Title
Differential Inflammatory Vascular Cytokine Profiles Associated with Angiotensin II Type 1 Receptor Antibodies and Human Leukocyte Antibodies in Pediatric Renal Transplantation

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A dissertation submitted in partial satisfaction of the
requirements for the degree Master of Science
in Clinical Research

by

Meghan Pearl

2018
ABSTRACT OF THE DISSERTATION

Differential Inflammatory Vascular Cytokine Profiles Associated with

Angiotensin II Type 1 Receptor Antibodies and

Human Leukocyte Antibodies in Pediatric Renal Transplantation

by

Meghan Pearl

Master of Science in Clinical Research

University of California, Los Angeles, 2018

Professor Janet S Sinsheimer, Chair

Background: Both human leukocyte antigen donor specific antibodies (HLA DSA) and non-HLA autoantibodies have been implicated in antibody-mediated rejection (AMR), allograft dysfunction, and allograft failure in kidney transplantation. Angiotensin II type 1 receptor antibody (AT1R-Ab), is a non-HLA antibody implicated in poor renal allograft outcomes, although its actions may be mediated through a different mechanistic pathway than HLA DSA.

Objective: Our aim was to examine serum cytokine profiles associated with AT1R-Ab and distinguish them from those associated with HLA DSA in serially collected blood samples from a cohort of pediatric renal transplant recipients.
Methods: 65 pediatric kidney transplant patients were monitored for 2 years post-transplant. Blood samples from early post-transplant and at 6, 12, and 24 months post-transplant and during suspected episodes of kidney transplant rejection were tested for AT1R-Ab, HLA DSA, and a panel of 6 cytokines (TNF-α, IFN-γ, IL-8, IL-1β, IL-6, and IL-17). Associations between antibodies and cytokines were evaluated.

Results: AT1R-Ab, but not HLA DSA, was associated with elevations in TNF-α, IFN-γ, IL-8, IL-1β, IL-6, and IL-17. This relationship remained significant even when controlling for relevant clinical factors and potential confounders, and was consistent across time points.

Conclusion: In contrast to HLA DSA, AT1R-Ab was associated with elevations in vascular inflammatory cytokines in the first 2 years post-transplant. This profile of vascular cytokines may be informative for designing further studies to understand the distinct pathophysiology of AT1R-Ab mediated allograft injury in kidney transplantation.
The thesis of Meghan Pearl is approved.

David Elashoff

Elaine F. Reed

Janet S Sinsheimer, Committee Chair

University of California, Los Angeles

2018
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INTRODUCTION

Antibody-mediated rejection (AMR) remains a leading cause of allograft failure and subsequently contributes to the ongoing organ shortage in renal transplantation.\textsuperscript{1-3} Both human leukocyte antigen donor specific antibodies (HLA DSA) and non-HLA autoantibodies have been implicated in AMR, allograft dysfunction, and failure.\textsuperscript{2,4-7} Angiotensin II type 1 receptor antibody (AT1R-Ab), in particular, is a non-HLA antibody, that has gained more recognition for its detrimental effects on the renal allograft, although its actions may be mediated through a different mechanistic pathway than HLA DSA.\textsuperscript{8,9}

AT1R-Ab activates the AT1R, a G-protein coupled receptor which mediates the vasoconstrictive and salt retention actions of angiotensin II.\textsuperscript{7} In addition to these classical effects, activation of the AT1R also triggers inflammatory and pro-fibrotic pathways.\textsuperscript{10,11} In contrast, HLA-DSA alloantibodies bind to Class I and II HLA receptors on the allograft endothelium. This interaction provokes endothelial cell activation, proliferation, and migration leading to the histological changes associated with acute and chronic AMR.\textsuperscript{12-15} Both AT1R-Ab and HLA DSA can directly injure endothelial cells and some studies have suggested an interplay between AT1R-Ab and HLA DSA in promoting allograft injury.\textsuperscript{16,17}

Given AT1R-Ab and HLA DSA activate different receptors, we hypothesized that their cytokine profiles may be distinct. Therefore, our aim was to analyze serum cytokines associated with activation of the AT1R and vascular inflammation\textsuperscript{18-23} and distinguish them from those that associate with HLA DSA in serially collected blood samples from a cohort of pediatric renal transplant recipients.\textsuperscript{9} Insight into cytokine signatures associated with these antibodies in renal transplant patients, may inform further mechanistic studies of HLA and non-HLA antibody-mediated injury.
MATERIALS AND METHODS

