used. 2) The fluorescence range established is very sensitive in predicting a positive XM.

Therefore, we **hypothesized** that using the highest FlowXM positive serum dilution it is possible to establish a clinically relevant correlate on SAB that could predict positive FlowXM. Positive FlowXM as a clinical correlate was used in this study as this method is the current clinical gold standard for DSA detection and a positive test is considered strong contraindication to transplant. Furthermore, it was hypothesized that SAB tests can provide information about sub-clinical DSA levels in relation to positive FlowXM. To test for these hypotheses, following **objectives** were undertaken:

1. DSA concentration was measured on SAB by testing the highest FlowXM positive serum dilution from a number of variably sensitized patients. This was used to establish a fluorescence range on SAB that correlated to positive FlowXM (harmful DSA levels).

2. SAB were used to measure DSA below the FlowXM positive level to obtain standard curve that approximates subclinical DSA in the serum.

3. Test the established range from objective 1 in predicting positive FlowXM. This was done by carrying out FlowXM tests using various serum/cell combinations.

CHAPTER 2- METHODS

2.1 Patient serum samples:

For this study, a number of potential kidney recipients that had been tested in the Histocompatibility lab between the years 2010 to 2012 were initially screened. Only those patients for whom FlowXM titration test results and antibody specificity reports were available were selected to be used. FlowXM titration tests helped approximate DSA titre in the patient, which allowed to identify high titre and low titre patient samples. Antibody specificity reports provided with a comprehensive list of all anti-HLA antibodies present in each patient, in addition to identifying DSA. Knowing specificities of anti-HLA antibodies in the serum was important in order to test these patient samples with lymphocytes of known HLA during the third phase of the study.

The selection criteria included a pool of variably sensitized patients, with low as well as high titre DSA, based on their FlowXM titration test results. The selected samples also consisted of patients that contained DSA against single as well as multiple HLA antigens. Furthermore, patients were selected to include HLA antigens from Class I and Class II that are most commonly present in the normal population. Detailed characteristics of patient samples used in the study including DSA and other anti-HLA antibodies present in the serum are presented in Table 2.1.

Thus, for the selected set of patients, historic serum samples that had been previously collected by the histocompatibility lab and stored during their routine pre-transplant workup were obtained, and used in the study. It is also worth noting that in some cases multiple serum samples from the same patient were obtained. These samples

were drawn at different times during the patient's pre-transplant clinical treatment with immunosuppressive agents. These sera were considered as separate samples, independent of one another, as they represented varying amount of DSA titre in the patient. Appropriate Research Ethics Board (REB) approval and patient consent for their serum samples to be used in the study was also obtained prior to using serum samples. Once obtained from the Histocompatibility lab, the serum samples were stored at -80 degree Celsius in the lab and aliquots were made in order to minimize freeze-thaw cycles.

Serum#	Date sample collected	DSA	Other Class 1 ab.	Other Class 2 ab.
S001 [1]	26/09.2011	a29,b44	a1,11,25,26,29,34, b13,18,27,35,44,45,49,51,52, 55,57,62,65	dr4,8,9,10,11,15,16,17,18, dr52
S002	8/3/2010	b55	None	none
S003	17/06/10	a23, b60	a2, 3, 23, 24 b7, 13, 18, 27, 34, 44,45, 49,51,52,57,60,62	dr53
S004	17/05/11	b18	b18,49,51,52,59,63	dr10, dq9
S005	29/04/11	b61	a1,2,3,23,24,25,26,29,30,31,32,68, b13,18,27,38,44,45,49,51,52, 53,57,58,59,61 b62,63,64,65, bw4	none
S006	18/02/10	a3	a1,2,3,11,25,26,29,30,31,33,34,68	dr1,103,10,15, 51,53 dq2,5
S007	15/11/10	b27,dr1.01.01,dq5	None	dr103,12 dq6
S008	29/11/11	b52, dq8	a1,23,24,29,32, b27,38,44,45,49,51,52,57	Anti DP-alpha ab., dr103,dr11,12,13,15,16,51, dq4,5,6,7,8,9
S011	20/07/11	a11,cw7, dq5	a3,b7,13,27,42,48,54,56,55, 60, 61,67,73,81,82, bw6	dr12,15,16 dq4,6
S012 [1]	1/9/2012	b62	a1,11,25,26,29,34, b13,18,27,35,44,45,49,51,52, 55,57,62,65	dr4,8,9,10,11,15,16,17,18, dr52
S013 [3]	28/09/11	b8	None	none
S015	9/11/2010	a2	b57	none
S016	30/06/11	b56,dr1,dq7	b13,35,46,49,50,51,52,53,56, 57,62,63	dr1,10,103,7,9,14,17,51,53, dq7,8,9
S017 [4]	11/7/2010	a3	None	None
S018 [4]	25/07/2011	a3	None	None
S019 [4]	8/8/2011	a3	None	none
S020 [2]	25/08/10	dr7,dr11, dq7, cw2,6	a31	dr7,8,11,12,13,16,51,52, dq 7,8,9
S022	10/11/2011	a29,dr7, dr53	a1,11,25,26,3,30,31,32,33,68, b27,35,49,52,53,54,55,56,62, 63,7	dr1,10,7,9
S024	15/06/10	dr15, dq6, B65	a1,2,23,24,25,31,32, b18,27,35,38,44,45,49,51,52, 55,57,62,65	dq 4,5,6,7,8,9 dr1,103,4,8,9,10,11,12,13,1, 15,16,17,18, 51,
S025 [2]	8/9/2010	dr7,dr11, dq7,	a31	dr7,8,11,12,13,16,51,52, dq 7,8,9

		cw2,6		
S027 [2]	13/9/2010	dr7,dr11, dq7, cw2,6	a31	dr7,8,11,12,13,16,51,52, dq 7,8,9
S028 [3]	19/09/11	b8	None	none
S029	17/11/10	a24,dr10	a23,a31,a32, b47,49,52	dq2
S031	22/08/11	a2, b51, dr9	b18, b35, b52, b8	dr1,103, 10

Table 2.1: Anti-HLA antibody characteristics of serum samples used in the study. DSA as well as other anti-HLA antibodies identified in the specificity reports are presented along with the serum#. [1,2,3,4] Samples collected from the same patients on a different date.

2.2 SAB reagents:

SAB are available from the manufacturer for their use on a Luminex instrument as well as on a Flow cytometer. Both beads are manufactured similarly and coated using purified Class I or Class II HLA. However, Luminex based HLA-SAB contains more HLA antigens per test compared to Flow based bead tests. In order to measure anti-HLA antibodies using SAB, we decided to use Flow based beads instead of Luminex as using Flow cytometer was more convenient due to availability of the instrument on our facility. As a result, FlowPRA® Single Antigen assays were purchased from One Lambda Inc. (CA, U.S.A).

FlowPRA® HLA Class I Single Antigen assay contains Phycoerythrin (PE) labeled beads coated with 32 different antigens from Class 1 HLA (HLA-A and HLA-B). These antigens are divided into four groups, each group containing eight different HLA-SAB. Beads coated with different HLA can be distinguished within each group based on varying amounts of PE present on each SAB, resulting in separate bands being produced on PE channel (Figure 2.1). Thus, each HLA bead is identified by its level of PE fluorescence, and the binding of fluorescein isothiocyanate (FITC) anti-IgG antibody can be calculated for each HLA-SAB separately. A control bead, with no HLA antigens bound to its surface, is also included in each group as a check for any background FITC fluorescence produced by IgG binding non-specifically from the test serum. Similarly, FlowPRA® HLA Class II Single Antigen assay contains 32 different Class II HLA (HLA-Dr and HLA-Dq) coated SAB, divided into four groups of eight different HLA each.

Additionally, supplementary test assays (FlowPRA® Single Antigen HLA Class I Supplement - Group 1 through Group 9) were also purchased for the detection of antibodies against less common antigens of HLA-A, HLA-B, HLA-Bw or HLA Cw. As well as supplementary test assays for Class II DP antigens were also purchased. Comprehensive list of Class 1 and Class II antigens present on the SAB Test assays are available at (http://www.onelambda.com/product-attachment.aspx?c1=antibody-detection&c2=flowpra-sup-sup-&c3=3&c4=10&c5=37). In addition, FlowPRA® Negative Control Serum, deficient in alloantibodies, was purchased from One Lambda Inc. This was used as a negative control when performing SAB experiments, in order to measure background FITC signal produced by SAB as well as non-specific IgG binding to the beads. This background FITC fluorescence was used to calculate increased fluorescence signal produced when anti-HLA antibodies in the patient serum reacted with the beads. Negative control serum was also used to perform 'negative FlowXM tests' in order to measure background auto-fluorescence on B and T cells. This was required to establish a negative cutoff for B and T cells beyond which FlowXM test could be considered positive due to binding of DSA.

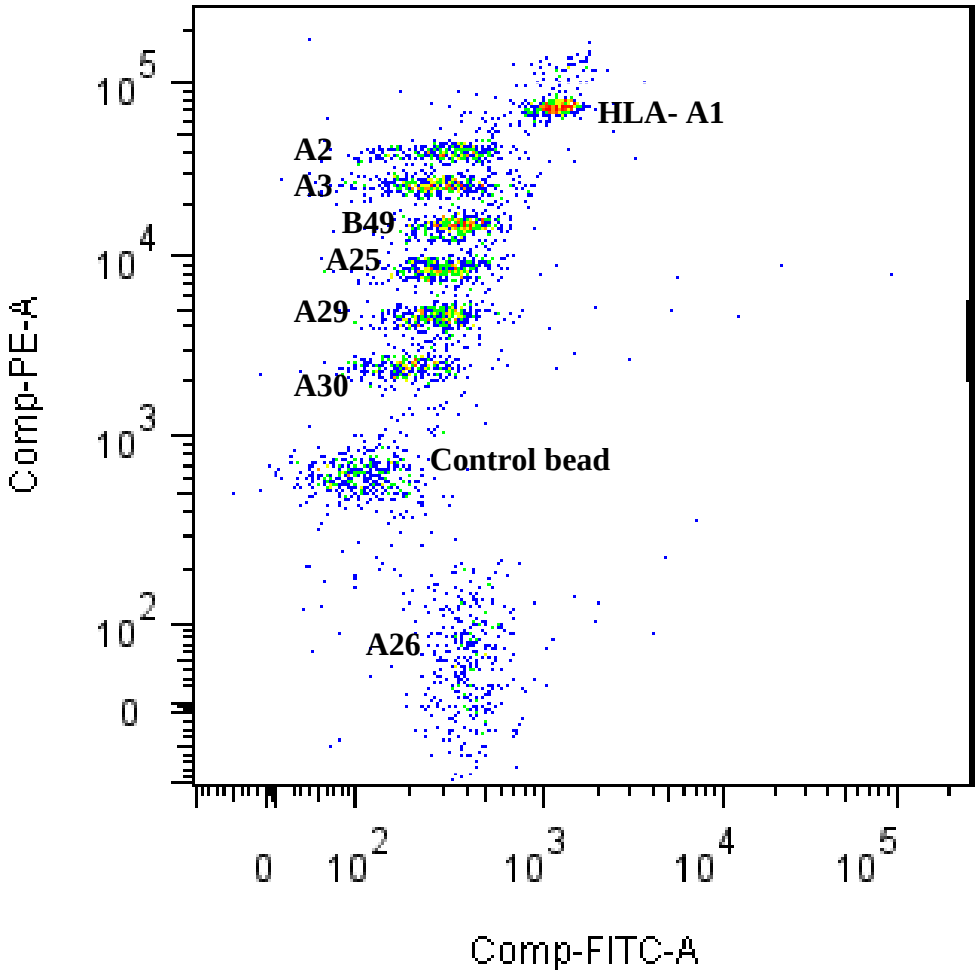


Figure 2.1: Eight different HLA-SAB in Group 1 FlowPRA® HLA Class I Single Antigen assay, as seen on FlowJo Analysis Software. Different HLA-SAB are labeled on the figure and can be distinguished based on their PE fluorescence. Control bead is present in each group to assess non specific IgG binding.

2.3 SAB preparation to detect anti-HLA antibodies in patient serum:

Standard operating procedures (SOP) developed by HRLMP were followed precisely in order to detect anti-HLA antibodies in the patient serum, using FlowPRA SAB. 20ul of neat patient serum or diluted serum sample was incubated with 4ul of appropriate SAB in 2ml plastic ependorff tubes for 30 minutes in dark at room temperature; allowing anti-HLA antibodies present in the patient serum to bind to HLA antigens present on the beads. Serum dilutions were carried out in IMDM-10 media. Similarly, 12ul of FlowPRA® Negative Control Serum was incubated with 4ul of SAB separately in ependorff tubes. Following the incubation period, SAB were washed twice in 1.5ml of 1x FlowPRA® Washing Buffer diluted in distilled water (10x FlowPRA® Washing Buffer, One Lambda). Strict adherence to wash procedures, time in wash buffer and proper aspiration was performed for each sample. This is important in order to maintain a reproducible background and to avoid any blocking effect of residual serum IgG antibodies when anti-IgG secondary antibody is added during the next step.

After washing, the beads were incubated in 100ul of diluted FITC anti-Human IgG antibody for 30 minutes in dark at room temperature. Stock FITC anti-Human IgG antibody was diluted to 1/101 in 1x washing buffer. The addition of FITC anti-human IgG antibody detects for the presence of any anti-HLA IgG antibodies that reacted with the SAB when these beads were incubated with patient serum. The beads were then washed again twice in 1.5ml of 1x washing buffer in order to remove any unbound antibody. Following the wash, beads were re-suspended in 0.5% formaldehyde and stored

at 4 degree Celsius in dark. Samples were analyzed using BD LSRII Flow cytometer available at McMaster Flow Cytometry Facility within 12 hours.

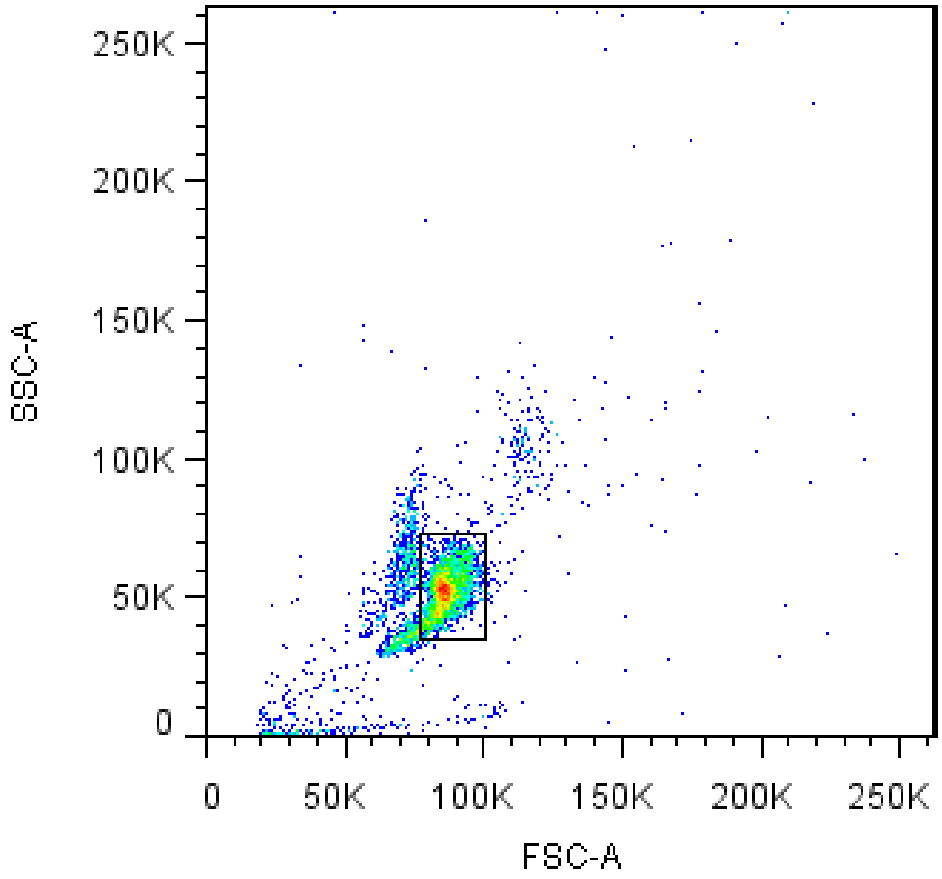
2.4 Instrument setup and data acquisition for SAB analysis:

In order to set up LSRII instrument, appropriate compensation was drawn using a group of SAB incubated in negative control serum. Instructions provided with FlowPRA® Single Antigen assays on compensation setup were followed when setting up compensation values. A template was created and saved on the instrument for data acquisition and used throughout the project. To account for daily variations in LSRII readouts and to keep the relative fluorescence same between different experiments, control bead incubated with negative serum was targeted every time to obtain a MFI value of around 100 on the FITC channel by adjusting FITC voltage, prior to acquisition of data. This allowed comparison between FITC values obtained from different experiments performed using SAB. Instrument calibration and quality control was also routinely performed by Flow Facility lab technician using calibration beads. SAB were gated based on their Forward (FSC) and Side scatter (SSC) and data was recorded for negative control serum and patient serum. Acquisition was considered complete when more than 50 beads for each SAB were collected or each band in the group was visible on the PE channel.

