The clinical utility and thresholds of Virtual and Halifaster Flow crossmatches in lung transplantation

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Abbreviations

BFXM	B-cell Flow cytometry
CDC	complement dependent cytotoxicity
DTT	dithiothreitol
DSA	Donor Specific Antibodies
EDTA	Ethylenediamine tetraacetic acid
FXM	flow cytometry crossmatch
HLA	Human Leukocyte Antigen
LTx	lung transplantation
MFI	mean fluorescent intensity
NGS	Next Generation Sequencing
TFXM	T-cell Flow cytometry
VXM	virtual crossmatch
XM	crossmatch

Keywords

Antibodies, flow cytometry, virtual crossmatch, DSA

Authors' contributions

S.J.H., G.I.S., L.C.S., and G.P.W., designed the study. S.J.H., B.L, R.C., M.M., I.N., and M.B.D., collated the data, carried out data analyses, and produced the initial draft of the article. G.I.S., L.C.S., and G.P.W. contributed to drafting the article. All authors have read and approved the final submitted article.

Abstract

Introduction: Immune sensitization, defined as the presence of alloreactive donor-specific antibodies (DSA), is associated with increased wait-times and inferior transplant outcomes. Identifying pre-transplant DSA with a physical cell-based assay is critical in defining immunological risk. However, improved solid phase antibody detection has provided the potential to forgo this physical assay. Here, we evaluated the association between DSA mean fluorescence intensity (MFI) and the recently introduced Halifaster Flow cytometry crossmatch (FXM) to determine if MFI could predict the outcome of FXM and whether a virtual crossmatch (VXM) would provide an accurate risk assessment.

Methods: Sera from 134 waitlisted lung patients was retrospectively assessed by Halifaster FXM against lymphocytes preparations from 32 donors, resulting in 265 FXMs. HLA typing was performed to 2-field allelic level and Luminex single antigen beads (SAB) used to identify DSA. The association between FXM and Luminex MFI was calculated using ROC analysis. MFI threshold accuracy was confirmed using a separate validation cohort (174 recipient sera and 34 donors), whereby both virtual crossmatch (VXM) and FXMs were compared.

Results: From the 265 FXM performed, 48 (18%) T-cell (TFXM) and 56 (21%) B-cell (BFXM) were positive. In the evaluation cohort, MFI thresholds of 2000 for HLA-A, B, DRB1 and >4000 for DQB1, were predictive of a positive FXM. The validation cohort of 233 paired FXM and VXM confirmed these MFI thresholds for both TFXM and BFXM with an accuracy of 91.4% and 89.3% respectively.

Conclusion: A positive VXM, defined with HLA-specific MFI thresholds predicts Halifaster FXM reactivity, and can potentially expedite organ allocation, by minimising the need for the more time-consuming flow cytometry crossmatch.

Introduction

The pre-lung transplant work-up for patients with end-stage lung disease includes an assessment of the presence of anti-HLA antibodies. Identifying these anti-HLA antibodies is an important step in accurately determining immunological risk of transplantation, recognizing that sensitized patients typically wait longer for immunologically compatible donor lungs and have worse outcomes following transplantation[1-4].

In addition to using solid phase assays to detect anti-HLA antibodies, many centers also employ functional, cell-based assays to define pre-transplant immunological risk. Historically, a Complement Dependent Cytotoxicity (CDC) crossmatch (XM), wherein serum from the recipient is mixed with donor lymphocytes in an in-vitro assay was utilized to identify any alloreactive HLA donor specific antibodies (DSA). These HLA DSA define immunological incompatibility and, if present, are associated with poorer post-transplant clinical course. Although the sensitivity of the CDC assay is substantially less than other assays[5], and in some cases fails to identify some potentially harmful non-complement fixing DSA, it is still performed in many laboratories worldwide, including those within Australia.

