Enduring Hearts Award: Vanderbilt University Department of Pediatrics

Antibody Mediated Allograft Injury Following Pediatric Heart Transplantation: Mechanistic Insights and Predictive Modeling

Year 1 Progress Report: 7/1/18- 7/31/19

Time line of proposed studies for each aim. Mechanistic studies, blue; data analysis, red:

Year	1	2
Aim 1: Antibody	Perform detailed phenotyping of anti-HLA antibodies in the pre- and early post-transplant period after pediatric heart transplantation 	
Aim 2: Pathology	To develop an automated, transport-based morphometry system that can be easily and widely applied to routine EMB early after transplant with the goal of predicting late graft outcomes 	
Aim 3: Predictive Models	To develop a risk prediction model for late graft outcomes 	

.

We report below on the study progress in Year 1 by Specific Aim:

<u>Specific Aim 1</u>: Perform detailed analysis of DSA in the pre and early post-transplant period after pediatric heart transplantation

<u>Hypothesis and Rationale.</u> We hypothesize that HLA DSA phenotype and HLA mismatch, determined by epitope load, can predict adverse allograft outcomes including acute rejection (ACR and AMR), as well as severity of rejection in the first 3 years after transplantation. Specifically, we hypothesize: i) the timing (early) and phenotype of DSA (high titer, complement fixing and/or IgG3) is more predictive of clinical events than DSA strength by MFI alone; ii) HLA epitope assessment better reflects the level of mismatch than traditional matching by HLA alleles. We will also explore impact of dense DSA phenotyping and epitope matching on late graft function and transplant free survival.

DSA will be characterized by strengths (MFI), titer by performing serial dilutions and complement fixing. In addition, IgG subtype analysis and the identification of IgG3 may be predictive of “a more pathogenic DSA”.

Year 1 Accomplishments

1. **DSA TITER**
    - Pre-formed (at transplant) and early post-transplant DSA were identified in 109 / 273 patients screened from the parent CTOTC-09 parent study. An additional 43 patients are in the progress of being screened for the presence of DSA.
    - Longitudinal analysis of DSA titer early post-transplant was performed by the single antigen bead assay with patients' samples diluted at 1:4 and 1:16. We performed 160 Class I and 120 Class II tests in 30 patients.

Representative examples and interpretation of the tests are illustrated in Figure 1,2 and Table 1.

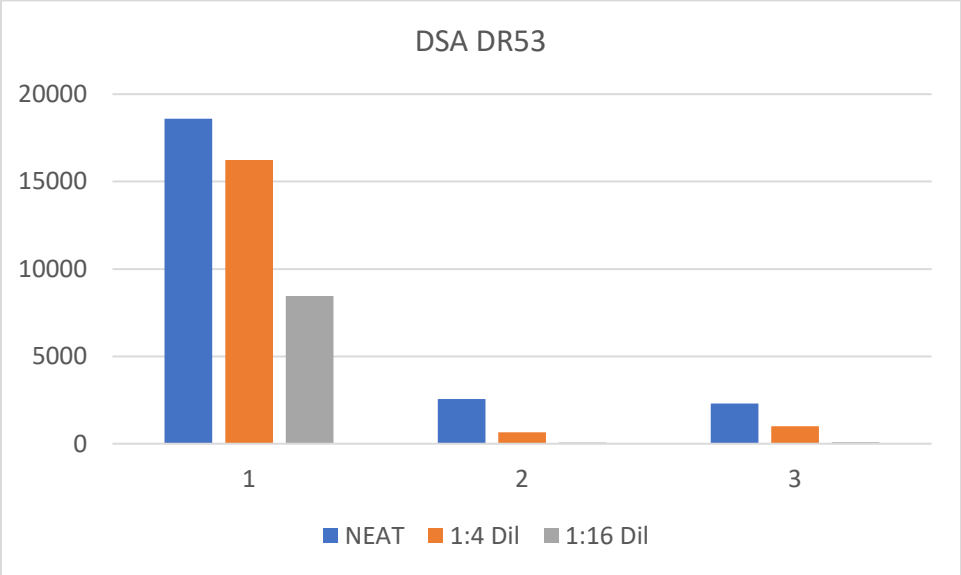

Figure 1: The DSA DR53 present pre-transplant has significantly dropped at 4 weeks and 6 weeks post-transplant as evidenced by neat MFI and titer. Time 0 at 1:4 and 1:16 dilution the DSA was strong>8000 MFI. The next 2 periods at 1:4 dilution was <1000 MFI.