Patients and Study Design

In this retrospective study, 65 pediatric kidney transplant patients were monitored for 2 years post-transplant. From August 2005 to November 2014, 83 patients were enrolled in the UCLA Pediatric Kidney Transplant Immune Monitoring Study, and 18 patients were excluded from analysis secondary to missing > 1 study sample at the specified time points. This study was approved by the UCLA Institutional Review Board (#11-002375) and conforms with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and the Principles of the Declaration of Istanbul. Informed consent and when appropriate patient assent was obtained for all patients. Blood samples were analyzed from early post-transplant and at 6, 12, and 24 months post-transplant and during suspected episodes of kidney transplant rejection. In longitudinal analyses, blood samples were grouped by time-point to allow for analysis of both protocol and clinically indicated samples. Demographic and clinical data including age, race, ethnicity, HLA mismatch, transplant type (deceased/living donor), time on dialysis, immunosuppression regimen, and viremia (cytomegalovirus, Epstein Barr virus, or BK Virus) was collected. Study data were collected and managed using a secure REDCap (Research Electronic Data Capture) electronic data capture tool hosted at UCLA. Of the 65 patients, 54 patients had complete 2 year follow up, 7 patients suffered allograft loss, and 4 patients transferred care to a different institution. No patients died during the study period. Patient level outcomes have been previously reported. A total of 232 blood samples at the above described time points were analyzed.
Clinical Protocols and Biopsy Evaluation

Immunosuppressive strategies at our institution included induction with either anti-thymocyte globulin (ATG) for PRA ≥ 30%, delayed graft function, or rapid-steroid withdrawal protocol or anti-CD25 monoclonal antibody for those with PRA<30%. Maintenance immunosuppression consisted of steroid free or steroid based immunosuppression, a calcineurin inhibitor, and an anti-metabolite. Acute and chronic rejection were treated with previously described protocols.25

Patients underwent biopsies at 6, 12, and 24 months post-transplantation per protocol or for clinical indication. Biopsies were evaluated using 2013 Banff Criteria by a blinded pathologist.26 Though our primary analysis was based on all 232 blood samples, we did a sub-analysis of 63 blood samples collected 7 days prior or 3 days after the time of biopsy. This window was chosen to account for rapid changes in serum cytokines both in general and in response to immunomodulatory therapy.

AT1R and HLA Antibodies and Cytokine Testing

HLA typing of recipient and donor was performed using molecular methods as previously described.25 HLA antibodies were detected using a Luminex single antigen bead (SAB) assay (Immucor, Stanford, CT) and quantified by mean fluorescence intensity (MFI). Antibodies were considered positive when MFI was ≥1000 for HLA-A, -B, -DR, -DQ, and ≥2000 for HLA-C and –DP.27 AT1R-Ab was measured by enzyme-linked immunosorbbent based assay (One Lambda, Canoga Park, CA). Sera were diluted 1:100, tested in duplicate and AT1R-Ab concentrations were determined by a standard curve. AT1R-Ab IgG >17 units/ml was considered positive.9,28,29 Cytokines were selected based on a literature review of cytokines that
have been associated with activation of the AT1R\textsuperscript{18-23} and measured in serial post-transplant samples to avoid effects of dialysis and end-stage renal disease. A custom magnetic bead kit including TNF-α, IFN-γ, IL-8, IL-1β, IL-6, and IL-17 (EMD Millipore, Darmstadt, Germany) was used per manufacturer's instructions. Fluorescence was quantified using a Luminex 200TM instrument.