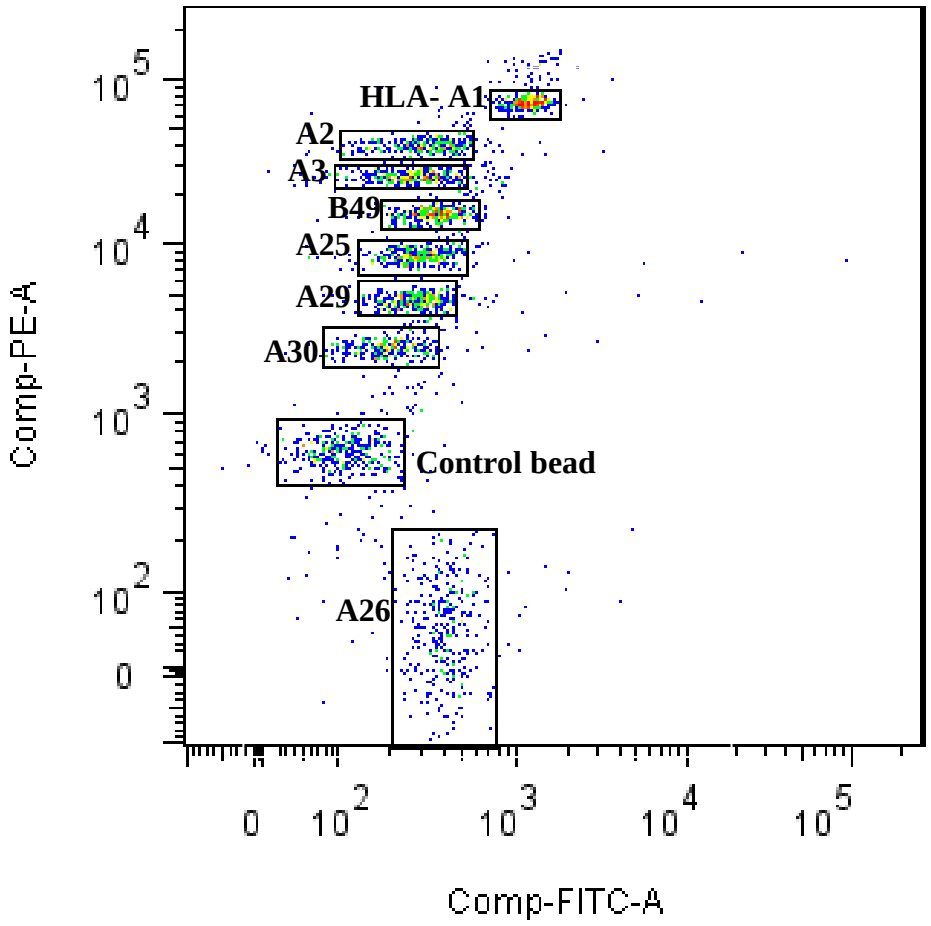
2.5 SAB analysis

Tree Star FlowJo Analysis software was used in order to analyze the acquired data. The majority of SAB were gated based on their FSCxSSC (Figure 2.2 a). The gated population was then analyzed for its PE and FITC fluorescence on SAB (Figure 2.2 b). Different SAB in a group could be distinguished based on their PE fluorescence. Each SAB were boxed individually to include majority of the beads and FITC Median Fluorescence Intensity (MFI) was calculated for each SAB. Thus, results were obtained from SAB experiments performed with negative control serum (Figure 2.2 b). As seen in the figure, FITC signal on the SAB incubated with the negative control serum was very low. However, when SAB were incubated with the patient serum, there was an increase in FITC signal on SAB (Figure 2.2 c). This was due to antibodies reacting with corresponding HLA on SAB. Since, SAB tests are highly specific, increased FITC signal was seen on only those SAB that were bound by anti-HLA antibodies present in the serum. Furthermore, increase in FITC signal on SAB was dependent on the titre of anti-HLA antibodies present in the serum. This increase in FITC fluorescence was quantified by calculating Delta MFI values for each SAB. Delta MFI was calculated by subtracting FITC MFI on SAB incubated with negative control serum from SAB incubated with the patient serum. This provided with a SAB MFI measure for various anti-HLA antibodies present in the patient serum. Results from SAB tests that produced a Delta value of greater than 100 MFI on the control bead were considered invalid due to high background noise produced by non-specific serum IgG binding.

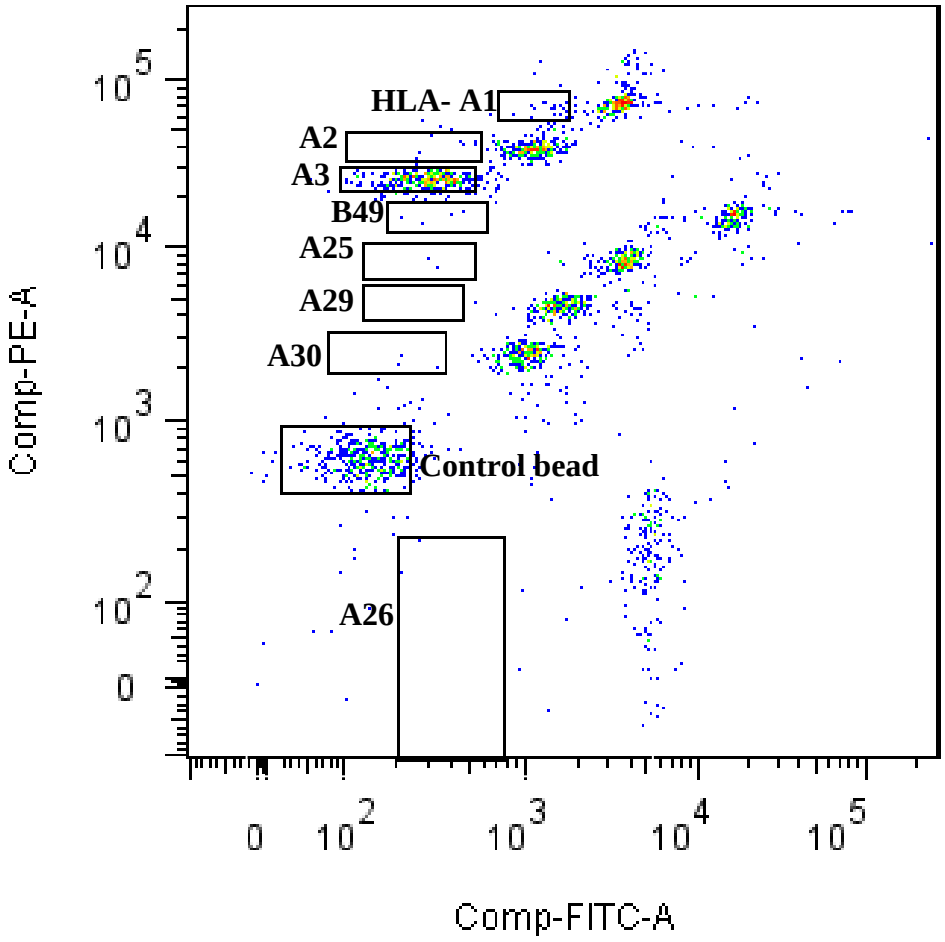
Figure 2.2: Gating strategy used to analyze the data acquired from SAB experiments. **a)** FSC and SSC gate was used to target the majority of SAB. **b)** The gated population was analyzed for its PE and FITC fluorescence. Figure shows FITC MFI produced by Class I Group I SAB incubated with 'negative control' serum. **c)** Increase in FITC signal in comparison to 'negative control' could be seen when various anti-HLA antibodies in patient serum S001 reacted against antigens on SAB. No shift in FITC MFI on the control bead is present, indicating very low background non-specific IgG binding.



a)


HLA- A1
A2
A3
B49
A25
A29
A30
Control bead
A26
Comp-PE-A
Comp-FITC-A
0
10^2
10^3
10^4
10^5

b)


HLA- A1
A2
A3
B49
A25
A29
A30
Control bead
A26
Comp-PE-A
Comp-FITC-A
0
10^2
10^3
10^4
10^5

c)

2.6 Measuring HLA density on the beads

In order to measure density of various HLA coating Class I FlowPRA SAB, we used FITC conjugated mouse W6/32 anti-human monoclonal antibody. W6/32 monoclonal antibody recognizes a common epitope shared by all Class I HLA, which allows it to bind various antigens present on Class I SAB. Prior to its use, the antibody was titrated using SAB in order to establish an optimal concentration that was just below the amount required to saturate the beads. The titrations were carried out in IMDM-10 media and optimal concentration was calculated at 1/400ul W6/32 antibody. Thus, 1/400ul W6/32 antibody was incubated with 4ul of different Class I SAB in separate ependorf tubes for 30 minutes in dark. As a negative control, beads were also incubated in IMDM-10 in the absence of W6/32 antibody. Following the incubation period, beads were washed twice in 1.5ml of 1x FlowPRA Washing Buffer and then re-suspended in 0.5% formaldehyde. Samples were analyzed using LSRII Flow cytometer. Instrument setup and SAB analysis was carried out as mentioned previously in Section 2.4 and 2.5, respectively. Delta MFI values were calculated as a measure of FITC W6/32 antibody bound to the beads and results were obtained from three separate experiments. Thus, varying degree of fluorescence was produced on different SAB depending on the amount of bound FITC W6/32 antibody.

Similarly, HLA density on Class II FlowPRA SAB was measured using a FITC conjugated mouse L243 anti-human monoclonal antibody. Since, L243 antibody only

reacts with HLA-Dr and HLA-Dq of Class II MHC, this antibody could not be used to measure antigen density for HLA-DP.

To quantify FITC fluorescence produced on SAB, Quantum FITC-5 MESF beads were used. Quantum FITC-5 MESF kit includes one blank and five different population of fluorescently labeled beads, each with known levels of FITC fluorescence; this allows development of a FITC calibration curve when these beads are run simultaneously on the flow cytometer. To obtain a calibration curve, 1 drop from each population of beads was added to 0.5 ml of 0.5% formaldehyde as per manufacturer's instructions. Samples were then analyzed on LSRII flow cytometer setup using SAB incubated with negative control as mentioned in Section 2.4. MESF beads were identified based on their FSCxSSC pattern and acquisition was considered complete when 100,000 events were recorded. FITC signal on MESF beads was used to produce a calibration curve on Bangs Laboratories' quantitative software QuickCal V5, available at www.bangslabs.cm/products/quickcal. This software was also used to quantify FITC fluorescence on different Class I and Class II SAB.

2.7 Performing FlowXM tests

In order to perform FlowXM tests, SOP developed by the HRLMP to carry out FlowXM tests were followed precisely. Same day peripheral blood collected by the Histocompatibility lab from potential kidney donors was used in performing FlowXM. Whole blood was diluted in equal amounts of phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated by layering the diluted blood over Ficoll-Paque PLUS and centrifuging at 2500 rpm for 20 minutes. The interface layer, consisting mainly of PBMC, was carefully isolated. The sample was then washed in 10ml of PBS and cell pellet was obtained by centrifuging at 1500 rpm for 10 minutes. The supernatant was discarded and donor cells were re-suspended in RPMI in two 1ml volumes. Since platelets express Class I HLA, cells were washed twice in order to remove any platelet contamination by spinning the sample at 1300rpm for 2 minutes. This was important since anti-HLA antibodies could be absorbed by the platelets, resulting in a false negative or weak positive FlowXM. Cell count was then adjusted to 1 million cells/ml. 50 ul of donor cells were incubated in 30 ul of appropriate neat or diluted patient serum at room temperature for 30 minutes. In order to set up a 'negative FlowXM', 50 ul of donor cells were also incubated in 30 ul of 'negative control' serum. Following incubation period, cells were washed three times in 2ml of PBS. Adherence to proper wash procedures was important as residual IgG antibodies in the patient serum can bind to anti-IgG secondary antibody and adsorb it. This can result in a false negative or weak positive FlowXM. Next step involved addition of anti-CD3, anti-CD19 and anti-IgG secondary antibodies. 5 ul of

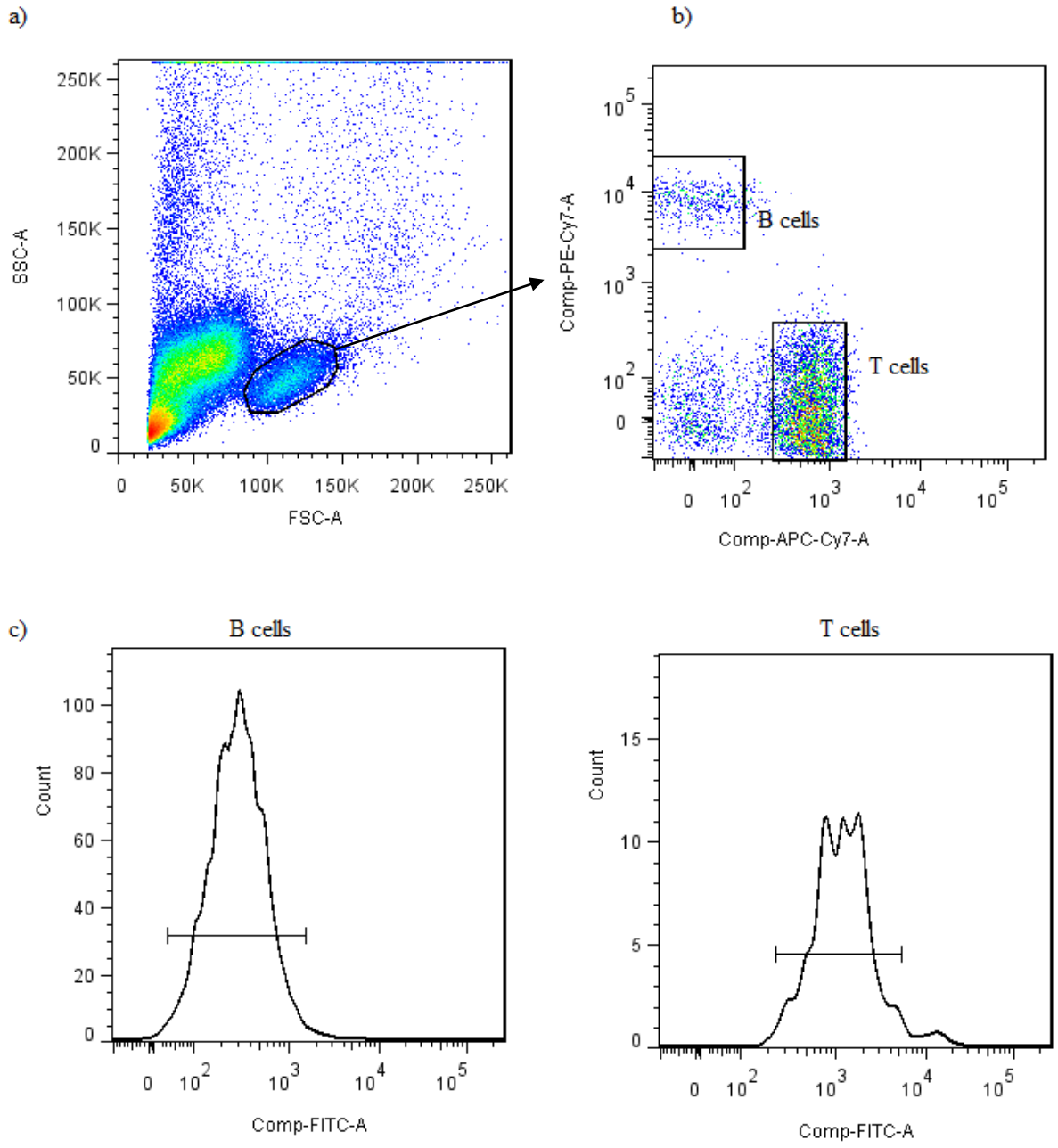
APC-cy7 anti-CD3 antibody and 2 ul of PE-cy7 anti-CD19 antibody were used to label T cells and B cells, respectively. In order to detect for the presence of anti-HLA antibodies bound to donor cells, FITC anti-IgG antibody was also added. The cells were then incubated at 4 degree Celsius for 30 minutes in dark. Following incubation, the cells were washed twice in 2ml of PBS to wash away any unbound antibodies. The cells were re-suspended in 50ul PBS, filtered and analyzed on LSR II Flow cytometer within 2 hours of sample preparation.

Prior to acquisition of data, unstained donor lymphocytes were targeted to obtain a MFI value of around 100 on the FITC channel by adjusting FITC voltage. This was done for every experiment in order to account for differences in cell auto fluorescence between different donors, and to keep the relative FITC fluorescence same in each experiment. In order to set up appropriate compensation, single color controls were used in each experiment. These were donor cell samples stained separately with APC-cy7, PE-cy7 or FITC antibodies individually. Following compensation setup, data was recorded on the flow cytometer. Minimal acquisition for proper measurement was 100 events for B cells and T cells, even though higher numbers were recorded for each experiment.

In order to analyze the acquired data, Tree Star FlowJo Analysis software was used. Lymphocytes were identified based on their FSC and SSC parameters; strict gating was carried out to exclude any debris, platelet or monocyte contamination (Figure 2.3 a). Cell doublets were also excluded based on their FSC-height and SSC-height profile. The gated population was then analyzed for its PE-cy7 (CD19) and APC-cy7 (CD3) signal. B cells were identified as CD19 high CD3 negative and T cells were identified as CD3 high

CD19 negative (Figure 2.3 b). FITC MFI signal, as a measure of cell bound anti-HLA IgG antibody, was analyzed on B cells and T cells (Figure 2.3 c). Thus, depending on the B and T cell FITC MFI produced, a FlowXM test was considered either T cell positive or B cell positive or both. A higher MFI value indicated larger amounts of anti-HLA antibodies reacting against donor cells.

Figure 2.3: Gating strategy used to analyze FlowXM tests. Figures show results from 'negative control' serum reacting with donor B and T cells. **a)** Lymphocytes were gated based on their FSCXSSC parameters; platelets, debris and monocytes were excluded from this initial mononuclear gate. **b)** B cells were identified as CD19 high CD3 negative; T cells were identified as CD3 high CD19 negative. **c)** FITC MFI signal on B cells and T cells was analyzed as a measure of anti-HLA antibody reacting against donor cells.



2.8 Statistical analysis

In order to detect outliers in the results obtained from SAB experiments, the InterQuartile method for outlier detection was used. Value(s) was declared outlier if it was smaller than (1st Quartile – 1.5 x InterQuartile Range) or larger than (3rd Quartile + 1.5 x InterQuartile Range). This method was deemed appropriate as multiple outliers were suspected in the data set and the population was non-normally distributed. The normality of the population was checked using Anderson darling normality calculator. In order to compare MFI values between various serum groups, non paramateric Mann-whitney U test was used ($p<0.05$). Categoric results from FlowXM tests were analyzed using Fischer's exact test ($p<0.05$).

CHAPTER 3- RESULTS

3.1 Objective 1: Calculating antibody strength that corresponds to positive FlowXM using SAB

In order to establish a fluorescence range on SAB that equates positive FlowXM, individual anti-HLA antibody levels for known DSA in twenty one different patient serum samples were quantified. All serum samples used in the study had previously been tested by FlowXM titration test in the clinical laboratory. Results from these FlowXM titration tests detailed the highest dilution, for each serum, that resulted in a positive FlowXM on either T cells or B cells or both. In many cases, serum dilution that resulted in a positive FlowXM differed for T cell XM and B cell XM, as expected.

The highest serum dilution that resulted in a positive FlowXM, for B and T cells separately was used when testing with SAB. This allowed quantification of DSA that was just above the level required to produce a positive FlowXM, below which the FlowXM test was negative. Using the highest serum dilutions was important in producing a stringent MFI range on SAB. These dilutions varied, from neat to as high as 1/64, for different serum samples depending on the DSA titre in the patient serum.

Each of the sera used had single or multiple DSA (range 2-5 DSA). Thus, the positive FlowXM produced at the highest serum dilution was the result of a single or multiple DSA binding on the cell surfaces. In cases where multiple DSA were present in the patient serum, SAB signal strength for total DSA was calculated as the sum of MFI for individual DSA present in the serum. Previous work by others has indicated that low-titer DSA could be detected on SAB, but would only cause a weak or negative

FlowXM if individual targets for DSA were present on donor cell surface. However, if several of these low titre DSAs were targeted against multiple HLA on donor cells, the increased binding of total DSA would result in a positive FlowXM (Warner 2009). The positivity of FlowXM increased with the number of HLA targets for these low titre DSA. Thus adding of multiple DSA signals on the respective HLA beads, should be equivalent to the total DSA binding in the FlowXM.

3.1.1 SAB signal strength measured for DSA in sera positive B cell FlowXM

Several issues are apparent in defining SAB positive signals for DSA in relation to a positive B cell FlowXM. In a clinical setting, FlowXM on B cells is not always interpretable, especially near the established negative cutoff fluorescence for B cells. Due to higher background auto fluorescence seen on B cells compared to T cells, and due to presence of low affinity Fc-gamma receptor that increases IgG binding on B cells, there is a high variability in the negative control range for B cell FlowXM. This results in a higher negative threshold beyond which a positive XM can be attributed entirely due to presence of DSA. This can potentially result in false negatives, especially when low titre DSA is present. Furthermore, since B cells express both Class I and Class II HLA, measuring signal strength for DSA that results in a positive B cell FlowXM could be highly useful in deciphering the role of both Class I and Class II anti-HLA antibodies in FlowXM. Thus, DSA signal strength on SAB was measured in relation to positive B cell FlowXM.