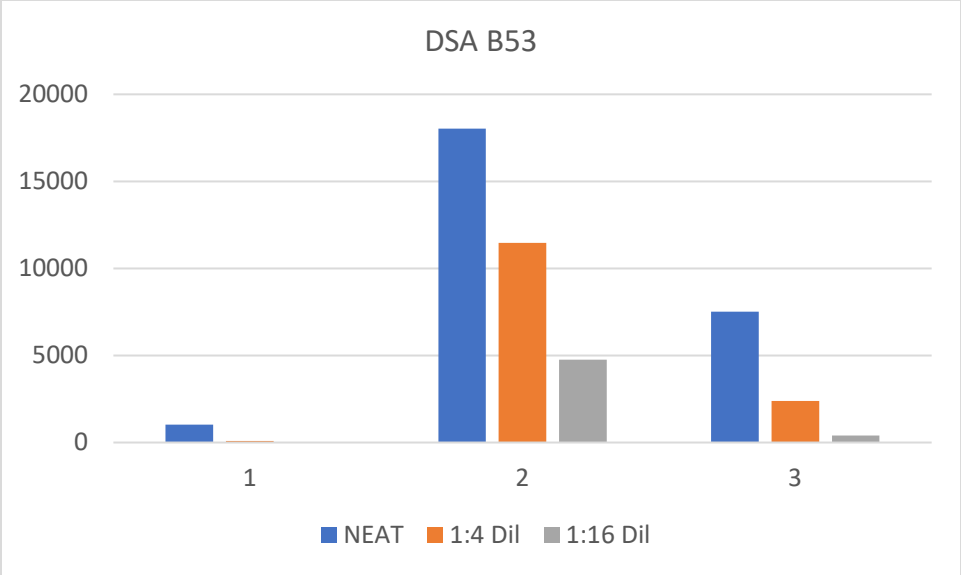

Figure 2: At 2 weeks post-Tx the B53 DSA increased significantly (memory response) and was strong at 1:4 and moderate at 1:16 dilution. At 6 weeks, the neat MFI was still in the strong range (8000 MFI) while at 1:4 dropped to weak range (2000 MFI) and was negative at 1:16.

These 2 examples illustrate how we can estimate the longitudinal change by comparing not only the neat MFI but also the significant drop in titer. Analysis of the entire cohort in Aim 3 will be required to determine how serial titer determinations over time (rather than just MFI strength or single determinations and preformed vs. memory responses) correlate with clinical outcomes.

Table 1. Example of Multiple DSA High Titer

Time 0	A2	A3	DQ2	DQ7	DR52
Neat	17462	7824	23600	18491	12900
1:4 Dil	10002	11287	16354	12047	12522
1:16 Dil	4974	7506	4330	17161	7920
Time 1w					
Neat	7555	8851	22800	12600	5500
1:4 Dil	9374	4734	15662	11024	11143
1:16 Dil	8855	4400	18305	5094	5640
Time 3w					
Neat	8500	4400	23400	18700	10700
1:4 Dil	5296	8178	10200	14135	10680
1:16 Dil	7690	7733	20367	10113	9202

This subject's antibody profile illustrates the limitation of single antigen bead testing (SAB) in the presence of high titer antibody. The neat serum may show inhibition and lower MFI than the subsequent dilutions. In this case, the dilution may unveil the inhibition and provide a better tool to estimate the longitudinal changes. We also performed SAB after treating the serum with EDTA (see below) to remove the native complement that may interfere with the assay with high

titer antibody. All these high titer DSAs were also complement binding (defined as SAB-C1q test positive >500 MFI).

2. Complement binding activity

Patients that exhibit DSA and have a titer positive at 1:16 are tested for C1q-binding. Patients with DSA below the certain titer threshold (<1:16) have been shown to be negative. To date, C1q testing has been performed on 49 patients who are DSA positive at 1:16 dilution. Of these, 17 (35%) DSA were found to be C1q positive. This has been correlated with worse clinical outcomes in prior adult studies. The presence of positive C1q binding will be correlated with the pre-specified clinical outcomes in the final multivariable models.

3. IgG subtype analysis

Patients that exhibit DSA and are positive >2000 MFI at 1:4 dilution are tested for IgG subtype. IgG subtype analysis has been shown to be negative (assay sensitivity) in patients with DSA <4000 MFI and negative at 1:4 dilution. Kits for IgG DSA subtype have been obtained (One Lambda) and assay has been validated on test samples per manufacturers instructions. Studies on actual CTOT-C-09 samples will commence in the next few weeks, this completing the final detailed phenotyping of DSA. As has been demonstrated in adult kidney transplantation, we believe that detailed and serial phenotyping of DSA in the early post-operative period will provide incremental information that will help predict short and mediate term post-transplant outcomes.

YEAR 2 Plan

1. We will complete all the DSA characteristics in the next 4 months.
2. Results of DSA titer, complement binding activity and IgG subclass will then be added to the existing CTOT-C09 DSA database which also contains all clinical outcome data for this population. The clinical sites are currently responding to clinical queries on all enrolled subjects and follow-up will end this October 31. By the end of the calendar year 2019, the database will be locked, including the results of all the studies supported under this proposal.
3. The analysis of DSA characteristics and clinical outcomes will be done within the 18-24-month period as initially proposed.
4. Similarly, next-generation HLA typing of the 270 available pairs of donor/recipient will be completed in the next 6 months and the correlation of eplet load with clinical outcomes will be done during the 18-24 month period (see number 3 below). We have determined that this strategy is superior to conversion from antigen-level to allele level analysis based on the NMDP online haplotype determination tool (which can be quite inaccurate, especially for class II DQ antigens). Analysis will then be performed using the HLAMatchmaker tool as previously described.