**Statistical Methods**

Prior to statistical analysis, cytokines were transformed using the log\((x+1)\) transformation. The value of the lowest lower limit of detection (LLD) per cytokine was used for values below the limit of detection. For cytokines where \(<50\%\) of the samples were below the LLD (IL-8, TNF-α, IFN-γ), data was analyzed as a continuous outcome. For cytokines where \(>50\%\) of the samples were below the LLD (IL-1β, IL-6, IL-17) data was analyzed as a categorical variable (\(<\text{LLD vs.}\ >\text{LLD}\) ). Kruskal-Wallis and chi square tests were used to compare cytokine levels in blood samples with and without AT1R-Ab and HLA DSA. Mixed effects linear and logistic regression models using a random effect for patients were used to evaluate the effect of AT1R-Ab and HLA DSA positivity on cytokines over time. Models were summarized using regression coefficients for linear models and odds ratios for the logistic regression models. Covariates potentially relevant to elevations in serum cytokines were included in the models and included age, sex, mean HLA mismatch, living vs. deceased donor, viremia (presence or absence of cytomegalovirus, Epstein-Barr virus, or BK Virus during the follow up period), and rejection (presence or absence of biopsy proven rejection including borderline rejection during the follow up period). Interaction between time by AT1R-Ab and HLA DSA by AT1R-Ab were evaluated using separate mixed effect logistic regression models.
controlled for patient level random effect. Time was modeled as a categorical variable to ease interpretation of regression coefficients. A p-value below 0.05 was considered statistically significant and all tests were two-sided. The R Statistical Computing Environment was used for analysis (R Core Team; Vienna, Austria).

RESULTS

Demographics and Immunological Characteristics of Cohort and Samples

Clinical characteristics of the cohort are briefly described in Table 1 and comprehensively detailed elsewhere. The rate of rejection during the follow-up period (including borderline rejection) was 45% and the rate of viremia with CMV, EBV, or BK Virus was 49%. AT1R-Ab > 17 units/mL was present in 92/232 (40%) blood samples from 38/65 (58%) patients, and HLA DSA > 1000 MFI was present in 25/232 (11%) blood samples from 19/65 (29%) patients. The majority of patients with HLA DSA developed DSA to Class II antigens and median MFIs were low. The median AT1R-Ab level in AT1R-Ab positive patients was 24 units/mL.

Cytokines Elevations Differentiate AT1R-Ab from HLA-DSA

The distribution of cytokine levels in blood samples positive for AT1R-Ab and HLA DSA are shown in Figures 1a and 1b respectively. AT1R-Ab was a predictor of higher levels of serum inflammatory vascular cytokines TNF-α, IFN-γ, IL-8, IL-1β, IL-6, and IL-17. In comparison, HLA DSA was not a predictor of any of the six cytokines. IL-1β, IL-6, and IL-17 had 61%, 68%, and 76% of values below the LLD for the assay respectively, therefore, samples
were treated as dichotomous (< or > LLD). In contrast, only 5%, 32%, and 14% of samples were outside the limits of detection for the assay for TNF-α, IFN-γ, and IL-8 respectively. Therefore, these cytokines were analyzed as continuous outcomes using the quantified value of each cytokine for each sample.

Given the lower prevalence of HLA DSA positivity when compared to AT1R-Ab in our population, we conducted a 4 group sub-analysis of samples based on AT1R-Ab and HLA DSA status to examine the differential effect of AT1R-Ab and HLA DSA on serum cytokines. Overall, the effect of AT1R-Ab on elevations of serum cytokines remained consistent (Table 2) in samples with and without HLA DSA. Notably, IL-8 and IL-1β levels appeared higher in AT1R-Ab positive and HLA DSA negative samples while TNF-α and IL-17 were higher in samples positive for both.

**Adjusted Analysis of Determinants of Increased Cytokine Levels**

Given serum cytokines may also be influenced by a number of other inflammatory events, we examined models controlled for relevant demographic and clinical factors including age, sex, mean HLA mismatch, living vs. deceased donor, time post-transplant, viremia, and rejection. Viremia and rejection were assessed as presence or absence at any time during the follow up period (see Methods). Even after controlling for these factors, the relationship between AT1R-Ab and all 6 cytokines remained significant (Table 3). Further, we tested for any interaction effects between HLA DSA and AT1R-Ab on cytokine levels, and there were no significant interactions (Figure S1). Therefore, this interaction was not included in the final model.
Given the potential importance of rejection in influencing cytokine levels and the possibility of serum cytokine levels changing fairly rapidly over the course of days, we conducted a sub-analysis of samples that were collected within 7 days prior and 3 days after a biopsy (see methods). Of the 232 samples, 63 met this criteria. An analysis of these samples revealed a potential association between IL-8 and acute biopsy proven rejection, however, these results were inconclusive secondary to poor model fit (Table S1). On this sub-analysis, there was also no association between any of these cytokines and the presence of any acute or chronic vascular finding (Table S1).