Using the highest serum dilution that produced a positive FlowXM test on B cells, FlowPRA SAB testing was performed on 19 different serum samples. These serum samples were taken from variably sensitized patients with single as well as multiple DSA against Class I and/or Class II HLA antigens with varying titre (Table 3.1). Highly sensitized patients with very high DSA titre (serum# S008 and S011), required a higher serum dilution to be tested on SAB because previously titred FlowXM results indicated that higher dilutions still produced a positive FlowXM test. Conversely, patients with low DSA titre (serum# S001, S002, S005, S013, S016 and S029) required lower serum dilutions to be tested on SAB.

Serum#	DSA	B cell FlowXM positive dilution
S001	A29, B44	Neat
S002	B55	Neat
S003	A23, B60	1/2
S005	B61	Neat
S007	B27, Dq5, Dr1	1/2
S008	B52, Dq8	1/64
S011	Dq5, A11, Cw7	1/32
S012	B62	Neat
S013	B8	Neat
S015	A2	1/2
S016	Dr1, B56, Dq7	Neat
S017	A3	Neat
S020	Dr11, Dr7, Cw2, Cw6, Dq7	1/8
S022	A29, DR7, DR53	1/2
S025	Dr11, Dr7, Cw2, Cw6, Dq7	1/2
S027	Dr11, Dr7, Cw2, Cw6, Dq 7	Neat
S028	B8	1/8
S029	Dr10, A24	Neat
S031	B51, Dr9, A2	1/2

Table 3.1: Serum samples used in SAB experiments in order to establish a DSA MFI that correlates to positive B cell FlowXM. Sera included single as well as multiple DSA against Class I and/or Class II HLA. Higher B cell FlowXM positive dilution corresponds to high titre sera.

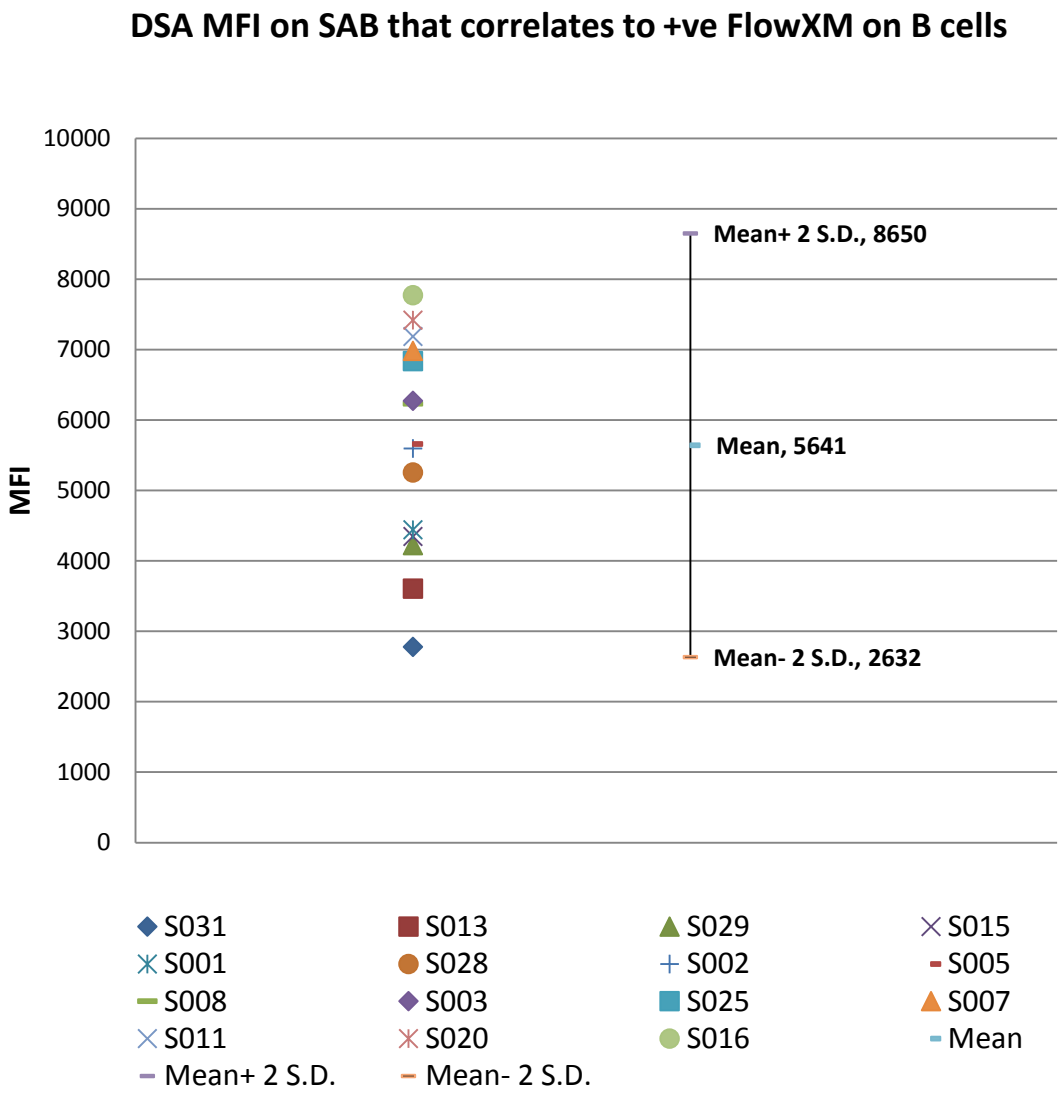


Figure 3.1: SAB MFI values produced by DSA positive sera at dilutions that correspond to B cell positive FlowXM. Mean value was calculated as 5,641 MFI (S.D. 1504; n=15). Mean MFI+2S.D. and Mean MFI-2S.D. values represent 95% confidence interval.

MFI values produced by DSA on SAB from these tests are presented in Figure 3.1, excluding outliers. In cases where multiple DSA were present in the serum, MFI values are presented as the sum of individual DSA. MFI values for the tested sera ranged from 2780 to 7772 MFI. Based on the data, a mean fluorescence value of 5641 MFI (S.D. 1504; n=15) as a correlate to minimal DSA required to produce a positive B cell FlowXM (highest dilution for FlowXM positive) was calculated. MFI values of 8650 (mean+2S.D.) and 2632 (mean-2S.D.) was calculated as upper and lower thresholds with 95% confidence interval, respectively. This represents the range of MFI produced by DSA on SAB from 95% of all positive B cell FlowXM. Below the lower threshold of 2632 MFI, only 2.5% of all B cell positive FlowXM should produce a DSA signal on SAB.

To further verify that adding SAB signal strength for sera with multiple DSA was appropriate, MFI values produced by serum samples with single DSA and multiple DSA were compared (Figure 3.2). Mean MFI of 4892 (S.D. 1644; n=5) was calculated for serum samples with single DSA; whereas, sera containing multiple DSA specificities produced a mean MFI value of 6015 (S.D. 1644; n=10), which was slightly higher. However, there was no statistically significant difference between the two data set ($p=0.126$).

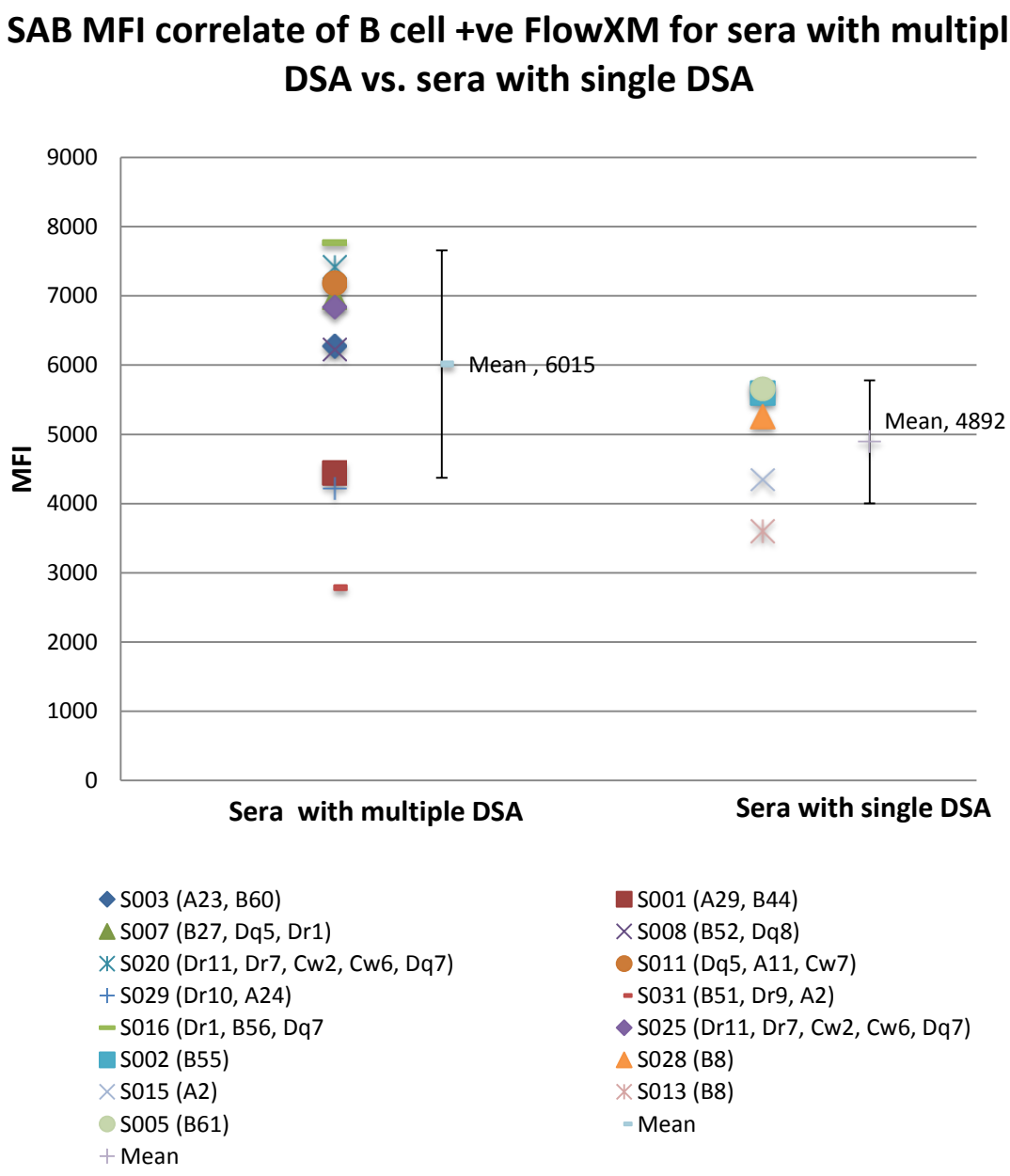


Figure 3.2: Comparison between SAB MFI produced by serum samples containing single DSA (n= 5) and multiple DSA (n= 10) at dilutions that were B cell FlowXM positive. Mean MFI was calculated for sera with multiple DSA as well as single DSA; 1S.D. error bars are also shown for the calculated means. There was no statistically significant difference between the two groups, p=0.126.

3.1.2 Comparing SAB MFI produced by neat serum samples and diluted serum samples with positive FlowXM results

In this study the highest serum dilutions that resulted in positive FlowXM were used when testing sera with SAB. For many serum samples this equated to neat sera whereas other serum samples required dilution in IMDM-10 before the FlowXM positive dilutions could be achieved. The idea behind using the highest FlowXM positive dilution was that various serum samples would have equivalent amount of DSA above XM level, regardless of patient sensitization or neat DSA titre. Thus, in order to validate if DSA concentrations were equivalent between neat sera and diluted sera at FlowXM positive levels, comparison was drawn between SAB MFI values produced by these two types of sera. However, no difference was seen between MFI values produced by neat sera and diluted sera (Figure 3.3). This validated that diluted serum samples, that was just sufficient for positive FlowXM, had an equivalent DSA levels as neat sera. Thus, there was no effect of dilution on the resulting range produced by various sera on SAB.

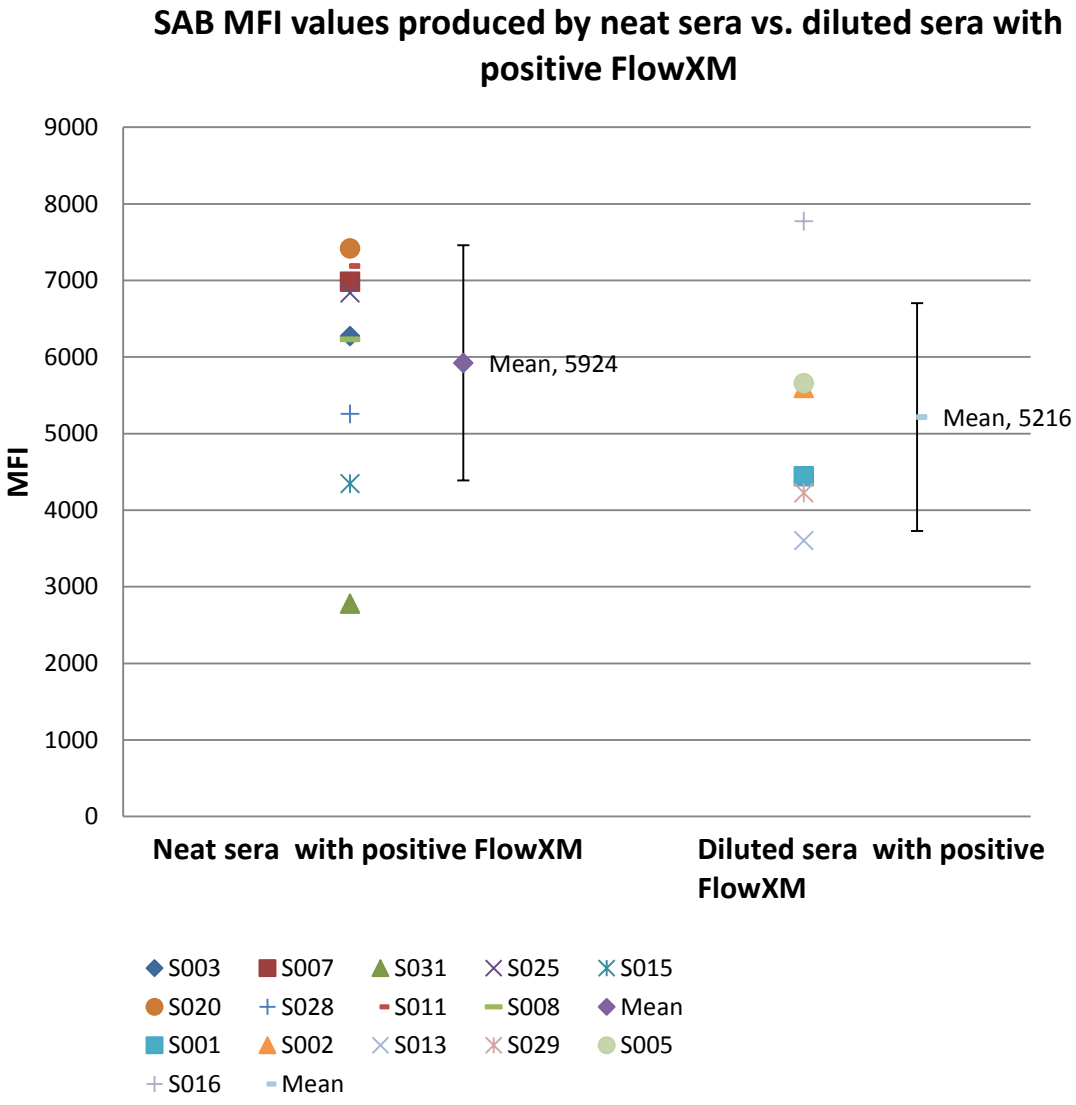


Figure3.3: Comparison between DSA MFI values produced by neat sera (n= 9) and diluted sera (n= 6) at highest FlowXM positive dilution. Mean MFI and 1 S.D. error bars for both groups are also shown. No significant difference exists in MFI values produced.

SAB could also be helpful in cases where FlowXM does not provide a definite result. Two serum samples with single Class I DSA for which FlowXM titration tests were negative were analyzed using SAB. As seen in the XM results, neat dilution of these two serum samples produced a negative FlowXM on B and T cells (Table 3.2). However, when lower dilutions for these sera were tested, FlowXM tests produced higher MFI than neat values. This could have been due to very high concentration of DSA in these serum samples producing a 'prozone effect'. Conversely, high concentrations of 'natural' IgM antibodies could have competed with IgG binding on cell surface; thus producing a lower signal on B and T cells at high serum concentrations. These serum samples were tested using SAB to see if DSA MFI produced by these sera corresponded with the previously established MFI for positive B cell FlowXM. Interestingly, DSA MFI produced by both of these sera was within the established range. Serum# S004 produced a MFI value of 6450 whereas serum# S006 produced a MFI value of 7474

		FlowXM test results			
Serum #	**DSA**	**B cell MFI**	**T cell MFI**	**Serum dilutions tested**	**SAB MFI**
		Cutoff= 751	Cutoff= 215		
S004	B18	434	169	Neat	6450
		N/A	N/A	1/2	3300
		680	172	1/4	1235
		Cutoff= 709	Cutoff= 273		
S006	A3	404	240	Neat	7474
		N/A	N/A	1/2	4754
		471	177	1/4	2302

Table 3.2: Two serum samples that were FlowXM negative on B and T cells. Cutoff MFI values beyond which a FlowXM test is considered positive are shown for B and T cells. SAB test results for different serum dilutions tested are also shown; these values corresponded to previously established SAB MFI range for B cell positive FlowXM.

3.1.3 Outlier analysis

Four serum samples, Serum# S027, S012, S017 and S022 were excluded from our calculated mean and S.D. for B cell positive FlowXM. Serum# S027 produced a very high background noise due to non-specific IgG binding as could be seen by high FITC signal produced by the control bead. Therefore, SAB test results from this serum sample were considered invalid. Serum # S012, S017 and S022 were excluded from the calculations since these sera produced a very high bead signal compared to the rest (Table 3.3). Results from these serum samples were considered outliers when tested using IQR outlier test. Two of the serum samples, S017 and S012, contained single DSA specificity with DSA against A3 and B62 HLA antigens, respectively. Serum# S022 contained DSA against three different HLA antigens: A29, DR7 and DR53, of which DSA against A29 produced the highest signal on the bead. However, similar background noise as seen previously with serum S027 was not evident on these three serum samples. To rule out technical issues in sample preparation, SAB tests were repeated on Serum# S022, S017 and S012. But no significant difference was seen in the bead MFI as the MFI values still remained very high.

Serum#	DSA	Dilution tested	DSA MFI (Test 1)	DSA MFI (Test 2)
S012	B62	Neat	11,178	12,056
S017	A3	Neat	20,562	17,900
S022	A29, DR7, DR53	1/2	21,550	20,717

Table 3.3: Very high SAB MFI produced by serum# S012, S017 and SO22. These serum samples were considered outliers and excluded from the calculated mean SAB MFI that correlates to B cell positive FlowXM.