The flow cytometry crossmatch (FXM) is a more sensitive cell-based assay, which involves incubating recipient serum with donor lymphocytes and analyzing for the binding of antibodies to the lymphocytes using a flow cytometry analyzer. However, reliance on donor cell quantity and quality, as well as logistical issues in performing the assay has meant the FXM is often performed retrospectively. Recently, the development of the faster Halifaster Flow Cytometry protocol demonstrated that a FXM could be performed without the problem of extending allograft ischemic times[6].

However, even with improved workflow and use of fewer donor lymphocytes, performing a FXM for all waitlisted patients is not possible.

A Virtual crossmatch (VXM) is the assessment of immunogenic compatibility based on the patients' solid phase HLA antibody profile and compared with the donor HLA type. A positive VXM is defined by the presence pre-transplant anti-HLA DSA and is used to predict a physical XM result. The use of VXM has allow the circumventing of a prospective physical crossmatch[5, 7-10]. Often, the results of a VXM can be attained faster than a physical XM, as it only requires the donor HLA type and recipient HLA antibody profiles to be assessed.

In the current study, our primary aim was to determine the correlation between the MFI of HLA antibodies with the ability to produce a positive FXM, with the objective to define DSA MFI thresholds which could be adopted for an Australian VXM protocol. We further aimed to define patient groups which could be safely managed with either a VXM only or prospective FXM for the assessment of potential harmful anti-HLA antibodies.

Methods

Cohorts

The Alfred Hospital Ethics Committee (478/19) and Australian Red Cross Blood Service (06022019) approved this study.

The derivation cohort was used to define MFI thresholds that were associated with FXM positivity. This primary cohort consisted of 32 lung transplant recipients and their corresponding donors, who had stored pre- and post-transplant sera available, as well as corresponding access to stored donor cells. Retrospective FXM were performed between these 32 donor samples and the corresponding recipient sera who received the organ from that donor. Additionally, these same 32 donors were assessed against

70 other sera from historical waitlisted patients that did not receive a transplant from these donors. These patient sera were selected based on the presence of anti-HLA antibodies with varied Luminex MFI values, with the aim to include a range of weak, moderate and strong MFIs and all HLA loci. In total, FXMs were performed between the 32 donor cell samples and 134 unique patient sera (including the 32-transplanted pairs) (Figure 1A). The CDC-XMs group consisted of 32 transplanted donor-recipient pairs, and a further 21 waitlist patients, who at time of transplant were considered for that donor. In total, the derivation cohort consisted of 53 prospective CDC-XMs and 265 retrospective FXMs (Figure 1B).

A separate validation cohort was established to confirm the accuracy of predicting FXM using these MFI thresholds. This cohort comprised of a further 34 fresh donor cell populations and 174 unique sera from recent waitlisted patients. The combinations of donor cells and patient sera in the validation cohort were selected on HLA antibody specificities and MFI strengths, resulting in a total 233 FXM and VXM.

HLA Typing

HLA typing on recipients and donors was performed using either Luminex SSO (One Lambda, Inc., USA), or Next Generation Sequencing (NGS) (MIA FORA flex11, Immucor, USA on Miseq platform) for HLA-A -B -C -DRB1 -DRB345 -DQB1 -DQA1 -DPB1 and -DPA1 loci and reported to 2-fields, using CWD[11] (SSO results). The Victorian Transplantation and Immunogenetics Service (Melbourne, Australia) performed all testing.

HLA antibody testing

Sera was tested by Luminex Single Antigen beads (SAB) (One Lambda Inc. USA) and Ethylenediamine tetraacetic acid (EDTA) treatment used to negate potential prozone effect. Any Luminex normalized MFI>500 was considered as positive for identifying

HLA DSA as per routine lab assignments, however HLAMatchmaker (http://www.epitopes.net/) was used to confirm HLA antibody profiles. If antibody profiles did not fit matchmaker eplets or known sensitization, reactions >500 MFI was considered false and not reported as DSA.

