De Novo Donor-Specific Antibodies in Renal Transplantation

by:

Dr. Chris Wiebe

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University of Manitoba
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Abbreviations

AMR	Antibody-Mediated Rejection
APC	Antigen Presenting Cell
cAMR	Chronic Antibody-Mediated Rejection
cg	Chronic Glomerulopathy
ci	Interstitial Fibrosis
cPRA	Calculated Panel Reactive Antibody
Cr	Creatinine
CRP	C-reactive protein
ct	Tubular Atrophy
cv	Vascular Fibrous
DAMP	Damage Associated Molecular Pattern
***dn*DSA**	de novo Dono-Specific Antibody
DSA	Donor-Specific Antibody
ELISA	Enzyme-Linked Immunosorbent Assay
ESRD	End-Stage Renal Disease
FITC	Fluorescein Isothiocyanate
FK	Tacrolimus
g	Glomerulitis
HLA	Human Leukocyte Antigen
i	Interstitial Inflammation
IFN-γ	Interferon Gamma
IFTA	Interstitial Fibrosis and Tubular Atrophy

IL	Interleukin
IVIg	Intravenous Immunoglobulin
MBL	Mannose Binding Lectin
MFI	Mean Fluorescence Intensity
MM	Mismatches
MMF	Mycophenolate Mofetil
NFAT	Nuclear Factor of Activated T-cells
NFkB	Nuclear Factor kappa B
PBS	Phosphate Buffered Saline
PCR	Polymerase Change Reaction
PRA	Panel Reactive Antibody
PRR	Pattern recognition receptor
ptc	Peritubular Capillaritis
SAPE	Phycoerythrin-conjugated Streptavidin
SSO	Sequence Specific Oligonucleotide
t	Tubulitis
TCMR	T-cell Mediated Rejection
TerEps	Terasaki Epitopes
TNF	Tumor Necrosis Factor
v	Vasculitis

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Chapter 1

Abstract

Abstract

The natural history for patients with *de novo* donor-specific antibodies (*dn*DSA) and the risk factors for its development have not been well defined. Furthermore, clinical and histologic correlation with serologic data is limited. We studied 315 consecutive renal transplants without pre-transplant donor-specific antibody (DSA), with a mean follow-up of 6.2 ± 2.9 years. Protocol (n = 215) and for cause (n = 163) biopsies were analyzed. Solid phase assays were used to screen for *dn*DSA post-transplant. A total of 47 out of 315 (15%) patients developed *dn*DSA at a mean of 4.6 ± 3.0 years post-transplant. Independent predictors of *dn*DSA were HLA-DRβ_1 MM > 0 (OR 5.66, $p < 0.006$); and non-adherence (OR 8.75, $p < 0.001$); with a strong trend toward clinical rejection episodes preceding *dn*DSA (OR 1.57 per rejection episode, p=0.061). The median 10-year graft survival for those with *dn*DSA was lower than the No *dn*DSA group (57% vs. 96%, $p < 0.0001$).

Pathology consistent with antibody-mediated injury occurred and progressed in patients with *dn*DSA in the absence of graft dysfunction. Furthermore, non-adherence and cellular rejection contributed to both *dn*DSA development and the risk of progression to graft loss.

(Human leukocyte antigen) HLA epitope matching is a novel strategy that may minimize *dn*DSA development. HLAMatchmaker software was used to characterize epitope mismatches at 395 potential HLA-DR/DQ/DP conformational epitopes for a subset of 286 donor–recipient pairs in which samples were available for high-resolution HLA-

typing. Epitope specificities were assigned using single antigen HLA bead analysis and correlated with known monoclonal alloantibody epitope targets. Locus-specific epitope mismatches were more numerous in patients who developed HLA-DR *dn*DSA alone (21.4 vs. 13.2, $p<0.02$) or HLA-DQ dnDSA alone (27.5 vs. 17.3, $p<0.001$). An optimal threshold for epitope mismatch (10 for HLA-DR, 17 for HLA-DQ) was defined that was associated with minimal development of Class II *dn*DSA using a receiver operating characteristic analysis. Applying these thresholds, 0% and 2.7% of patients developed *dn*DSA against HLA-DR and HLA-DQ, respectively, after a median of 6.9 years follow-up. Epitope specificity analysis revealed that 3 HLA-DR and 3 HLA-DQ epitopes were independent multivariate predictors of Class II *dn*DSA when mismatched between the donor and recipient.

HLA-DR and DQ epitope matching outperforms traditional low-resolution antigen-based matching and has the potential to minimize the risk of *de novo* Class II DSA development, thereby improving long-term graft outcome.

Chapter 2

Introduction

Introduction

Study Rational

Renal transplantation is the treatment of choice for patients with End-Stage Renal Disease (ESRD) due to improved quality of life, prolonged survival, and reduced costs when compared to dialysis.(1-3) In the modern era of renal transplantation sensitive cross-match techniques and potent immunosuppression have reduced the rate of acute rejection from 20-30% during the first post-transplant year during the 1980's to 10-15% by 2011.(4) http://srtr.transplant.hrsa.gov/annual_reports/2011/Default.aspx. Nevertheless, long-term graft survival has seen only modest improvement, limited primarily by immune-mediated injury and in particular chronic antibody mediated rejection (cAMR).(5-8) For those whose grafts fail and return to dialysis, patient survival declines by three fold in both Canadian and American cohorts that have been studied. (9,10) Therefore, it is imperative that the causes of late allograft loss be identified to target treatment and prevention strategies.

The development of *de novo* donor-specific HLA antibodies (*dn*DSA) post-transplantation has been associated with higher graft failure rates.(11-20) Moreover, *dn*DSA can appear before graft loss suggesting that *dn*DSA may represent a mechanism of repetitive injury and a potential prognostic biomarker.(12,21-23) Unfortunately, early studies of *de novo* antibodies employed less sensitive and accurate cytotoxicity or enzyme-linked immunosorbent assay (ELISA), which may have misclassified patients, and often did not determine the donor specificity of the *de novo*

HLA antibody, which has recently been shown to be important.(16,17) In addition, most studies evaluate sera for HLA antibodies at a single time point with no serial evaluation to determine when they develop and if they persist at the time of graft failure. Furthermore, the true importance of *dn*DSA may be underestimated as they frequently appear late post-transplant, whereas many studies have focused serum antibody assessment on the early post-transplant period (i.e. first 5 years).(24-26)

Importantly, there has been limited correlation of clinical and serologic events with histopathology in patients with *dn*DSA to infer causal relationships.(27) Even the most comprehensive pathologic analysis by Hidalgo et al. examined patients with graft dysfunction and therefore was unable to determine the frequency and timing of *dn*DSA onset relative to the onset of graft dysfunction and had no information as to the impact of *dn*DSA occurring in the absence of graft dysfunction.(17) Because clinical status at the time of *dn*DSA development may vary from normal function to acute dysfunction with rapid graft loss, the relevance of *dn*DSA across this spectrum warrants further study.

Part one of this study describes the sequential evaluation of sera for *dn*DSA in a consecutive cohort of kidney transplants from a single center utilizing the most sensitive HLA antibody detection techniques. The risk factors for *dn*DSA development, the correlation of *dn*DSA with clinical and pathologic outcomes, and the significance of *dn*DSA in patients with normal graft function are examined.

An important observation in part I of the study was that HLA-DR mismatch was an independent risk factor for the development of *dn*DSA.(28) In addition we an others

have observed that the majority of dnDSA are HLA Class II, either in isolation or concurrent with HLA Class I.(28,29) However, it is unknown if current HLA typing strategies by low-resolution antigen or high-resolution allele methods allows for adequate risk stratification to minimize *dn*DSA development. An alternative approach would be to replace the current HLA-based method of allocation by a more refined epitope-based method in which multiple potential immunogenic sites of the HLA molecule are evaluated. In this model, individual HLA epitopes contribute collectively to the overall immunogenicity of the mismatch, with epitope load or specific immunogenic HLA epitopes driving the formation of *dn*DSA post-transplant. This information could be used to assign a risk score to a given donor-recipient mismatch when allocating organs.

To address these questions the second part of the study examined a subset of the original prospective cohort in whom the necessary samples were available for high resolution HLA typing of both the donors and the recipients. Within this cohort of 286 donor-recipient pairs, 45 developed *dn*DSA against Class II HLA (HLA-DR or HLA-DQ). Epitope matching was assessed against traditional matching methods as a predictor for the development of *dn*DSA post-transplant. Models of epitope mismatch load were developed for HLA-DR and HLA-DQ to predict *dn*DSA risk, and specific epitope mismatches were evaluated for their relative immunogenicity.

Immunobiology of renal allograft rejection

Transplant rejection has been broadly classified into 3 phases; allorecognition, lymphocyte activation, and effector responses. However, in all cases of transplantation organs are first exposed to ischemic injury that can initiate non-specific innate immune system mediated damage. In fact it has been known for decades that the simple act of inducing ischemia and restoring blood flow to an organ results in the production of inflammatory cytokines, and the recruitment of macrophage and other cells into the organ.(30) In the transplant setting innate activation may occur locally in the graft as a consequence of ischemic injury during organ harvesting and cold storage, and in the case of deceased donors, systemically in response to the cause of death.(31-33) Tissue injury or stress may be sensed by immune cells through pattern recognition receptors (PRR's) which detect the expression of invariant damage associated molecular patterns (DAMPs) in the setting of tissue stress, injury, and necrosis.(34,35) DAMPs may include reactive oxygen species, heat shock proteins, heparin sulfate, and others,(34,36) and can be sensed by receptors present on antigen presenting cells (APC) such as macrophage, monocytes, and dendritic cells, as well as other immune cells.(31) The binding of DAMPs to DAMP receptors results in the production of pro-inflammatory cytokines interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), and type I interferons, in addition to the increased expression of chemokines and P-selectin. (37,38) The production of interferon-gamma (IFN-γ) may be particularly important in the transplant setting since HLA Class I and Class II molecules, which are present at low levels constitutively on the renal endothelium, are up-regulated in the presence of IFN-γ,

and serve as the targets for allorecognition.(39-41) Thus, DAMPs may act as a bridge that links early ischemic injury and necrosis to innate immune activation and ultimately the up-regulation of adaptive immune targets.(31,42,43)

Another feature of the non-specific injury response is complement activation. Activators of the complement pathway in this setting may include: 1) natural antibodies to normally cryptic antigens,(44,45) 2) acute phase reactants such as C-reactive protein (CRP) which bind to phospatidycholine and sphingomyelin in damaged membranes,(46-48) and 3) mannose-binding lectin (MBL), which has been shown to co-localize with complement in an ischemia-reperfusion injury model of cardiac allografts.(45) Once activated complement can lyse cells directly via the membrane attack complex (C5-9) and also produces opsonins (C3b, C4b) and chemokines (C3a, C5a) that attract other immune cells to the sight of the inflammation.(49)

In the post-transplant setting other inflammatory events such as infections, immunizations, procedures, or trauma may act non-specifically to initiate immune alloreactivity through IFN-γ mediated HLA up-regulation.(40,50) Importantly, non-specific injury activating the innate immune system is not required for transplant rejection as T-cell deficient mice who are allowed to heal and recover for 100 days post-transplant will still have prompt rejection of skin or cardiac grafts when reconstituted with T-cells.(51) However, non-specific innate activation helps to explain two commonly observe phenomena in transplant rejection. First, rejection occurs at the highest rate in the first few months after transplant.(52,53) Second, fully HLA matched deceased donors, in whom cold ischemic time is greater and DAMP's associated with the cause of death may be present, have worse graft survival compared to fully HLA mismatched living

donors.(54) Thus, although non-specific innate immune activation is not required for rejection to occur, its presence increases endothelial HLA expression, co-stimulatory molecules, and cytokines. These effects may alter the threshold of T-cell activation, promote recruitment of lymphocytes and influence the cytokine profile of the adaptive immune response. Thus, the transplant can now be identified as a site of inflammation and injury setting the stage for adaptive allorecognition and activation.

Allorecognition and Transplant Rejection

Understanding how the immune system recognizes alloantigen is central to an appreciation of *dn*DSA development. The primary targets of the immune system in the transplant setting are HLA Class I (HLA-A, -B, -C) and Class II (HLA-DR, -DQ, and -DP). The renal endothelial cells express both classes of HLA molecules constitutively, and as stated above, expression is greatly increased by inflammatory cytokines, especially IFN-γ. Three different pathways exists in which APCs may activate the adaptive immune system in secondary lymphoid tissues.(55,56) The first pathway, referred to as the “direct pathway” occurs when donor APCs, with intact donor HLA, are transported within the graft and are recognized by the recipient T-cell receptors (Figure 1A). By this mechanism up to 1:100 to 1:100,000 of recipient lymphocytes are able to recognize and respond to allogeneic HLA.(57-59) After activation, allo-specific T-cells undergo clonal expansion and migrate from the secondary lymph tissues back to the graft in order to bind target donor HLA directly on the surface of parenchymal or endothelial cells instigating T-cell mediated rejection (TCMR). This type of rejection is

Figure 1. Immune allorecognition pathways

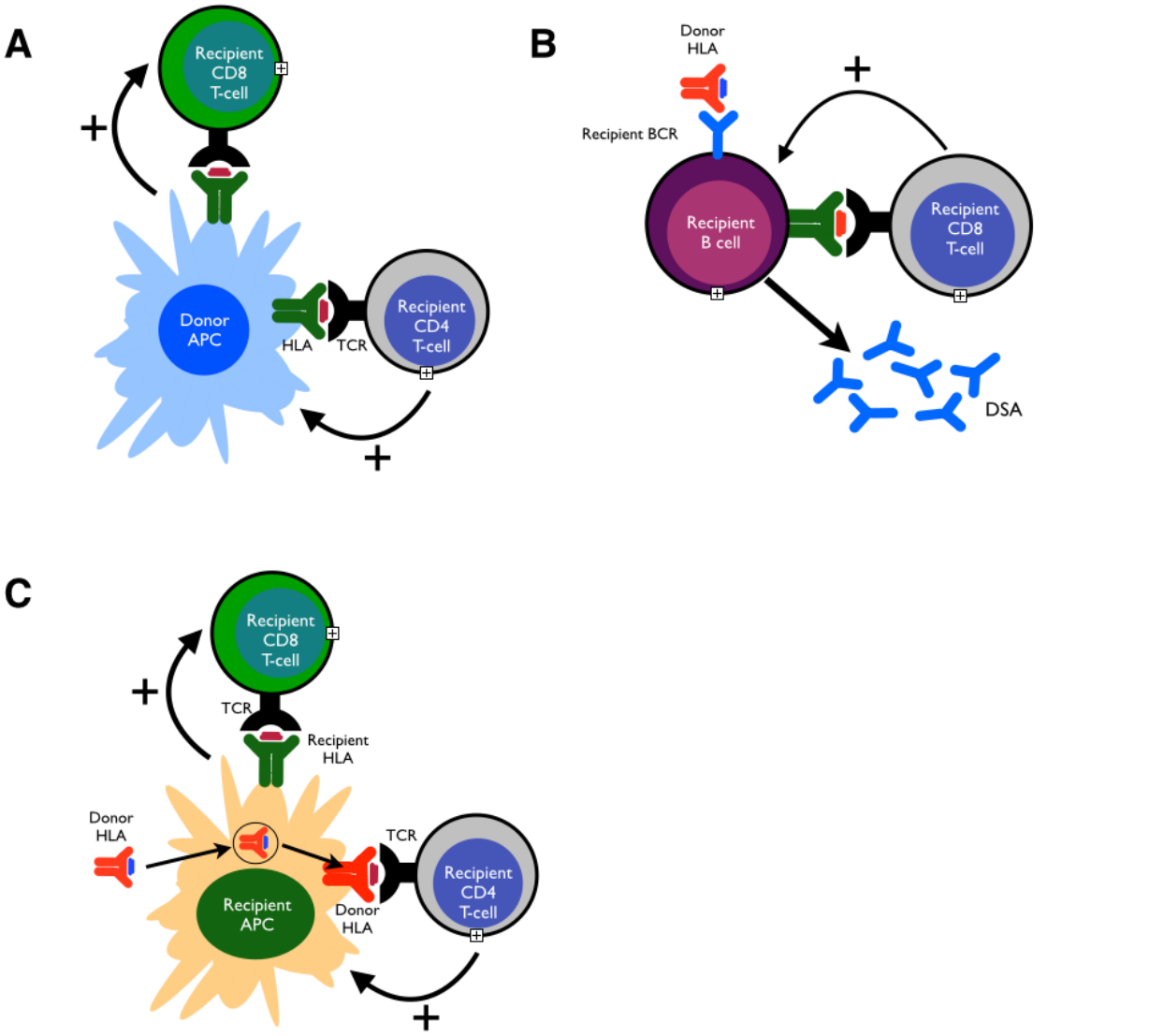


Figure 1. In the **Direct pathway** recipient CD4 and/or CD8 T-cells interact with donor antigen-presenting cells **(A).** In the **Indirect Pathway** recipient CD4 T-cells interact with recipient antigen-presenting cells **(B)**. In the **Semi-Direct Pathway** recipient antigen-presenting cells acquire donor HLA via membrane transfer**.** APC, Antigen Presenting Cel; HLA, Human Leukocyte Antigen; TCR, T cell Receptor; BCR, B cell Receptor; DSA, Donor-Specific Antibody

characterized by the infiltration of mononuclear cells into the renal interstitial space or the renal tubules.(60) Tissue injury may occur through the release of perforin, granzyme B, reactive oxygen species, and other degradative enzymes.(49)

The “indirect pathway” involves the recipient APCs, including B-cells, engulfing and processing allogeneic antigens before presenting them complexed to recipient HLA to recipient T-cells in the secondary lymph tissues (Figure 1B). By this mechanism 1:100,000 to 1:1,000,000 CD4 T-cells can be activated to undergo clonal expansion. Unlike the direct pathway, CD4+ T-cells produced by the indirect pathway are not able to bind specifically to allogeneic HLA targets, which express only donor HLA. However, alloantigen specific CD4 T-cells can differentiate into T follicular helper cells (Tfh) which are a specialized subset of CD4 T-cells that localize to the germinal center and induce B-cells to undergo class-switching and differentiation into long lived memory cells and DSA secreting plasma cells.(61-63) Secreted DSA can then initiate antibody-mediated rejection (AMR) by targeting the donor HLA molecules on the surface of the endothelium and parenchyma of the allograft.(64-66) Bound DSA can activate complement leading to the generation of the membrane attack complexes and the lysis of cells.(65,67) As mentioning above, the complement cascade intermediates may also act as opsonins (C3b, C4b) and chemokines (C3a, C5a). Bound DSA also can act as the activating ligand for Fc receptors on macrophage, granulocytes, and NK cells.(66)

The third pathway referred to as the “semi-direct” pathway involves the recipient APCs migrating to the graft and acquiring donor HLA through membrane transfer from donor cells.(55,56) These APCs expressing both donor and recipient HLA provide a

mechanism by which donor CD4 T-cells could theoretically provide indirect help (via the recipient APC) to donor CD8 T-cells in a three-cell cluster model (Figure 1C).

Notably, while the direct pathway can lead to a robust response in the early post-transplant period, it is limited by the lack of renewable donor APCs which are targeted for destruction by recipient cytotoxic T-cells and NK-cells.(68,69) A series of elegant experiments by Conlon et al. revealed that the indirect pathway, but not the direct, can induce B-cell class-switching and the production of long-lived plasma cells in response to alloantigen.(61) Thus, the direct pathway is unlikely to be a relevant mechanism for *dn*DSA development, which tends to develop late post-transplant and require antibody secreting plasma cells.(70) In this context it is not surprising that alloantibody is the dominant cause of late graft loss given: 1) the continual presence of donor endothelium, 2) the endless supply of recipient APCs, and 3) the inability of the direct pathway to operate after donor APC's have been lost.