3.1.4 Testing HLA density on SAB

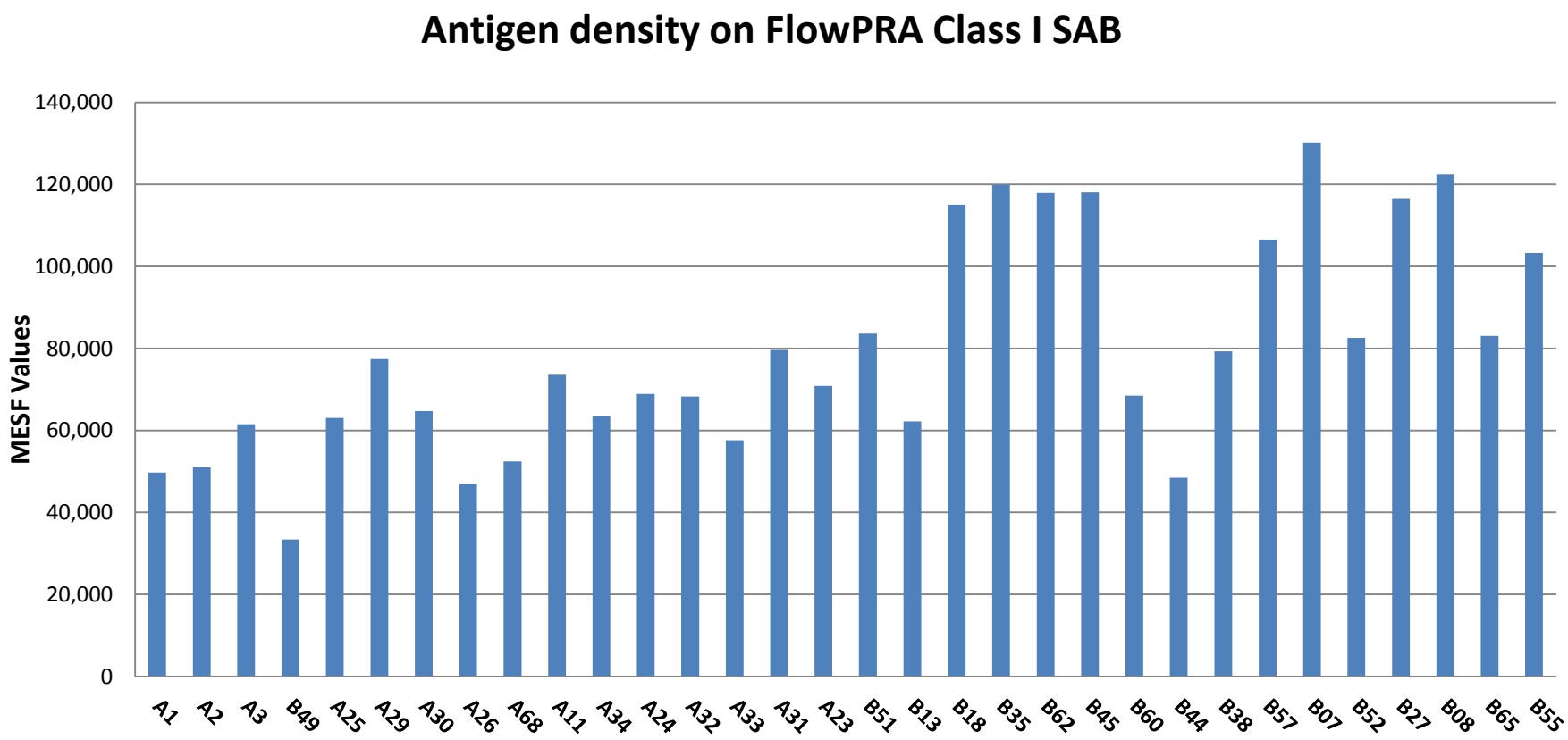
Previous work by others has shown that differences in densities among various antigens coating SAB exist (Warner 2009). This can result in differences in signal strength on beads, with certain anti-HLA antibodies producing higher signal. In order to see if these differences could be contributing to high bead signal produced by the outlying DSA, we quantified the amount of HLA molecules present on FlowPRA Class I and Class II SAB. FITC MFI values, as a measure of HLA bound W6/32 antibody, were used as an approximation of HLA density on the beads. This varied among different antigens depending on the amount of HLA molecules present on SAB for W6/32 antibody to bind. Thus, results from three separate experiments were obtained and average values for different antigens were calculated. These MFI values were then quantified using Quantum MESF beads. MESF beads allowed conversion of MFI values into MESF units, so that a direct comparison could be drawn between different antigens based on their MESF values. This was done by correlating fluorescence intensity on the sample (FITC MFI on SAB) to a calibration curve obtained from MESF beads with known concentration of fluorophores.

Figure 3.4 shows MESF values obtained for different antigens present on FlowPRA Class I and Class II SAB, respectively. As evident from the results, there are significant differences in antigen densities between various HLA molecules, with certain HLA molecules present on SAB more than two times in number compared to other HLA. Average HLA density on Class I SAB was 79,377 MESF units and 73,676 for Class II

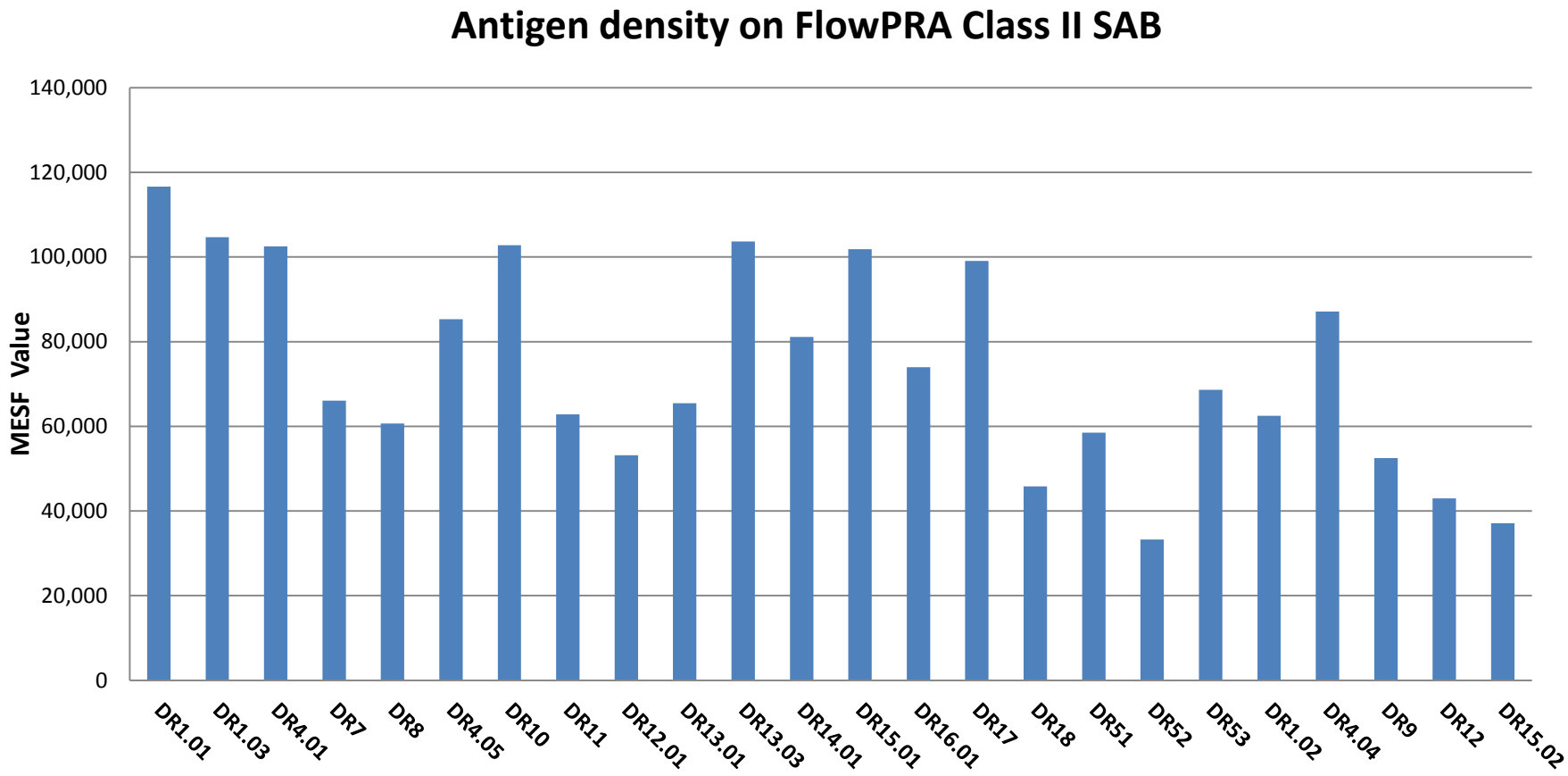
SAB. Among Class I HLA antigens, HLA B49 was present in the lowest density on SAB (33,400 MESF units), whereas HLA B07 was present in the highest amount (130,154 MESF units). Similarly, among Class II HLA antigens, HLA DR1.01 was present in the highest density (116,657 MESF units) and HLA DR52 was present in the least amount (33,260 MESF units).

However, there was no correlation between antigen density on SAB and DSA producing unusually high MFI values. Even though HLA B62 (117,971 MESF units) was present in a very high density among Class I HLA, HLA A29 (77,411 MESF units) and HLA A3 (61,516 MESF units) were present at the mean and below mean values, respectively. Thus, difference in antigen density on SAB as a contributing factor to high bead MFI produced by A3, A29 and B62 DSA was not supported by the results.

Figure 3.4: HLA antigen density on FlowPRA Class I **(a)** and Class II **(b)** SAB. Antigen densities are presented in MESF units for different HLA beads. Many HLA molecules were present in twice as much density that others.



a)


Antigen density on FlowPRA Class II SAB
MESF Value
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
DR1.01
DR1.03
DR4.01
DR7
DR8
DR4.05
DR10
DR11
DR12.01
DR13.01
DR13.03
DR14.01
DR15.01
DR16.01
DR17
DR18
DR51
DR52
DR53
DR1.02
DR4.04
DR9
DR12
DR15.02

b)

3.1.5 SAB signal strength measured for DSA in sera positive T cell FlowXM

Multiple studies have shown the importance of T cell crossmatch and its utility in predicting adverse graft outcomes (Rebibou et. Al. 2004, Karpinski et. Al. 2001, Ocura et. Al. 1993). As a result, positive T cell crossmatch is invariably considered a contraindication to transplantation. Thus, establishing a separate MFI value on SAB that correlates to a positive T cell FlowXM could be highly useful in crossmatch prediction.

Since T cells do not express Class II HLA antigens, we evaluated only Class I DSA on SAB in order to establish a separate MFI for positive T cell FlowXM. Using the same principles as mentioned previously for B cell FlowXM, we gathered data from thirteen different serum samples for which results from T cell FlowXM titration tests were available. Table 3.4 shows DSA and serum dilutions for different samples used in SAB testing. Results from Serum# S027 were excluded due to high IgG background noise as mentioned previously. The MFI values for T cell FlowXM ranged from 1089 to 6731 (Figure 3.5). Mean MFI of 3226 (n=12; S.D. 1931) was calculated as a correlate to minimal Class I DSA required to produce a positive T cell FlowXM. In order to establish similar thresholds as were calculated for B cell FlowXM, to obtain MFI range that includes 95% of all positive T cell FlowXM, we used Mean+2S.D. and Mean-2S.D for upper and lower cutoffs, respectively. The upper threshold was calculated at 7087 MFI. However, lower threshold was below the SAB positivity range due to high S.D. for the T cell data set (MFI value of -636 as lower cutoff). Thus, lower cutoff above which 97.5%

of T cell FlowXM would be positive (or below which 2.5% of the FlowXM tests would be positive) could not be established.

No outliers in the data set were identified when examined with the IQR outlier test. Furthermore, similar comparison between serum samples with multiple DSA and single DSA, as performed for B cell FlowXM, could not be carried out due to small sample size for multiple DSA in T cell positive FlowXM. To see if DSA MFI values used to establish B cell positive FlowXM differed from that of T cell positive FlowXM, comparison was carried out between the two data set. Mann-Whitney U test showed a significant difference between the two DSA MFI as expected (p=0.0014), indicating that different DSA MFI values on SAB corresponded to positive FlowXM on B and T cells.

Serum#	DSA	T cell FlowXM positive dilution
S008	B52	Neat
S012	B62	1/2
S016	B56	Neat
S017	A3	1/64
S018	A3	1/2
S019	A3	1/4
S020	Cw2, Cw6	1/8
S022	A29	1/8
S024	B65	Neat
S025	Cw2, Cw6	1/4
S027	Cw2, Cw6	Neat
S029	A24	Neat
S031	B51, A2	1/4

Table 3.4: Serum samples used in SAB experiments in order to establish a DSA MFI that correlates to positive FlowXM on T cells. Higher dilution corresponds to high titre sera.

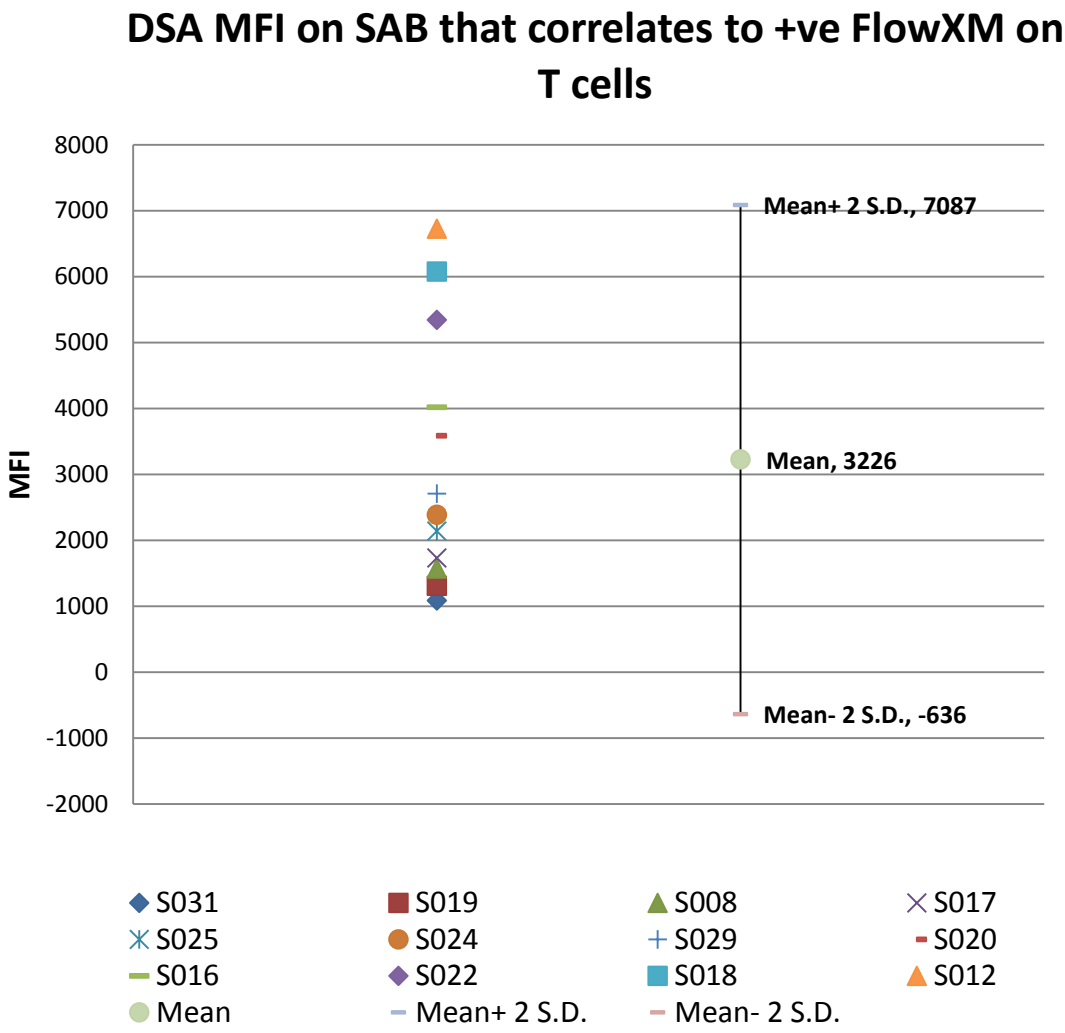


Figure 3.5: MFI values produced by DSA at serum dilutions that correspond to positive FlowXM on T cells (n=12; S.D. 1931). Mean MFI as well as 95% confidence intervals are shown (Mean+/- 2S.D.). Due to higher variability, Mean-2S.D. is a negative MFI value (below SAB detection threshold).

3.1.6 SAB signal strength measured for DSA in a B cell positive and T cell negative FlowXM with only Class I anti-HLA antibodies.

The significance of a positive B cell XM with a negative T cell XM is highly controversial with evidence against as well as in favor of successful transplantation across B cell positive XM (Lobashevsky et. Al. 2000, Eng et. Al. 2008, Goh et. Al. 2012). Furthermore, since B cells express more Class I HLA on their surface compared to T cells (Pellegrino et. Al. 1978), this could potentially result in a higher signal on B cells even in the presence of low amounts of Class I DSA. Thus, T cell negative B cell positive FlowXM might not always be seen as a contraindication to transplant when only Class I DSA is present. Consequently, it is important to analyze the significance of Class I DSA signal strength measured on SAB in such situations.

In order to see if MFI values for Class I DSA in B cell positive T cell negative XM varied from Class I DSA in B cell positive T cell positive FlowXM, comparison was carried out between two sets of MFI values. Mean MFI for Class I DSA in B cell positive T cell positive FlowXM was 5426 (S.D. 986; n=3); whereas mean MFI for Class I DSA in B cell positive T cell negative FlowXM was 4725 (S.D.890; n=4) (Figure 3.6). However, there was no significant difference between Class I DSA MFI between two groups ($p= 0.4$).

Furthermore, comparison was also made between SAB MFI for B cell positive T cell negative Class I DSA and B cell positive T cell positive Class I and Class II DSA, to

see if any difference existed. However, there was no statistically significant difference between the two sets of MFI (p=0.138).