Cells

All donor cells were isolated from fresh blood at the time of transplant using a lymphoprep gradient. Cells were frozen and stored up until the day of FXM preparation. Prior to performing the FXMs, all frozen cells were thawed in 10% FCS media and centrifuged at 500g for two minutes, before a rewash in 10% FCS media for a second centrifuge. Cells were then isolated using the EasySep negative selection kit (Stemcell, Canada) with a final concentration of 1 x 10^7 cells per ml in a solution of PBS-FCS 2% solution.

CDC Crossmatch

CDC T- and B-cell crossmatches were prospectively performed for all recipients who received transplants (n=32) and a select few who were also considered for transplants with same donor (n=21). The CDC-XM was scored on a scale of 1-8 with any score >1 considered positive, that is when >11-20% cells were reactive against patient sera, and any positive results were also confirmed following treatment with dithiothreitol (DTT) to exclude the presence of IgM antibodies.

Flow cytometry Crossmatch

The Halifaster FXM protocol was used for FXM assays and has been previously described[6], however the major modifications to other FXM assays are shorter incubation times and cell/sera volume ratios. Halifaster FXMs were performed using both T-cell and B-cells isolated from donors. Both T-cell FXM (TFXM) and B-cell FXM (BFXM) were positive when the Median channel shift (MCS) was three deviations

above the fluorescence of the negative control. A positive result, used for all subsequent analyses, was recorded with a fluorescence of >50 MCS above this negative control (plus three deviations). This was to remove any potential weak interferences from non-HLA interactions. HLA Class I is expressed on both T- and B-cells, so a TFXM and BFXM could be affected with HLA Class I DSA, while HLA Class II is only expressed on B-cells so would not affect a TFXM.

Virtual Crossmatch

VXM was performed on the validation cohort to determine the accuracy of the calculated MFI thresholds from the derivation cohort. Using the MFIs thresholds established in this study, each donor-recipient pairing in the validation cohort had a VXM performed. Any HLA DSA greater than the defined MFI thresholds for each HLA loci was deemed as a positive VXM (VXM+). For any donor HLA allele, which was not represented on the Luminex SAB kit used, the closest allele on panel was used. For example, the C*07:02 bead was used for inferring DSA to C*07:01. All donor-recipient combinations had corresponding FXMs performed, under same conditions as the derivation cohort. The comparison between the validation VXM and FXMs was used to calculate the overall true and false rates of predicting the FXM.

Clinical

Recipient selection for transplant was along standard lines, requiring a negative T-cell CDC crossmatch and preferably no DSA present. The majority of patients received a triple immunosuppressant regimen consisting of tacrolimus, azathioprine or mycophenolate and prednisolone, as has been previously described[12]. Induction therapy with an IL-2 antagonist reserved for patients with limited renal reserve.

Statistical analysis

Luminex MFI thresholds were calculated using receiver-operating characteristics (ROC) analyses where >95% of DSA+ above any the given threshold returned a positive FXM. All data was analyzed using SPSS v21.

Results

Derivation Cohort

Presence of anti-HLA antibodies

Of the 265 sera analyzed in the derivation cohort, HLA DSA was identified in 154 (58%) of patient sera. From these, DSA directed towards HLA-DQB1 was represented the most (27%). Overall, 45 (17%) sera of the derivation cohort had both HLA class I and II DSA identified (Table 1).

CDC

Of the 53 CDC-XM performed, there were 2 T-cell and 10 B-cell positive XM. Of these, only an A*68 (MFI 12608) was present for both a T-cell and B-cell CDC+, the other T-cell CDC+ returned a B-cell CDC- and had no HLA DSA and therefore presumed to be false positive. There was also a DQ7 (MFI 2000) which was seen for a B-cell positive CDC (Table 2). Seven B-cell positive CDC-XM occurred in the absence of any HLA DSA with the presumption that these were false positive reactions and possibly due to non-HLA interactions. There was no association between Luminex MFI and CDC positivity from this small CDC cohort (Table 2).