As is the case with all antigen recognition, T-cell activation also requires a number of co-stimulator molecules, of which the most well described are CD80 and CD86 on the APCs interacting with cognate proteins on the T-cell (e.g. CD28 for positive feedback, CTLA-4 for negative feedback).(71,72) When antigen specific helper T-cells become activated a cascade of phosphorylating events and second messengers lead to the nuclear translocation of transcription factors including: nuclear factor of activated T-cells (NFAT), nuclear factor-kappa B (NFkB), and activator protein-1.(36)

Defining when donor-specific antibodies are *de novo*

To define DSA as being *de novo*, it is necessary to exclude the presence of DSA at the time of transplant using highly sensitive assays. Widespread adoption of highly sensitive solid phase assays has allowed for the detection of clinical relevant antibodies; however, the definition of a positive test using the mean fluorescence intensity (MFI) generated in flow cytometry assays lacks standardization. This is important since a low level MFI (<1000), considered insignificant by many centers, may be due to: 1) the presence of low titer DSA, or 2) the presence of the antibody epitope spread across multiple beads that each bind the DSA and dilute the individual bead MFI. Low-level DSA missed or called negative at the time of transplant may increase post-transplant as memory B-cells become activated leading to the false impression of *dn*DSA. Zachary et al. used tetramers with donor HLA-specific peptides to show that donor-specific memory B-cells can exist in DSA-negative (MFI cutoff 1000) serum, and that a B-cell recall response could result in DSA positivity within 2 months post-transplant.(73)

To avoid this problem we used two pieces of evidence to argue that DSA detected by post-transplant monitoring were in fact *de novo*.(28) First, all historical and current (time of the transplant) sera had to be negative using a low MFI cutoff of 300 with the most sensitive solid phase assays, and special attention given to the possibility of epitope spread across multiple beads. Second, protocol biopsies performed at six months were used to confirm the absence of antibody-mediated injury prior to the subsequent detection of a *dn*DSA in the serum.

Diagnosis of antibody mediated rejection

Banff renal allograft pathology conferences have resulted in a standardized classification schema to categorize and score acute and chronic histopathology affecting specific compartments of the renal allograft (Table 1).(60) The development of criteria for the pathologic diagnosis of both acute and chronic forms of AMR in 2003 was a major advancement towards understanding and studying the effect of DSA on the allograft (Table 2).(74-76) Currently, a diagnosis of acute AMR requires: 1) the presence of a morphologic evidence of tissue inflammation: glomerulitis (g), peritubular capillaritis (ptc), acute tubular necrosis or severe vasculitis (v); 2) immunopathologic evidence of complement deposition (C4d) in the microvasculature; and 3) serologic evidence of circulating DSA (Figure 2). The diagnosis of chronic antibody-mediated rejection (cAMR) requires the same serologic and immunofluorescence criteria plus the presence of chronic tissue injury: glomerular basement membrane thickening and reduplication (cg), peritubular capillary basement membrane multi-layering, interstitial fibrosis/tubular atrophy (IFTA), and/or fibrous intimal thickening in arteries (Figure 2).

Molecular Diagnosis of Antibody Mediated Rejection

The diagnosis of AMR is limited by the robustness of the individual diagnostic tests that are required to meet the criteria. Microcirculatory lesions for example lack specificity as they can be seen in 46% of borderline and 68% of T-cell mediated rejection.(77,78) Furthermore, histopathology scores are limited by subjectivity and reproducibility

Table 1. Quantification of Banff Histologic Scores

Acute	Chronic
Glomerulitis • g0: No glomerulitis • g1: Glomerulitis in < 25% of glomeruli • g2: Glomerulitis in 26-75% of glomeruli • g3: Glomerulitis in >75% of glomeruli	**Glomerulopathy** • cg0: <10% double contours in any glomerulus • cg1: ≤25% double contours in any glomerulus • cg2: 26-50% double contours in any glomerulus • cg3: >50% double contours in any glomerulus
Interstitial Inflammation • i0: No interstitial inflammation or < 10% • i1: 10-20% of parenchyma inflamed • i2: 26-50% of parenchyma inflamed • i3: > 50% of parenchyma inflamed	**Interstitial fibrosis** • ci0: <5% interstitial fibrosis • ci1: 6-25% interstitial fibrosis • ci2: 26-50% interstitial fibrosis • ci3: >50% interstitial fibrosis
Tubulitis • t0: No mononuclear cells in tubules • t1: 1-4 cells / tubular cross section • t2: 5-10 cells / tubular cross section • t3: >10 cells / tubular cross section or tubular basement membrane destruction	**Tubular Atrophy** • ct0: No tubular atrophy • ct1: ≤25% cortical tubules atrophied • ct2: 26-50% cortical tubules atrophied • ct3: >50% cortical tubules atrophied
Vasculitis • v0: No Arteritis • v1: mild-moderate intimal arteritis • v2: severe arteritis with ≥25% luminal area lost • v3: transmural arteritis or fibrinoid change and medial necrosis	**Vascular Fibrous Intimal Thickening** • cv0: No chronic vascular changes • cv1: ≤25% of luminal area narrowed • cv2: 26-50% of luminal area narrowed • cv3: >50% of luminal area narrowed
Peritubular Capillaritis • ptc0: <10% peritubular capillaries (ptc) inflamed • ptc1: ≥10% ptc inflamed and ≤ 4 cells / lumin • ptc1: ≥10% ptc inflamed and ≤ 10 cells / lumin • ptc1: ≥10% ptc inflamed and ≥ 10 cells / lumin	

Table 2. Current Banff Diagnostic Criteria for Antibody Mediated Rejection

Acute

• Morphologic Evidence (≥ 1 of the following required)
  • Acute tubular necrosis
  • Glomerulitis
  • Peritubular capillaritis
  • Severe vasculitis
• Immunopathologic Evidence
  • Positive staining for C4d in the peritubular capillaries
• Serologic Evidence
  • Presence of donor-specific antibodies in the blood

Chronic

• Morphologic Evidence (≥ 1 of the following required)
  • Transplant glomerulopathy
  • Peritubular basement membrane multilayering
  • Interstitial fibrosis and tubular atrophy
  • Arterial fibrous intimal thickening
• Immunopathologic Evidence
  • Positive staining for C4d in the peritubular capillaries
• Serologic Evidence
  • Presence of donor-specific antibodies in the blood

Figure 2. Common Pathologic Features Associated with Acute and Chronic Antibody Mediated Rejection

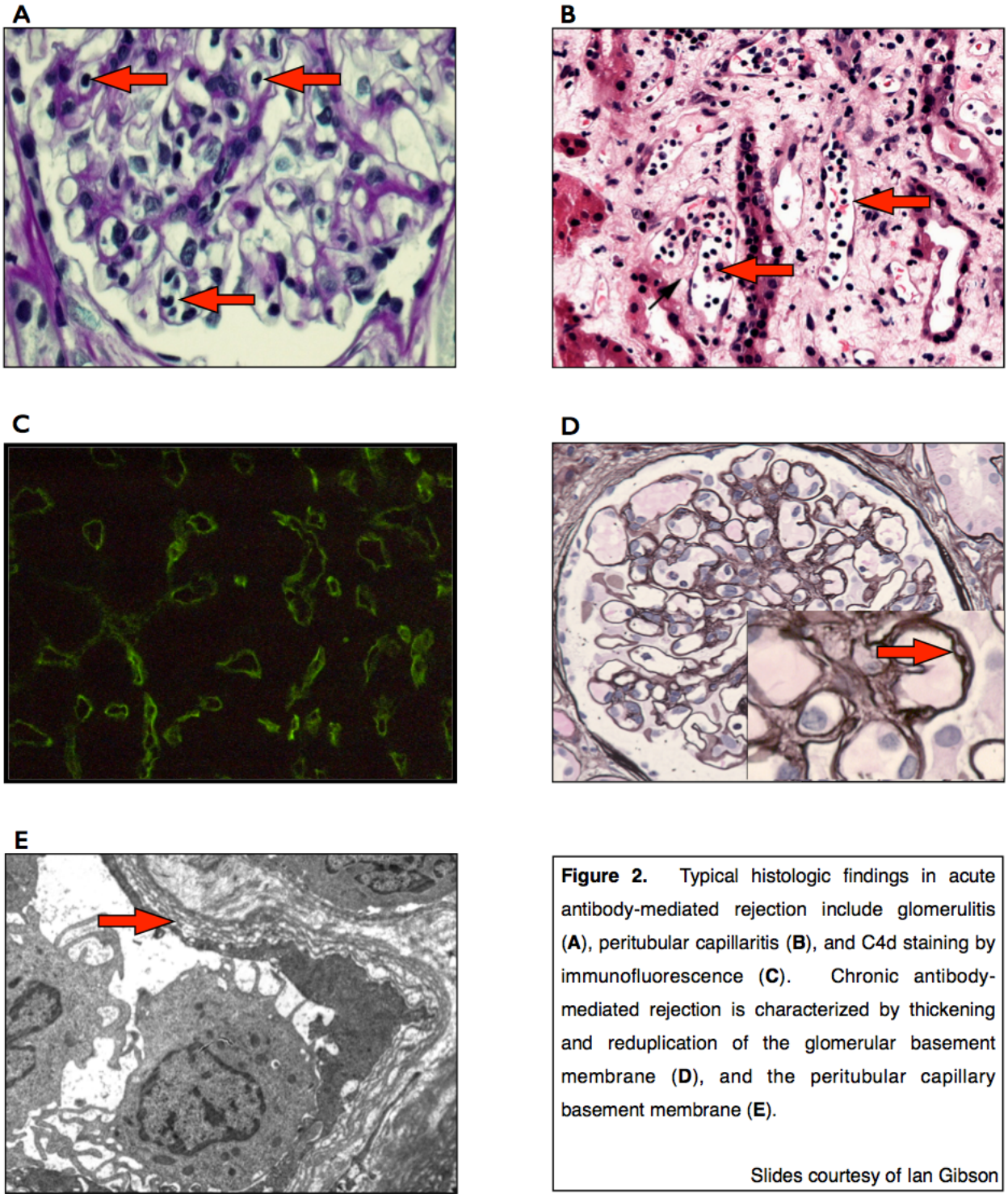


Figure 2. Typical histologic findings in acute antibody-mediated rejection include glomerulitis (**A**), peritubular capillaritis (**B**), and C4d staining by immunofluorescence (**C**). Chronic antibody-mediated rejection is characterized by thickening and reduplication of the glomerular basement membrane (**D**), and the peritubular capillary basement membrane (**E**).

Slides courtesy of Ian Gibson

between individual pathologists.(77,79,80) In addition, multiple authors have now published case series showing that C4d testing may be insensitive for the diagnosis of AMR.(27,81,82) Finally, HLA antibody assessment is both sensitive and specific, however, as stated above there is a lack of agreement on cutoffs to define positivity. (83,84)

In an attempt to address some of the limitations noted above, Sellarés et al. have published a molecular classifier score designed to aid in the diagnosis of AMR.(85) This score was developed using a microarray analysis of renal biopsy RNA transcripts acquired at the time of kidney biopsy for dysfunction and the previously developed classifiers known to be relevant in AMR (endothelial transcripts, INF-γ transcripts, and NK cell transcripts).(86-88) By this method the authors were able to show that the molecular score correlated with histologic scores, DSA status, and was an independent predictor of graft failure. These findings were validated in an independent international multicenter cohort.(89)

Due to the insensitivity of C4d testing, and the pathologic and molecular evidence that patients with Cd4 negative AMR have worse outcomes than patients without features of AMR, updated Banff 2013 guidelines (unpublished) will include C4d negative AMR if one of the following is present: 1) elevated molecular AMR score, or 2) at least moderate microvascular inflammation (g+ptc ≥ 2).(http://www.myast.org/T3)

Chapter 3

Methods

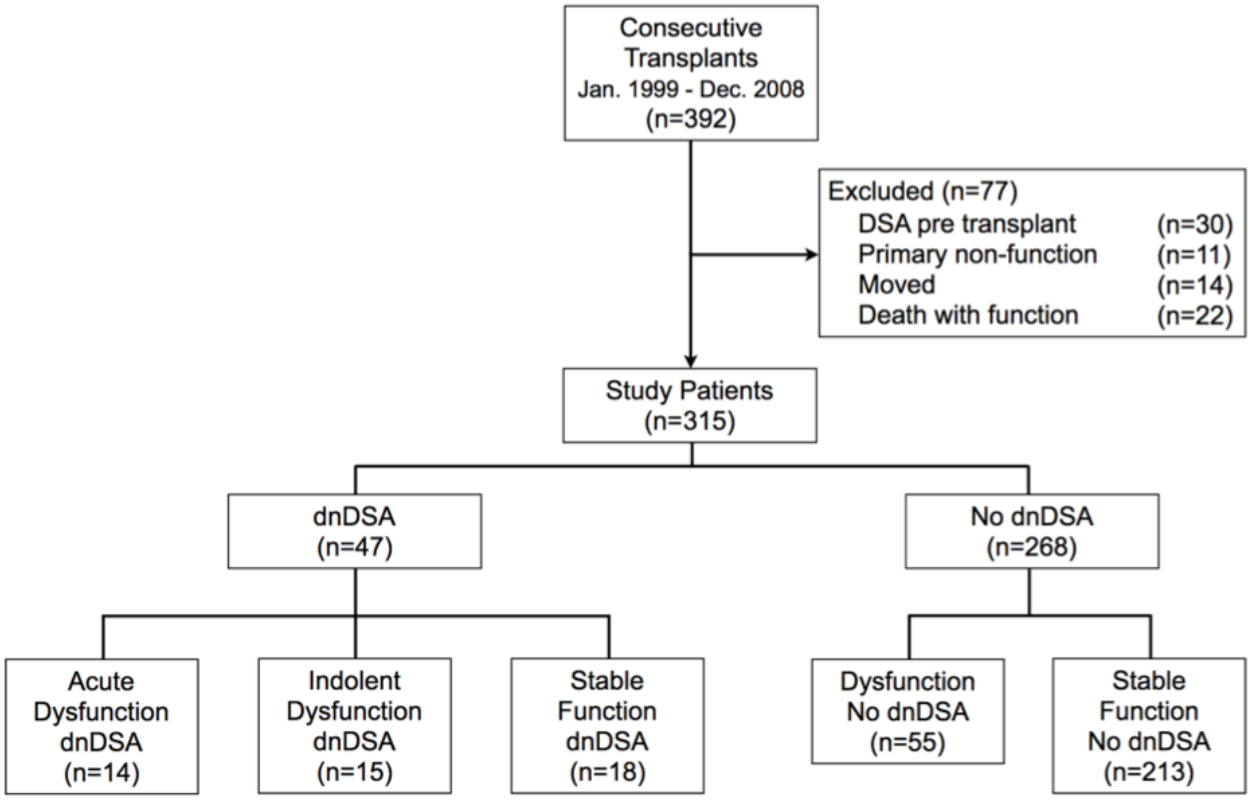
Methods

Patient Cohort Studied

Approval was obtained from the Institution Health Research Board (H2011: 211). Three hundred and ninety-two consecutive patients received renal transplants at the Health Sciences Centre, Winnipeg, Manitoba between January 1999 and December 2008. Seventy-seven patients were excluded: pre-transplant DSA (n = 30), death with a functioning graft (n = 22), moved and lost to follow-up (n = 14), primary non-function (n = 11), leaving 315 patients (adult n = 270, pediatric n = 45) for analysis (Figure 3). This cohort was largely Caucasian (72%) but also included Aboriginal (16%), Asian (8%) or African-American (2%) patients. Standard immunosuppression consisted of a calcineurin inhibitor (tacrolimus [n = 249] or cyclosporin [n = 65]), an antiproliferative (mycophenolate mofetil [n = 313] or azathioprine [n = 1]) and prednisone (n = 314). Induction therapy with thymoglobulin (n = 30) or basiliximab (n = 68) was used in 98/315 (31%) of patients. There was one transplant recipient with an HLA identical twin living donor who was not on immunosuppressive medication.

Part two of the study represented a subset of the original consecutive cohort in which the necessary DNA samples were available for high-resolution HLA typing of both the donor and the recipient. Of the 392 in the original cohort 106 were excluded for pre-transplant DSA (n = 30), death with a functioning graft (n = 22), moved and lost to follow-up (n = 14), primary non-function (n = 11), and no available sample for high-

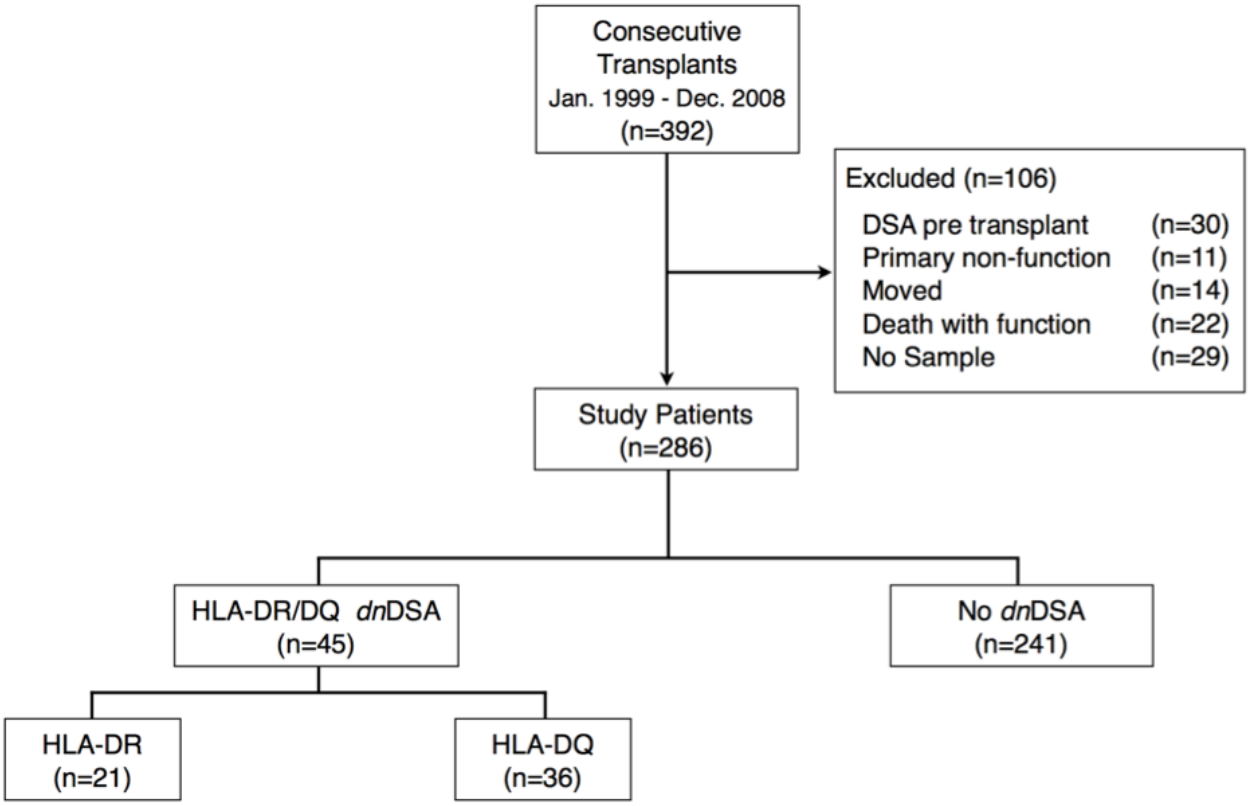
Figure 3. Patient Flow (Part I)



resolution HLA donor and/or recipient typing (n = 29) leaving 286 patients (adult [n = 247], pediatric [n = 39]) for analysis (Figure 4). This cohort was largely Caucasian (72%) but also included Aboriginal (16%), Asian (9%) or African-American (2%) patients. Standard immunosuppression consisted of a calcineurin inhibitor (tacrolimus [n = 237], cyclosporine [n = 48]), mycophenolate mofetil (n = 285) and prednisone (n = 285). Induction therapy with thymoglobulin (n = 29) or basiliximab (n = 56) was used in 85/286 (30%) patients. There was one transplant recipient with an HLA identical twin living donor not treated with immunosuppressive medication.

All patient clinical and lab data were stored and managed in a central statistical database. All patient charts were reviewed and additional information extracted as needed. Donor and recipient phlebotomy samples were centrifuged to isolate the buffy coat, which was then stored at -82°C. Serial serum samples obtained for DSA monitoring were collected at 0, 1, 3, 6, 9, 12, and 18 months and then yearly. Serum samples were frozen and stored at -82 °C for retrospective testing. Biopsies were analyzed by experienced renal pathologists, and scored according to the Banff 1997 classification with incorporation of the updates from 2009 (Table 1). The acute Banff score determines acute glomerular (g 0-3), interstitial (i 0-3), tubular (t 0-3), and vascular (v 0-3) changes, whereas the chronic Banff score assesses chronic glomerular (cg 0-3), interstitial (ci 0-3), tubular (ct 0-3), and vascular (cv 0-3) changes. Biopsies were judged adequate when ≥ 7 glomeruli and ≥ 1 vessel were available for analysis.