The Relationship between Time Post-Transplant and Cytokine Levels

Time post-transplant was significantly associated with levels of all cytokines except for IL-6 (Figure 2 a-f). To assess if time impacted the relationship between AT1R-Ab and elevated cytokines, we created a separate model to test for any interaction effect. On this analysis, we found no significant interaction between time and AT1R-Ab on cytokine levels (Table S2). Therefore, though cytokine levels in the overall population varied by time point (for example in early vs late post-transplant samples), the increase in serum cytokines in AT1R-Ab positive vs negative samples within any given time point was consistent.

DISCUSSION

We show that in a cohort of pediatric renal transplant recipients, AT1R-Ab and HLA DSA have distinct associations with vascular inflammatory cytokines TNF-α, IFN-γ, IL-8, IL-1β, IL-6, and IL-17. The association between AT1R-Ab and these serum cytokines remained
significant even when controlling for HLA DSA, in addition to time post-transplant, age, sex, mean HLA mismatch, living vs. deceased donor, presence of rejection, and viremia. Further, our longitudinal samples allowed us to demonstrate the consistency of the relationship between AT1R-Ab and cytokine elevations over time. The distinct cytokine profiles suggests that AT1R-Ab and HLA DSA may exert renal allograft damage by activating different pathways and, therefore, may have unique pathophysiology.

Since the original paper describing AT1R-Ab in patients with acute AMR and hypertension\textsuperscript{8}, multiple (but notably not all\textsuperscript{30}) studies\textsuperscript{9,16,17,31-38} have indicated an association between AT1R-Ab with vascular inflammation and poor clinical outcomes. There continues to be a relative paucity of work with longitudinal measurements of AT1R-Ab\textsuperscript{9,16,34}. Furthermore, though AT1R-Ab has been reported to provoke HLA sensitization\textsuperscript{17,34} and synergize with HLA DSA\textsuperscript{16,38}, how these pathways interact remains unclear. Our initial work revealed an association between AT1R-Ab and IL-8, IL-1β, and TNF-α in patients with AT1R-Ab at any time during the first 2 years post-transplant. Given cytokines may fluctuate rapidly, we undertook this additional analysis to examine individual samples for the relationship between AT1R-Ab, HLA DSA, and cytokines with the goal of elucidating differences in inflammatory profiles.

Given AT1R-Ab has been shown to be an allosteric agonist\textsuperscript{7,8} of the AT1R, we chose to measure cytokines associated with activation of the AT1R and vascular inflammation. Overall, activation of AT1R is considered to be pro-inflammatory and clinical benefits have been shown with AT1R blockade in multiple disease states\textsuperscript{39}. Angiotensin II has been shown to stimulate TNF-α production in glomerular endothelial cells in rats,\textsuperscript{40} and both IL-17 and IFN-γ are involved in angiotensin II mediated increases in blood pressure and salt retention in mice\textsuperscript{19}. Angiotensin II treated renal proximal tubular cells and mouse macrophages secrete IL-1β, IL-6,
IL-8, and TNF-α,\textsuperscript{20,41} and human PBMCs exposed to sera with AT1R-Ab produce IL-8.\textsuperscript{18} However, the complicated feedback loops involved in angiotensin II mediated inflammation remain poorly understood. For example, immune cells (T Cells and macrophages) also express AT1R and activation in this setting may be involved in immunomodulation in normal physiology.\textsuperscript{39,42,43} Cytokines in our samples were measured in patient serum, therefore, we cannot determine if the elevations were primarily related to tissue or circulating immune cell release. Furthermore, how immunosuppression directed against T-cells impacts AT1R signaling in immune cells requires further investigation.