Flow cytometry

Of the 265 FXMs performed, there were 48 (18%) TFXM+ and 56 (21%) BFXM+. 93 (35%) patient serum had at least one identified class I DSAs, and 99 (37%) at least one Class II DSA (Table 3) while 47 (18%) had both class I and II DSA+. A positive FXM was associated with the presence of HLA class I DSA in 40 of 48 (83%) TFXM+

and 32 of 56 (57%) BFXM+. While, HLA class II DSA was associated with 33 of 56 (59%) BFXM+. Overall, 50 of 56 (89%) BFXM+ had either an associated class I, class II or both DSA identified. For TFXM+ but BFXM-, the T-cell positivity was interpreted as FXM+ for ROC and other analysis.

ROC Analysis

In the ROC analysis from the derivation cohort, the highest identified (Top MFI) MFI for HLA-A and HLA-B (HLA-AB), and cumulative class I were the strongest predictors of a TFXM+ and BFXM+ with Area Under Curve (AUC) of .846 and .851 respectively (Figure 2). HLA-A, HLA-B and cumulative class I were also predictive of the BFXM (AUC .731 and .743). Of the class II loci, HLA-DRB1 and -DQB1 were both shown to be predictors of the BFXM with AUC of .626 and .676 respectively, although cumulative class II MFI was also an accurate predictor (AUC .745) (Figure 2). Using the ROC analysis (Table 4), which determines the optimal balance between sensitivity and specificity of the MFI in predicting FXM, thresholds of MFI>2000 for HLA-A and HLA-B, were calculated found to be best predictive for TFXM. For the BFXM, thresholds of MFI>2000 for HLA-A, -B and -DRB1, and MFI>4000 for HLA-DQB1 demonstrated the most favorable predictive measures.

Using the defined MFI thresholds established above (>2000 for HLA-A, -B, and DRB1; and >4000 for HLA-DQB1) FXM positivity could be predicted in >87% for each loci (Table 5). Although, HLA-DQB1 with the MFI>4000 threshold demonstrated 88% accuracy. FXMs with HLA DSA for the other loci also returned positive in HLA-A, -B and DRB1 demonstrated 96%, 93% and 88% respectively. Overall, 92.4% of TFXM and 90.5% of BFXM were concordant with the expected results with the use of HLA-A,-B,-DRB1>2000 and HLA-DQB1>4000 MFI thresholds.

Validation Cohort

Assessing Luminex MFI for VXM thresholds

Using the defined Luminex MFI thresholds from the derivation cohort (>2000 HLA-A; -B; -DRB1 and >4000 HLA-DQB1), 233 VXMs were performed on the validation cohort, followed by FXMs on the same 233 paired samples to determine the accuracy of these MFI thresholds for FXM prediction. Overall, the MFI thresholds for the VXM correctly predicted the FXM result in 90.5% of all FXMs performed, with 91.4% for TFXM and 89.3 for BFXM respectively (Table 6). The majority of the disparity between VXM and FXM arose from positivity for a FXM in the absence of identified HLA antibodies above these thresholds (FXM+ but VXM-), which occurred for 13 (6%) T-cells and 12 (5%) B-cells. Of the 13 TFXM+ but VXM-, three had HLA-A or HLA–B DSA identified with MFIs below the threshold, while four had HLA-C DSA (all MFI>6000), HLA-C was not including in the modelling due to not being a good predictor in the ROC, however these HLA-C DSA demonstrated clear FXM+. These also accounted for three of the 12 BFXM+ but VXM-. Of the remaining nine BFXM+ but VXM-, two had identified HLA-DRB4 DSA >2000, and one had a DPB1 DSA with MFI 16701 (DPB1 thresholds were not calculated from derivation cohort). Overall, there were 6 (2.5%) TFXM+ and 6 (2.5%) BFXM+ in the absence of any identified HLA DSA from the validation cohort.