Figure 4. Patient Flow (Part II)



High Resolution HLA Typing by Sequence Specific Oligonucleotide (SSO) Methods

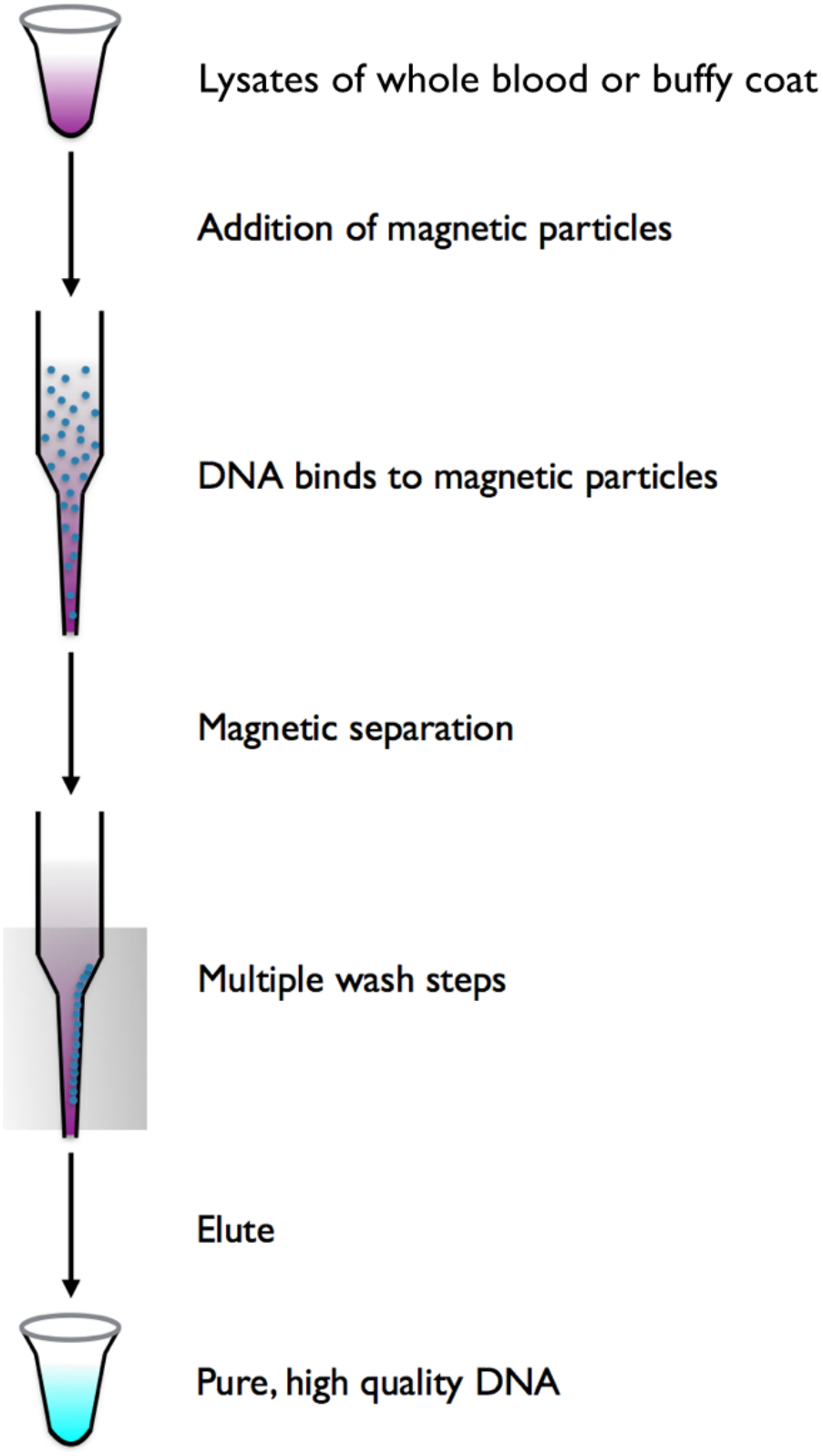
DNA Extraction

Stored buffy coat or whole blood samples were used for DNA extraction. The BioRobot EZ1 instrument (Precision System Science Co., Ltd., Japan) was used in with the EZ1 DNA Blood or Spleen kits (Lot #142329648, 139311736, 142336878, 142317117, 142322804). In this protocol 150 μL of buffy coat or 350 μL of whole blood was added to sample tubes. The EZ1 instrument was then loaded with reagent cartridges, sample tubes, and tubes for the eluted DNA. During the fully automated protocol: 1) cells were lysed, 2) in the presence of a chaotropic salt, DNA bound the silica surface of the magnetic particles, 3) the DNA was separated from the lysate using a magnet, 4) the DNA was then washed repeatedly while magnets held the beads in place, 5) and finally purified DNA was eluted and collected using elution buffer (Figure 5).

DNA Amplification and Hybridization

Amplification primers and D-mix were thawed immediately before use. Room temperature extracted DNA (2 μL) or control (2 μL reagent water) was added to each well of a 96 well PCR tray. Next, 0.2 μL of Taq Polymerase (Lot# 033), 13.8 μL D-mix, and 4.0 μL of group specific biotin labeled amplification primer were added to each well. Trays were covered and sealed before being placed into the Perkin Elmer (PE) 9600 Thermalcycler (PE Biosystems, Mississauga, ON, Canada) that was set to run the

Figure 5. DNA Extraction Overview



LABType® SSO PCR program (Table 3).

Once amplification was complete the presence or absence of biotinylated PCR product was visually confirmed using argose gel electropheresis with 5.0 μL of sample concurrent with positive and negative controls, following by staining with ethidium bromide and photo documentation with ultraviolet light.

Next 2.5 μL of denaturation solution was added to each well of a 96 well PCR tray before adding 5 μL of each amplified DNA sample into the corresponding well locations. Samples were covered and sealed then vortexed for 15 seconds before incubating for 10 minutes at room temperature. Then 5 μL of neutralization buffer was added to each well and mixed thoroughly. Next 4.0 μL of LABType SSO® bead mixture (LABType SSO $DR\beta_1$ Lot#07A, LABType SSO $DR\beta_{3/4/5}$ Lot#010, LABType SSO $DQ_{\alpha1\beta1}$ Lot#008, LABType SSO $DP_{\alpha1\beta1}$ Lot#003) was added to each well followed by 34 μL of hybridization buffer. Trays were sealed again and vortexed for 15 seconds. PCR trays were placed into the pre-heated (60°C) thermal cycler for 15 minutes. After removing the trays and seals 100 μL of wash buffer was added followed by centrifugation for 5 minutes at 1000 g. After removing the supernatant the wash was repeated twice with 100 μL of wash buffer followed by 5 minutes of centrifugation at 1000 g, for a total of 3 washes. Next, 50 μL of phycoerythrin-conjugated streptavidin (SAPE) solution was added to each well and mixed well. Trays were again sealed and placed into the thermal cycler for 5 minutes at 60°C. Upon removal from the thermal cycler, 100 μL of wash buffer was added to each well, followed by 5 minutes of centrifugation at 1000 g. After removing the supernatant, 70 μL of wash buffer was added to the bead pellet, which, was mixed thoroughly before being transferred to a microplate to be acquired using the

Table 3. LABType® SSO PCR Program for DNA Amplification

Step	Temperature	Time (min:sec)	Number of Cycles
1	96°C	03:00	1
2	96°C	00:20	5
	60°C	00:20	
	72°C	00:20	
3	96°C	00:10	30
	60°C	00:15	
	72°C	00:20	
4	72°C	10:00	1
5	4°C	forever	1

Luminex 100 instrument (Qiagen, Germantown, MD, USA).

Raw fluorescent intensity data acquired by the Luminex instrument was then imported and analyzed using HLA fusion™ software version 2.0. The fluorescent intensity of each bead was normalized and then compared to a reference set of positive and negative controls determined by the manufacturer (One Lambda, California, USA). The typing assignment was then determined by the pattern of positive and negative reactions associated with published HLA gene sequences.(90) Ambiguities were resolved by using published HLA frequency tables used to exclude rare alleles and select the most frequent alleles.(91)

Testing for Donor Specific Antibody

FlowPRA® Screening

Initial screening of patient serum for *dn*DSA was performed using FlowPRA® Screening beads. These beads consist of a pool of 30 different bead preparations each containing multiple HLA Class I and Class II antigens. All common HLA antigens, as well as many rare antigens are represented in the pool (Table 4).

A bead mixture consisting of 4.5 μL of Class I beads, 4.5 μL of Class II beads, and 0.8 μL of control beads was added to each tube for testing. Next 20 μL patient serum or control serum was added and mixed thoroughly. The samples are incubated for 30 minutes at room temperature. Tubes were washed with 1 mL of wash buffer then centrifuged at 9000 g for 2 minutes before removal of the supernatant. This wash was repeated for a total of two washes. Next 1 μL of 100X fluorescein isothiocyanate (FITC)

Table 4. HLA Alleles present on FlowPRA® Screening Beads

CLASS I					
HLA-A		**HLA-B**			**HLA-C**
A1	A66	B7	B48	B63	Cw1
A2	A68	B8	B49	B64	Cw2
A3	A69	B13	B50	B65	Cw4
A11	A74	B18	B51	B67	Cw5
A23	A80	B27	B52	B71	Cw6
A24		B35	B53	B72	Cw7
A25		B37	B54	B73	Cw8
A26		B38	B55	B75	Cw9
A29		B39	B56	B78	Cw10
A30		B41	B57	B81	Cw12
A31		B42	B58	BW4	Cw14
A32		B44	B59	BW6	Cw15
A33		B45	B60		Cw16
A34		B46	B61		Cw17
A36		B47	B62		Cw18

CLASS II					
HLA-DR		**HLA-DQ**		**HLA-DP**	
DRB1*01	DRB1*14	DQA1*01	DQB1*03:01	DPB1*01	DPB1*14
DRB1*01:03	DRB1*15	DQA1*02	DQB1*03:03	DPB1*02	DPB1*17
DRB1*04	DRB1*15	DQA1*03		DPB1*03	DPB1*18
DRB1*07	DRB1*16	DQA1*04		DPB1*04	DPB1*19
DRB1*08	DRB1*03:01	DQA1*05		DPB1*05	DPB1*20
DRB1*09	DRB1*03:02	DQA1*06		DPB1*08	DPB1*21
DRB1*10	DRB5*(51)	DQB1*02		DPB1*09	DPB1*40
DRB1*11	DRB5*(52)	DQB1*04		DPB1*10	
DRB1*12	DRB5*(53)	DQB1*05		DPB1*11	
DRB1*13		DQB1*06		DPB1*13	

labeled anti-human IgG was mixed with 99 μL of wash buffer to make a 1X solution. Then 100 μL of 1X FITC was added to each tube, vortexed, and incubated for 30 minutes. During the incubation samples were covered to avoid light exposure. Next, samples were fixed with 300 μL of 0.05% Sodium Azide before the fluorescence intensity of each bead was acquired on a Becton Dickinson (BD) FACSCalibur™ Flow Cytometer (BD Biosciences, Mississauga, ON, Canada). The percentage of beads with a fluorescent intensity greater than the negative control gate, also referred to as the panel reactive antibody (PRA), is determined by the software after taking into account peak architecture. All samples with greater than 0% PRA were further evaluated with single antigen beads to determine antigen specificity.

FlowPRA Single Antigen

All samples with a PRA > 0% on the FlowPRA® screening test or those patients known to be at increased risk of HLA antibodies development (e.g. pregnancy, previous blood transfusion, previous transplant) were tested by FlowPRA® single antigen beads for specificity analysis. Methods for the FlowPRA® single antigen bead analysis were the same as those described above for the FlowPRA® screen with the exception of the detection beads added. FlowPRA® single antigen beads consist of 32 beads divided into four groups each coated with a unique purified HLA antigens. Therefore, beads in which the fluorescent intensity is shifted to the right of the negative control represent a specific HLA antibody against the antigen represented on that bead. If the HLA antigens mismatched between the donor and recipient were not present in the FlowPRA® single

antigen 32 bead panel, additional bead panels were used. By this method we identified HLA antigen specificities before our laboratory switched to LABScreen® single antigen bead testing routinely in 2011 (see below). For the purpose of this study all *dn*DSA positive patient sera was retested by LABScreen® single antigen beads for validation and to maintain consistency for results reporting.

LabScreen Single Antigen

Using a 96 well microplate 4.5 μL of LABScreen single antigen beads and 20 μL of test serum was added to the bottom of each well. All common, and some rare, HLA alleles were represented on the Class I LABScreen single antigen beads (Table 5) and Class II LABScreen single antigens beads (Table 6). After mixing, trays were covered and sealed then incubated for 30 minutes at room temperature. Following incubation, 150 μL of wash buffer was added to each well and the tray was centrifuged at 1300 g for 5 minutes. Supernatant was removed and the wash was repeated twice with 150 μL of wash buffer and centrifugation at 1300 g for 5 minutes for a total of three washes. Following the third wash, supernatant was removed and 100 μL of phycoerythrin (PE)-conjugated anti-human IgG was added to each well and was mixed thoroughly before it was incubated for 30 minutes. At the end of the 30-minute incubation trays were centrifuged for 5 minutes at 1300 g and the supernatant was removed. A total of three washes were performed with 200 μL of wash buffer followed by centrifugation for 5 minutes and removal of supernatant. Next 80 μL of D-PBS with 0.05% Sodium Azide was added to each well and the trays were covered, sealed and vortexed. Fluorescence

Table 5. HLA Class I Alleles Represented on LABScreen Single Antigen Beads*

HLA-A		HLA-B		HLA-C
A*01:01	A*66:02	B*07:02	B*42:01	C*01:02
A*02:01	A*68:01	B*08:01	B*44:02	C*02:02
A*02:03	A*68:02	B*13:01	B*44:03	C*03:02
A*02:06	A*69:01	B*13:02	B*45:01	C*03:03
A*03:01	A*74:01	B*14:01	B*46:01	C*03:04
A*11:01	A*80:01	B*14:02	B*47:01	C*04:01
A*11:02		B*15:01	B*48:01	C*05:01
A*23:01		B*15:02	B*49:01	C*06:02
A*24:02		B*15:03	B*50:01	C*07:02
A*24:03		B*15:10	B*51:01	C*08:01
A*25:01		B*15:11	B*51:02	C*12:03
A*26:01		B*15:12	B*52:01	C*14:02
A*29:01		B*15:13	B*53:01	C*15:02
A*29:02		B*15:16	B*54:01	C*16:01
A*30:01		B*18:01	B*55:01	C*17:01
A*30:02		B*27:05	B*56:01	C*18:02
A*31:01		B*27:08	B*57:01	
A*32:01		B*35:01	B*57:03	
A*33:01		B*37:01	B*58:01	
A*33:03		B*38:01	B*59:01	
A*34:01		B*39:01	B*67:01	
A*34:02		B*40:01	B*73:01	
A*36:01		B*40:02	B*78:01	
A*43:01		B*40:06	B*81:01	
A*66:01		B*41:01	B*82:01	

*Beads represented in LABScreen® Single Antigen Lot 6.04.

Table 6. HLA Class II Alleles Represented on LABScreen® Single Antigen Beads*

HLA-DR	HLA-DQ	HLA-DP
DRB1*01:01	DQA1*01:01 DQB1*03:02	DPA1*01:03 DPB1*01:01
DRB1*01:02	DQA1*01:01 DQB1*05:01	DPA1*01:03 DPB1*02:01
DRB1*01:03	DQA1*01:01 DQB1*06:02	DPA1*01:03 DPB1*03:01
DRB1*03:01	DQA1*01:02 DQB1*05:02	DPA1*01:03 DPB1*04:01
DRB1*03:02	DQA1*01:02 DQB1*06:02	DPA1*01:03 DPB1*04:02
DRB1*04:01	DQA1*01:02 DQB1*06:04	DPA1*01:03 DPB1*20:01
DRB1*04:02	DQA1*01:02 DQB1*06:09	DPA1*01:04 DPB1*03:01
DRB1*04:03	DQA1*01:03 DQB1*06:01	DPA1*01:04 DPB1*11:01
DRB1*04:04	DQA1*01:03 DQB1*06:03	DPA1*01:04 DPB1*13:01
DRB1*04:05	DQA1*02:01 DQB1*02:01	DPA1*01:04 DPB1*18:01
DRB1*07:01	DQA1*02:01 DQB1*02:02	DPA1*01:04 DPB1*28:01
DRB1*08:01	DQA1*02:01 DQB1*03:01	DPA1*01:05 DPB1*18:01
DRB1*09:01	DQA1*02:01 DQB1*03:02	DPA1*02:01 DPB1*01:01
DRB1*09:02	DQA1*02:01 DQB1*03:03	DPA1*02:01 DPB1*02:02
DRB1*10:01	DQA1*02:01 DQB1*04:01	DPA1*02:01 DPB1*03:01
DRB1*11:01	DQA1*02:01 DQB1*04:02	DPA1*02:01 DPB1*05:01
DRB1*11:04	DQA1*03:01 DQB1*02:01	DPA1*02:01 DPB1*06:01
DRB1*12:01	DQA1*03:01 DQB1*03:01	DPA1*02:01 DPB1*09:01
DRB1*12:02	DQA1*03:01 DQB1*03:02	DPA1*02:01 DPB1*10:01
DRB1*13:01	DQA1*03:01 DQB1*03:03	DPA1*02:01 DPB1*11:01
DRB1*13:03	DQA1*03:02 DQB1*03:02	DPA1*02:01 DPB1*13:01
DRB1*14:01	DQA1*03:02 DQB1*03:03	DPA1*02:01 DPB1*14:01
DRB1*15:01	DQA1*03:03 DQB1*04:01	DPA1*02:01 DPB1*15:01
DRB1*15:02	DQA1*04:01 DQB1*02:01	DPA1*02:01 DPB1*17:01
DRB1*15:03	DQA1*04:01 DQB1*04:02	DPA1*02:01 DPB1*18:01
DRB1*16:01	DQA1*05:01 DQB1*02:01	DPA1*02:01 DPB1*19:01
DRB1*16:02	DQA1*05:03 DQB1*03:01	DPA1*02:01 DPB1*23:01
DRB3*01:01	DQA1*05:05 DQB1*03:01	DPA1*02:02 DPB1*28:01
DRB3*02:02	DQA1*06:01 DQB1*03:01	DPA1*04:01 DPB1*13:01
DRB3*03:01		
DRB4*01:01		
DRB4*01:03		
DRB5*01:01		
DRB5*02:02		

*All DRB1 alleles are associated with DRA1*01:01; Beads representative of LABScreen® Single Antigen beads Lot 8.05

intensities were then acquired using the Luminex 100 instrument (Qiagen, Germantown, MD, USA). Raw data was interpreted using HLA Fusion® software. To be considered adequate samples had to meet the following requirements: 1) negative control bead mean fluorescent intensity (MFI) < 500; 2) positive control bead MFI > 500; 3) positive control to negative control ratio > 2; and 4) at least 50 of each bead had to be acquired. The spread of HLA epitopes across multiple beads, which could lower individual bead MFI, and the pattern of background noise due to non-specific binding were also considered. After evaluating the above factors, beads with an MFI > 300 who met the adequacy requirements were considered positive for DSA.

Epitope Mismatch Identification

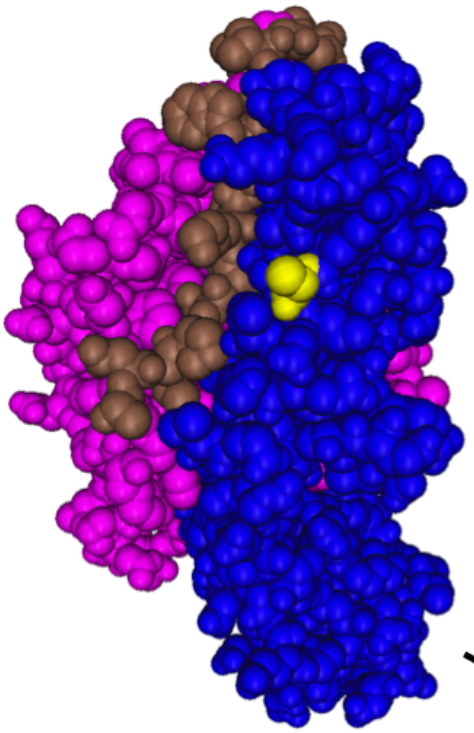
HLAMatchmaker software (HLA- Matchmaker DRDQDP Matching version 3.0, http://www.hlamatchmaker.net) was used to define potential epitope mismatches between donors and recipients.(92) In brief, this method is based on two underlying principles: (1) the immune system recognizes and develops antibodies against non-self antigens, or more specifically the epitopes on those antigens, while ignoring self-antigens/epitopes; (2) epitope binding affinity is largely determined by a small number of polymorphic amino acids near the center of the epitope. The evidence and the rationale for these postulates have been reported previously.(92-94) HLAMatchmaker software compares amino acid sequences between donor and recipient alleles to identify and quantify differences. Only polymorphic amino acids are of interest since these amino acids by definition may be different between the donor and recipient, therefore be

perceived as non-self. Furthermore, only amino acids at or near the molecules surface where they are accessible to antibody binding are considered. HLAMatchmaker version 3.0 incorporates the three-dimensional location of the amino acids within the HLA structure and identifies surface exposed patches of polymorphic amino acids that are continuous or discontinuous in the linear sequence but are brought into proximity on the tertiary structure (Figure 6). Patches of polymorphic amino acids on the surface are called eplets. By these methods a catalogue of potential eplet-derived epitopes has been described for each HLA locus (170 HLA-DR$\beta_{1/3/4/5}$, 89 HLA-DQα_1, 76 HLA-DQβ_1, 17 HLA-DPα_1 and 43 HLA-DPβ_1), which may be present on the donor and/or the recipient HLA alleles. We used HLAMatchmaker DRDQDP Matching (version 3.0) software to identify the subset of eplet-derived epitope mismatches that were present in each donor–recipient pair. We quantified the number of epitope mismatches between donors and recipients and correlated the epitope mismatch load with the risk of developing *dn*DSA. In addition we analyzed the individual of risk each epitope mismatch contributed in a multivariate model to assess whether certain epitope mismatches were more likely to lead to *dn*DSA development than others.