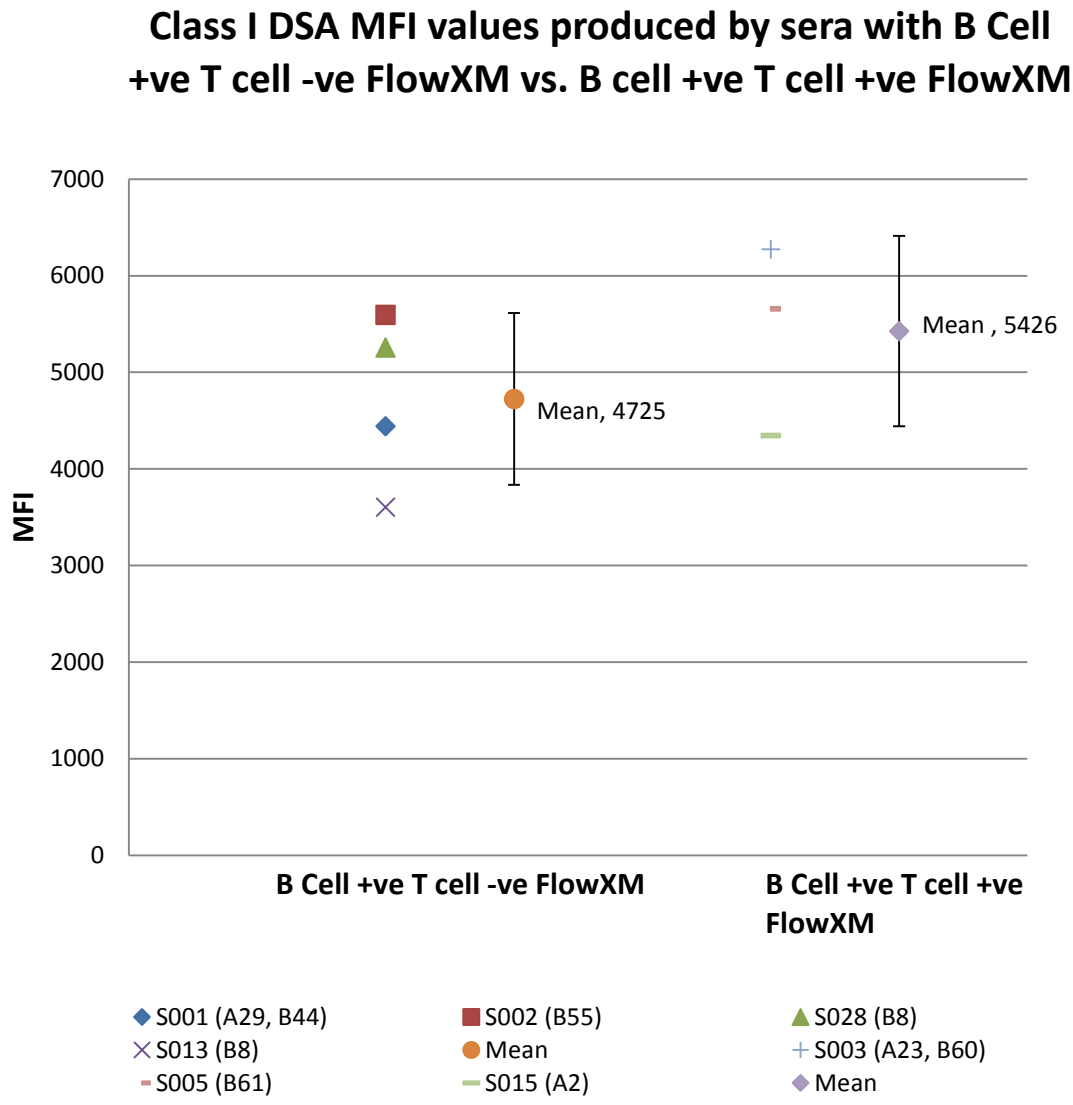


Figure 3.6: Comparison between Class I DSA MFI values produced by serum samples with B cell positive T cell positive FlowXM (n=3) and B cell positive T cell negative FlowXM (n=4). Mean MFI values and 1 S.D. error bars are also shown. No significant difference was seen between the two groups, p=0.4.

3.1.7 Establishing lower thresholds for DSA detection on Class I and Class II SAB.

A cutoff MFI on SAB in relation to XM positive quantities of DSA was also established. This was important as it provided with the lower limits for DSA detection on SAB below which sera could not be considered positive for DSA. In order establish this threshold, SAB tests were carried out on serum sample deficient in anti-HLA antibodies. Essentially, this was 'Negative control serum' which was commercially purchased for its use in SAB testing and assessing background IgG noise on the beads. Thus, results were gathered from 'negative control' experiments performed on FlowPRA Class I and Class II SAB (n= 4 to 8). Mean MFI and 3S.D. values from these experiments were calculated for each HLA bead (Table 3.5). 3S.D. represented the variation in MFI above the mean for each HLA bead in Class I and Class II groups. An average of 3S.D. values from Class I beads was calculated as 168 MFI. This value was used as an absolute cutoff below which signal on Class I bead (delta MFI) could not be considered a true positive due to presence of DSA. Similarly, a separate cutoff for Class II beads was established as 123 MFI.

Table 3.5: Mean MFI and 3S.D. values used in establishing lower thresholds for DSA detection on Class I (a) and Class II (b) SAB.

a)

Group I SAB HLA	**Mean MFI (n=8)**	**3 S.D.**
A1	1112	182
A2	284	118
A3	257	70
B49	352	114
A25	263	117
A29	234	113
A30	180	73
A26	320	327
Group II SAB HLA	**Mean MFI (n=5)**	**3 S.D.**
A68	1113	338
A11	275	181
A34	419	370
A24	419	253
A32	247	120
A33	224	150
A31	178	96
A23	239	312
Group III SAB HLA	**Mean MFI (n=7)**	**3 S.D.**
B51	1150	219
B13	287	139
B18	278	125
B35	354	99
B62	216	15
B45	205	58
B60	180	94
B44	296	370
Group IV SAB HLA	**Mean MFI (n=7)**	**3 S.D.**
B38	1136	261
B57	314	201
B07	261	117
B52	362	115
B27	240	76
B08	300	300

B65	247	166
B55	199	93
	Cutoff	**168**

b)

Group I SAB HLA	**MFI (n=9)**	**3 S.D.**
DR1.01	767	324
DR1.03	196	166
DR4.01	198	94
DR7	299	91
DR8	193	64
DR4.05	198	95
DR10	146	69
DR11	173	149
Group II SAB HLA	**MFI (n=5)**	**3 S.D.**
DR12.01	826	267
DR13.01	216	126
DR13.03	232	99
DR14.01	291	87
DR15.01	227	141
DR16.01	165	93
DR17	135	114
DR18	172	105
Group III SAB HLA	**MFI (n=4)**	**3 S.D.**
DRB5.01.01	912	321
DRB3.02.02	246	66
DRB4.01:03	167	69
DR1.02	240	101
DR4.04	189	105
DR9	229	100
DR12	143	73
DR15.02	156	77
Group IV SAB HLA	**MFI (n=4)**	**3 S.D.**
Dq2	1162	297
Dq4	253	122

Dq5	256	110
Dq6	295	58
Dq7	221	73
Dq8	234	88
Dq9	186	70
Dp	234	117
	Cutoff	**123**

3.2 Objective 2: Determining standard curve for DSA signal strength on SAB below FlowXM positive levels.

SAB signal strength that corresponds to positive FlowXM could potentially help predict a positive T or B cell FlowXM when DSA levels reach the established MFI. However, in many cases sub clinical DSA levels are present in the patients. Thus, in order to measure these subclinical DSA levels, it is important that a standard titration curve is developed on SAB that allows measuring of DSA levels below FlowXM positive levels.

In order to measure DSA levels below the FlowXM positive range, we measured SAB signal strength for DSA by further diluting the previously tested serum samples. Beginning with the highest dilution that resulted in a positive FlowXM, two fold serial dilutions were carried out; and DSA levels at each dilution point were measured using SAB. It was expected that sera will be positive for DSA in the bead assay at the starting dilution, and at several dilutions below that; this is because the SAB test is more sensitive than the FlowXM test. In cases where multiple DSA were present in the patient serum, MFI values on the beads were added at corresponding dilutions. Thus, titration curves from several different serum samples were generated and plotted on a *xy* log graph. An average titration curve for the data set was obtained by calculating average *y* values at different dilution points.

3.2.1 Standard curve for measuring DSA on SAB below B cell FlowXM positive level

In order to produce a standard curve for DSA below B cell FlowXM positive levels, fifteen serum samples that were previously used to calculate B cell FlowXM positive MFI in Objective 1 were analyzed. Using same serum dilutions tested in Objective 1 as our starting points, three more serial dilutions were carried out for each serum sample and tested using SAB. Thus, SAB results were generated for four different dilution points for majority of the samples, and titration curves were plotted (Figure 3.7); dilution data could be generated for only three dilution points for serum# S003, S007 and S015 due to technical difficulties. Majority of the titration curves show a linear decrease in DSA concentration below the FlowXM positive levels as seen in the graph. DSA curves are more closely packed together at higher serum dilutions than lower dilutions, indicating less variability. Furthermore, these curves stay above the lower threshold for DSA detection established for Class I and II SAB, indicating that signal produced on SAB at lower serum dilutions was due to presence of DSA.

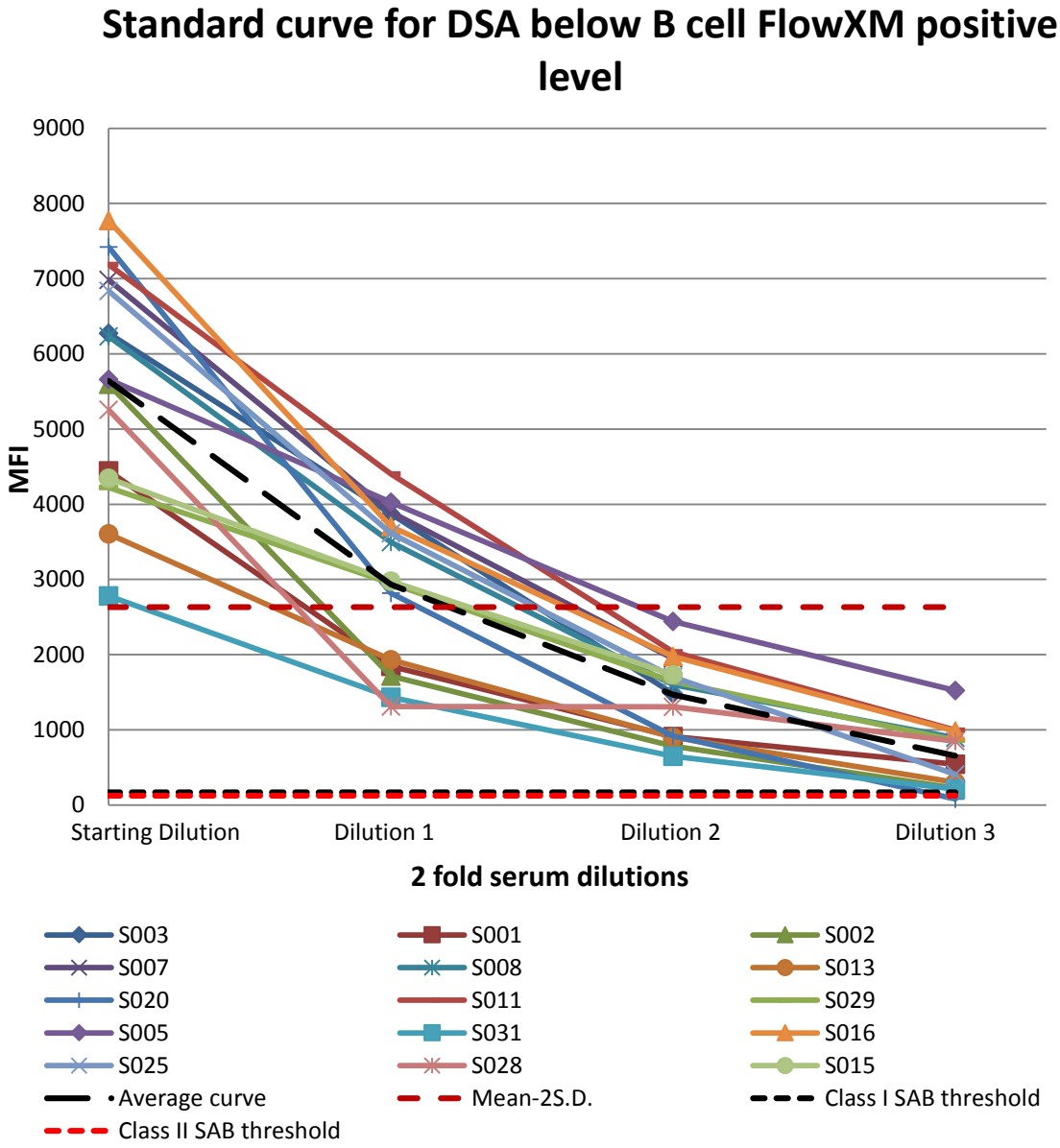
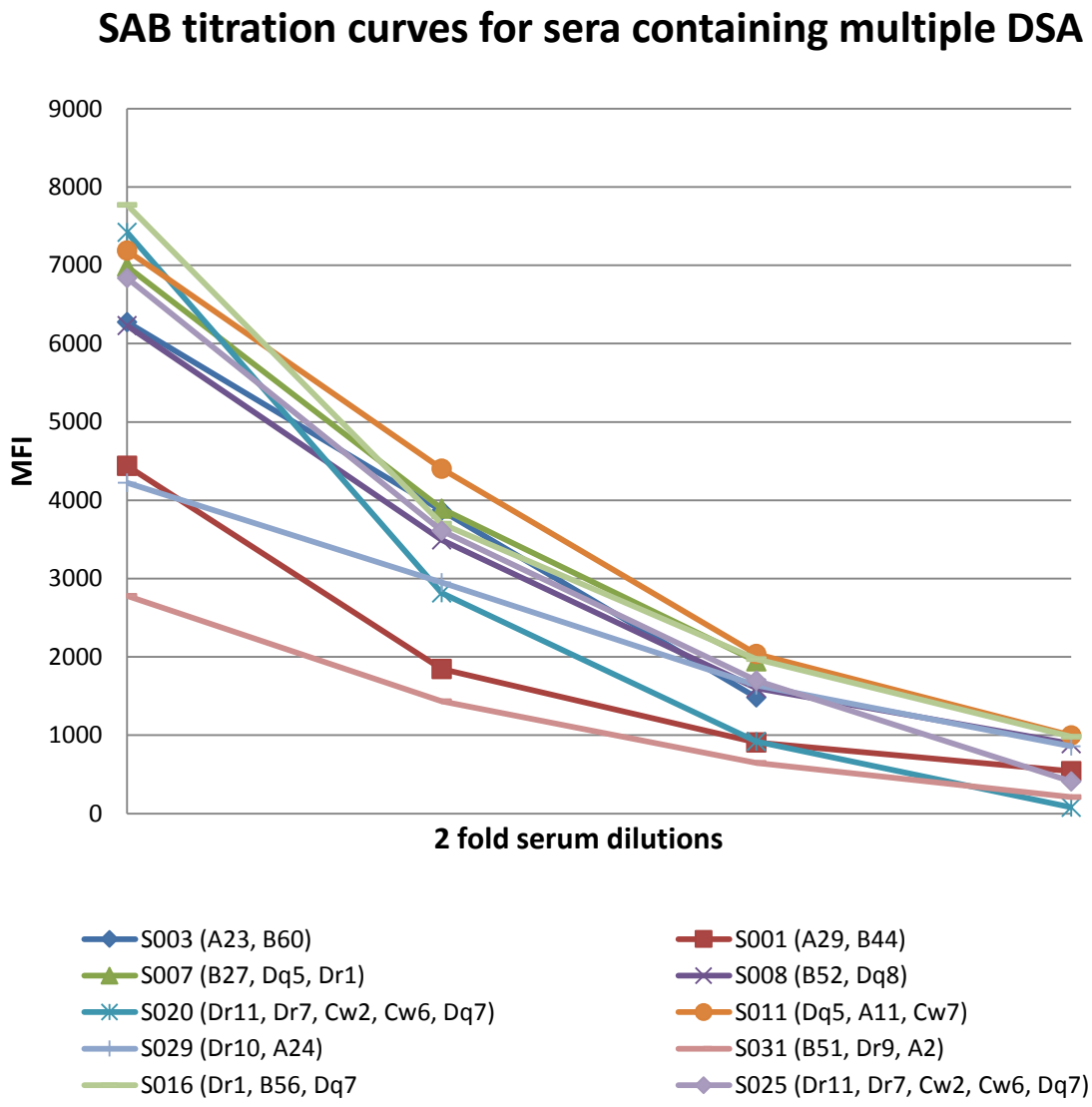


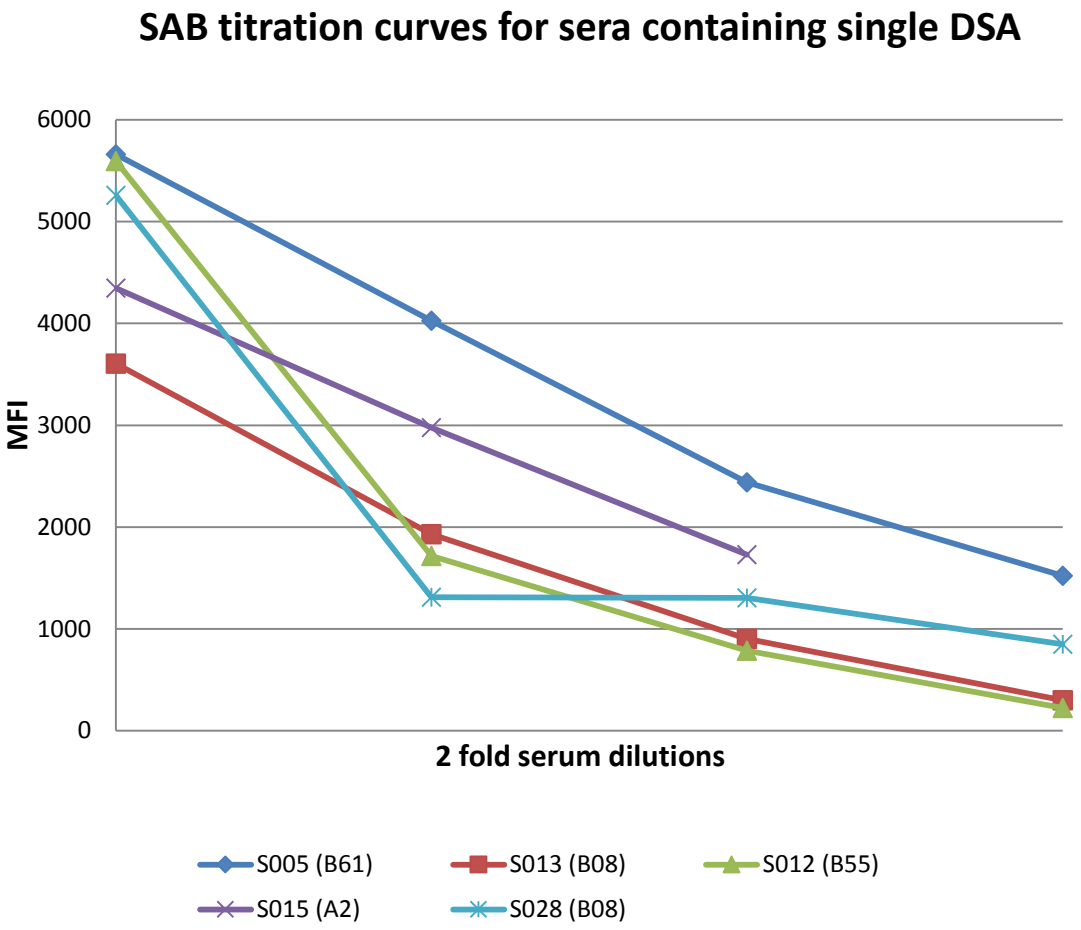
Figure 3.7: Sera titration curves on SAB below B cell positive FlowXM level. Starting serum dilution corresponds to positive FlowXM on B cells. An average curve reflective of all titration curves is also shown. Lower thresholds for DSA detection for Class I and II SAB are also plotted, indicating that the signal produced on SAB at lower serum dilutions is due to the presence of DSA.