Discussion

Our study demonstrates that a positive virtual XM, determined by HLA-specific MFI thresholds, predicts a positive Halifaster FXM. Moreover, in our cohort a positive FXM was only seen in transplants with poorer outcomes. The utility of Luminex MFI thresholds to predict FXM results has been previously studied [8, 13, 14]. However, Luminex MFI are only semi-quantitative as their values may vary between centers,

kits, and even between lab technologists [15-17], therefore, it is imperative that centres perform a similar exercise to establish their own relevant MFI thresholds. The use of a MFI threshold is the first step in the evolution towards a VXM approach for LTx and away from a cell-based assay as the final checkpoint for clinically significant DSA. Historically, and even currently in Australia, a prospective CDC assay has been utilized in the pre-transplant assessment in transplantation. Although CDC can identify complement fixing DSA, its reduced sensitivity does mean it may miss some HLA DSA which could generate a significant FXM result and potentially be detrimental to transplant outcomes (see Table 2).

We have demonstrated that different Luminex MFI thresholds are required for different HLA loci and specifically DQB1 DSA requires an increased MFI to produce consistent positivity in the FXM. Furthermore, both HLA-C and DQA1, were unable to reliably be used to predict FXM reactivity, however there were rare instances (4x HLA-C MFI>6000 and 1x DQA1*05 MFI=18185) where these were the only HLA antibodies present and could explain the FXM positivity. Although there were DSA against the HLA-DRB345, -DPB1 and -DPA1 in this study, there was no FXM+ reaction that could not be explained with the use of the thresholds we defined (HLA-A, -B, -DRB1 and –DQB1) and no DSA isolated to only these loci only were seen in this study, therefore thresholds for these loci were unable to be determined. Although the clinical significance of antibodies to HLA-DP has been questioned, other studies have reported antibody-mediated rejection attributable to preformed HLA-DP specific antibodies[18]. Therefore, in the case of DSA directed exclusively towards HLA-DR345 or -DP, it can be argued a FXM is warranted.

The variation in reactivity between HLA gene targets has been previously shown[19]. The increased DQB1 Luminex MFI required to elicit a positive FXM in this cohort could

be due to varied expression of DQB1 on the Luminex bead sets, or potentially due to HLA expression on donor cells. Previous work have demonstrated variation in HLA expression between living and deceased donors which could further impact the accuracy of any FXM[20, 21]. Given the inability in our cohort to establish a Luminex MFI threshold for DQA1 and C loci, we recommend that HLA-C and DQA1 antibodies be evaluated on an individual patient basis using sera titers and surrogate FXMs to determine which antibodies represent immunologic risk.

Luminex MFI values may vary considerably between patient samples and time points, troubling clinicians in determining which DSA to classify as unacceptable and which to cross for transplant. We found the highest individual Luminex MFI (Top MFI), and therefore immunodominant DSA, the simplest and most accurate to use for a VXM. An average Luminex MFI of alleles over multiple beads proved a lot more cumbersome to determine for each HLA specificity and was not as accurate. The cumulative effect of multiple weaker DSA can on occasion provide a positive FXM[6], however, it has been reported that the use of cumulative MFI for FXM prediction is less effective for multiple weaker DSA[6]. Thus, as we have demonstrated, using a Top MFI threshold provides the most accurate way in predicting the FXM reactivity. Multiple DSA could be reacting to different HLA epitopes, or towards a shared epitope within the HLA molecule, which complicates the use in any VXM.

Furthermore, special consideration must be made to the needs of highly sensitized patients. Even the use of VXM thresholds MFI>2000 (HLA-A, -B, -DRB1) and MFI>4000 (DQB1) to rule out moderate positive FXMs will lead to highly sensitized patients being excluded from offers, and preference given to less-sensitized patients, which is problematic. We now, with communication between treating teams and the testing labs, personalize risk profile for highly sensitized patients above these VXM

thresholds. The use of surrogate FXM and serum dilution tests to confirm HLA titer allow us to cross HLA antibodies above the stated VXM thresholds without affecting early outcomes.

A limitation of FXM is the use of lymphocytes as the target as this incurs the risk that antibodies maybe recognizing non-HLA targets, and potentially transplant irrelevant molecules on the donor cell surface. Here we had a proportion of FXM positive assays which had no identified HLA antibodies (TFXM 4% and BFXM 3%). Clinically, the importance of this FXM positivity is unknown in the absence of any HLA DSA should not exclude a patient to transplant. Some studies have looked at non-HLA auto-antibodies as an explanation to this FXM reactivity in the absence of any HLA DSA[22, 23]. However, the role of non-HLA auto-antibodies on risk of rejection in lung transplantation needs to be further investigated.