Activation of endothelial cells by HLA Class I has also been associated with increases in IL-1β, IL-6, IL-8, and TNF-α in vitro.\textsuperscript{44,45} HLA class II binding has been associated with increased IL-6 production in endothelial cells and promotion of TH17 lymphocyte expansion.\textsuperscript{46} In PBMCs isolated from transplant patients, some studies have shown HLA antibodies increase cytokines (TNF-α, IFN-γ, and IL-6)\textsuperscript{47} while others have not.\textsuperscript{48} Based on this, we hypothesized HLA DSA may also be associated with elevations in some or all of these serum cytokines. Though as a whole HLA DSA were also associated with increases in cytokines, the relationships were weaker than the associations with AT1R-Ab and did not meet statistical significance in our cohort. HLA DSA were less prevalent in our samples than AT1R-Ab, which reduced our power to detect differences, however, we chose to use a model with both HLA DSA and AT1R-Ab to mitigate this. We also found no interaction between HLA DSA and AT1R-Ab in increasing serum cytokines. Most of the HLA DSA in our cohort were low to moderate MFI and our samples were drawn relatively early post-transplant. It is possible that we may have observed an effect of HLA DSA on these cytokines in samples with higher MFIs.
All 6 cytokines measured in our study are involved in vascular inflammatory pathways and may be involved with linking antibody-mediated injury to poor allograft outcomes. Endothelial cells can produce IL-1β and IL-8, which are known to promote leukocyte chemotaxis and artherogenesis. Elevations in IL-6 and TNF-α have also been associated with rejection, mortality, and allograft loss in renal transplantation. Interestingly, in this analysis, the association between AT1R-Ab and these cytokines remained significant even when controlling for rejection. This design does not allow us to draw mechanistic conclusions. Taken together, however, this data suggests AT1R-Ab may cause vascular inflammation that is not necessarily exclusively associated with acute rejection. This is consistent with our clinical finding in this cohort of decline in eGFR in patients with AT1R-Ab both with and without rejection. The relationship between AT1R-Ab, vascular inflammation, rejection, and clinical outcomes requires further investigation.

Our study has some limitations. The majority of blood samples did not have time matched biopsy samples per our above described criteria. This limited our ability to effectively analyze direct relationships between cytokine profiles and biopsy findings. Cytokine levels fluctuate quickly and may be rapidly impacted by treatment for rejection. Therefore, we limited our primary analyses to relating antibodies with cytokines since they were all tested in the same blood samples. We used statistical approaches in our model to limit the impact of the different event rates for AT1R-Ab and HLA DSA positivity on our ability to detect factors with greater associations with elevations in serum cytokines. The low event rate for HLA DSA positive samples also limited our ability to do sub-analyses by HLA Class or MFI. The availability of longitudinal samples, however, allowed us to assess relationships at multiple time points post-
transplant. We were also able to take advantage of the rich clinical data available on our cohort to control for potential confounders.