De novo Donor-Specific Epitope Specificity Assignment

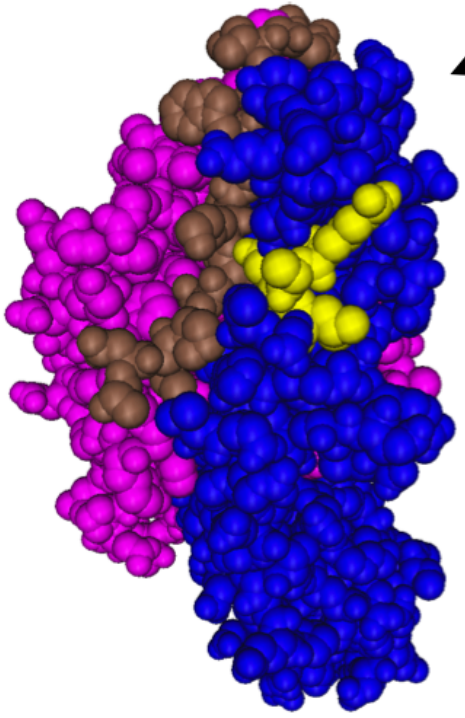
For transplant recipients who developed *dn*DSA, HLAMatchmaker Single DRDQ allele antibody screen version 2.0 was used to analyze LABScreen™ single antigen bead reactivity patterns to determine eplet-derived epitope specificities of *dn*DSA (http://

Figure 6. Eplet discovery



Surface exposed, polymorphic amino acid residue 71, glycine

Cn3D software used to select amino acid residues within a 3 angstrom radius



Amino acids within a 3 angstrom radius include: 70 arginine, 71 glycine, 72 glutamate, and 73 leucine. Only amino acids 70, 71, and 73 are polymorphic for HLA-DQ and therefore are including in the naming of the eplet.

www.hlamatchmaker.net).(95) In brief, each bead in the LABScreen® single antigen panel contains one HLA allele, and each of these alleles contains many different eplet-derived epitopes potentially responsible for the *dn*DSA developed by that patient. By examining the negative single antigen beads, which by definition have not bound the antibody, we could exclude any eplets present on those beads from the potential pool of eplet specificities. Since some eplets on the negative beads were also present on some positive beads, this also eliminated many eplet candidates from the repertoire of eplets represented by alleles on the positive beads. We then examined the remaining eplets on the positive beads for those present across all positive beads, which could explain the pattern of bead reactivity. When two or more eplet sites were possible (because they always occur together on the positive beads) they are separated by a slash (e.g. 52PQ/84EV).

A second approach to HLA epitope assignment based on the established knowledge of observed antibody binding reactivity patterns was also used to correlate with the HLAMatchmaker eplets.(96,97) In this strategy, El-Awar et al. isolated alloantibodies or monoclonal antibodies generated by hybridoma cells lines and tested them against LABScreen® single antigen beads.(96) The amino acid sequence of the alleles on all beads were analyzed to look for surface exposed polymorphic amino acids that were exclusively shared on the beads which reacted with the antibody in question. These amino acids define the putative epitopes and have been called Terasaki epitopes (TerEps). Final assignment of epitope specificity to *dn*DSA in our study integrated HLAMatchmaker eplet-derived epitopes with corresponding TerEps after sequence alignment of reactive beads using the Immunogenetics project HLA database along with

published reports of eplet/TerEp correlation.(90,98-101) Three-dimensional models of HLA structures were created and analyzed using freely available Cn3D software (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

Patient phenotypes at the time of *dn*DSA detection

Patients were classified into five phenotypes based on clinical presentation at the time *dn*DSA was first detected. Patients in the "Acute Dysfunction *dn*DSA" group were those with *dn*DSA and an acute rise in Cr ≥ 25% from baseline in ≤ 2 months (n = 14). The "Indolent Dysfunction *dn*DSA" group were patients with *dn*DSA and graft dysfunction (proteinuria ≥ 0.5 g/day or Cr ≥ 25% baseline) whose Cr increased by 25% in > 2 months (n = 15). The group "Stable Function *dn*DSA" had *dn*DSA detected by routine surveillance but had no graft dysfunction (proteinuria ≥ 0.5 g/day or Cr ≥ 25% baseline, n = 18). Patients with no *dn*DSA who had persistent graft dysfunction (proteinuria ≥ 0.5 g/day or Cr ≥ 25% baseline) were categorized as "Dysfunction No DSA" (n = 55), and 35 of 55 were biopsied. Finally, the "Stable Function No DSA" group had neither *dn*DSA nor persistent graft dysfunction (n = 213); 27 of these patients had 24 month protocol biopsies as part of a previous study and have now remained free of dysfunction for ≥ 5 years, thus serving as a control group for histologic comparisons.(102)

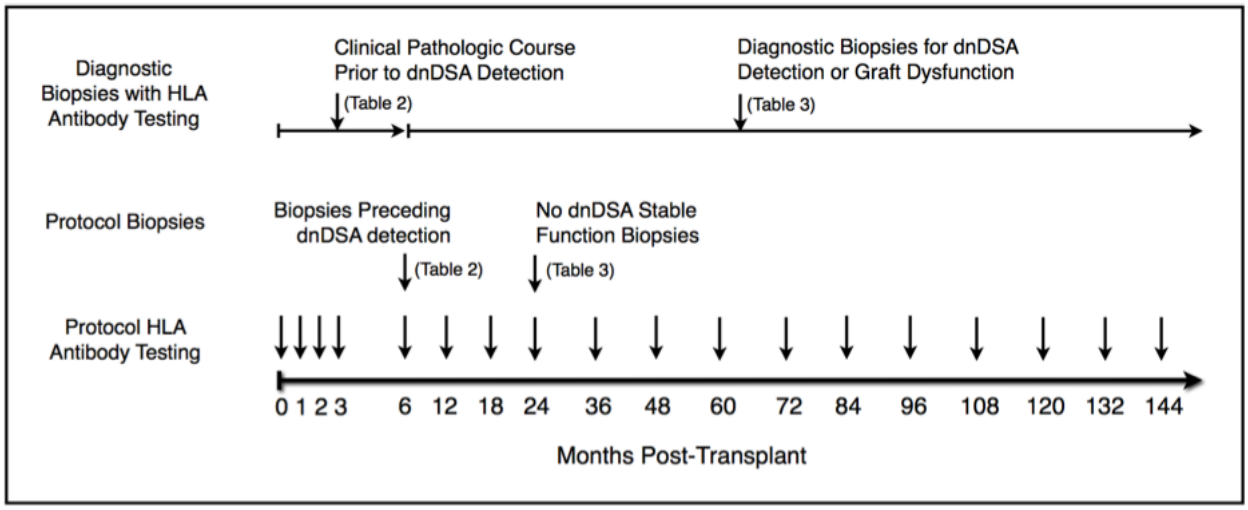
Definition of Non-Adherence

Non-adherence was defined as patient admission of medication non-adherence documented by clinic staff and/or drug levels below the detectable limit. Repeated failure to attend clinic visits or perform laboratory evaluations (i.e. blood draws for medication levels) constituted a pattern of behavior defined as non-adherence in a minority of patients.

Clinical and pathologic monitoring

Study patients were followed at a single center in the adult or pediatric transplant clinic. Protocols beyond 6 months include serum creatinine (Cr) measurement every four to eight weeks, and quarterly urine collections for proteinuria assessment. Six-month protocol biopsies were performed on all consenting patients (Figure 7). Renal biopsy was offered to all newly detected *dn*DSA patients since January 2008 as standard of care. Clinically indicated allograft biopsies were performed if proteinuria was ≥ 0.5 g/day or the serum Cr rose ≥ 25% from baseline without a known cause. Clinical rejections were biopsied proven in 92%, including 100% of the clinical rejections in the *dn*DSA subgroup. Biopsies were evaluated using the Banff criteria by a single pathologist who was blinded to DSA status in most but not all cases.(103)

Figure 7. Serial Serum and Histology Sampling Protocol



Statistical analysis

Part I

Comparisons between baseline predictors and clinical outcomes were done using Student's *t*-test for parametric continuous variables and Wilcoxon Rank-Sums test for non-parametric data. Chi-squared or Fisher's exact tests were used to test categorical variables. Survival analysis was done by the Kaplan–Meier method using the log-rank test for significance. Linear regression analysis was used to determine the association between two continuous variables. Nominal logistic regression analysis using a step-wise approach was performed to search for significant predictors of *dn*DSA and graft loss. All univariate correlations with a p-value <0.2 were initially considered in the models.

Part II

Stepwise nominal logistic regression was performed to search for significant clinical predictors of *dn*DSA. Nonparametric univariate analysis of patient demographics were compared using the Wilcoxon Rank-Sums test. *De novo* DSA free survival analysis was done by the Kaplan–Meier method using the log-rank test for significance. Receiver operating curve analysis was used to determine the optimal eplet-derived epitope mismatch threshold which best predicted *dn*DSA development. Logistic regression was used to assess the significance of individual eplet-derived epitope mismatches as predictors of *dn*DSA.

Chapter 4

Results

Results

Part I: Evolution and Clinical Pathologic Correlations of *De Novo* Donor-Specific HLA Antibody Post Kidney Transplant

Patient outcomes and risk factors for *dn*DSA and graft loss

Overall the entire patient cohort represented a low-risk group with 97% receiving their first transplant and 90% of patients having a calculated panel reactive antibody (cPRA) < 10%. However, post-transplant antibody surveillance during a mean follow-up of 6.2 ± 2.9 years found that 47 of 315 (15%) patients developed *dn*DSA. The mean time from transplantation to development of *dn*DSA was 4.6 ± 3.0 years. No patient had *dn*DSA before 6 months post-transplant. Thirty-two of 47 patients with *dn*DSA had class II antibodies alone, 3 had class I alone and 12 had both class I and II *dn*DSA. Once present the *dn*DSA persisted, or in some cases underwent epitope expansion (Table 7). An additional 18% of patients formed *de novo* HLA antibody that was not donor-specific (class I, n = 38; class II, n = 13; classes I and II, n = 5).

The 10-year graft survival for patients with *dn*DSA was lower than that of the no *dn*DSA group (59% vs. 96%, $p < 0.0001$, Figure 8). There was no difference in 10-year graft survival between the group with *de novo* HLA antibodies, pre-transplant HLA antibodies (n = 39), and those with no antibodies ($p = 0.817$, Figure 9). Patients with *dn*DSA had a

Table 7. *dn*DSA Specificities Detected within Phenotype Groups

Acute Dysfunction (n=14)		Indolent Dysfunction (n=15)		Stable Function (n=18)	
Class I	**Class II**	**Class I**	**Class II**	**Class I**	**Class II**
A2	DQ2	A31*	DR13, DR52, DQ6*	B58	DR8, DR12, DQ4, DQ7
B44	DR8*, DQ5	A2*, A11*	DR9*, DQ9	A1	DQ6
A30, B61	DR17, DR52, DQ2	A68, B65	DQ7		DR15*, DR51, DQ6
A34	DR15, DR51, DQ5	B62	DR17, DR53*, DQ2		DR53, DQ8, DQ9
A24	DR9		DQ6		DR13, DR52*
A24	DQ4		DR53		DR11
	DR17, DQ5		DQ5		DR16, DR51*
	DQ2		DQ5		DQ7
	DQ7		DQ5		DQ5
	DP13		DR13		DQ5
	DQ5		DR53*, DQ2		DQ2
	DR53, DQ2		DQ7		DQ8
	DR15, DR51*		DR13, DR52, DR53, DQ2		DQ7
	DQ6		DR53		DQ7
		A1			DQ9
					DQ4
				A30	
				A1	

Each row represents a patient within one of the three phenotype groups; *dn*DSA, de novo donor-specific antibody
**de novo* DSA that developed subsequent to the first positive serum sample

Figure 8. Kaplan-Meier estimates of graft survival of patients with dnDSA versus those without dnDSA

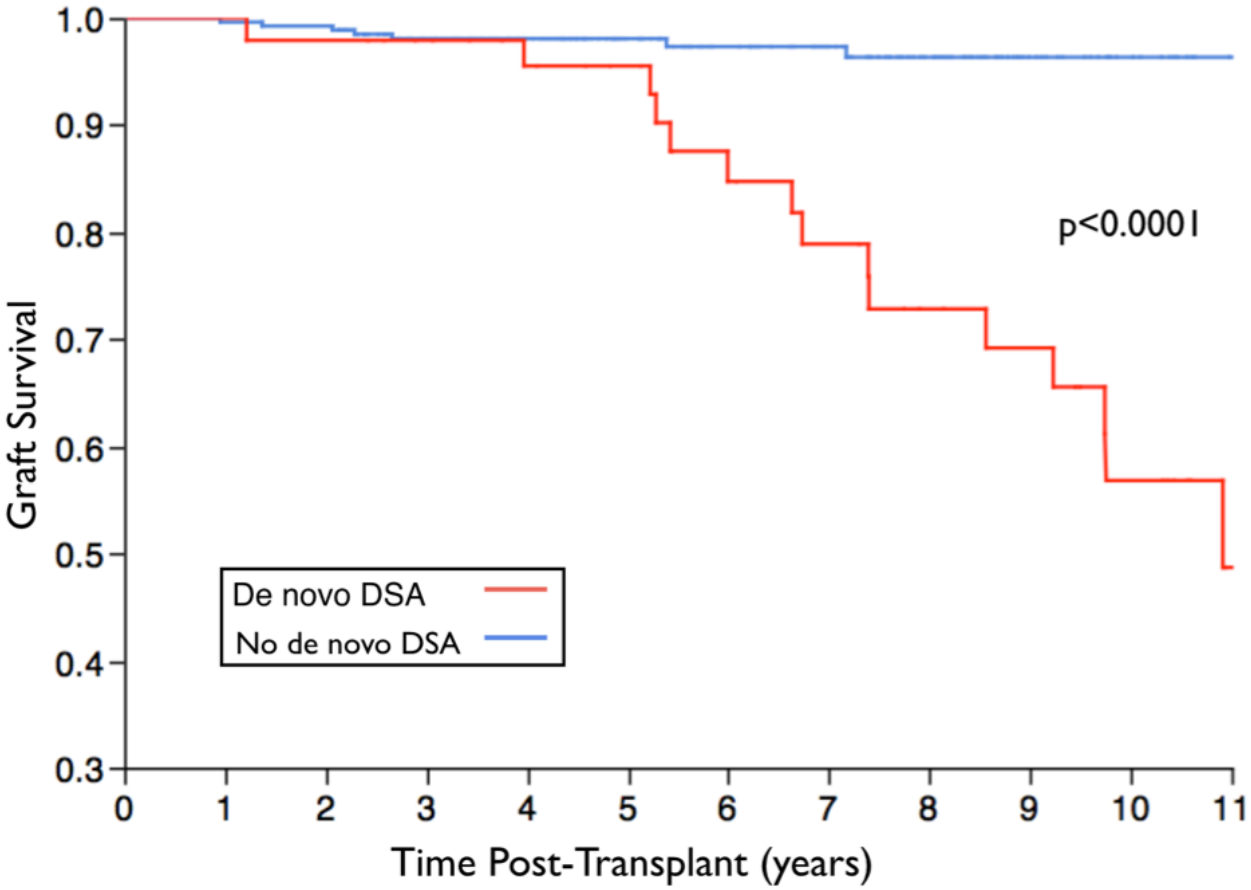
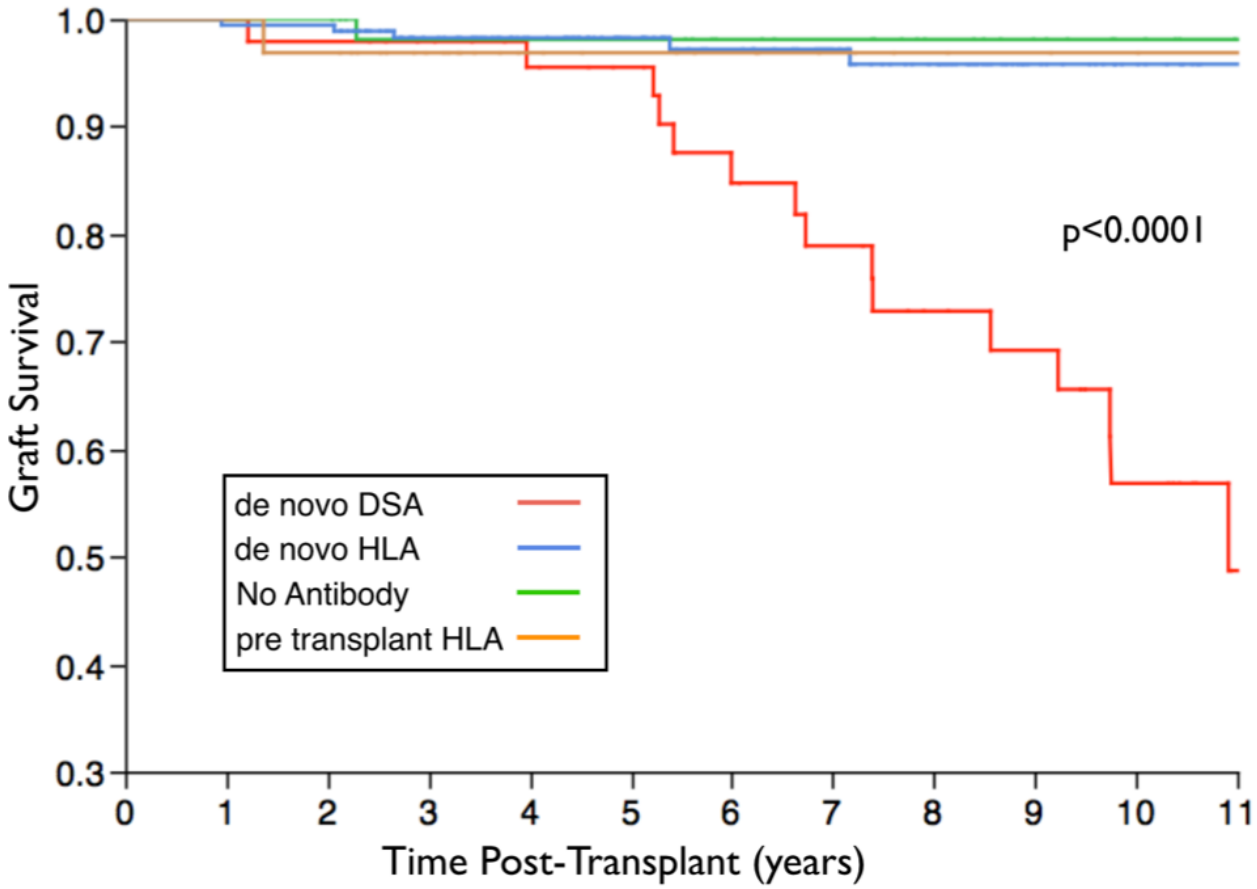


Figure 9. Kaplan-Meier estimates of graft survival of patients with donor-specific and non-donor-specific HLA antibodies



non-significant albeit strong trend toward a worse 10-year graft survival (p = 0.197, Figure 10) compared with patients with graft dysfunction from other causes. No patient developed *dn*DSA prior to 6 months after which there was a gradual rise in prevalence to 26% by 10 years (Figure 11). Non-adherent patients had a higher prevalence of *dn*DSA at all time points and by 10-years post-transplant was 61% versus 10% (p < 0.0001) in adherent patients (Figure 12). Patients in the *dn*DSA group were younger (33 years vs. 42 years, p = 0.008, Table 8) and had longer cold ischemic times (8.9 h vs. 7.5 h, p = 0.020) than those with no *dn*DSA. The *dn*DSA group had a higher number of total HLA mismatches (MM) compared to the no *dn*DSA group (3.28 vs. 2.84, p = 0.009) driven largely by HLA-DRβ_1 MM (1.15 vs. 0.87, p = 0.005). Other pre-transplant risk factors were similar between the two groups.