Furthermore, within our validation cohort, there were instances where HLA DSA was likely misidentified. A subsequent review showed these antibodies did not fit known epitopes and failed to elicit reactions on all FXMs, i.e. they were false positive Luminex results. This highlights the importance of accurate identification of HLA antibody specificities, and the potential use for surrogate cell-based assay like FXM could be used to confirm antibody status prior to listing. The use of HLA epitopes to aid in the classification of antibodies defined by Luminex does reduce errors[24]. We believe the use of Luminex MFIs for a pre-transplant VXM could provide a faster and more accurate immunological risk assessment by identifying HLA antibodies present at the time of transplant. Furthermore, additional work is required to investigate FXM positives DSA negatives in this, and other cohorts, to determine the clinical relevance of these results.

Although we have shown the reliability of using Luminex MFI to predict FXM reactivity, there are limitations to using a VXM. When multiple weaker DSAs are present, or for highly sensitized patients where crossing a known DSA is the only option, a pre-transplant FXM may provide clarity of the strength of FXM reactivity therefore help decide if transplant proceeds and whether pre-transplant treatments are warranted. We suggest that in the absence of comprehensive surrogate FXMs which can aid in determining FXM reactivity of an individual patient level prior to organ offers, a prospective FXM could be performed for the group of patients with multiple weak DSAs just below thresholds to more accurately define risk.

A limitation to this study was the selection of transplants towards T-cell negative CDC-XMs. As the clinical practice is to select for these negative T-cell CDC crossmatches, much of this limitation cannot be avoided. We understand this limitation may have influenced the conclusions of the DSA associations with the CDC results, however several studies have demonstrated MFI thresholds which correlate to CDC positivity[14, 25-27] and the lack of sensitivity seen in CDC when compared to FXM. Here, we believe the bias towards T-cell negative CDC did not affect the conclusions made with Luminex MFI use in predicting FXM reactivity and the benefit of using a VXM in pre-transplant assessments.

We have shown that Luminex MFI values can reliably predict clinically relevant reactivity in a Halifaster FXM. That a VXM result is a reliable pre-transplant assessment providing fast and accurate immunological risk for lung transplantation for the majority of waitlist patients. Further work needs to be undertaken, examining the specificity driving of HLA antibody-negative FXM positive results.

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Conflict of Interest

The authors have declared no conflicting interests.

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Figure legend

Figure 1: Derivation FXM and CDC cohorts. Composition of cells and sera used for both the derivation FXM (A) and derivation CDC (B) cohorts. For the FXM cohort 32 donor cells were crossmatched against the Lung transplant recipient (pre- and post-transplant sera) as well as 70 selected sera from sensitized waitlist patients chosen for the HLA antibody profile. Not all waitlist sera was crossmatched against all donor cells in the FXM cohort. All CDCs were performed at time of donation against patient waitlist sera considered at time of transplant.

Figure 2: ROC curves of the relationship between HLA DSA and FXM positivity. The ROC curve represents the relationship between the specificity and sensitivity of using MFI to predict the FXM. The greater the Area under curve (AUC) the stronger the relationship. HLA class I Cumulative MFI and Top HLA A/B MFI was predictive of both TFXM and BFXM reactivity. Of the HLA class II, DRB1 and DQB1 Top MFI were able to predict BFXM positivity. HLA-DRB345 and HLA-C DSA, in isolation did not accurately predict FXM reactions and reliable Luminex MFI thresholds were unable to be determined.