In conclusion, we describe a unique inflammatory cytokine profile for AT1R-Ab in renal transplant patients. Currently, the differences in non-HLA and HLA antibody-mediated allograft injury are poorly understood. This profile of vascular cytokines may be informative for designing further studies to understand the distinct pathophysiology of AT1R-Ab mediated injury in kidney transplantation.
Table 1: Demographic and Clinical Characteristics. Blood samples analyzed were from a cohort of 65 pediatric kidney transplant recipients. These 65 patients yielded 232 blood samples for analysis. SD, standard deviation; IQR, interquartile range; ESRD, end stage renal disease; FSGS, focal segmental glomerulosclerosis; PKD, polycystic kidney disease; EBV, Epstein-Barr virus; CMV, cytomegalovirus; BKV, BK virus; MFI, mean fluorescence intensity.
Table 2: Comparison of cytokine levels in blood samples with and without AT1R-Ab and HLA DSA. For cytokines with <50% of samples above the lower limit of detection (LLD) (TNF-α, IFN-γ, IL-8) data are summarized as median and IQR and compared using the Kruskal Wallance test. For cytokines where ≥50% of samples were below the LLD (IL-1β, IL-6, IL-17) data are summarized as percent positive where positive is >LLD and compared using chi-square test. In HLA DSA negative patients, cytokine levels were higher in the AT1R-Ab positive group. In HLA DSA positive patients, cytokine levels were higher in the AT1R-Ab positive group with the exception of IFN-γ and IL-1β. The comparison of all 4 groups was statistically significant for all cytokines except IL-6 and IL-17.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>HLA DSA – AT1R-Ab - (n = 126)</th>
<th>HLA DSA – AT1R-Ab + (n = 81)</th>
<th>HLA DSA + AT1R-Ab - (n = 14)</th>
<th>HLA DSA + AT1R-Ab + (n = 11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/mL</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>8.8 (5.76-14.4)</td>
<td>11.11 (7.83-16.56)</td>
<td>7.8 (5.15-8.48)</td>
<td>20.44 (5.33-43.05)</td>
<td>0.023</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.57 (0.29-11.37)</td>
<td>8.52 (4.07-19.84)</td>
<td>5.88 (0.92-17.5)</td>
<td>4.13 (0.58-13.35)</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-8</td>
<td>6.11 (2.06-16.72)</td>
<td>19.94 (7.67-46.56)</td>
<td>7.03 (0.18-41.75)</td>
<td>10.22 (4.46-65.69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% &gt; LLD</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>34 (26.98%)</td>
<td>44 (54.32%)</td>
<td>5 (35.71%)</td>
<td>3 (27.27%)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6</td>
<td>32 (25.4%)</td>
<td>34 (41.98%)</td>
<td>4 (28.57%)</td>
<td>5 (45.45%)</td>
<td>0.069</td>
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<tr>
<td>IL-17</td>
<td>26 (20.63%)</td>
<td>21 (25.93%)</td>
<td>2 (14.29%)</td>
<td>5 (45.45%)</td>
<td>0.249</td>
</tr>
<tr>
<td>Cytokine</td>
<td>AT1R-Ab + (n=92)</td>
<td>p-value</td>
<td>HLA DSA + (n=25)</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
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<td>---------</td>
<td></td>
</tr>
<tr>
<td><strong>Linear Models</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Coefficient (95% CI)</td>
<td>Coefficient (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.38 (0.14 - 0.63)</td>
<td>0.002</td>
<td>-0.01 (-0.42 - 0.39)</td>
<td>0.883</td>
<td></td>
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<tr>
<td>IFN-γ</td>
<td>0.71 (0.25 - 1.18)</td>
<td>0.002</td>
<td>0.35 (-0.38 - 1.08)</td>
<td>0.360</td>
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</tr>
<tr>
<td>IL-8</td>
<td>1.04 (0.61 - 1.47)</td>
<td>&lt;0.001</td>
<td>0.45 (-0.24 - 1.14)</td>
<td>0.221</td>
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</tr>
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<td><strong>Logistic Models</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>3.01 (1.32 - 6.96)</td>
<td>0.008</td>
<td>0.86 (0.25 - 2.96)</td>
<td>0.817</td>
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<td>IL-6</td>
<td>3.89 (1.34 – 11.36)</td>
<td>0.012</td>
<td>2.74 (0.55 - 13.58)</td>
<td>0.155</td>
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<tr>
<td>IL-17</td>
<td>2.56 (0.99 – 6.62)</td>
<td>0.049</td>
<td>1.26 (0.32 - 4.89)</td>
<td>0.714</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> **Linear regression models** adjusted for patient level random effect, time, age, sex, mean HLA mismatch, living vs. deceased donor, viremia, and presence of rejection. Cytokines log transformed for analysis.

<sup>b</sup> **Logistic regression models** adjusted for patient level random effect, time, age, sex, mean HLA mismatch, living vs. deceased donor, and presence of rejection.

**Table 3: Multivariable Models for Factors Effecting Increased Cytokine Levels in AT1R-Ab vs. HLA DSA Positive Samples** Individual linear and logistic regression models were created for cytokines with <50% of samples below the LLD (TNF-α, IFN-γ, IL-8) and ≥50% of samples below the LLD (IL-1β, IL-6, IL-17) respectively. Viremia and rejection were assessed on the patient level as any viremia or rejection during the follow-up period. Viremia was included in the linear, but not logistic regression models because of model instability. However, sensitivity analysis using a linear model for IL-1β, IL-6, and IL-17 did not reveal any effect of viremia on these relationships. Time post-transplant was significant for all cytokines except IL-6. For IL-6, living donor (p=0.013) and Mean HLA mismatch (p=0.04) were also associated with detectable IL-6 levels. OR, odds ratio.
Figure 1: Distribution of cytokine data in a) AT1R-Ab Positive (>17 units/mL) vs negative and b) HLA DSA Positive (MFI>1000) vs negative blood samples. Differences between the groups were evaluated using linear mixed effects regression models with each cytokine as the outcome variable and AT1R-Ab and HLA DSA as the predictors. All models include a random effect for patient
Figure 2: Cytokine Levels by AT1R-Ab Status Over Time. Relationship of a) TNF-α b) IFN-γ c) IL-8 d) IL-1β d) IL-6 and f) IL-17 and AT1R-Ab Status in Blood Samples by Time Post-Transplant. Time post-transplant (except in the case of IL-6) was significantly associated with elevation in serum cytokine levels. The interaction between time and AT1R-Ab evaluated in separate models was not significant (Table S2). This may indicate that the effect of AT1R-Ab status on each cytokine was constant over time.
STATISTICAL APPENDIX