***Clinical pathologic correlations before the onset of dn*DSA**

There was no difference between the two groups with regard to delayed graft function. Post-transplant there was a large difference in the rate of non-adherence between those with and without *dn*DSA (49% vs. 8%, p < 0.001, Table 9). Non-adherence was more prevalent in the pediatric cohort (24% vs. 13%, p = 0.035).

The onset of *dn*DSA was ≥ 6 months in all patients (range 6–130). Interestingly, zero to six month clinical rejection episodes (borderline or Banff 1A/1B cellular rejections) occurred more commonly in the *dn*DSA group compared with the no *dn*DSA group (28% vs. 13%, p = 0.015, Table 9). Moreover, despite a median acute glomerulitis (g) score of zero in both groups, the *dn*DSA group had significantly higher peritubular capillaritis

Figure 10. Kaplan-Meier estimates of graft survival of those with *dn*DSA versus those with dysfunction from other causes

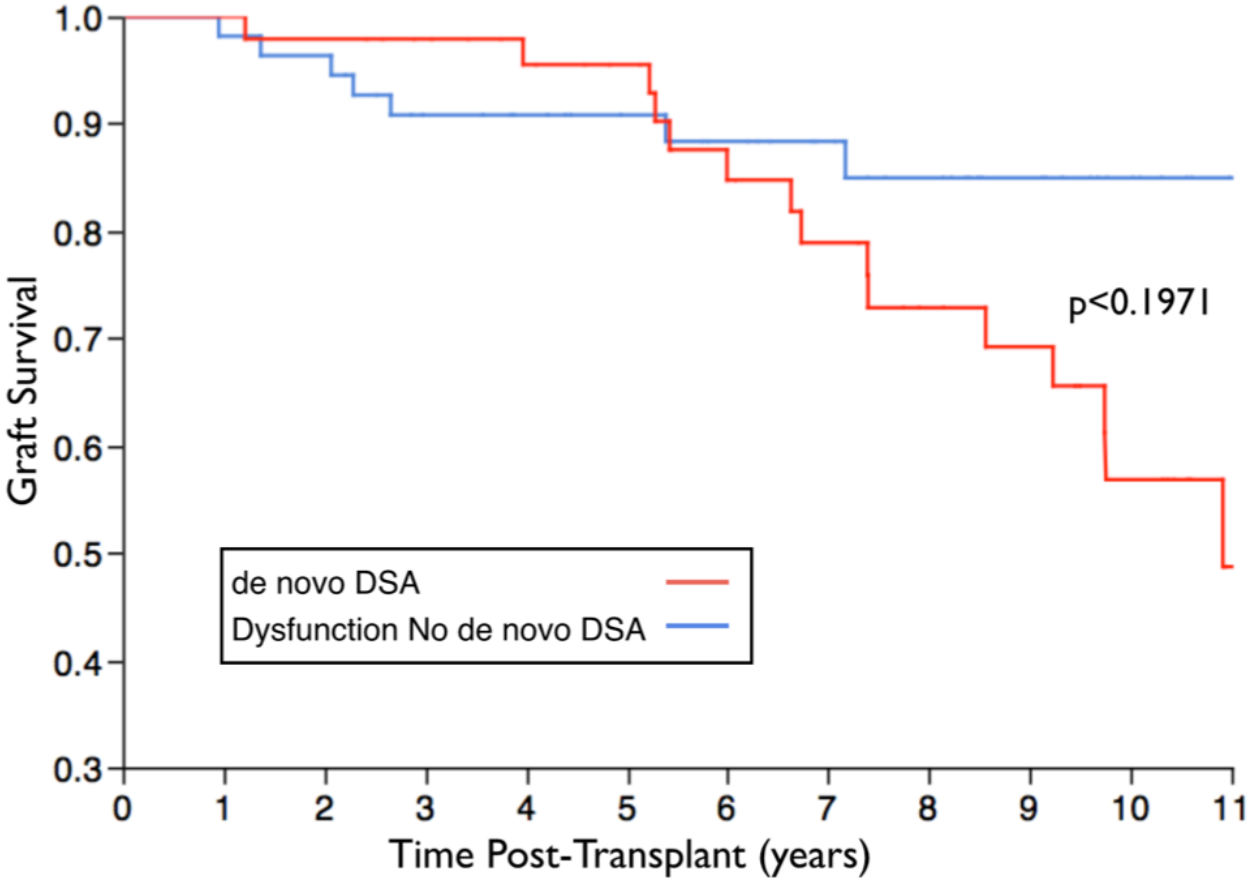


Figure 11. *dn*DSA Prevalence Post-Transplant

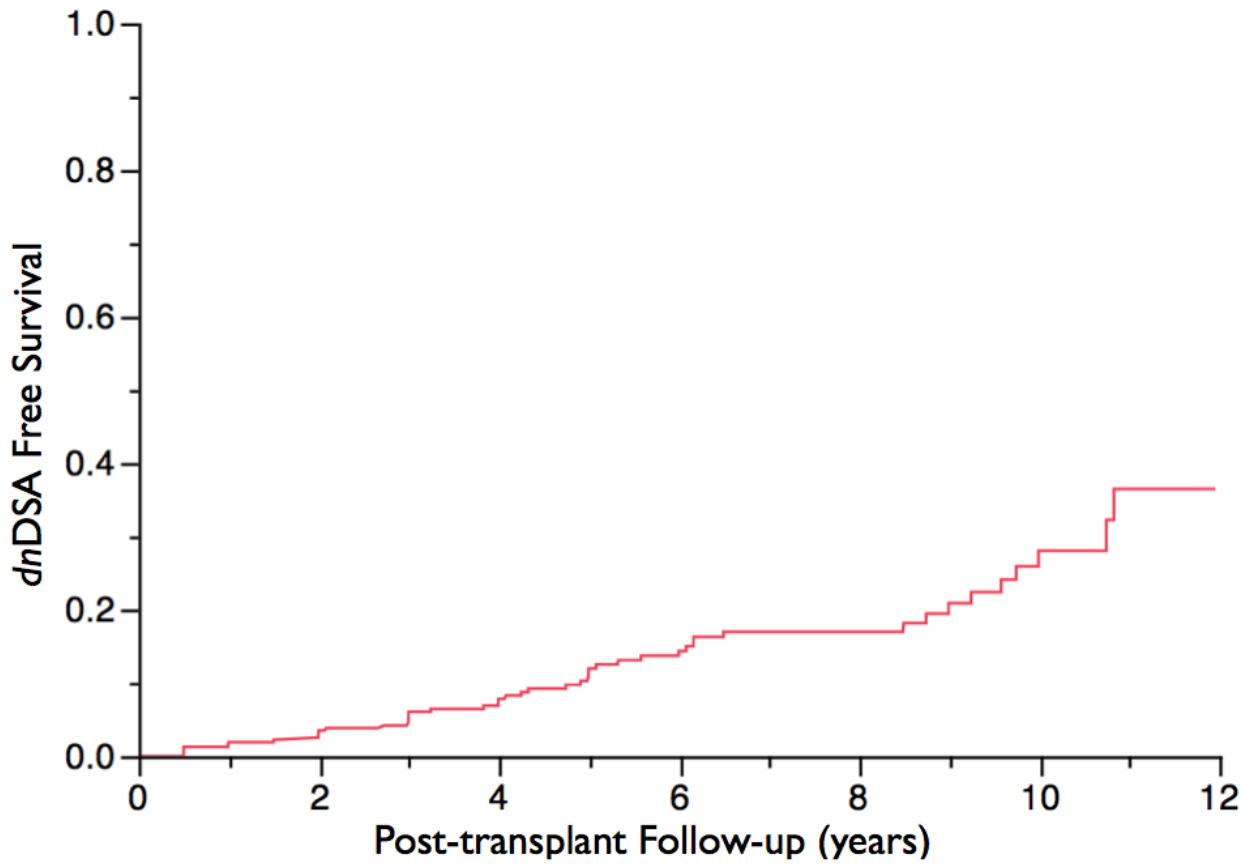


Figure 12. *dn*DSA Prevalence in Adherent versus Non-Adherent Patients Post-Transplantation

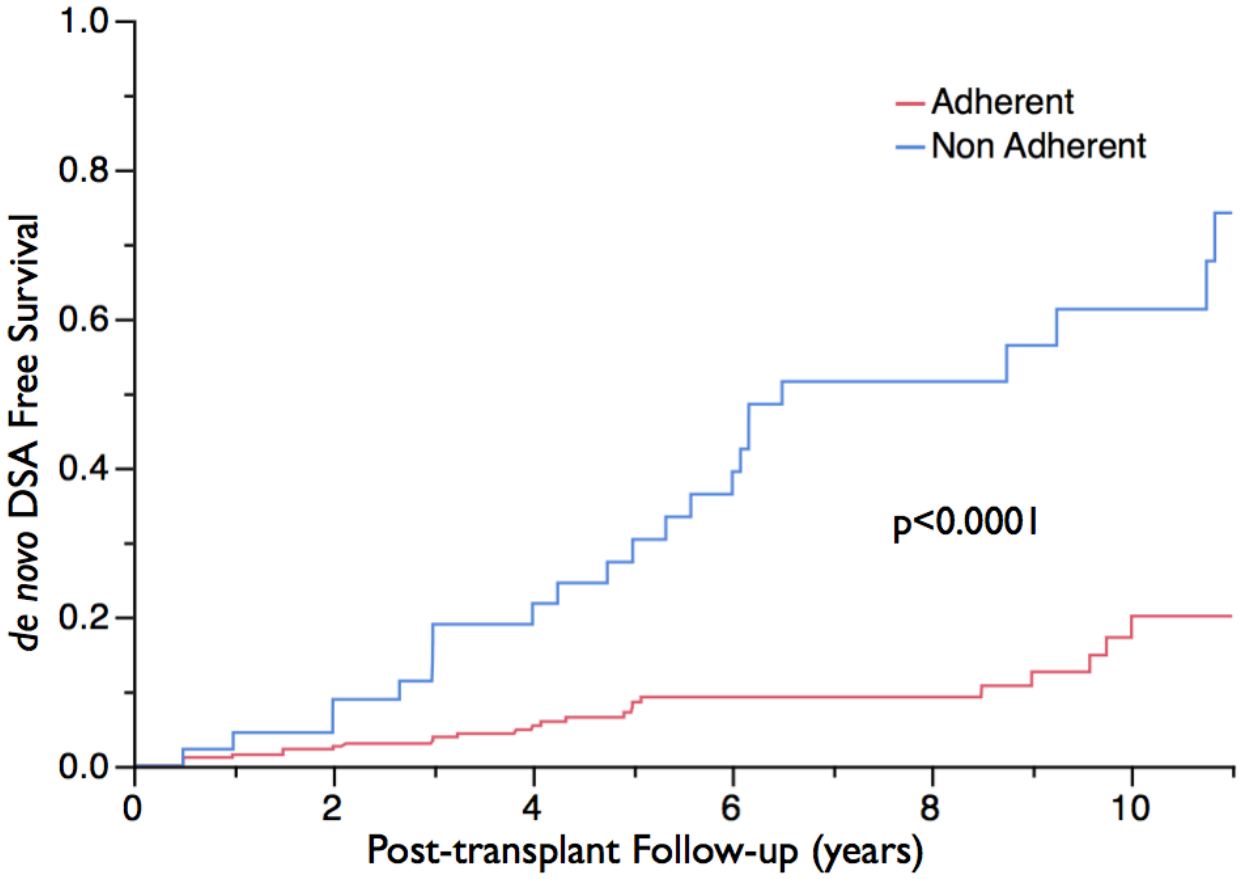


Table 8. Baseline Characteristics

	dnDSA (n=47)	No dnDSA (n=268)	p value
First Transplant	96%	98%	0.555
Adult Recipient	72%	88%	0.009
Recipient Age (years)	33 ± 17	42 ± 16	0.008
Donor Age (years)	36 ± 15	40 ± 14	0.053
Living Donor	43%	53%	0.211
cPRA%	3.4 ± 14	5.3 ± 17	0.371
HLA-A Mismatch	1.04 ± 0.6	0.97 ± 0.7	0.469
HLA-B Mismatch	1.17 ± 0.6	1.08 ± 0.7	0.432
HLA-DRβ1 Mismatch	1.15 ± 0.5	0.87 ± 0.7	0.005
HLA-DRβ3 Mismatch	0.26 ± 0.4	0.16 ± 0.4	0.097
HLA-DRβ4 Mismatch	0.21 ± 0.4	0.13 ± 0.3	0.118
HLA-DRβ5 Mismatch	0.15 ± 0.4	0.15 ± 0.4	0.979
HLA-DQβ1 Mismatch	0.96 ± 0.5	0.80 ± 0.7	0.085
Total Mismatch	3.28 ± 0.9	2.84 ± 1.6	0.009
Cold Ischemic Time (hours)	8.9 ± 6	7.5 ± 6	0.020

dnDSA, de novo Donor-Specific Antibody; cPRA, calculated Panel Reactive Antibody; HLA, Human Leukocyte Antigen

(ptc) scores in zero to six month clinical rejection biopsies compared to the no *dn*DSA

Table 9. Clinical Pathologic Course Preceding *dn*DSA

	No *dn*DSA (n=268)	Total *dn*DSA (n=47)	*dn*DSA Adherent Subgroup (n=24)	*dn*DSA Non-Adherent Subgroup (n=23)
Non-Adherence	8%	49%‡	0%	100%
DGF Requiring Dialysis	12%	11%	8%	13%
Clinical Rejection 0-6 months	13%	28%*	29%	26%
Subclinical Rejection 0-6 months	15%	26%	30%	22%
6 Month Protocol Biopsy n	151	37	18	19
g	0.02 ± 0.2	0.03 ± 0.2	0.05 ± 0.2	0.0 ± 0.0
i	0.37 ± 0.6	0.62 ± 0.8*	0.33 ± 0.6	0.90 ± 0.9†
t	0.41 ± 0.7	0.62 ± 0.9	0.28 ± 0.7	0.95 ± 1.0†
v	0.01 ± 0.1	0.03 ± 0.2	0.06 ± 0.3	0.0 ± 0.0
ptc	0.11 ± 0.4 (n=46)	0.60 ± 0.9 (n=30)†	0.14 ± 0.5 (n=14)	1.0 ± 1.0 (n=16)‡
C4d+	0% (n=16)	10% (n=31)	7% (n=14)	12% (n=17)
cg	0.02 ± 0.2	0.03 ± 0.2	0.05 ± 0.2	0.0 ± 0.0
ci	0.53 ± 0.6	0.57 ± 0.7	0.56 ±0.7	0.58 ± 0.7
ct	0.65 ± 0.6	0.62 ± 0.6	0.61 ± 0.6	0.63 ± 0.6
cv	0.36 ± 0.6	0.36 ± 0.6	0.44 ± 0.7	0.29 ± 0.5
Clinical Rejection 7-12 months	3%	6%	0%	13%*
12 month Serum Cr. (µmol/L)	113 ± 44	116 ± 44	121 ± 44	110 ± 45
*dn*DSA onset (months)	-	56 ± 36	51 ± 37	60 ± 34
Month Proteinuria ≥ 0.5 gm/day	51 ± 40 (n=43)	67 ± 34 (n=25)	70 ± 40 (n=7)	66 ± 33 (n=18)
Month Cr ≥ 25% baseline	34 ± 31 (n=33)	68 ± 31 (n=29)‡	79 ± 28 (n=7)‡	65 ± 32 (n=22)‡

Significance level compared to the No *dn*DSA group *p<0.05, †p<0.01, ‡p<0.001 DGF, Delayed Graft Function; g, glomerulitis; i, interstitial inflammation; t, tubulitis; v, vasculitis; ptc, peritubular capillaritis; cg, glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular fibrous intimal thickening; Cr, creatinine, dnDSA, de novo Donor-Specific Antibody

group (2 vs. 1, p = 0.049). Both the clinical rejection frequency and their ptc scores were higher independent of adherence in the *dn*DSA patients. Similarly, *dn*DSA patients had a trend toward more subclinical rejections (Banff 1A or higher) in the first 6 months compared to the no *dn*DSA group (26% vs. 15%, p = 0.100).

Before the onset of *dn*DSA, the only significant differences found in the 6-month protocol biopsies between the no *dn*DSA and the *dn*DSA groups were the interstitial inflammation (i) (0.37 vs. 0.62, $p < 0.05$) and ptc (0.11 vs. 0.60, $p < 0.01$) scores (Table 9). However, a sub-analysis of the *dn*DSA group found that these differences were confined to the non-adherent *dn*DSA subgroup. Indeed, in the non-adherent *dn*DSA group 9 of 19 had borderline or Banff 1A/1B subclinical cellular rejections in their 6-month protocol biopsies.

A stepwise logistic regression analysis identified two predictors of *dn*DSA after adjustment with a strong trend for a third: HLA-DRβ1 MM > 0 (OR 5.66, $p < 0.006$); and non-adherence (OR 8.75, $p < 0.001$); and clinical rejection episodes preceding *dn*DSA (OR 1.57 per rejection episode, p = 0.061).

Pathologic correlations with patient phenotypes at the time of dn*DSA *detection

Table 10 summarizes the three different patient phenotypes based on graft function at the time of *dn*DSA detection. Non-adherence was documented in 100% of the Acute Dysfunction *dn*DSA group, in 53% of the Indolent Dysfunction *dn*DSA group and in only 6% of the Stable Function *dn*DSA groups ($p < 0.001$). These groups were compared to

Table 10. Pathologic Correlations with Patient Phenotypes at the time of dnDSA Detection

	Acute Dysfunction dnDSA	Indolent Dysfunction dnDSA	Stable Function dnDSA	Dysfunction No dnDSA	Stable Function No dnDSA
n	14	15	18	55	213
Clinical Rejection 0-6 months	36%*	27%*	22%	24%*	10%
Non-Adherence	100%‡	53%‡	6%	16%*	6%
Month dnDSA Positive	60 ± 35	61 ± 31	49 ± 31	-	-
Month Protein ≥ 0.5 gm/day	63 ± 38	70 ± 33	-	51 ± 40	-
Month Cr ≥ 25% baseline	63 ± 34	73 ± 28	-	34 ± 31	-
Biopsy n	12	13	14	35	27
Month of Biopsy	63 ± 34	71 ± 26	53 ± 46	27 ± 21	24 ± 2
Creatinine at biopsy	490 ± 420‡	156 ± 59†	118 ± 44	189 ± 180†	106 ± 31
g	0.92 ± 0.8‡	0.92 ± 0.8‡	0.14 ± 0.4	0.20 ± 0.5	0.04 ± 0.2
i	2.0 ± 1.1‡	1.07 ± 0.8†	0.50 ± 0.8	0.74 ± 1.0	0.37 ± 0.6
t	2.0 ± 1.0‡	0.54 ± 0.5†	0.35 ± 0.6	0.60 ± 0.9†	0.11 ± 0.3
v	0.08 ± 0.3	0 ± 0	0.21 ± 0.8	0.03 ± 0.2	0 ± 0
ptc	2.20 ± 0.7‡	1.92 ± 1.0‡	0.93 ± 1.0‡	0.27 ± 0.6	0.04 ± 0.2
C4d+	80%‡	39%†	57%‡	0%	4%
cg	0.25 ± 0.5†	0.92 ± 1.2‡	0 ± 0	0.14 ± 0.4	0 ± 0
ci	1.17 ± 0.6*	1.62 ± 0.5‡	0.50 ± 0.7	1.37 ± 0.7‡	0.67 ± 0.6
ct	1.25 ± 0.6	1.85 ± 0.7‡	0.93 ± 0.5	1.46 ± 0.6†	0.93 ± 0.6
cv	0.75 ± 0.8	0.78 ± 0.6	0.57 ± 0.7	0.67 ± 0.7	0.41 ± 0.6
Months of follow-up post dnDSA detection	29 (1-69)	45 (1-88)	19 (0-128)	-	-
Graft Failure	57%‡	40%‡	0%	15%‡	0%

Significance level compared to the Stable Function No dnDSA group *p<0.05, †p<0.01, ‡ p<0.001.
g, glomerulitis; i, interstitial inflammation; t, tubulitis; v, vasculitis; ptc, peritubular capillaritis; cg, glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular fibrous intimal thickening; Cr, creatinine, dnDSA, de novo Donor-Specific Antibody

patients with Stable Function and No *dn*DSA and to a group with persistent graft dysfunction with no *dn*DSA.