	CDC (*n*=53)		FXM (*n*=265)	
	DSA+ (%)	MFI range	DSA+ (%)	MFI range (Mean)
No HLA DSA	29(55%)		111(42%)	-
Class I only	12(23%)	533–12308	48(18%)	508–19980 (4962)
HLA A	3(6%)	2254–12308	38(14%)	935–12629 (5609)
HLA B	3(6%)	508–5867	44(17%)	508–19980 (6762)
HLA C only	3(6%)	2186–11843	25(9%)	533–16644 (6244)
Class II only	7(13%)	1175–13133	61(23%)	639–23210 (5392)
HLA DRB1	6(11%)	1673–13133	32(12%)	1076–21275 (3322)
HLA DR345	3(6%)	1103–3640	33(12%)	909–11355 (4925)
HLA DQB1	5(9%)	1175–13133	72(27%)	639–23210 (5711)
DPB1 only	-	-	4(2%)	2867–3423 (2535)

Table 1: Range of MFI for HLA DSA+ for CDC and FXM used in derivation cohort. The derivation cohort consisted of 53 prospective CDC and 265 retrospective FXM assays, with all the Luminex MFI of identified HLA DSA ranges listed. All identified HLA DSA >500 MFI was determined to be DSA+. All CDC crossmatches included in this cohort were performed with donor cells and recipients who were considered for transplant at the time of donation.

Class I	MFI	Class II	MFI	Tx'ed	T-CDC	B-CDC	TFXM	BFXM
A3	6865	None	-	Yes	-	+	-	-
Cw7	4973	None	-	Yes	-	-	-	-
Cw6	6000	DQ7	2000	Yes	-	+	+	+
None	-	DQ2 DR3	4990 4530	Yes	-	-	-	+
None	-	DR4	1217	Yes	-	-	-	+
None	-	DQ5	3915	Yes	-	-	-	-
A2	2254	None	-	No	-	-	+	-
A68	12608	None	-	No	+	+	+	+
B35 B39	2309 1716	None	-	No	-	-	+	-
B44	3897	None	-	No	-	-	+	+
B51	5867	None	-	No	-	-	+	-
Cw6	11483	None	-	No	-	-	-	-
Cw7	2186	None	-	No	-	-	-	-
None	-	DR7	2547	No	-	-	-	-
None	-	DR7	2867	No	-	-	-	-
None	-	DR3	1673	No	+	-	-	-
No HLA DSA Identified				No	-	+	+	-
No HLA DSA Identified				No	-	+	-	-
No HLA DSA Identified				No	-	+	-	-
No HLA DSA Identified				No	-	+	-	-
No HLA DSA Identified				No	-	+	-	-
No HLA DSA Identified				No	-	+	-	-
No HLA DSA Identified				No	-	+	-	-

Table 2: Identified DSA MFI, CDC positivity and corresponding FXM results of patients in the derivation cohort. All identified HLA DSA used for the derivation CDC-XM cohort. All CDCs were performed prospectively at time of transplant, DTT used to confirm positivity and all sera re-tested with EDTA treatment to confirm HLA specificity. Only HLA DSA>1000 MFI listed. A HLA-A*68 DSA (MFI 12608) was the

only DSA present for both a T-cell and B-cell positive CDC. Standard clinical practice is to not transplant against a T-cell CDC-XM.

	HLA Class I		*HLA Class II*	
	DSA-	*DSA+*	*DSA-*	*DSA+*
TFXM-	164	53	-	-
TFXM+	8	40	-	-
BFXM-	164	62	156	70
BFXM+	12	32	11	33
Total	172	93	166	99

Table 3: HLA DSA association with FXM results in derivation cohort. A summary of all the FXM performed in the derivation cohort with the identified HLA DSA. A DSA+ was reported if any HLA Class I (A, B, C) or Class II (DRB1, DQB1, DRB345 or DPB1) DSA was identified. The presence or absence of any DSA from the other HLA loci was not accounted for here, however was accounted for DSA MFI threshold calculations.