In order to see if serum samples with single DSA produced similar titration curves as serum samples with multiple DSA, titration results for these sera were plotted separately (Figure 3.8). However, comparison between average curves of two groups could not be drawn due to very few titration curves generated on sera with single DSA. Nevertheless, majority of the sera with single DSA showed a titre effect similar to that seen in samples with multiple DSA, except for serum# S028 in single DSA group. Serum# S028 showed a very sharp decrease in MFI initially, while leveling off at lower dilutions. This could be due to presence of polyclonal DSA against more than one epitopes of the antigen. In such sera, low affinity antibody is present against an epitope of the antigen in high titre, while high affinity antibody against different epitope of the same antigen is present in low titre. Thus, high affinity antibody in low titre results in a high MFI at a higher serum concentration. Whereas, at lower serum concentration low affinity antibody present in high titre continues to persist, resulting in a low MFI value.

Figure 3.8: Comparison between SAB titration curves obtained from sera with multiple DSA (n=10) **(a)** and single DSA (n=5) **(b)**. Starting dilution corresponds to positive FlowXM on B cells. Curves show a similar titration profile.



a)


SAB titration curves for sera containing single DSA
6000
5000
4000
3000
2000
1000
0
MFI
2 fold serum dilutions
S005 (B61)
S013 (B08)
S012 (B55)
S015 (A2)
S028 (B08)

b)

To further validate that adding MFI values for low titre DSA in sera with multiple specificities was appropriate, individual titration curves obtained from DSA in these sera were also plotted (Figure 3.9). These DSA showed a similar titre effect individually, with decreasing MFI values when serial dilutions of these serum samples were carried out. Furthermore, the MFI values produced were well above the minimum threshold for DSA detection on SAB, as established in section 3.1.6. This confirmed that the very low signal produced by these DSA on SAB was not the result of any background noise on the beads and could be titrated even at very low serum concentrations.

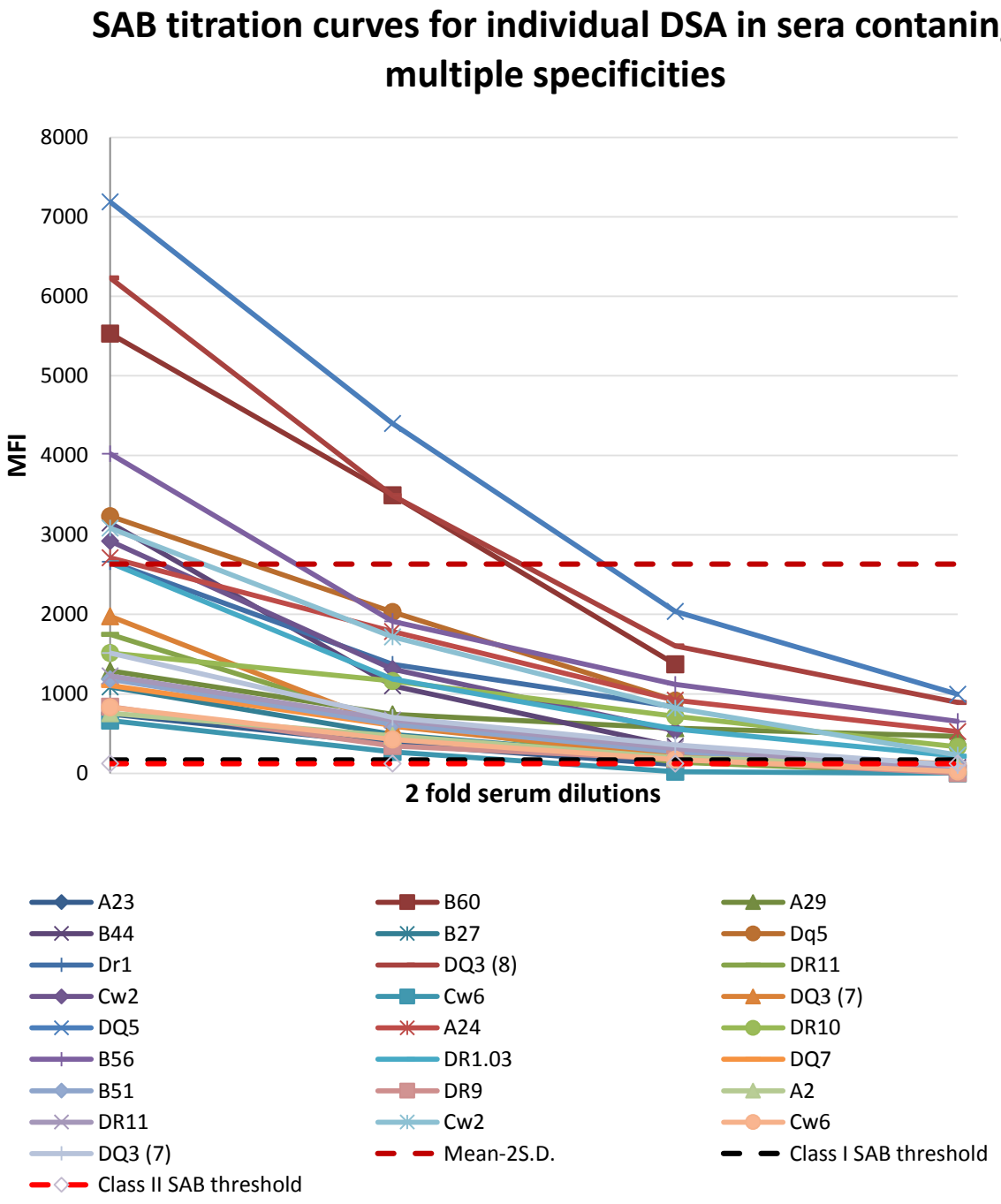


Figure 3.9: Titration curves for individual DSA in sera containing more than one specificity. Starting dilution corresponds to positive FlowXM on B cells. Lower thresholds for DSA detection by Class I and Class II SAB are also shown. Individual DSA detected by SAB are well above these thresholds, indicating SAB accurately detect low levels of DSA.

3.2.2 Comparison between titration curves obtained from sera with only Class I DSA and sera with Class I and II DSA.

In a clinical setting, many times only Class I DSA is present in the patient serum, without the additive effect of Class II DSA. For the standard curve to be used clinically, it must accurately measure anti-HLA antibody levels in patients with only Class I DSA. Since SAB titration for Class I DSA is unknown, there could be difference in dilution curves of sera with only Class I DSA. In order to see if titration curves for sera with Class I DSA below B cell positive FlowXM level differed from those with both Class I and Class II DSA, titration curves from these two groups were plotted separately and average titration curve for each group was obtained (Figure 3.10). However, no difference was seen in the titration curves generated for these two groups of sera. As seen in the figure, average titration curves were within one standard deviation of each other.

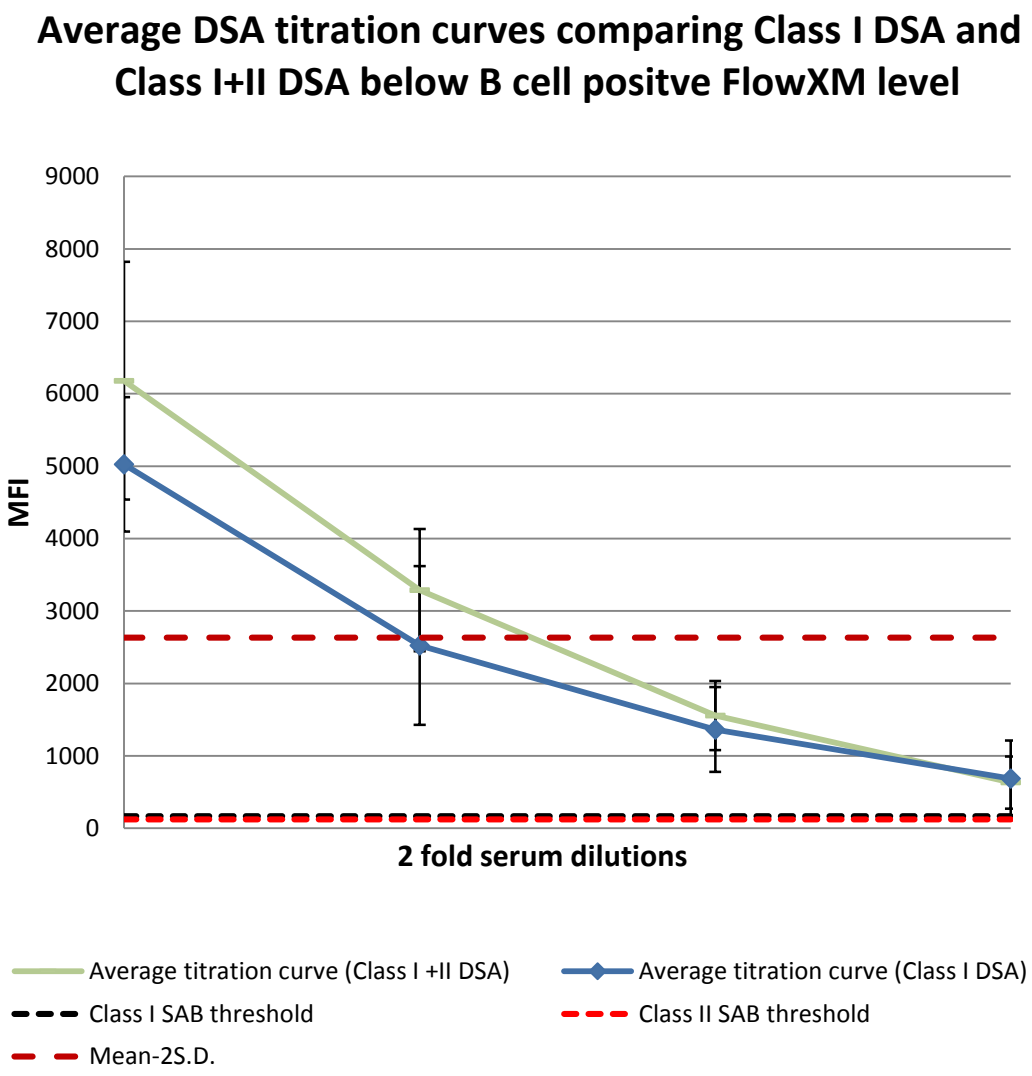


Figure 3.10: Comparison between average titration curves obtained from sera containing only Class I DSA (n=7) and sera containing Class I+II DSA (n=8) below positive B cell FlowXM. Error bars representing 1S.D. are shown for both curves at all serum dilutions. DSA detection thresholds for Class I and Class II SAB are also shown.

3.2.3 Standard curve for measuring DSA below FlowXM positive level on T cells

Since a positive T cell crossmatch predicts worse graft outcomes in kidney recipients (Rebibou et. Al. 2004), a separate standard that allows approximation of DSA in relation to T cell FlowXM is required. Thus, a separate standard curve, similar to that generated for B cells, was also produced for T cells that approximated DSA concentration below FlowXM positive levels. In order to establish this curve, serum samples that produced a positive T cell FlowXM were used. Serial dilutions were carried out for these sera, starting with the highest dilution that resulted in positive T cell FlowXM. MFI values were calculated as a sum of individual bead MFI in cases where multiple DSA were present in the serum. Thus, titration curves were obtained from twelve different serum samples and a standard curve was generated (Fig 3.11). This could help decipher Class I DSA levels below positive T cell FlowXM. Comparison between serum samples with single DSA and sera with multiple DSA could not be drawn due to very few serum samples with multiple DSA.

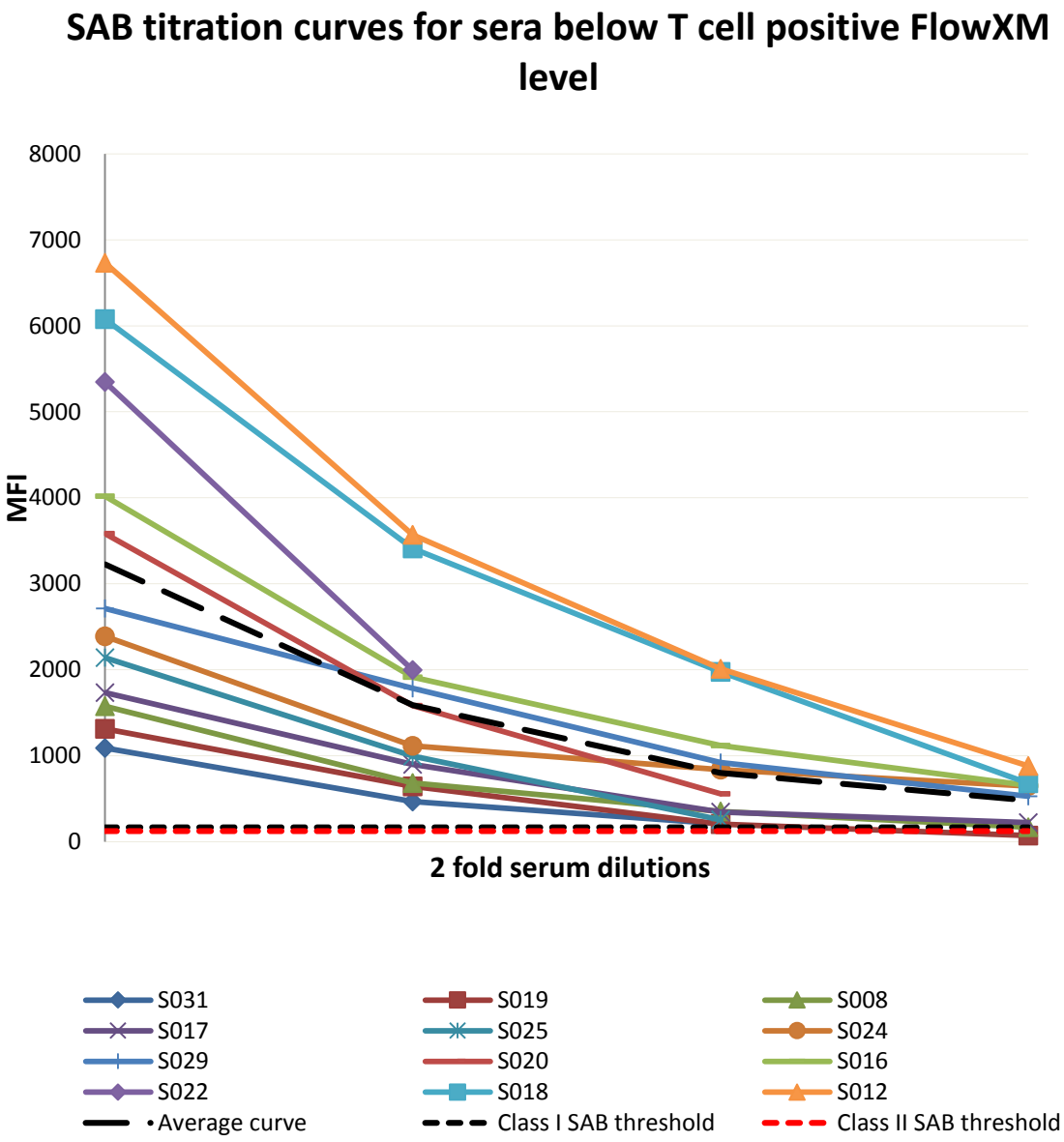


Figure 3.11: Titration curves for sera below T cell positive FlowXM level. Starting dilution represents T cell positive FlowXM. Curves for sera containing multiple DSA are shown as sum of individual DSA curves. An average curve representative of eleven different titration curves is shown. Lower thresholds for DSA detection for Class I and Class II SAB are also plotted.

3.3 Objective 3: Testing MFI cutoffs that can reliably predict positive FlowXM.

In order for SAB tests to be used clinically, it is important that these tests can reliably predict a positive XM test and adverse graft outcomes. This is possible if a MFI cutoff on SAB can be established above which DSA is likely to produce a positive FlowXM. Thus, in order to establish such cutoff, various MFI values were tested by carrying out FlowXM tests. The tested MFI values were within our range established from Objective 1as this range included 95% of all FlowXM positive DSA.

Thus, FlowXM tests were performed on serum samples that were previously used in objective 1. Since these sera also contained many other Class I and Class II anti-HLA antibodies apart from DSA (Table 2.1), it was possible to match these sera against cognate antigens on third party donor cells for which HLA typing was available. Furthermore, SAB signal strength for these other anti-HLA antibodies was also available at various dilutions. This allowed SAB titration curves, similar to those generated for DSA in objective 2, for many other anti-HLA antibodies present along with DSA. These titration curves were used to approximate serum dilution that would produce a MFI value of interest (MFI value being tested as a potential cutoff).

Thus, using various dilutions of the same serum sample different SAB MFI values could be tested on donor cells for their predictability of FlowXM. When choosing serum/cell combination all HLA present on the donor cells were used as potential targets. Thus, single as well as multiple HLA targets were available in different serum/cell combinations. In cases where multiple HLA were targeted on lymphocytes, the serum

dilution required to produce a MFI of interest was dependent on the sum of individual antibodies targeting those HLA.

3.3.1 Establishing a negative threshold for B and T cells above which a FlowXM test can be considered positive.

In order to carry out FlowXM tests and interpret results, it was essential to establish negative thresholds, for B cells and T cells, beyond which a FlowXM test could be considered positive due to presence of DSA. These negative thresholds account for the variability in background auto fluorescence of B cells and T cells and due to non-specific IgG binding. Since these values vary from lab to lab and instrument to instrument, 30 different FlowXM tests were carried out in our lab by testing healthy donor cells with 'negative control' serum. Thus, MFI values were obtained from these tests and negative thresholds were calculated as Mean MFI+ 2S.D (Table 3.6). These thresholds were 2061 MFI for B cells and 237 MFI for T cells.

Table 3.6: MFI values on B cells and T cells obtained from n=29 negative FlowXM. Mean+2S.D. were used to calculate thresholds beyond which FlowXM on B cells and T cells were considered positive.

Sample #	B Cell MFI	T Cell MFI
D1	1786	170
D12	834	117
D13	1800	170
D14	1426	142
D2	1198	145
D15	1115	285
D3	536	252
D16	931	177
D4	1300	139
D5	460	204
D17	926	116
D18	806	140
D19	626	153
D6	1009	198
D20	1395	197
D21	2017	146
D22	575	130
D7	639	170
D23	2052	138
D24	864	140
D8	1037	172
D25	1206	117
D26	2030	136
D9	1050	125
D27	422	133
D10	879	123
D28	1349	109
D29	775	131
D11	438	108
Mean	1086	155
S.D.	488	41
Mean+2S.D.	2061	237

3.3.2 Establishing a MFI cutoff that predicts B cell positive FlowXM.

SAB MFI correlate for B cell positive FlowXM established in Objective 1 ranged from 8650 MFI (mean+2S.D.) to 2632 (mean-2S.D.), with a mean MFI of 5641. Since this range represented 95% of B cell positive FlowXM DSA, it was hypothesized that MFI values within this range would be suitable in achieving a SAB cutoff that would predict positive B cell FlowXM with high sensitivity. Thus, SAB MFI values of 3000, 6000 and 9000 were used as potential cutoffs in order to test their sensitivity in predicting a positive FlowXM. Serum dilutions that corresponded to these MFI values were used in performing FlowXM tests.