Acute Dysfunction *dn*DSA group

In this group, the onset of *dn*DSA was essentially concurrent with the onset of clinical dysfunction and the mean serum Cr at biopsy was higher than that of the Indolent Dysfunction *dn*DSA group (490 μmol/L vs. 156 μmol/L, $p = 0.016$, Table 10). The Acute Dysfunction *dn*DSA group had higher g (0.92 vs. 0.04, $p < 0.001$), ptc (2.20 vs. 0.04, $p < 0.001$), C4d+ (diffuse or focal) (80% vs. 4%, $p = 0.001$) and cg (0.25 vs. 0, $p = 0.008$) scores in comparison to the Stable Function No *dn*DSA group consistent with a diagnosis of acute and chronic antibody-mediated injury. It should be noted that i, t and ci scores were also significantly higher in the Acute Dysfunction *dn*DSA group. In fact, 8 of 12 patients met the criteria for Banff 1A/1B cellular rejection and 1 of 12 patients met criteria for borderline cellular rejection.

Indolent Dysfunction *dn*DSA group

In this group, the onset of *dn*DSA preceded the onset of clinical dysfunction by an average of 9 months for proteinuria, and 12 months for serum Cr elevation. This group had higher g (0.92 vs. 0.04, $p < 0.001$), ptc (1.92 vs. 0.04, $p < 0.001$), C4d (39% vs. 4%, $p = 0.009$) and cg (0.92 vs. 0.0, $p < 0.001$) scores in comparison to the Stable Function No *dn*DSA group consistent with acute and chronic antibody mediated injury. The g and

ptc scores were not significantly different compared to the Acute Dysfunction group, however, the percentage of biopsies that were C4d positive was higher in the Acute Dysfunction group ($p = 0.047$). Biopsies were less likely to meet the criteria for Banff 1A/ 1B cellular rejection compared to the Acute Dysfunction group (1/13 vs. 8/12, $p = 0.004$), whereas 5 of 14 had borderline rejection.

Stable Function *dn*DSA group

Compared to the Stable Function No *dn*DSA group, the Stable Function *dn*DSA group had significantly higher ptc (0.93 vs. 0.04, $p < 0.001$), and C4d (57% vs. 4%, $p < 0.001$) scores. By light microscopy there was no evidence of chronic antibody mediated injury. Only 1 of 14 Stable Function *dn*DSA patients biopsied had Banff 1A/1B cellular rejection and 2 of 14 patients had borderline cellular rejection. Subsequently, patient's baseline immunosuppression was increased by targeting FK trough levels of 8 ± 2 μg/L and MMF trough levels of ≥ 2.0 μg/L. The first four patients with Stable Function *dn*DSA and microvascular inflammation were also treated with both pulse steroids and 2 g/kg intravenous immunoglobulin (IVIg) monthly for three cycles followed by repeat biopsy after a median follow-up of 5.5 months (range 4–8 months). In three of these patients, there was a progression in Banff g, ptc, C4d or cg scores (Table 11).

Dysfunction No *dn*DSA group

Biopsies were performed in 35 of 55 (64%) patients in the Dysfunction No *dn*DSA group. Recurrent glomerular disease accounted for 16 of 35 (46%), which included focal

Table 11. Repeat Biopsies

Biopsy Month	Serum Cr (μmol/L)	g	i	t	v	ptc	C4d	cg	ci	ct	cv
Patient 1											
Protocol (6 months)	98	0	0	0	0	0	n/a	0	0	0	0
Protocol (24 months)	82	0	0	0	0	0	Neg.	0	0	0	0
*dn*DSA +ve (61 months)	82	0	0	0	3	0	Neg.	0	0	0	1
Post IVIg (66 months)	91	1	1	1	1	2	Neg.	0	1	1	2
Patient 2											
Protocol (6 months)	128	0	0	0	0	0	Neg.	0	1	1	0
*dn*DSA +ve (27 months)	123	1	0	0	0	2	Pos.	0	0	1	0
Post IVIg (33 months)	125	2	2	1	0	2	Pos.	0	1	0	0
Patient 3											
*dn*DSA +ve (6 months)	130	0	2	2	0	2	Pos.	0	0	1	1
Post IVIg (14 months)	185	2	2	1	0	2	Neg.	2	2	2	0
Patient 4											
Protocol (3 months)	90	0	0	0	0	0	Neg.	0	0	0	0
*dn*DSA +ve (15 months)	93	0	1	1	0	2	Pos.	0	0	1	0
Post IVIg (19 months)	103	0	1	1	0	2	Pos.	0	0	1	0

*dn*DSA, *de novo* donor-specific antibody; IVIg, intravenous immunoglobulin

segmental glomerulosclerosis (n = 3), IgA nephropathy (n = 10) and other (n = 3). Interstitial fibrosis and tubular atrophy (IFTA) with (n = 8) or without (n = 6) cellular rejection occurred in 14/35 (40%). The remainder included acute tubular necrosis secondary to rhabdomyolysis (n = 1) and BK virus nephropathy (n = 2). There was no significant increase in g, ptc, C4d or cg scores compared to the Stable Function No *dn*DSA group consistent with the lack of an antibody mediated injury in either group.

Long-term graft outcomes

Graft loss occurred in 22 of 315 patients during the study period (Table 10), of which 14 had *dn*DSA. Biopsy proven causes of graft loss in the No *dn*DSA group included IFTA with (n = 3) or without (n = 2) cellular rejection, membranoproliferative glomerulonephritis (n = 1), diabetic nephropathy (n = 1) and BK nephropathy (n = 1). In the *dn*DSA group, biopsy proven causes of graft loss were chronic active antibody-mediated rejection (AMR, n = 10), IFTA with *de novo* membranous nephropathy (n = 1), and IFTA with concomitant recurrent IgA nephropathy (n = 1). Two patients with *dn*DSA did not have a biopsy at the time of graft loss.

After adjustment independent predictors for graft loss included: recipient age (OR 1.06 per year younger, p = 0.005), delayed graft function requiring dialysis (OR 5.21, p = 0.023), clinical rejection episodes preceding *dn*DSA (OR 1.95 per rejection episode, p = 0.015), non-adherence (OR 4.34, p = 0.016) and *dn*DSA (OR 6.34, p = 0.004).

Part II: Class II HLA Epitope Matching—A Strategy to Minimize De Novo Donor-Specific Antibody Development and Improve Outcomes

Clinical Predictors of de novo DSA

The patient cohort represented a low risk group overall with 97% receiving their first transplant and 89% of patients having a cPRA <10%. During a median follow-up of 6.9 years 45 out of 286 (16%) of patients developed HLA-DR *dn*DSA (n = 9), HLA-DQ *dn*DSA (n = 24) or both (n = 12) at a mean onset of 55 months (median 48, range 6–130 months)(Table 12). The median MFI on initial detection was 5266 (range 776–18,282), and all patients were monitored serially to ensure persistence of the DSA. While three patients had an initial MFI < 1000 (776, 890, 995), two of these individuals had biopsy-proven AMR and the third's subsequent MFI increased to 1224. No patients developed HLA-DP *dn*DSA. Our analysis was performed exclusively in patients who developed Class II *dn*DSA; however, Class I HLA *dn*DSA coexisted with Class II *dn*DSA in 14 out of 45 (31%) of patients, which was associated with a nonsignificant trend toward worse graft survival (39% vs. 61%, p = 0.2). At the time of initial detection the MFI of Class II *dn*DSA was significantly greater than the MFI of Class I *dn*DSA whether it occurred in isolation or in combination with Class I *dn*DSA (Table 13).

Patients who developed Class II *dn*DSA were younger (31 vs. 42 years, p < 0.01, Table 12), had younger donors (35 vs. 40 years, p < 0.02) and were more likely to have received a deceased donor graft (62% vs. 47%, p = 0.05) compared to those who did not develop Class II *dn*DSA. Non-adherence (n = 39/286) was more common in those

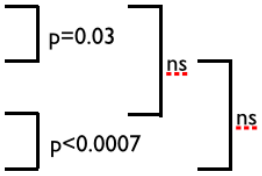
Table 12. Clinical Demographics for Part II

	No Class II *dn*DSA (n=241)	Class II *dn*DSA (n=45)	DR *dn*DSA Alone (n=9)	DQ *dn*DSA Alone (n=24)	Both DR & DQ *dn*DSA (n=12)
First Transplant	98%	96%	100%	95%	92%
Adult Recipient	89%	71%*	78%	71%*	67%
Recipient Age (years)	42 ± 16	31 ± 17*	32 ± 16	30 ± 15**	34 ± 22
Donor Age (years)	40 ± 14	35 ± 15*	38 ± 18	35 ± 15	32 ± 13*
Living Donor	53%	38%*	11%*	46%*	42%
Panel Reactive Antibody (cPRA)	6%	4%	4%	2%	7%
HLA-DR$\beta_{1/3/4/5}$ High Resolution Mismatch	1.8 ± 1.2	2.1 ± 0.9*	2.4 ± 0.9	1.8 ± 0.9	2.6 ± 0.7*
HLA-DR$\beta_{1/3/4/5}$ Epitope Mismatch	13.2 ± 13.5	17.6 ± 10.6**	21.4 ± 8.4*	12.8 ± 9.6	24.2 ± 9.9**
HLA-DQ$_{\alpha1/\beta1}$ High Resolution Mismatch	1.9 ± 1.4	2.3 ± 0.8*	2.0 ± 1.0	2.3 ± 0.7	2.7 ± 0.9*
HLA-DQ$_{\alpha1/\beta1}$ Epitope Mismatch	17.3 ± 16.7	24.4 ± 12.1***	11.0 ± 10.5	27.5 ± 11.7***	28.2 ± 6.0**
HLA-DP$_{\alpha1/\beta1}$ High Resolution Mismatch	1.2 ± 1.1	1.4 ± 0.9	1.7 ± 1.0	1.1 ± 0.9	1.6 ± 0.8
HLA-DP$_{\alpha1/\beta1}$ Epitope Mismatch	5.7 ± 6.6	6.9 ± 5.9	9.3 ± 4.1*	5.9 ± 5.7	7.2 ± 4.7
Rejection Episodes Preceeding dnDSA	0.2 ± 0.6	0.6 ± 0.8*	0.8 ± 1.0**	0.4 ± 0.6*	0.8 ± 0.8***
dnDSA Onset (months)	-	55 ± 34	79 ± 37	51 ± 20	45 ± 33
Delayed Graft Function	12%	11%	22%	4%	17%
Cold Ischemic Time (hours)	7 ± 6	9 ± 6*	12 ± 6*	7 ± 6	9 ± 6*
Non-Adherence	8%	47%***	33%*	46%*	58%***
Median 10-year Graft Survival	93%	44%***	43%***	64%**	30%***

Plus-minus values, means ± SD; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to No dnDSA group. dnDSA, de novo Donor-Specific Antibody; HLA, Human Leukocyte Antigen

Table 13. MFI values by dnDSA Class at initial detection

	Median MFI	Range MFI
dnDSA Class I Alone	2973	1964 - 5256
dnDSA Class II Alone	4979	776 - 18282
dnDSA Class I in patients with dnDSA Class II	1396	407 - 8672
dnDSA Class II in patients with dnDSA Class I	8663	1397 - 13496



*dn*DSA, *de novo* Donor-Specific Antibody

who developed Class II *dn*DSA (47% vs. 8%, $p < 0.001$). There was no significant difference with regard to transplant number, cPRA or delayed graft function between the two groups. Median 10-year graft survival was significantly lower in those who developed Class II *dn*DSA (44% vs. 93%, $p < 0.001$).

Molecular Predictors of de novo DSA

To quantify HLA-DRβ mismatches HLA-DRβ_1 and HLA-DR$\beta_{3/4/5}$ were considered together for a total high-resolution HLA-DR$\beta_{1/3/4/5}$ mismatch score, which could range from zero to four. Similarly, both HLA-DQα_1 and β_1 chains were considered as a combined HLA-DQα_1/β_1 score, which could range from zero to four mismatches. Both high-resolution and epitope mismatches were greater in the Class II *dn*DSA group compared to the no Class II *dn*DSA group (Table 12). However, on subgroup analysis high-resolution typing revealed a trend, but no significant difference in locus-specific mismatches for those who developed HLA-DR *dn*DSA alone (2.4 vs. 1.8, $p = 0.1$, Table 12) or HLA-DQ *dn*DSA alone (2.3 vs. 1.9, $p = 0.2$) compared to those who did not develop *dn*DSA. In contrast, locus-specific epitope mismatch was significantly greater for those developing HLA-DR *dn*DSA alone (21.4 vs. 13.2, $p < 0.02$) or HLA-DQ *dn*DSA alone (27.5 vs. 17.3, $p < 0.001$). For patients who developed *dn*DSA against both HLA-DR and DQ loci, high-resolution mismatches were significantly elevated for both HLA-DR (2.6 vs. 1.8, $p < 0.02$) and HLA-DQ (2.7 vs. 1.9, $p < 0.05$), which was also the case for the epitope mismatch at both HLA-DR (24.2 vs. 13.2, $p < 0.01$) and HLA-DQ (28.2 vs. 17.3, $p < 0.01$) loci. No patients in the study developed *dn*DSA against HLA-DP, and

there was a low number of high-resolution (1.2 - 1.1) and epitope mismatches (5.9 - 5.7). Low-resolution, high-resolution and epitope mismatch were each analyzed for their ability to predict *dn*DSA development in Figure 13 where epitope mismatch was found to provide better resolution compared to the other methods.

In a multivariate model, independent clinical predictors of HLA-DR *dn*DSA were non-adherence (OR 6.0, $p < 0.001$, Table 14A), HLA-DR epitope mismatch (OR 1.06 per mismatch, $p < 0.001$) and clinical rejection episodes preceding *dn*DSA onset (OR 2.6 per rejection episode, $p < 0.001$). The independent predictors of HLA-DQ *dn*DSA were non-adherence (OR 8.5, $p < 0.001$), HLA-DQ epitope mismatch (OR 1.04 per mismatch, $p < 0.001$) and younger age (OR 1.03 per year younger, $p < 0.01$).

Determining an Optimal Mismatch Threshold

The effect of different epitope mismatch load cutoffs was analyzed using Kaplan-Meier *dn*DSA free survival curves (Figure 14). Dividing epitope mismatch by quartiles revealed a significant difference in *dn*DSA free survival for both HLA-DR ($p < 0.01$, Figure 14A) and HLA-DQ ($p < 0.01$, Figure 14B), which was driven by the difference between the first or second quartile compared to either of the third or forth quartiles. Figures 14(C) and (D) show locus-specific epitope mismatches analyzed using an optimal cutoff, determined by a receiver operating curve analysis, of 10 mismatches for HLA-DR ($p < 0.001$) and 17 mismatches for HLA-DQ ($p < 0.001$). In contrast, apart from a zero antigen mismatch at each locus, attempts to define a threshold using high-resolution typing for HLA-DRβ_1, HLA-DR$\beta_{3/4/5}$, HLA-DQα_1 or HLA-DQβ_1 to minimize *dn*DSA

Figure 13. HLA Typing Methods used to Predict *dn*DSA Development

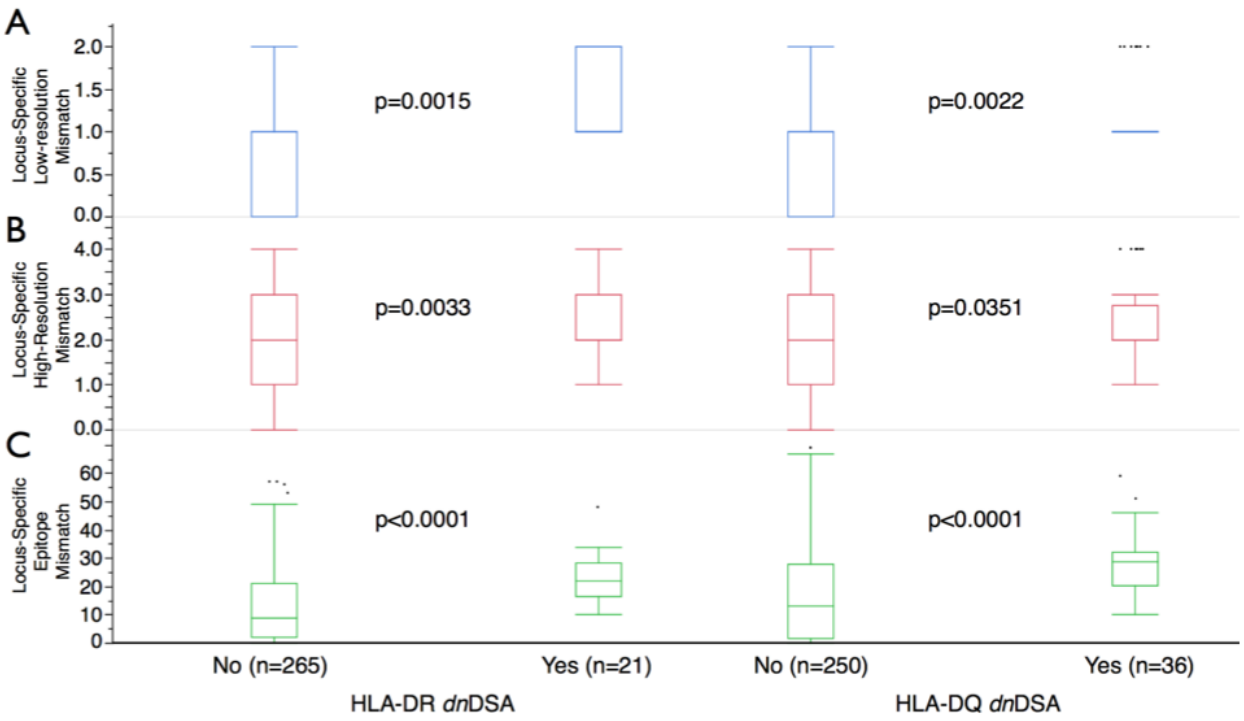


Figure 12. Panel (**A**) shows the number of low-resolution HLA-DRβ_1 or HLA DQβ_1 mismatches for patients who developed or did not develop *dn*DSA posttransplant. Panel (**B**) shows the HLA-DR$\beta_{1/3/4/5}$ or HLA-DQα_1/β_1 high-resolution mismatches. Panel (**C**) shows the HLA-DR$\beta b_{1/3/4/5}$ or HLA-DQα_1/β_1 eplet-derived epitope mismatches. *dn*DSA, de novo donor-specific antibody; HLA, human leukocyte antigen.