TFXM				BFXM			
	MFI threshold	Sensitivity	Specificity		MFI threshold	Sensitivity	Specificity
HLA-AB	1000	0.729	0.069	*HLA-AB*	1000	0.494	0.064
	1500	0.667	0.041		1500	0.442	0.037
	1750	0.667	0.037		1750	0.442	0.032
	2000	**0.646**	**0.023**		2000	0.416	0.021
	2250	0.625	0.018		2250	0.416	0.011
	2500	0.604	0.018		2500	0.403	0.011
	3000	0.563	0.014		3000	0.364	0.011
HLA-C	1000	0.188	0.106	*HLA-DRB1*	1500	0.208	0.011
	1500	0.188	0.083		1750	0.195	0.011
	2000	0.188	0.065		**2000**	**0.182**	**0.011**
	3500	0.188	0.055		2250	0.169	0.005
	5000	0.167	0.051		2500	0.156	0.005
	6000	0.146	0.051		3000	0.130	0.000
Class I	1000	0.813	0.184	*HLA-DQB1*	2500	0.325	0.064
	1500	0.750	0.166		3000	0.312	0.037
	1750	0.750	0.115		3500	0.286	0.011
	2000	0.729	0.111		**4000**	**0.286**	**0.005**
	2250	0.729	0.092		4500	0.260	0.000
	2500	0.708	0.092	*HLA-DRB345*	3000	0.117	0.032
	3000	0.708	0.074		3500	0.078	0.032
	3500	0.646	0.069		4000	0.065	0.016
	4000	0.625	0.069		5000	0.065	0.011
				Class I	2000	0.494	0.112
					2500	0.494	0.080
					3000	0.494	0.064
					3500	0.442	0.064
					4000	0.416	0.064
				Class II	2000	0.571	0.191
					2500	0.519	0.181
					3000	0.481	0.133
					3500	0.455	0.090
					4000	0.442	0.069

Table 4: ROC analysis table. The sensitivity and specificity of MFI thresholds in predicting FXM+ assays. For TFXM positivity, a MFI>2000 for HLA-A or HLA-B was deemed the best predictor. For BFXM, the same HLA-A and HLA-B threshold remained predictive, while HLA-DRB1 MFI>2000 and DQB1 MFI>4000 was able to predict BFXM+.

		TFXM-	TFXM+	concordant	BFXM-	BFXM+	concordant
HLA-A MFI	<2000	216	33	87%	207	42	83%
	>2000	1	15	96%	2	14	88%
HLA-B MFI	<2000	215	23	87%	208	30	88%
	>2000	2	25	93%	1	26	96%
Cumulative HLA-A&B MFI	<3000	213	19	88%	202	30	87%
	>3000	4	29	88%	7	26	79%
HLA-DRB1 MFI	<2000	-	-	-	207	42	83%
	>2000	-	-	-	2	14	88%
HLA-DQB1 MFI	<4000	-	-	-	209	34	86%
	>4000	-	-	-	0	22	100%
Overall	<threshold	213	16	92.4%	195	11	90.5%
	>threshold	4	32		14	45	

Table 5: HLA DSA MFI thresholds association with FXM positivity (derivation cohort). Luminex DSA MFI thresholds of >2000 for HLA-A, B, DRB1 and MFI>4000 for DQB1, as calculated by the ROC. A concordant result was determined where the >MFI threshold correlated to a FXM+ result. For the overall concordance, thresholds for all HLA loci were considered (HLA-A and –B for TFXM and HLA-A, -B, -DRB1 and -DQB1 for BFXM).

	TFXM-	TFXM+	BFXM-	BFXM+	Total
VXM-	145	13	108	12	
VXM+	7	68	13	100	
Concordant	91.4 %		89.3%		90.5%

Table 6: Accuracy of defined VXM MFI thresholds in predicting Halifaster FXM (Validation cohort). The VXM, with MFI thresholds >2000 for HLA-A/B and DRB1, and MFI>4000 for DQB1 predicted 90.5% of all FXMs (91.4% TFXM, 89.3% BFXM). Overall, a FXM+ occurred in the presence of a VXM- result 13 (6%) for TFXM and 12 (5%) for BFXM, while a FXM- where it was predicted to be positive (VXM+) occurred 7 (3%) TFXM and 13 (6%) BFXM.