Specific Aim 2: To develop an automated transport based-based morphometry system that can be easily and widely applied to routine endomyocardial biopsies (EMB) early after heart transplant with the goal of predicting late graft outcomes.

Hypothesis and Rationale

We hypothesize that capillary injury from AMR (with/without co-existent T cell-mediated rejection) leads to interstitial capillary remodeling that can be reliably detected using a combination of endothelial cell (CD34) staining and automated transport-based morphometry on whole slide imaging (WSI). This combined approach can be widely applied and will enable us to detect early (<6 months) capillary shape changes characteristic of graft syndromes that occur before, during, and can persist after clinical/subclinical AMR episodes that lead to microvascular remodeling and eventually loss, and that contribute to chronic graft dysfunction. We further hypothesize that these early shape changes occur as a consequence of injury and microvascular angiogenic signaling, which will eventually lead to capillary loss and chronic graft dysfunction. Once validated, this approach can be widely applied and will enable clinicians to detect early (<6 months) capillary shape changes characteristic of various graft syndromes. Consequently, earlier intervention to prevent irreversible microvascular pathology, typical of late graft dysfunction, may be possible.

Year 1 Accomplishments:

1. All slides logged into core laboratory, and standard stains performed per protocol.
2. Core laboratory diagnostic evaluation performed (ACR, AMR, etc.) and entered into database.
3. CD34 staining protocols fully developed and validated on our automated slide stainer (Ventana Discovery ULTRA).
4. A new technician has been trained on our automated staining platform and slide scanning.
5. IT infrastructure required to handle storage of WSI for all CTOT04/09 cases and stains has been upgraded.

Year 2 Plan:

1. Outstanding patient sample timepoints shall be received (anticipated through end October 2019). CD34 Staining will be completed on all specimens in months 13-18.
2. As staining is conducted, central pathologist, A. Jake Demetris, shall score cases according to established scoring template.
3. TBM analytics shall be prepared, validated, and executed on defined patient samples as outlined in initial grant. TBM shall build upon programming designed and reported from our program (Feingold, et al. J Heart Lung Transplant. 2017 Dec;36(12):1336-1343. PMID 29055602).

Specific Aim 3: To develop a risk prediction model that incorporates all clinical information from CTOTC-09, combined with results from aims 1-2 above, and that can be applied in the first six months after transplantation to predict late (3 and 5 year) graft outcomes among early post-transplant survivors.

We hypothesize that a prediction model can be developed based on donor and recipient characteristics, early post-transplant events and laboratory findings that will allow for robust prediction of 3 and 5 year graft outcomes.

This aim was planned for Year 2 (see timeline above).

Year 2 Plan:

Analysis for this aim will occur during the last 6 months of the study period, when all data from Aims 1-2 is complete and all data checks and queries have been resolved. Manuscript preparation will follow data analysis.

Year 1 Progress Report: Lay Summary

Our long-term goal is to understand early graft events that lead to the shortened lifespan of children receiving heart transplants. Understanding the risk factors for adverse graft outcomes will help determine the best opportunities for prevention and management of factors that lead to shortened life expectancy. In broad terms, we know that donor-specific antibodies (DSA; circulating proteins in the blood that attach to the graft and damage it) lead to poorer long-term graft outcomes. However, not all antibodies are equally damaging. In **Aim 1** of this study, we determine which pediatric heart transplant recipients have DSA in their blood before and after transplant and in year 1 of the study, we have performed multiple additional specialized tests to determine the special characteristics of all these antibodies in order to help us determine which ones are most damaging to the new heart. We believe this will help define a target population for more intensive therapies to prevent and treat "bad" antibody development. In **Aim 2,** we use highly sophisticated and novel pathology techniques to look at biopsies of the transplanted heart of transplant recipients. We believe that studying the small blood vessels in the heart (graft capillaries) within 6 months of the transplant using automated pathology techniques with special stains, we may be able to identify those patients with early damage to blood vessels that may indicate the potential for adverse longer-term outcomes. We also believe that these damaged capillaries will be associated with certain antibodies as identified in Aim 1. In year 1, we obtained all the biopsy slides, stained them with special stains and made whole slide images of all materials for automated computer analysis that will occur in year 2. **Aim 3** will use the results of the tests in Aims 1 and 2 to create a computer model to predict which recipients are at highest risk for adverse outcomes such as rejection or graft loss. This analysis will occur in the last 6 months of the study per the original study timeline. In summary, we achieved the goals we set out in year one and anticipate that the studies will be completed on time at the end of year 2. We are hopeful that this model can help the child's care team earlier in the post-transplant period to personalize their care to improve the child's long-term outcomes.