Fifty eight FlowXM tests using thirteen different sera were performed. These sera were tested against lymphocytes from eleven different donors, for whom HLA typing was available. Class I or Class II anti-HLA antibodies or a combination of two were used to target HLA on the donor cells. Detailed characteristics of the serum samples used and antigens targeted by anti-HLA antibodies are presented in Table 3.7. Five FlowXM tests were performed with sera indicating 9000 MFI on SAB, eleven FlowXM tests were carried out in 6000 range and eighteen FlowXM tested 3000 MFI on SAB. FlowXM tests were also carried out on serum dilutions that indicated SAB MFI of less than 3000 on the titration curves.

Results for B cell FlowXM from these tests are detailed in Table 3.7 FlowXM tests on B cells were considered positive if the resulting MFI on B cells was greater than the negative cutoff value of 2061 MFI, established in section 3.3.1. Repeat FlowXM

testing for some samples was also performed where possible. This was used to confirm FlowXM tests in majority of the cases, except one. FlowXM test performed on Serum# S020 against donor# D1 produced a weak positive result for one of the samples whereas the second sample produced a negative FlowXM. Since the MFI value for the positive sample was not significantly higher than the established negative cutoff, this FlowXM test was considered negative overall. The results showed that 40% of the FlowXM tests were B cell positive at 9000 SAB MFI; whereas 18% and 11% were B cell positive at 6000 and 3000 MFI, respectively. Below 3000 MFI, 13% of the tests were B cell positive. SAB MFI value of 9000 was most sensitive in predicting a positive FlowXM on B cells.

Table 3.7: FlowXM tests performed using various combinations of donor cells and patient sera to test sensitivity of SAB MFI of 9000, 6000 and 3000 in predicting positive FlowXM. Serum anti-HLA antibodies targeting antigens on donor cell surfaces are also listed. Test results are shown as MFI values produced on B cells when anti-HLA antibodies react against donor cells. Higher MFI values on B cells represent greater amount of antibodies reacting against donor cells. Tests producing MFI values of greater than 2061 were considered positive. SAB MFI of 9000 had the highest sensitivity (40%) in predicting a positive FlowXM on B cells.

SAB MFI range			**9000**	**6000**	**3000**	**<3000**
Donor #	Serum #	HLA target for ab.	B cell FlowXM MFI	B cell FlowXM MFI	B cell FlowXM MFI	B cell FlowXM MFI
D1	S008	Dq8		5359/6047	2301/3543	3119
D1	S020	Dq8				2372/1546
D1	S031	B35				1216
D2	S003	B7, B44, A2	2715			
D3	S013	B8			609/1004	
D3	S015	A2			934/984	564/609
D4	S001	B44, B51	3191	1924	1435	1398
D4	S011	Dq5, Cw7		759	630	699
D5	S003	B51, B60	740	721	504	395
D6	S013	B8			661	477
D6	S028	B8		772	480	527
D7	S008	Dq8		13600	11000	8938
D7	S020	Dq8				957
D7	S015	A2			552	403
D8	S003	A2, A3			1449/1155	1012/950
D8	S016	Dq7				751/749
D8	S007	Dq6				727/517
D8	S022	A3			788/643	683/473
D9	S001	A26, B49, DR52		1046	979	629
D9	S003	B49		1711	1021	730
D10	S003	B52				828
D10	S007	Dq5			1141	1072
D11	S007	Dq6				320
D11	S003	B45,B51, Dr 53	523	363	375	304
D11	S001	A29, B45, B51, Dr15	797	474	508	418
D11	S029	A31		818	515	442
#of total FlowXM tests			5	11	18	24
# of +ve FlowXM tests			2	2	2	3

3.3.3 Establishing a MFI cutoff that predicts T cell positive FlowXM.

In order to establish a similar MFI cutoff that predicts T cell positive FlowXM with certainty, serum dilutions with MFI values of 9000, 6000 and 3000 were used. Single HLA as well as multiple HLA on donor cells were used as targets. The anti-HLA antibodies belonged to HLA-A or HLA-B or both.

Thus, forty one different FlowXM tests were carried out using various dilutions from nine different serum samples. Five FlowXM were performed to test the predictability of 9000 MFI, six FlowXM were performed in the 6000 range and fourteen FlowXM were used to test MFI value of 3000 on SAB. FlowXM tests were considered T cell positive if the resulting MFI values were greater than the negative threshold of 237 MFI, established for T cell FlowXM. Results obtained from these FlowXM tests are presented in Table 3.8. At 9000 MFI, 100% of the FlowXM tests were T cell positive. At 6000 MFI only 33% tests produced a positive result, whereas at 3000 MFI 64% were T cell FlowXM positive. Below 3000 SAB MFI, only 25% of the serum samples resulted in a positive FlowXM on T cells. Furthermore, many of the serum samples that produced a positive FlowXM at 3000 MFI, showed a negative result when dilutions corresponding to a lower SAB MFI were tested. These results indicate that for sera resulting in MFI values of greater than 3000 on SAB, there is a significantly higher risk of positive FlowXM on T cells (64% for MFI >3000 vs. 25% for MFI <3000).

SAB MFI range			9000	6000	3000	<3000
Donor #	Serum #	HLA target for ab.	T cell FlowXM MFI	T cell FlowXM MFI	T cell FlowXM MFI	T cell FlowXM MFI
D1	S031	B35				328
D2	S003	B7, B44, A2	544			
D3	S013	B8			239/744	
D3	S015	A2			394/568	278/304
D4	S001	B44, B51	300	226	170	143
D4	S011	Cw7				139
D5	S003	B51, B60	635	606	360	253
D6	S013	B8			278	215
D6	S028	B8		180	163	199
D7	S015	A2			312	206
D8	S003	A2, A3			632/555	388/356
D8	S022	A3			302/202	219/177
D9	S001	A26, B49			297	140
D9	S003	B49		620	253	184
D10	S003	B52				139
D11	S003	B45,B51	243	164	176	118
D11	S001	A29, B45, B51	250	146	178	131
D11	S029	A31			105	99.9
#of total FlowXM tests			5	6	14	16
# of +ve FlowXM tests			5	2	9	4

Table 3.8: T cell FlowXM tests performed to test sensitivity of SAB MFI of 9000, 6000 and 3000 in predicting a positive FlowXM. Antigen targets for anti-HLA antibody binding for different donor cells are also listed. FlowXM results are shown as MFI values produced on T cells. Higher MFI values represent greater amount of antibodies reacting against donor T cells. Tests were considered positive if T cell MFI was greater than 237. SAB MFI of 9000 had the highest sensitivity in predicting a positive FlowXM on T cells; sera indicating >3000 MFI on SAB generated higher frequency of T cell positive FlowXM (64%) compared to sera <3000 SAB MFI (25%).

CHAPTER 4- DISCUSSION

4.1 MFI range on SAB that correlates to positive FlowXM on B cells and T cells

SAB provide with a powerful tool to detect low levels of DSA that might otherwise go undetected by cell based techniques such as FlowXM or CDCXM. Because of their high sensitivity and specificity, these assays are being increasingly used in the transplant clinics. However, in the present setting, the use of these assays is limited to mainly as a tool for detecting anti-HLA antibody specificities in the patient serum. One of the major barriers that prevent the use of SAB as a predictor of graft damage is the uncertainty about low levels of DSA measured by these beads. It has been argued that SAB might be overly sensitive in measuring low levels of DSA that might not otherwise be disadvantageous to the graft outcome. Thus, for these beads to be used clinically, it becomes imperative that a MFI range on SAB is established that approximates the level of DSA required to cause AMR, or cause injury to graft structure and function.

In this study, a correlate that predicts graft damage with high sensitivity was used in order to establish such MFI range on SAB. FlowXM is the current clinical standard for crossmatch testing; and a positive FlowXM test is considered as a strong predictor of early graft damage as well as late graft loss post-transplant (Graff et. Al. 2009, Ocura et. Al. 1993). In addition, many studies have shown that DSA detected by positive FlowXM indicates poor graft outcomes, even in the presence of a negative CDCXM (Ilham et. Al. 2008, Karpinsky et. Al. 2001, Mahony et. Al. 1990). Thus, pre-transplant sera from variably sensitized patients that produced a positive B and/or T cell FlowXM were tested against FlowPRA SAB. Mean MFI values of 5641 (range 2780-7772) and 3226 (1089-

6731) were established as correlates to positive FlowXM on B cells and T cells, respectively. Mean+/-2S.D. (95% C.I.) values were also calculated that dictate MFI values produced by majority of the FlowXM positive DSA on SAB.

Previously, many studies have attempted to establish a similar range on SAB that correlates to a positive B cell or T cell XM, but the experimental approaches have been different among various groups resulting in a lack of consensus. Batal et. al. (2010) used neat patient serum to establish MFI value of 7810 (S.D.5400) on Luminex SAB as a correlate to positive T cell CDCXM. This value is much higher than the T cell correlate established in the current study as Batal et. al. used CDC positive XM, which is a much less sensitive method of DSA detection than FlowXM. Zachary et. al. (2009) found DSA MFI values of 6000 and 10000 on Luminex SAB as good predictors of positive FlowXM and CDCXM, respectively. Similarly, Moreno et. al. (2012) defined MFI value of 6500 on Luminex SAB as a cutoff that predicted positive T cell FlowXM. However, these previous studies used neat serum samples that produced a positive XM in order to establish such MFI values on SAB, unlike the current study which used the highest serum dilution that was FlowXM positive. Testing the highest serum dilution that results in a positive XM is important when samples from variably sensitized patients are considered. For example, if serum sample form a highly sensitized patient with a greater DSA titre is tested on SAB, it would invariably result in a higher MFI signal due to larger amounts of DSA binding to the beads. Conversely, if serum sample from a low sensitized patient with low amounts DSA is tested on SAB, such sample would produce a smaller signal on the bead. Thus, it is very important that serum dilutions that contain DSA concentrations that

are just above the level required to produce a positive XM test are used. This ensures that equal concentrations of DSA are tested on SAB regardless of patient sensitization history. Thus, MFI ranges established using diluted sera are very accurate without large variability in the results.

Even though the clinical relevance of positive B cell XM in transplantation is controversial, several studies have shown decreased graft function or graft loss associated to positive B cell XM (Eng et. Al. 2008, Kotb et. Al. 1999, Song et. Al. 2012). Furthermore, studies have shown higher prevalence of AMR (Pollinger et. Al. 2007) and increased incidence of transplant glomerulopathy (Issa et. Al. 2008, Eng et. Al. 2009) when DSA against Class II HLA are present in the patient serum. This indicates that accurately measuring Class II DSA along with Class I DSA might be important pre-transplant as well as post-transplant when considering long term graft survival. In the current study, it has been successfully shown that a MFI range on SAB can be established that correlates to a positive FlowXM on B cells. A positive FlowXM on B cell could occur due to presence of either Class I or Class II DSA, or a combined effect of both Class I and Class II DSA present in the serum sample. Thus, it is also important to see if differences in SAB MFI values could exist for these three different groups. When comparison was drawn between SAB MFI values produced by sera with only Class I DSA and Class I+II DSA, our results showed no statistically significant difference in these DSA MFI values. This indicates that the established MFI range of 2780- 7772 predicts a positive B cell FlowXM independent of the serum sample containing Class I or Class I+II DSA. In addition, it confirms that DSA from Class I and Class II can be added

on SAB to establish FlowXM positive range. However, a similar comparison could not be drawn for sera with only Class II DSA due to lack of serum samples.

Furthermore, this study compared the role of Class I DSA in B cell positive T cell negative FlowXM and B cell positive T cell positive FlowXM. Many studies have shown mixed graft outcomes when XM was positive on B cells but negative on T cells (Lobashevsky et. Al. 2000, Eng et. Al. 2008, Goh et. Al. 2012). Thus, a B cell positive T cell negative XM might not always be considered a contraindication to kidney transplantation. Furthermore, higher expression of Class I HLA on B lymphocytes exists; which makes B cell XM more sensitive to Class I DSA than a T cell XM. Therefore, low levels of DSA that result in a positive XM on B cells might not be enough to produce a positive XM on T cells as higher levels of Class I DSA would be required to produce a positive XM on both B cells and T cells. Thus, SAB MFI values for DSA in sera from these two groups might also vary. However, when MFI values from these two serum groups were compared, no statistically significant difference was seen in Class I DSA. This is important clinically as it indicates that the SAB MFI range established in this study predicts a positive B cell FlowXM independent of a T cell FlowXM when only Class I DSA is present in the patient serum.

4.2 Significance of the established FlowXM positive range on SAB

The SAB range established in this study is important as it predicts DSA MFI values that will result in a positive FlowXM on B cell or T cells. Since a positive FlowXM is a good predictor of graft rejection, it is expected that DSA concentrations that produce MFI values within the established range will inevitably prove harmful to the graft. Thus, by measuring serum DSA levels using SAB, it can be possible to identify high risk patients pre- as well as post-transplant.

Measuring DSA on SAB pre-transplantation can be helpful in stratifying patients based on their risk of developing AMR (Gloor et. Al. 2010). SAB allow accurate measurement of serum DSA levels in the patients. It allows quantification of DSA titre based on the SAB MFI values produced. Thus, it is possible to stratify potential recipients using their serum MFI values based on their risk of developing AMR. This can be useful in identifying low risk patients that can potentially be transplanted using aggressive immunosuppressive therapies. In addition, SAB measure of DSA can be useful in performing 'virtual crossmatch' on B and T cells. The benefits of virtual crossmatch have previously been shown in pediatrics heart recipients (Zangwill et. Al. 2007) as well as in kidney recipients (Bielmann et. Al. 2007). Using 'virtual crossmatch' patient DSA that produces MFI values on SAB within the FlowXM positive range can be ruled out as unacceptable DSA; this can help determine acceptable donor-recipient pairs. Using virtual crossmatch can also help save valuable time spent performing actual XM, which is especially crucial in a deceased donor setting. Many studies have shown a link between

increased cold ischemia time and delayed graft function (Doshi et. Al. 2011, Kayler et. Al. 2011) as well as increased rate of chronic graft rejection (Giblin et. Al. 2005, Salahudeen et. Al. 2004).

Post-transplant, many kidney recipients develop de-novo anti-HLA antibodies or up-regulate DSA production due to potential memory mechanisms. Studies have shown increased graft dysfunction or graft loss in patients that developed low levels of DSA post-transplant which were absent before transplantation (Zhang et. Al. 2005, Piazza et. Al. 2006). Furthermore, it has been shown that anti-HLA antibodies in the patient sera precede chronic graft rejection (Lee et. Al. 2002); and presence of DSA detectable by SAB is significantly associated to decreased long term graft survival (Mao et. Al. 2007, Kimall et. Al. 2011, Lee et. Al. 2009). These studies indicate that routine patient monitoring and follow-up is required to check for early signs of graft rejection or development of DSA immediately following transplantation. However, traditional markers such as C4d deposition used to test the stability of graft might not necessarily detect for the presence of low level DSA. Conventional biopsy findings and testing serum creatinine levels can also overlook the presence of DSA that might not pose any immediate danger to the graft, but would require months or even years before vascular changes that result in graft failure are seen. These traditional markers test for graft dysfunction and markers that only become evident after substantial damage to the graft has already occurred.

Using SAB, it is possible to effectively monitor patients post-transplant and detect DSA before any physical damage to the graft occurs. This can help clinicians identify

high risk patients whose DSA MFI values are within the established FlowXM positive range. Decisions regarding early immunosuppressive intervention can be made in these patients in order to avoid any adverse graft outcomes. In addition, using the standard curves developed in this study, it can be possible to decipher low levels of DSA in relation to antibody concentration required to have a negative graft outcome (FlowXM positive level). By comparing DSA levels in patient serum to that of the standard curve, inferences such as patient serum is ½ the level required to produce a positive FlowXM can be drawn. This can provide better indication of the patient DSA status. In addition to predicting AMR, DSA monitoring using SAB can also help clinicians tailor patient specific immunosuppressive therapy needs; this might be important in prolonging graft survival as previously shown in small bowel and multivisceral transplantation (Tsai et. Al. 2011).

4.3 SAB MFI produced by sera containing single DSA vs. multiple DSA

Current literature lacks much evidence on the role of multiple DSA detected by solid phase assays, and whether adding individual DSA MFI values is an appropriate interpretation when considering total MFI of the serum. Previous work by Warner et. al. showed that when considering cell based assays such as FlowXM tests, presence of multiple DSA produced an additive effect on donor cells; however, this effect was not additive in a mathematical sense (2009). In order to verify these findings and test whether adding individual bead MFI values in sera with multiple specificities is appropriate, MFI values obtained from sera with single DSA were compared against the total MFI of sera with multiple DSA. It was expected that sera with multiple DSA and single DSA would produce very similar MFI values since titred serum samples were used. The results showed that even though the sera with multiple DSA had a higher total mean MFI value than sera with single DSA, there was no significant difference when MFI values from these two groups were compared using Mann-Whitney U test. This indicates that SAB MFI values from up to a total of five DSA can be added to reflect the total MFI of the serum sample. Thus, the B cell range established in the current study can predict a positive FlowXM for sera containing single as well as multiple DSA.

However, it is worth noting that within the multiple DSA group, higher total MFI values were associated to serum samples containing larger number of DSA (3 to 5 different DSA) and DSA against Dq antigens. Lower number of DSA were associated to lower total MFI values; and these values were much closer to MFI produced by sera with

single DSA. This confirms Warner et al's (2009) findings that when considering total MFI value of the serum, multiple DSA detected by single phase assays might not be additive in a mathematical sense even though there is a combined effect of all DSA. In addition, it has been proposed that Dq antigens on SAB might react with DSA with greater strength (Zachary et. Al. 2009). This might result in greater MFI value being produced by SAB for DSA against Dq antigens.

It is also not clear whether MFI values produced by a polyclonal antibody that binds different epitopes of the same allele on SAB should be added together, or the highest MFI value produced should be considered reflective of the total serum activity (Zachary et. Al. 2009). For instance, a Bw antibody that binds to different HLA- B alleles could react to multiple SAB coated with HLA-B antigens. This could result in a reduced signal due to spreading out of antibody on a larger number of beads, whereas if only one SAB was targeted by the antibody it would result in a higher signal. Even though this is a very important issue that needs to be addressed in order to correctly utilize SAB in XM prediction, this was beyond the scope of the current study. Larger studies with a greater sample size are required before appropriate conclusions can be drawn.

4.4 Differences in HLA density and high signal produced by some sera on SAB

The unusually high bead MFI produced by the three serum samples also warrants a large scale serum analyses in order to see if particular antigens from Class 1 and Class II MHC frequently result in a higher signal on SAB. It has been previously shown that significant differences in densities of various HLA coating SAB exists (Warner et. Al. 2009); this has been confirmed by our SAB density experiments. To see if difference in HLA density could potentially contribute to difference in bead signal produced by DSA, coefficient of correlation was calculated between DSA MFI and bead MESF units (Figure 4.1). However, no correlation between HLA density and DSA MFI was evident for various SAB ($R^2=0.0278$). This indicated that differences in HLA density on SAB did not affect MFI values produced by DSA targeting these antigens. This was because various serum dilutions that were tested on SAB had similar concentration of DSA required to produce a positive FlowXM, independent of their neat DSA titre. If DSA concentration in the serum samples were not equivalent or HLA density on SAB played a role in producing DSA MFI, MFI values would have a strong correlation to HLA density on SAB.