Table 14. Multivariate Models of *dn*DSA Predictors*

Model	HLA Loci	Predictors	Odds Ratio (per unit change)	Odds Ratio (per unit change)	p value
A		Non-Adherence	6.0 (2.1-17.0)	-	p<0.001
Clinical Predictors	**HLA-DR**	HLA-DR Epitope Mismatch Load	1.06 (1.03-1.10)	32.8 (4.6-258.7)	p<0.001
		Clinical Rejection Preceding *dn*DSA	2.6 (1.5-4.6)	120.4 (7.9-2138.0)	p<0.001
		Non-Adherence	8.5 (3.6-20.0)	-	p<0.001
	HLA-DQ	HLA-DQ Epitope Mismatch Load	1.04 (1.0-1.02)	14.0 (2.9-70.7)	p<0.001
		Younger Age	1.03 (1.0-1.10)	8.9 (1.7-47.9)	p<0.01
B		48YQ (TerEp# undefined)	4.7 (1.2-16.6)	22.1 (1.5-276.3)	p<0.02
Epitope Predictors Adherent Patients (n=247)	**HLA-DR**	14SEH (TerEp#1006)	1.6 (0.9-2.8)	7.3 (0.5-63.9)	NS
		71DRA/71DEA (TerEp#1018)	4.0 (1.4-10.4)	15.6 (2.0-108.8)	p<0.01
		45GE/52LL/71RKA (TerEp#2001)	0.7 (0.04-3.4)	0.4 (0.001-11.4)	NS
	HLA-DQ	52PL/140T/182N (TerEp#2014)	3.0 (1.2-7.4)	9.2 (1.4-52.3)	p<0.02
		52PQ/84EV (TerEp#2004)	2.4 (1.1-4.9)	5.8 (1.3-24.1)	p<0.02

* Odds ratios with 95% Confidence Intervals HLA, Human Leukocyte Antigen

Figure 14. Kaplan-Meier dnDSA Free Survival Curves Analyzed by Locus-Specific Epitope Mismatches

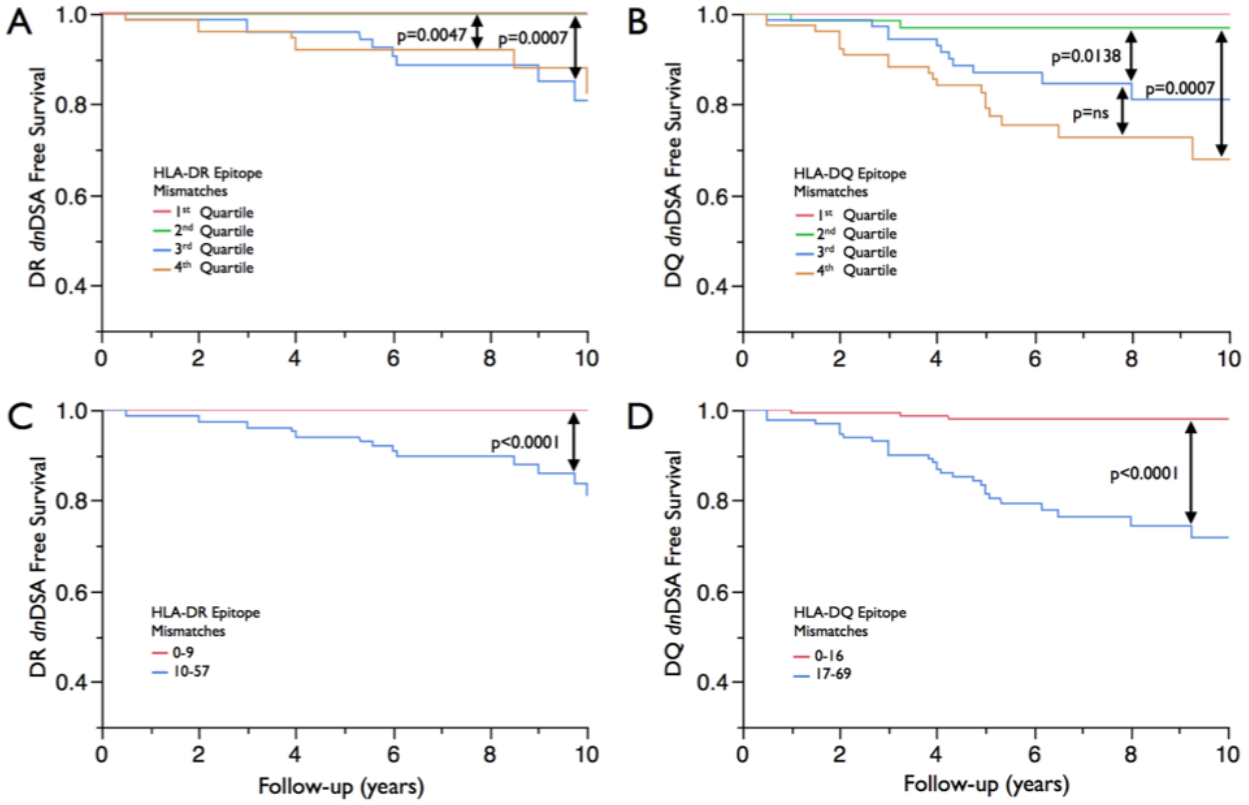


Figure 13. Panel (**A**) shows DR *dn*DSA free survival split by HLA-DRβ1/3/4/5 epitope mismatch quartiles. Panel (**B**) shows DQ *dn*DSA free survival split by HLA-DQα1/βb1 epitope mismatch quartiles. Panel (**C**) shows DR *dn*DSA free survival split by an optimal mismatch cutoff of 10 mismatches for HLA-DRβ1/3/4/5 and in Panel (**D**) an optimal mismatch cutoff of 17 for HLA-DQα1/β1. *dn*DSA, de novo donor-specific antibody; HLA, human leukocyte antigen.

development were uninformative (Figure 15).

Determining the Immuno-dominance of Individual Epitopes

Patients were monitored with serial serum tests and the first positive serum for each patient was used to assign epitope specificities for *dn*DSA against HLA-DR or HLA-DQ (Table 15). When analyzing the entire cohort the most common epitope specificities assigned to HLA-DR *dn*DSA were 14SEH (TerEp #1006) assigned to four *dn*DSA, 48YQ (no TerEp defined) assigned to three *dn*DSA and 71DRA/ 71DEA (TerEp #1018) assigned to three *dn*DSA. A multivariate logistic regression model identified 14SEH (OR 1.7, 95% CI 1.1–2.5, $p < 0.01$) and 71DRA/71DEA (OR 2.6, 95% CI 1.1–5.6, $p < 0.02$) as independent predictors of *dn*DSA while 48YQ (OR 2.4, 95% CI 0.8–6.3, $p = 0.08$) did not reach significance. In a subset analysis of adherent patients (n = 247, Table 14B), 71DRA/71DEA (OR 4.0, 95% CI 1.4–10.4, $p < 0.01$) and 48YQ (OR 4.7, 95% CI 1.2–16.6, $p < 0.02$) were independent predictors of *dn*DSA while 14SEH was no longer significant (OR 1.6, 95% CI 0.9–2.8, $p = 0.09$). However, it was noted that 4 out of 4 patients who developed HLA-DR *dn*DSA against the 14SEH epitope were non-adherent.

When analyzing the entire cohort the most common epitope specificities assigned to HLA-DQ *dn*DSA were 52PQ/84EV (TerEp #2004) assigned to nine *dn*DSA, 52PL/140T/ 182N (TerEp #2014) assigned to five *dn*DSA and 45GE/52LL/ 71RKA (TerEp #2001) assigned to five *dn*DSA. All three of these epitope specificities were significant

Figure 15. Kaplan-Meier dnDSA Free Survival Curves Analyzed by Locus-Specific High-Resolution Mismatches

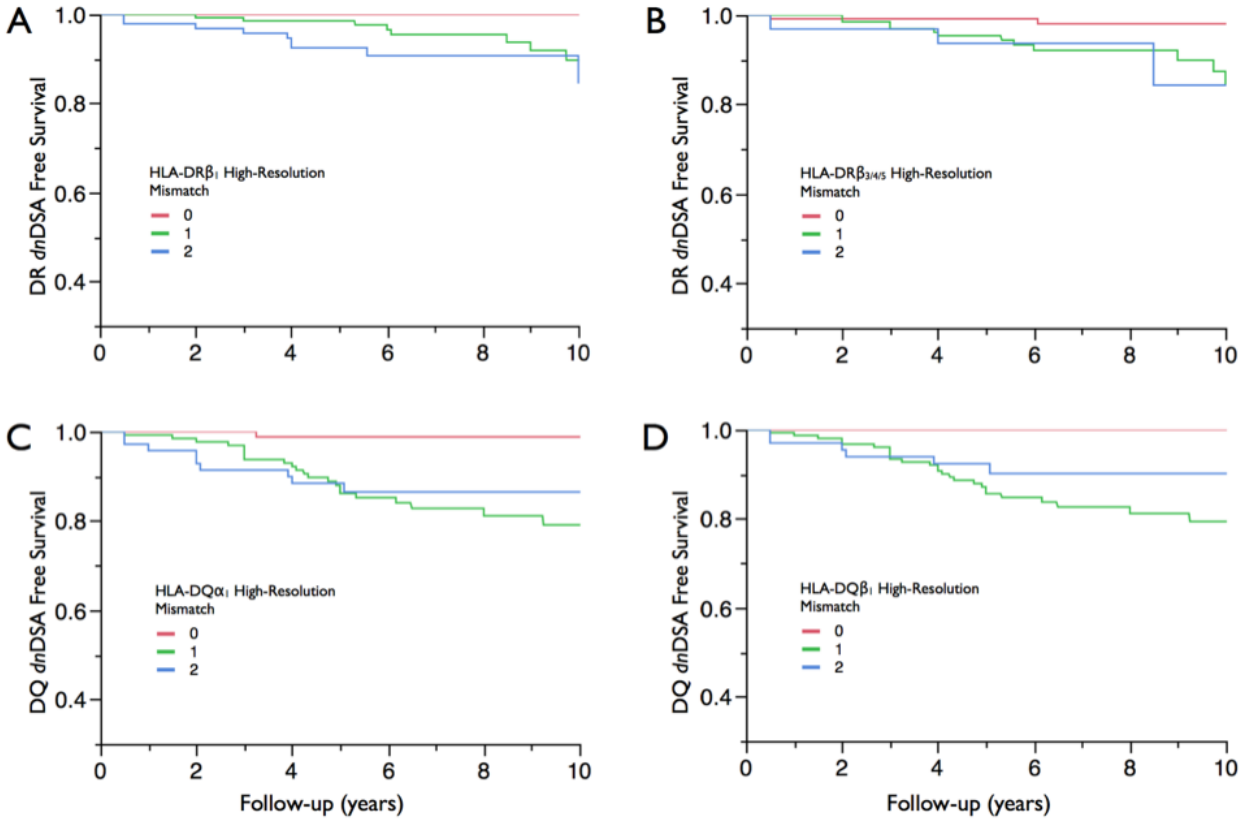


Figure 14. Panel (**A**) shows HLA-DR *dn*DSA free survival split by HLA-DRβ_1 mismatch. Panel (**B**) shows HLA-DR *dn*DSA free survival split by HLA-DR$\beta_{3/4/5}$ mismatch. Panel (**C**) shows HLA-DQ *dn*DSA free survival split by HLA-DQα_1 mismatch. Panel D shows HLA-DQ *dn*DSA free survival split by HLA-DQβ_1 mismatch.

Table 15. Epitope Specificities of de novo DSA on the Initial Positive Result

*dn*DSA	Eplet-Derived Epitope	Terasaki Epitope
DR13, DR52, DR53	14SEH, 4Q	TerEp#1006, TerEp #1001
DR17	14SEH	TerEp #1006
DR17	14SEH	TerEp #1006
DR13	14SEH	TerEp #1006
DR9	26KHY	TerEp #1401
DR53	48YQ	undefined
DR53	48YQ	undefined
DR53	48YQ	undefined
DR11	57DE	TerEp #1017
DR8, DR12	71DRA / 71DEA	TerEp #1018
DR13	71DRA / 71DEA	TerEp #1018
DR11	71DRA / 71DEA	TerEp #1018
DR15, DR51	71QAA, 108T	TerEp #1020, TerEp #1402
DR15, DR51	71QAA, 108T	TerEp #1020, TerEp #1402
DR17	71QKG	TerEp #1026
DR53	71RAE	TerEp #1023
DR52	98QS	TerEp #1036
DR16	142M	TerEp #1603
DR15	142M	TerEp #1603
DQ7	45EV	TerEp #2005
DQ7	45EV	TerEp #2005
DQ5	45G*	TerEp #2009
DQ8	45GV	TerEp #2010
DQ2	45GE / 52LL / 71RKA	TerEp #2001
DQ2	45GE / 52LL / 71RKA	TerEp #2001
DQ2	45GE / 52LL / 71RKA	TerEp #2001
DQ2	45GE / 52LL / 71RKA	TerEp #2001
DQ2	45GE / 52LL / 71RKA	TerEp #2001
DQ4 & DQ7	46VY† / 52P‡	TerEp #2003
DQA1*03	47QL / 52FRR / 187T	TerEp #2019
DQ7	52PL / 140T / 182N	TerEp #2014
DQ7	52PL / 140T / 182N	TerEp #2014
DQ4	52PL / 140T / 182N	TerEp #2014
DQ9	52PL / 140T / 182N	TerEp #2014
DQ4	52PL / 140T / 182N	TerEp #2014
DQ6	52PQ / 84EV	TerEp #2004
DQ5	52PQ / 84EV	TerEp #2004
DQ6	52PQ / 84EV	TerEp #2004
DQ5	52PQ / 84EV	TerEp #2004
DQ5	52PQ / 84EV	TerEp #2004
DQ5	52PQ / 84EV	TerEp #2004
DQ5	52PQ / 84EV	TerEp #2004
DQ6	52PQ / 84EV	TerEp #2004
DQ6	52PQ / 84EV	TerEp #2004
DQ7	55PPP	TerEp #2006
DQ7	55PPP	TerEp #2006
DQ5	55PR§	TerEp #2007
DQ5	70GA / 1161	TerEp #2015
DQ5	70GA / 1161	TerEp #2015
DQA1*05	75SL	Undefined
DQ2	77DR	TerEp#2026
DQ2	84QL	TerEp #2013
DQ6	125GQ	Undefined
DQ2	Undefined	Undefined

*This epitope is characterized by 45G, however, permissible surrounding amino acids include 46E/V; † This epitope is characterized by 46VY, however, permissible surrounding amino acids include 45E/G; ‡ This epitope is characterized by 52P, however, permissible surrounding amino acids include 53L/Q; § This epitope is characterized by 55PR, however, permissible amino acids include 56L/P

independent predictors of HLA-DQ *dn*DSA; 52PQ/84EV (OR 2.2, 95% CI 1.2–3.8, $p < 0.01$); 52PL/140T/182N (OR 2.1, 95% CI 1.0–4.3, $p < 0.05$); 45GE/52LL/71RKA (OR 2.5, 95% CI 1.0–5.8, $p < 0.05$). In the subset of adherent patients (n= 247) 52PQ/84EV (OR 2.4, 95% CI 1.1–4.9, $p < 0.02$, Table 14B), 52PL/ 140T/182N (OR 3.0, 95% CI 1.2–7.4, $p < 0.02$) were still significant predictors of *dn*DSA while 45GE/52LL/71RKA (OR 0.7, 95% CI, 0.04–3.4, $p = 0.7$) was no longer significant. However, 4 out of 5 patients who developed HLA-DQ *dn*DSA against 45GE/52LL/71RKA were non-adherent. The three-dimensional location of the epitopes found to be significant in the adherent patients is shown on the HLA-DR/DQ surface in Figure 16.

Figure 16. Three-Dimensional HLA Models with Immunogenic Epitopes

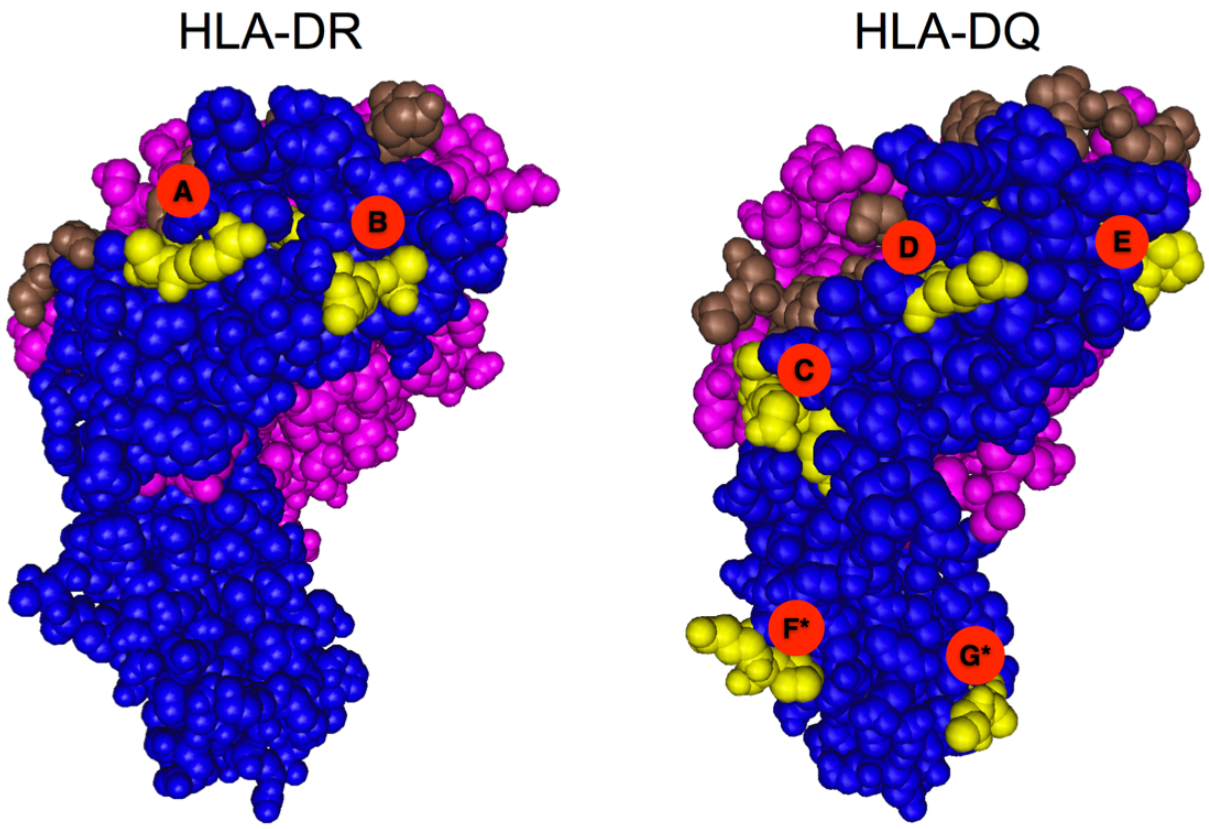


Figure 15. Amino acids highlighted in yellow are eplet-derived epitopes. These epitope specificities were the most commonly identified epitopes in the LABScreen® bead analysis based upon bead reactivity patterns and were significant independent predictors of *dn*DSA development in the multivariate model of adherent patients. **A** (71DRA, TerEp#1018), **B** (48YQ, TerEp# undefined), **C** (84EV, TerEp#2004), **D** (71RKA, TerEp#2001), **E** (52PL, TerEp#2014; 52PQ, TerEp#2004; 52LL, TerEp#2001), **F** (182N, TerEp#2014), **G** (140T, TerEp#2014).*Eplets E, F and G always occur together, therefore, the relevance of eplets F and G in terms of their antibody accessibility given their proximity to the cell membrane is uncertain. Blue, beta chain; pink, alpha chain; brown, peptide. *dn*DSA, de novo donor-specific antibody; TerEp, Terasaki epitope.

Chapter 5

Discussion

Discussion

Part I: Evolution and Clinical Pathologic Correlations of *de novo* Donor-Specific HLA Antibody Post Kidney Transplant

The principal findings in this part of this study were that *dn*DSA develops in 15% of low risk renal transplant recipients at a mean of 4.6 ± 3.0 years post-transplant and graft survival at 10-years is reduced by 40% in such patients. The independent risk factors for *dn*DSA development are HLA-DRβ_1 MM, non-adherence, and a strong trend toward clinical rejections prior to *dn*DSA onset. In particular, *dn*DSA patients were more likely to have preceding clinical and subclinical cellular rejections in the 0-6 month post-transplant period. Moreover, in those who develop graft dysfunction the *dn*DSA typically arises prior to the onset of proteinuria or a rise in creatinine. In non-adherent patients that present with *dn*DSA and dysfunction the histology is often a mixed cellular and antibody mediated rejection. Finally, allograft pathology consistent with antibody-mediated injury can occur in patients with *dn*DSA in the absence of graft dysfunction and the degree of injury can progress in these patients despite augmented immunosuppression.

Evidence for DSA being *de novo* is supported by the fact that in this low risk patient population all pre-transplant sera were negative for DSA using the most sensitive solid

phase and flow crossmatch assays, and by the late development of *dn*DSA at or after 6 months.

The decrease in graft survival for patients who develop *dn*DSA confirms previous reports.(11-18) In contrast we found that the presence of pre- and post-transplant non-donor-specific HLA antibodies had no correlation with graft survival in agreement with some,(16,17) but not all studies.(13-15,18,19,21,23,104) In patients with *de novo* HLA antibodies that are not donor specific it has been proposed that graft dysfunction may be due to *dn*DSA that is undetectable in the sera due to graft absorption.(22) However, in the current study this is unlikely as 10-year graft survival is >95% in these patients; only 9% (4/47) who develop detectable *dn*DSA had preceding *de novo* HLA antibodies; and HLA mismatch did not predict the development of *de novo* HLA antibodies in the absence of detectable *dn*DSA (data not shown).