1. **Sample selection**: This study was initially designed to examine the relationship between AT1R-Ab and clinical outcomes including biopsy findings. Therefore, in creation of our database, we grouped samples and biopsies occurring within 6 weeks of each other into the same event. Biologically, this was justified by the half-life of IgG being approximately 28 days. In our initial data analysis of cytokines, we kept sample and biopsy groupings in the same design as our initial database. In these analyses we looked at the differences in serum cytokine levels in samples associated with a non-rejection vs rejection biopsy and found no differences. These models were controlled for patient level random effects. We found this lack of association somewhat surprising given rejection would be expected to increase serum cytokines given the inflammatory nature of the event. On further review, we wondered if this analysis was flawed due to the inherent difference in biology of antibodies vs. cytokines. While the half-life of IgG is on the order of weeks, serum cytokines may shift quite rapidly over the course of days. Therefore, our initial database setup with a window of +/- six weeks between biopsy and blood sample may not have been optimized for an analysis of associations between serum cytokines and biopsy findings. Therefore, we designed a sub-analysis of “biopsy matched” samples which were defined as samples drawn 7 days prior to 3 days after a biopsy. This window was chosen to account for the potential of faster fluctuations in serum cytokines. The 3 day post-biopsy cut-off was chosen because rejection treatment, which would only be initiated if applicable after the biopsy, would be expected to alter cytokines fairly rapidly. Though this was a sound biological model for examining the relationships between rejection and cytokines, it presented a statistical challenge, as it resulted in only 63 of the 129 samples being relevant for analysis. The substantial reduction in samples size as well as the need to control for multiple samples from the same patient, resulted in model instability that made this analysis much less informative for the manuscript (Table S1). Given our hypothesis is that AT1R-Ab may mediate damage to the kidney through vascular injury, we also examined vascular inflammation in the biopsies. Vascular inflammation was determined by the pathologist scoring the biopsy based on consensus criteria. There are scores for both acute and chronic vascular findings in different vascular beds in the kidney (described below). Since these events were quite rare in our population, we compared samples associated with biopsies with any of these findings vs. none of these findings. Though there was an association between increased IL-6 and peritubular capillaritis (ptc) in the +/- 6 weeks window, there was not in the -7/+3 window (Table S1)
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>+/- 6 weeks OR (95% CI) n=129 samples</th>
<th>Overall p-value</th>
<th>-7d to +3 days from Biopsy OR (95% CI) n=63 samples</th>
<th>Biopsy p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biopsy Proven Rejection (yes/no)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.56 (0.81 - 3.01)</td>
<td>0.179</td>
<td>3.94 (0.82 - 18.95)</td>
<td>0.087</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.88 (0.65 - 1.18)</td>
<td>0.388</td>
<td>1.01 (0.62 - 1.62)</td>
<td>0.977</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.2 (0.84 - 1.73)</td>
<td>0.318</td>
<td>149.38 (148.88 - 149.89)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.02 (0.66 - 1.57)</td>
<td>0.928</td>
<td>3.08 (0.8 - 11.92)</td>
<td>0.103</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.06 (0.72 - 1.56)</td>
<td>0.766</td>
<td>4.1 (0.57 - 29.62)</td>
<td>0.162</td>
</tr>
<tr>
<td>IL-17</td>
<td>1.03 (0.69 - 1.53)</td>
<td>0.884</td>
<td>2.45 (0.72 - 8.37)</td>
<td>0.152</td>
</tr>
<tr>
<td><strong>Sum Vascular Score (g+ptc+v+cg+cv+ah) ≥ vs &lt; 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.17 (0.62 - 7.62)</td>
<td>0.225</td>
<td>1.45 (0.49 - 4.28)</td>
<td>0.498</td>
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<tr>
<td>IFN-γ</td>
<td>1.28 (0.65 - 2.5)</td>
<td>0.471</td>
<td>1.14 (0.7 - 1.85)</td>
<td>0.609</td>
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<tr>
<td>IL-8</td>
<td>3.27 (0.9 - 11.94)</td>
<td>0.073</td>
<td>1.3 (0.73 - 2.31)</td>
<td>0.376</td>
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<tr>
<td>IL-1β</td>
<td>2.06 (0.82 - 5.17)</td>
<td>0.125</td>
<td>1.65 (0.91 - 3)</td>
<td>0.101</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.6 (1.6 - 1.61)</td>
<td>&lt;0.001</td>
<td>1.22 (0.66 - 2.27)</td>
<td>0.53</td>
</tr>
<tr>
<td>IL-17</td>
<td>1.45 (0.7 - 3.02)</td>
<td>0.321</td>
<td>1.06 (0.58 - 1.93)</td>
<td>0.849</td>
</tr>
</tbody>
</table>