One explanation for high bead MFI produced by certain sera could be the presence of antibodies against ‘cryptic epitopes' on the beads. It has been argued that the manufacturing process of SAB can potentially alter the structure of HLA antigens, making ‘cryptic epitopes’ on certain antigens more readily available for antibody binding (Bray et. Al. 2004, El-Awar et. Al. 2009). Furthermore, it has been shown that natural antibodies against these ‘cryptic epitopes’ on certain HLA antigens exist in the normal

populations (El-Awar et. Al. 2009). Thus, it is possible that non-specific IgG binding to these 'cryptic' HLA antigens could have occurred, resulting in a higher than normal signal on SAB. Another possible explanation questions the results of FlowXM tests for these three serum samples. It is also possible that during XM testing serum factors might have hindered DSA binding to antigens on cell surface, resulting in a weaker FlowXM. However, similar interference was absent when testing with SAB due to inherent differences between beads and cells.

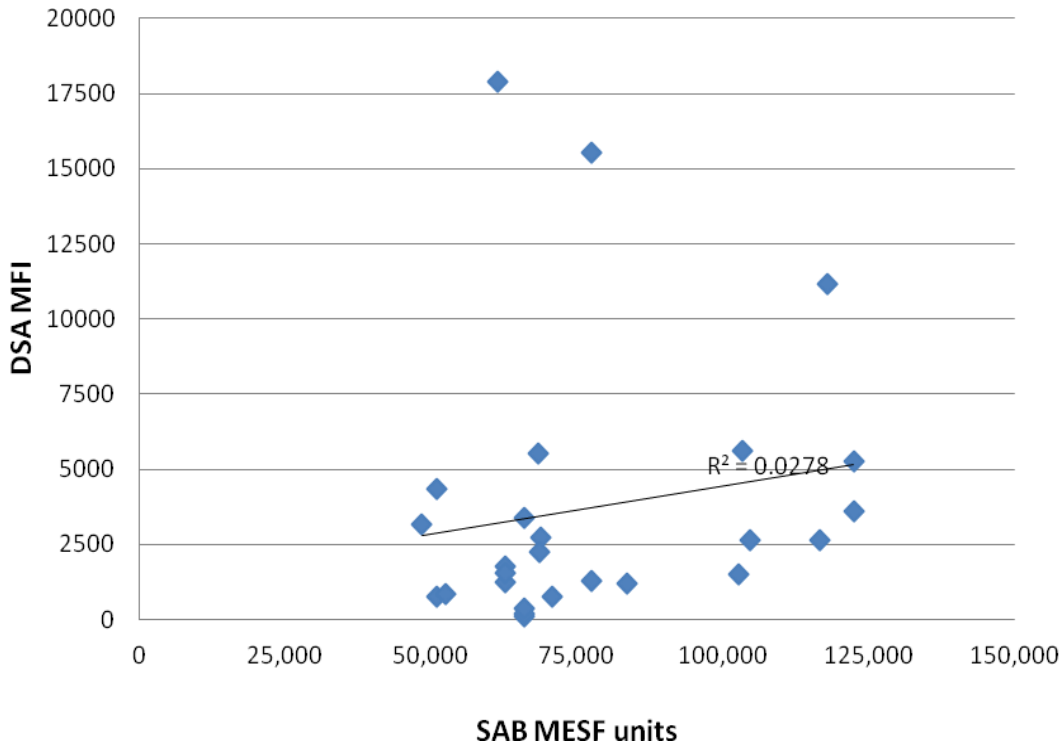


Figure 4.1: SAB MFI produced by various DSA and SAB MESF values obtained for the cognate antigens. No correlation was seen between DSA MFI and HLA density on SAB.

4.5 SAB MFI cutoffs used to predict B and T cell FlowXM

In order to test the predictability of SAB range established in the current study, FlowXM tests on B cells and T cells were also carried out. Since greater DSA concentrations were more likely to produce a positive XM than low levels of DSA, it was important to test different MFI values within the established range. These MFI values were chosen based on our results from Objective 1 which showed that 95% of DSA with positive FlowXM produced MFI values within 3000-9000 range. Thus, serum samples with SAB MFI values of 3000, 6000 and 9000, reflecting varying antibody concentrations were used to perform FlowXM on third party donor cells. As expected, higher MFI values correlated to a higher percentage of positive FlowXM on B cells and T cells (Figure 4.2). More importantly, serum MFI of >3000 produced a significant higher number of positive T cell FlowXM compared to sera <3000 MFI (64% at >3000 MFI vs. 25% at <3000 MFI, p=0.025). This value, which is much lower than the previously established Luminex MFI cutoffs of 6000 (Zachary et. al. 2009) and 6500 (Morino et. al. 2012), can serve as a potential cutoff beyond which DSA is expected to result in a significantly higher number of positive FlowXM.

In addition, positive predictive value (PPV) and negative predictive value (NPV) of 3000 MFI in predicting B cell and T cell FlowXM was also calculated (Table 3.9). PPV was calculated as:

$$PPV = \frac{\text{Number of true positives}}{\text{Number of true positives + Number of false positives}}$$

NPV was calculated as:

$$PPV = \frac{\text{Number of true positives}}{\text{Number of true positives + Number of false positives}}$$

PPV was calculated as 67% for B cells and 80% for T cells when MFI cutoff 3000 was used in predicting cross match. This indicates lower false positives for T cell test compared to B cell XM. NPV was calculated to be 75% for B cells and 57% for T cells, indicating higher false negatives for T cell tests. In addition, sensitivity and specificity of 3000 MFI cutoff in predicting B cell and T cell FlowXM test was also calculated. However, the established cutoff was not very sensitive in predicting either a T cell or a B cell FlowXM. Whereas, specificity was calculated at 87% for B cells and 75% for T cells, indicating that it is possible to achieve a high rate in predicting true negatives, when the established cutoff of 3000 MFI is applied. The lower sensitivity of crossmatch prediction by SAB could be due to several limiting factors as discussed in the following section.

A similar cutoff MFI that resulted in significantly higher positive FlowXM on B cells could not be established. Even though SAB MFI value of 9000 resulted in a higher percentage of positive B cell tests (40% positive tests) compared to serum samples with MFI values of below 9000 (13% positive tests), this difference was not statistically significant ($p>0.05$). This could be because B cell FlowXM test is more prone to producing a false negative FlowXM due to the issues discussed previously in section

3.1.1. Furthermore, there is an overlap between MFI values produced by FlowXM positive sera and FlowXM negative sera, especially at the lower thresholds. Thus, the sensitivity of the cutoff in predicting positive FlowXM might be compromised. Another possible explanation for the large number of unexpected negative FlowXM tests could be the low quality of the samples used in performing these tests as discussed below.

Table 3.9: Contingency tables showing FlowXM test results for a) B cells and b) T cells. MFI cutoff of 3000 had a PPV of 67% and 80% for B cells and T cells, respectively; NPV was calculated to be 75% and 57% for B cells and T cells, respectively. The established cutoff was not very sensitive in predicting positive FlowXM when it was applied to B cell tests. However, this cutoff was much more sensitive in predicting positive T cell FlowXM tests.

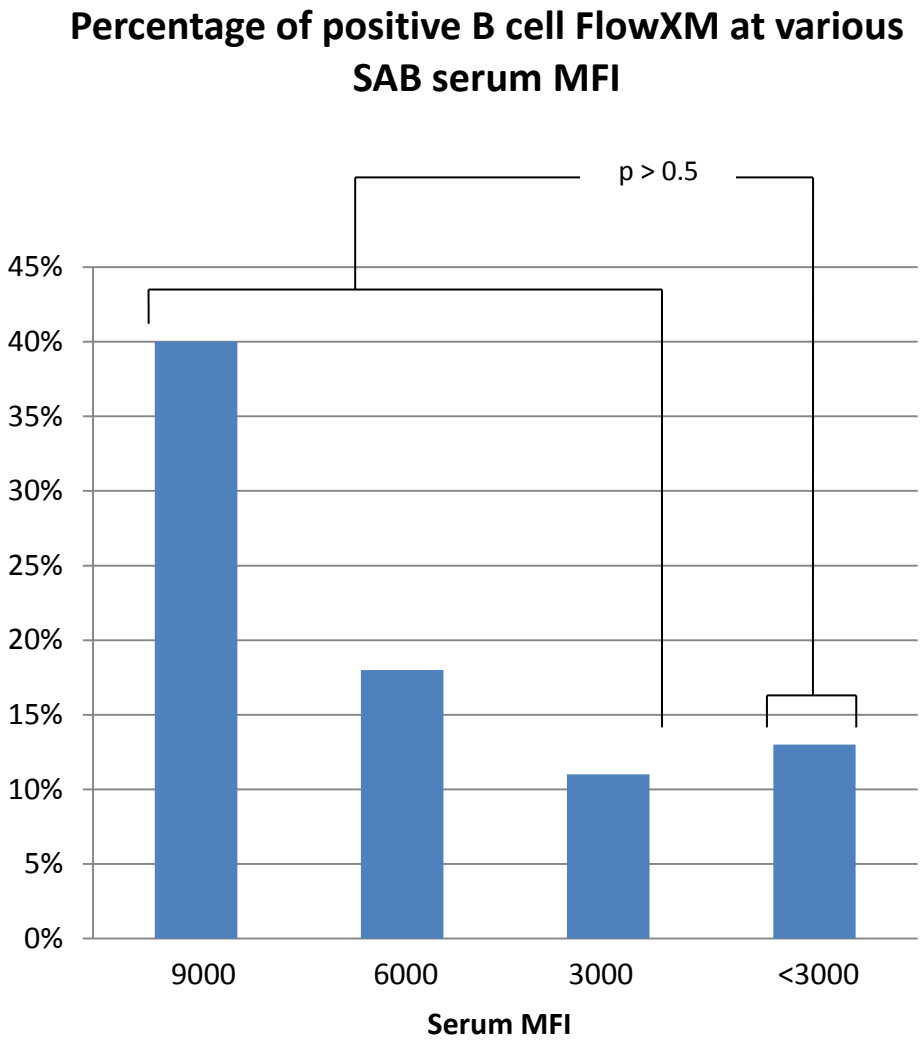
a)

	DSA MFI >3000	**DSA MFI <3000**
FlowXM +ve	6	3
FlowXM -ve	28	21
	PPV	6/9= 0.67
	NPV	21/28= 0.75
	Sensitivity	6/34= 0.18
	Specificity	21/24= 0.87

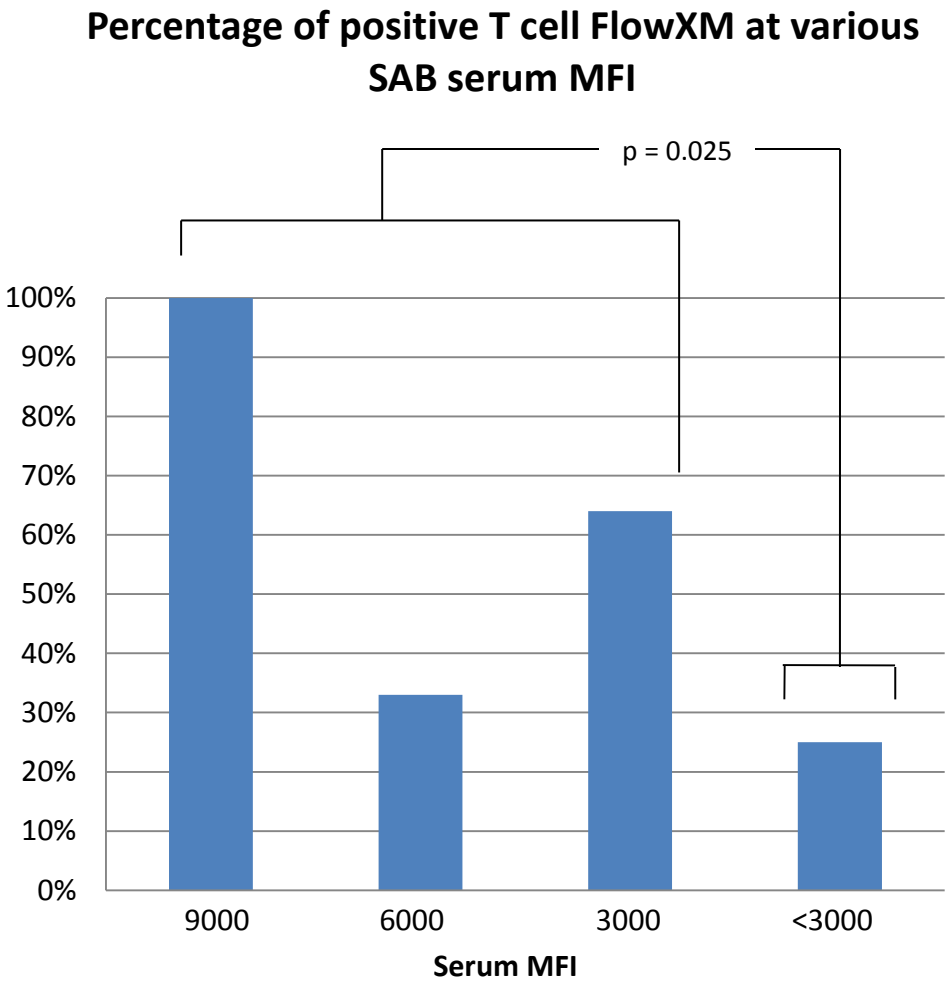
b)

	DSA MFI >3000	**DSA MFI <3000**
FlowXM +ve	16	4
FlowXM -ve	9	12
	PPV	16/20= 0.80
	NPV	12/21= 0.57
	Sensitivity	16/25= 0.64
	Specificity	12/16= 0.75

Figure 4.2: Percentage of positive (a) B cell and (b) T cell FlowXM at estimated DSA MFI values from cutoff sensitivity testing. Higher DSA MFI values resulted in higher percentage of positive FlowXM tests.



a)


Percentage of positive T cell FlowXM at various SAB serum MFI
p = 0.025
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
9000
6000
3000
<3000
Serum MFI

b)

4.6 Study drawbacks and issues with the results

As with any work, this study also carried some inevitable pitfalls. This study was limited in its scope due to the small number of serum samples analyzed using SAB. This was due to the fact that FlowXM titration test results were available for only a limited number of serum samples as these tests are not routinely performed on every serum sample in the Histocompatability lab. Therefore, this study failed to address whether any differences between MFI values from sera with single or multiple DSA exist on T cells.

Furthermore, the study used historic serum samples which were collected from patients from up to three years prior to beginning of this study and underwent multiple freeze-thaw cycles. Since, SAB MFI values for these sera were not available form when they were collected fresh, it was not possible to compare the results obtained three years later. Similarly, no repeat crossmatch on the same donors were available to compare the reactivity of the stored sera to XM results available from when it was tested fresh. Even though these samples were stored at -80 degree Celsius, the quality of the used samples could not be guaranteed.

Many technical challenges in the use of SAB tests also exist. SAB are very sensitive to the wash procedures used when performing these tests. Even though, strict adherence to the standard protocols for SAB testing was followed in order to avoid any differences in non-specific background IgG binding, repeat SAB testing on most serum samples was not possible due to limited quantity of the available sera. It would have been

ideal to test the serum samples with SAB at least twice to obtain FlowXM positive corresponding MFI values.

Furthermore, in this study FlowXM tests were carried out on donor cells isolated from blood samples that were more than six hours old in many cases. This could have had a significant effect on the background auto fluorescence signal produced by these cells. Since platelets also express Class I HLA, platelet contamination could have also resulted in a weaker or false negative FlowXM when testing MFI cutoffs.

4.7 Future directions

This study provides a proof of principle and a novel approach to establish SAB MFI range that predicts positive B or T cell FlowXM by using serum dilutions. However, this range is limited to Flow cytometry based SAB as these values were obtained using FlowPRA Single antigen assays. Nevertheless, a similar correlate on Luminex based SAB could also be developed for its use in a clinical setting. This would require a large scale study using serum samples collected from variably sensitized patients tested with FlowXM titration tests. It would also be important to include samples that produce positive FlowXM in various T cell and/or B cell settings to show that there are no differences between MFI values produced by these samples. Using serum samples that result in negative FlowXM would also be important in order to produce a lower threshold MFI on SAB below which sera will result in a negative FlowXM. Furthermore, it would be beneficial to do side by side testing of fresh serum samples with FlowXM titration test and SAB. This would avoid any effects of serum storage on the quality of the sample.

CHAPTER 5- CONCLUSION

5.1 Conclusion

This study provides evidence that further solidifies the use of SAB as an important tool in crossmatch testing and patient monitoring. Many important issues that challenge the use of SAB in DSA detection have been addressed in this study. This study has successfully shown that using highest FlowXM positive serum dilutions it is possible to establish fluorescence range on SAB that equates to positive XM, independent of patient DSA titre. When considering sera with multiple DSA, it is appropriate to consider the sum of individual DSA MFI on SAB as representative of total serum reactivity on cells. Furthermore, this study measured DSA MFI produced by serum samples obtained from various B and T cell Flow crossmatch settings. However, no difference between MFI produced by these serum samples was evident. This shows that using SAB it is possible to detect DSA level in the patient serum that would be harmful to the graft outcome in various situations.

In addition, this study has shown that it is possible to measure DSA concentration well below the FlowXM positive level, owing to the highly sensitive nature of SAB. A standard antibody curve can also be obtained that estimates sub-clinical DSA in patients in relation to positive FlowXM. This could prove highly useful in monitoring patients post-transplant and tailoring patient specific immunosuppressive therapies. By measuring DSA level on SAB sequentially, it would allow prediction of graft damage or AMR. This warrants for a clinical study that provides further evidence on the role of SAB in clinical monitoring of the patients.

Even though the correlate established in the study was not as sensitive in predicting a positive B or T cell FlowXM; this could be due to various limitations in carrying out this study as discussed earlier. When establishing a MFI threshold that correlates to harmful DSA levels, it is important to consider the implications of establishing a very low or a high threshold when it comes to crossmatch prediction and patient monitoring post-transplant. Pre-transplant, the goal is to increase access to all possible organs with appropriate exclusion of recipients that are at increased risk of graft rejection. The post-transplant objective is to avoid graft rejection by implementing appropriate immunosuppressive therapy when needed, without putting patients at increased risk of opportunistic infections. Therefore, care must be taken when establishing a cutoff MFI beyond which DSA can be considered harmful to the graft. Using the highest FlowXM positive serum dilution, as used in the current study, allows measuring DSA that is just above the FlowXM positive level. Thus, it is possible to establish a very narrow range on SAB that associates to harmful DSA levels and predicts positive FlowXM; this range should be very sensitive in predicting adverse graft outcomes. With its utility in 'quantitative virtual crossmatch' and monitoring patients post-transplant, such a range established on SAB could prove to be a boon in kidney transplantation.

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Detailed list of Class I and Class II HLA antigens present on SAB are available from the manufacturer at <http://www.onelambda.com/product-attachment.aspx?c1=antibody-detection&c2=flowpra-sup-sup-&c3=3&c4=10&c5=37>

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