HLA-DRβ_1 mismatch was an independent predictor of *dn*DSA confirming the work of Hourmant et al..(13) Moreover, this study supports the observation that *dn*DSA are predominantly directed at Class II donor HLA mismatches. Though the reason for this is unclear a number of mechanisms have been postulated.(14,15,17,20,23,105)

Previous studies have documented the link between non-adherence and late rejection, graft dysfunction, and loss.(106,107) In the current study, non-adherence differed significantly across the *dn*DSA subgroups and was a risk factor for both *dn*DSA and graft loss. The fact that clinical and subclinical rejection episodes occur equally in

adherent and non-adherent *dn*DSA patients in the first 6 months suggests that non-adherent behavior manifests after the early post-transplant period (Table 9). However, the elevated 6 month protocol biopsy Banff i, t and ptc scores in the non-adherent *dn*DSA subgroup as well as their higher frequency of clinical rejections from 7 to 12 months would suggest that as early as 6 months post-transplant the effects of non-adherence may become apparent. This is consistent with a report by Chisholm et al. who observed declining adherence levels beyond 5 months post-transplant.(108)

The frequency of clinical and subclinical rejections in the first 6 months is twice that of the no *dn*DSA patients regardless of whether the *dn*DSA patient was subsequently categorized as adherent or non-adherent (Table 9). Moreover, the higher ptc scores in the 0-6 month clinical rejection biopsies, a common feature of cellular rejection,(109) suggests that patients at risk for *dn*DSA have preceding cellular rejections with more intense inflammation within the microvasculature. We postulate that cellular rejection with peritubular capillaritis leads to increased HLA expression in the microcirculation thereby increasing the risk of allo-recognition by the recipient B-cell compartment.(64)

Once *dn*DSA was detectable in the serum, the intensity and frequency of concurrent cellular rejection varied in parallel with non-adherence suggesting cellular rejection may be both a result of non-adherence and a contributor to the degree of graft dysfunction observed in *dn*DSA patients. The Acute and Indolent Dysfunction *dn*DSA groups did not differ in g and ptc scores, however, they differed in C4d staining and incidence of Banff 1A/1B cellular rejection. This suggests that complement activation and cell mediated

rejection may account for the greater level of dysfunction observed in the Acute Dysfunction as compared to the Indolent Dysfunction *dn*DSA group. Finally, patients in the Acute or Indolent Dysfunction *dn*DSA groups who had either borderline or Banff 1A/ 1B cellular rejection combined with AMR were more likely to progress to graft loss compared to those with AMR alone (p=0.05).

In patients with stable graft function who have *dn*DSA, four lines of evidence would support the pathologic potential of *dn*DSA in this setting. First, in our study 10/14 Stable Function *dn*DSA patients had pathologic features consistent with antibody mediated microvascular injury. Einecke et al. emphasized that HLA antibody mediated microvascular injury was a major correlate of late graft loss in patients with graft dysfunction.(27) They also reported basement membrane duplication in the glomerular and/or peritubular capillaries occurred more commonly in patients with *dn*DSA, which implied as process of repetitive injury and repair. Unfortunately, electron microscopy was not routinely performed on our protocol biopsies – a clear limitation of the study. Second, *dn*DSA antibody would arise prior to the onset of graft dysfunction as it did in the Indolent Dysfunction *dn*DSA group of which 12/14 had features of antibody mediated microvascular injury. Third, histologic progression over time would provide evidence that Stable Function *dn*DSA patients are unlikely to remain clinically silent. To date we have repeat biopsies on four patients who showed no change in one and histologic progression in three despite augmentation in immunosuppression. Fourth, development of graft dysfunction would support that *dn*DSA has pathologic potential in Stable Function *dn*DSA patients. To date only 2/18 patients have progressed to graft

dysfunction. However, the median follow-up post-*dn*DSA in the group was only 19 months. A sub-group analysis of the adherent patients in the Indolent Dysfunction *dn*DSA group showed that the median time between *dn*DSA detection and an increase in creatinine was 20 months (data not shown). Given that the Stable Function *dn*DSA group was 94% adherent it is likely that the current follow-up is only now approaching the point when graft dysfunction might be expected. Other factors, not yet assessed, which may contribute to the slower rate of progression in the Stable Function *dn*DSA group include lower antibody titer, ability to activate complement, differences in immunogenicity or expression of the specific HLA epitopes, and the regulation of antibody responses. Nevertheless, while further natural history studies of this cohort are warranted, the available evidence supports the hypothesis that Stable Function *dn*DSA patients are at risk for progression to graft dysfunction/loss.

We propose a continuum of antibody-mediated damage based on a model adapted from the primate studies of Smith et al. (Figure 17).(110) Post-transplant *dn*DSA is preceded by an antibody-free period. It is likely that inflammatory events (e.g. preceding cellular rejection or graft infection) leads to enhanced IFN-γ levels which upregulate HLA expression on endothelial cells and stimulates B-cell allorecognition and subsequent long-lived plasma cells producing *dn*DSA.(39,40) At this point *dn*DSA onset may be overlooked without routine post-transplant monitoring of stable grafts. Nevertheless, biopsies in stable grafts with *dn*DSA generally reveal histologic changes consistent with microvasculature injury. *dn*DSA binding vascular endothelium is capable of inducing injury through the activation of complement (via C1q) or recruitment of

Figure 17. Proposed Natural History of *dn*DSA*

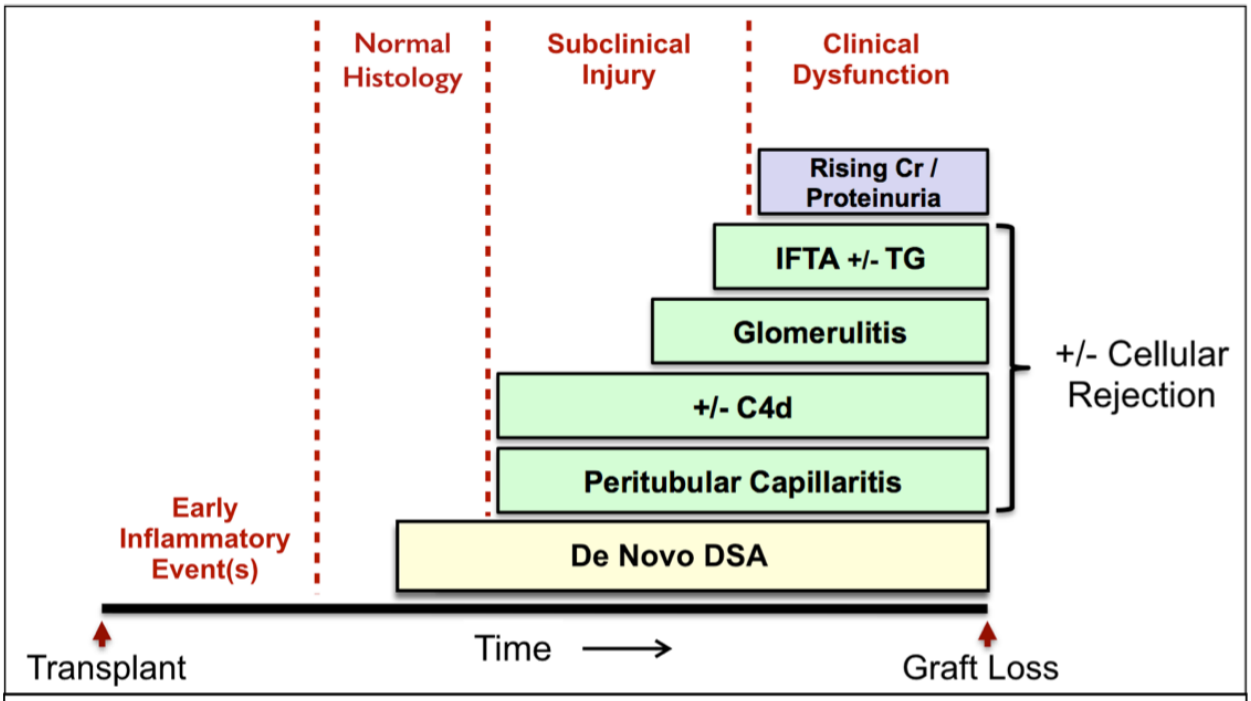


Figure 15. This figure shows a proposed model for patients developing *dn*DSA as they evolve from transplantation to graft failure. *dn*DSA, *de novo* donor-specific antibody; IFTA, interstitial fibrosis and tubular atrophy; TG, transplant glomerulopathy. Adapted from Smith et al. Am J Transplant 2008; 8: 1662-1672

neutrophils, macrophage or natural killer cells via Fc receptors.(64) Sustained microvascular inflammation (i.e. glomerulitis, peritubular capillaritis, and vasculitis) eventually leads to progressive tissue fibrosis (i.e. transplant glomerulopathy, loss of peritubular capillaries, and IFTA) resulting in graft dysfunction. Although *dn*DSA targets the microcirculation, there is often concomitant cellular inflammation, especially in the face of non-adherence, that may accelerate the development and severity of graft dysfunction and shorten the time to graft loss.

Limitations of part I of this study include: the time intervals (6 to 12 months) between HLA antibody screening, which may underestimate the time from initial detection of *dn*DSA to time of graft dysfunction; the separate pediatric and adult cohorts, which may have led to differences in practice confounding the analysis; the assignment of non-adherence may have resulted in false negatives; and the small number of patients in the Stable Function *dn*DSA group who have had repeat biopsies that may overestimate the risk of progression. Furthermore, because the Stable Function *dn*DSA patients who had repeat biopsy all received pulse steroids and IVIg we cannot be certain this therapy did not contribute to the observed progression.

Summary of Part I: Evolution and Clinical Pathologic Correlations of *de novo* Donor-Specific HLA Antibody Post Kidney Transplant

To improve long-term outcomes for transplant patients, strategies to prevent and address delayed graft function, cellular rejection and *dn*DSA associated microvascular injury are required. For example, avoiding HLA-DR mismatching in allocation, targeting

non-adherence early and, avoiding drug minimization protocols in patients at risk of developing *dn*DSA should be considered. Routine monitoring for *dn*DSA will identify patients for early interventional studies in an attempt to define effective therapies to alter their prognosis.(111) Finally, the importance of cellular rejection should continue to be recognized and treated aggressively given that it frequently precedes *dn*DSA, may be a sign of non-adherent behaviour, and itself may be causal in the induction of *dn*DSA. Moreover, when cellular rejection coincides with *dn*DSA and antibody mediated microvascular injury it may accelerate the time to graft dysfunction, and graft loss.

Part II: Class II HLA Epitope Matching—A Strategy to Minimize *de novo* Donor-Specific Antibody Development and Improve Long-Term Outcomes

The principal findings in this part of the study were that an epitope-based HLA mismatching approach out performs traditional low-resolution or high-resolution HLA antigen mismatching as a predictor for Class II *dn*DSA development and that some epitopes appear to be more immunogenic than others. While current antigen mismatching is limited by describing two HLA molecules as either matched or mismatched, epitope mismatching affords a more detailed assessment of the degree of difference between the donor and recipient. Indeed, there was no significant difference in the locus-specific high-resolution mismatch between those who developed HLA-DR *dn*DSA alone or HLA-DQ *dn*DSA alone. However, the level of locus-specific epitope mismatch was significantly higher for patients who developed *dn*DSA against either loci, and both HLA-DR and HLA-DQ epitope mismatch were elevated when patients developed *dn*DSA against both loci (Table 12).

Due to the limited range of possible values using traditional whole molecule mismatch (0,1, or 2 per locus), identifying patients at low risk for Class II *dn*DSA development required a mismatch threshold of zero at each locus (Figure 15). However, using epitopes to determine an optimal mismatch demonstrated that for the 134 patients with <10 HLA-DR epitope mismatches, none developed HLA-DR *dn*DSA and only 4/145 (2.7%) of patients with <17 HLA-DQ epitope mismatches developed HLA-DQ *dn*DSA after a median follow-up of 6.9 years (Figure 14). Interestingly high-resolution typing affords no additional benefit over low-resolution typing when it comes to predicting the development of *dn*DSA. Thus, spending additional time and money to perform routine high-resolution HLA typing seems to have little benefit unless those results are used as part of an epitope matching strategy.

Another limitation of current algorithms is that matching for HLA-DR does not ensure that the linkage disequilibrium with HLA-DQ is sufficient to prevent HLA-DQ *dn*DSA from arising. Multiple studies have now shown that HLA-DQ *dn*DSA occur more commonly than HLA-DR *dn*DSA(28,109,112-115) and are associated with antibody-mediated rejection, transplant glomerulopathy, and allograft failure.(112,113) In the current study patients who developed isolated HLA-DQ *dn*DSA had significantly elevated HLA-DQ epitope mismatch loads. Therefore, novel strategies whose aim is to prevent the development of Class II *dn*DSA should take into account both HLA-DR and HLA-DQ loci. Indeed, defining a threshold for HLA-DR and HLA-DQ epitope mismatch load could allow one to assign allocation points favoring low mismatch loads for both loci rather

than for just a zero HLA-DR or DQ high-resolution mismatch. The epitope mismatch load may also provide clinicians with a more detailed assessment of immunologic risk post-transplant to aid in clinical decision making regarding immunosuppressive sparing strategies or the need for post-transplant monitoring for *dn*DSA. Although the mechanism for how epitope load increases the risk of *dn*DSA development is unknown, the probability of allorecognition by a specific B-cell clone likely increases with an increasing number of mismatches, as would the likelihood of an immunodominant epitope being present.

Epitope-paratope interactions are influenced by the size, shape, and electrochemical properties of the amino acids present on the surface of the antigen and antibody.(93,116,117) Thus, it is not surprising that an *in silico* method of eplet-derived epitope assignment based on polymorphic amino acid positions on the three-dimensional surface of HLA models, such as HLAMatchmaker, would have varying degrees of antigenicity or immunogenicity. Indeed, Laux et al. defined six HLA-DP epitopes based on hypervariable amino acid regions and found that certain epitopes were more immunogenic than others and that mismatches at these epitopes outperformed low-resolution HLA-DP matching for predicting graft survival in retransplants.(118) Using an early version of HLA Matchmaker Dankers et al. showed that the number of Class I amino acid triplet mismatches correlated with the likelihood of Class I DSA at the time of graft failure.(119) Duquesnoy et al. reported the eplet HLA specificities of a heterogeneous group of 75 failed lung, heart, kidney, small bowel, liver, and pancreas transplants with Class II DSA.(95) However, the heterogeneity of the population, the

lack of a control group that did not develop DSA, and the assessment after graft failure make it difficult to assign a reliable risk to any individual eplet-derived epitope mismatch. Kosmoliaptsis et al. also reported that both the number of amino acid mismatches and their physiochemical properties were predictive of Class II antibody production.(120) However, this study focused on highly sensitized patients pre-transplantation. Our study cohort consisted of a consecutive series of patients without pre-transplant DSA, during an era of modern immune suppression that developed *dn*DSA prior to graft failure. In this context, it was possible to identify eplet-derived epitopes to which *dn*DSA were putatively directed (Table 15) and, using the group that did not develop *dn*DSA as a control, some of these same epitopes were significant independent predictors of *dn*DSA development in a multivariate model. Importantly, 5/6 of these immunogenic epitopes correlated with known monoclonal antibody or isolated alloantibody single antigen bead reactivity patterns used to define the Terasaki epitopes.(101) If validated in future studies, this information could be used to help clinicians avoid high risk epitope mismatches at the time of transplant, thus, minimizing the risk of *dn*DSA development and potentially improving long-term graft outcomes.

Non-adherence has been shown to be one of the strongest predictors of *dn*DSA.(28) Therefore, we examined a subset of adherent patients and found that 4 out of 6 of the most commonly assigned epitope specificities were also significant independent predictors of locus specific *dn*DSA in this group (Table 14B). The fact that these epitopes are associated with *dn*DSA in the presence of immunosuppressive therapy further support their potential immunodominance. Alternatively 2 of the 6 commonly

assigned epitope specificities were only significant independent predictors in non-adherent patients. Presumably, once patients are off immunosuppression, due to non-adherence or physician withdrawal after graft failure, the immune system is unencumbered from responding to all mismatched epitopes, even those that are relatively weak alloantigens.

Limitations of this study include the relatively small sample size and the associated risk for type II error, thus risk quantification should be interpreted with caution. However, the fact that Class II epitope mismatch can be demonstrated to be highly significant in this context suggests that the observed signal is real and strategies to minimize epitope load may have a tangible impact. The relatively small sample size may also account for the observation that combined Class I and II *dn*DSA had a non-significant trend towards worse graft outcome whereas a larger cohort would have likely detected significance. It is interesting that in these patients the MFI of the Class II *dn*DSA is significantly greater than the Class I *dn*DSA. However, one cannot attribute a dominant role for Class II *dn*DSA in these patients based on this MFI difference alone. Secondly, though highly suggestive, given the retrospective nature of the study we cannot prove a causal link between improved outcomes and epitope matching. Thirdly, the relatively limited ethnic diversity may restrict the observed immunodominant repertoire of HLA Class II epitopes, highlighting the opportunity to identify novel immunogenic epitopes in other populations.

Summary Part II: Class II HLA Epitope Matching—A Strategy to Minimize *de novo* Donor-Specific Antibody Development and Improve Long-Term Outcomes

To minimize the risk for developing *de novo* Class II DSA, the solid organ transplant community should reconsider the construct that a donor mismatched HLA molecule is a single entity. Viewed through the lens of the immune system, it is clear that allorecognition can generate antibodies to multiple non-self epitope mismatches even on a single molecule. In this context, HLA-DR and DQ epitope matching appears to outperform traditional low-resolution antigen-based matching and has the potential to reduce the risk of developing *de novo* Class II DSA thereby improving long-term graft outcome. Alternatively, a strategy, which focuses on avoiding a small number of highly immunogenic Class II epitope mismatches, may be the optimal approach to minimize risk while maximizing equitable access for all individuals. The latter will require a concerted effort by the transplant community to further inventory and validate which Class II epitopes are immunodominant.

Chapter 6

Future Directions

Future Research

We have demonstrated that patients whom develop *dn*DSA have significantly reduced graft survival and that both pathologic and clinical risk factors for its development can be identified. Some of these risk factors are potentially modifiable. Medication non-adherence for example may be preventable, or perhaps identified and treated early in an attempt to mitigate its effects. Class II HLA matching and/or epitope matching prior to transplant also represents an under-utilized opportunity to lower risk. Currently most transplant centers throughout Canada and the world only consider HLA-A, HLA-B, and HLA-DR when matching donors and recipients. However, we found that HLA-DQ *dn*DSA were the most common *dn*DSA to develop post-transplant and that isolated Class I *dn*DSA were rare and were less likely to be associated with a severe clinical phenotype. This highlights the need to consider and perhaps emphasize Class II HLA-DR and DQ in allocation algorithms. Importantly, a prospective study targeting one or both of these risk factors is needed to demonstrate that these risk factors can be modified in clinical practice, and that the incidence of *dn*DSA is lowered through intervention.

In individual patients we demonstrated that the short-term effect of *dn*DSA on clinical pathologic phenotypes ranges from acute graft dysfunction and histologic injury to long periods of clinical stability with subclinical inflammation.(28) To date the only non-invasive predictor of clinical pathologic phenotype at the time of *dn*DSA detection is medication non-adherence. This is problematic for therapeutic decision-making since

non-adherence occurs in the minority of patients and is difficult to measure. Furthermore, the treatments for antibody-mediated rejection (intravenous immunoglobulin (IVIg), plasmapheresis, rituximab, etc.) are expensive, invasive, and may increase the risk of infection or malignancy. Therefore, it is imperative that methods are developed to predict which patients with *dn*DSA are likely to progress and would benefit from early intervention.

To this end *dn*DSA have a number of features characteristic of a useful biomarker: 1) the assay is highly available and reproducible; 2) it is relatively inexpensive; 3) it is non-invasive; 4) it correlates with long-term outcomes and; 5) it can be detected before irreversible injury occurs in most adherent patients. However, little is known about the importance of *dn*DSA strength or its functional characteristics (e.g. the ability to bind complement), which may help to risk stratify individuals at the time of *dn*DSA detection. Thus specific questions that need to be addressed include: 1) how can antibody titer be accurately interpreted from currently available tests, and does it correlated with outcome for patients who present with a stable or indolent phenotype; 2) can *dn*DSA isotype subclasses (e.g. IgG1, IgG2, IgG3, IgG4) be used to predict outcomes; or 3) could the complement binding characteristics of *dn*DSA be used to predict which patients with newly identified *dn*DSA are likely to progress to graft failure. Since both antibody titer and their subclasses are known to effect the ability of antibodies to activated complement it is important that these questions be answered in a comprehensive way and not in isolation. These characteristics could help clinicians risk stratify patients at the time of *dn*DSA detection in order to make more educated decisions about treatment and prognosis.

Chapter 7

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Chapter 8

Appendix

- Published works

Published Works

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- Wiebe C, Nickerson P. Posttransplant monitoring of de novo human leukocyte antigen donor-specific antibodies in kidney transplantation. Curr Opin Organ Transplant 2013, Aug;18(4):470-7.3. Wiebe C, Gibson IW, Blydt-Hansen TD, Karpinski M, Ho J, Storsley LJ, et al.
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