Table S1: Effect of Sample Selection Window on Modelling the Relationship of Biopsy Findings and Serum Cytokines. Models evaluating the relationship between serum cytokines and 1) biopsy proven rejection and 2) vascular inflammation (as determined by presence or absence of any acute or chronic vascular findings). All models are controlled for patient level random effect. There was a 49% reduction in sample size with the narrower window between sample and biopsy. For Rejection vs. No Rejection, the odds ratio for serum IL-8 became uninterpretable in this model. g, glomerulitis; ptc, peritubular capillaritis; v, arteritis; cg, allograft glomerulopathy; cv, arterial fibrous intimal thickening; ah, arteriolar hyalinosis.

2. Challenges in Cytokine Data Analysis: Cytokine data were log + 1 transformed prior to analysis to allow for appropriate evaluation by linear regression model. The data analysis process presented multiple important additional data handling decisions. Firstly, the Luminex cytokine assay only allowed for up to 80 samples to be included on a given plate. Each run on a given plate measured all 6 cytokines and standard curves were generated to determine the lower and upper limits of detection (LLD and ULD) for each cytokine on each plate. The quantifiable
values obtained from each plate are directly comparable, but the cutoffs for the LLD and ULD are not consistent across plates. The samples used in this analysis were measured on 4 different plates. Therefore, we were presented with a challenge as to how to handle values outside the limit of detection, which in this case were mostly below the LLD. Strategies used by others including imputing “0” for these values, using the highest LLD obtained across plates, or using the lowest LLD obtained across plates. We tried all 3 of these strategies and it did not change the results. We chose to use the latter strategy as it was in our opinion the best of what were all flawed choices. Using the lowest LLD narrowed the gap between non-quantifiable and quantifiable results without allowing crossover between quantifiable and non-quantifiable values on different plates. Additionally, given 3 of the 6 cytokines had more than 50% of values below the LLD, we chose to treat them as a dichotomous variables to simplify the analysis and help mitigate the problem of choosing values for results <LLD. We completed analysis of the remainder of the cytokines as continuous data to avoid discarding potentially valuable information about their biology.

3. Time and Cytokine Levels: In our initial multivariable models examining factors that influence cytokine levels, we included time as a continuous variable. In these models we noted that time, specifically months post-transplant, seemed to have a significant influence on cytokine levels. This relationship is biologically plausible given there are known changes in biology and care that may influence inflammation over time in transplant patients. For example, inflammatory factors may be high in the immediate post-transplant period as part of carryover from the known high inflammatory state in dialysis patients and/or as a response to the surgical procedure. Immunosuppression level targets change over time as well, with the highest targets implemented peri-operatively and gradually lowered over the first year post-transplant. Certainly, as immunosuppression is lowered and the recipient immune system has additional opportunities to recognize the allograft, it is possible that certain cytokines may have later increases as this occurs. Though we can only speculate to the etiology, we did observe that the relationship between time and cytokine levels was not linear. Therefore, to further explore this relationship and avoid violating model assumptions for linear regression,
we changed time to a categorical variable in our model. As discussed in the main body of the paper, even including time in the model, the relationship between AT1R-Ab and cytokines remained significant. Furthermore, separate models were constructed to evaluate the interaction between AT1R-Ab and Time and these showed that the interaction term was not significant (Table S2).

4. Evaluating for Interaction between HLA DSA and AT1R-Ab: Given the great interest in HLA DSA in the transplant community, we wanted to further evaluate for any interaction between AT1R-Ab and HLA DSA. We constructed separate mixed effects regression models to evaluate this clustered on patient. As noted in the paper, these interactions were not significant. To supplement this discussion, the figures we created as part of this analysis are included below to further illustrate this point (Figure S1, below).
REFERENCES


18. Gunther J, Kill A, Becker MO, et al. Angiotensin receptor type 1 and endothelin receptor type A on immune cells mediate migration and the expression of IL-8 and CCL18 when


