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# Cytokine Profiles Associated With Angiotensin II Type 1 Receptor Antibodies



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**Introduction:** Angiotensin II type 1 receptor antibody (AT1R-Ab), is a non-human leukocyte antigen (HLA) antibody implicated in poor renal allograft outcomes, although its actions may be mediated through a different pathway than HLA donor-specific antibodies (DSAs). 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:** Blood samples from 65 pediatric renal transplant recipients drawn during the first 3 months post-transplant, at 6, 12, and 24 months posttransplant, and during suspected episodes of kidney transplant rejection were tested for AT1R-Ab, HLA DSA, and a panel of 6 cytokines (tumor necrosis factor [TNF]- $\alpha$ , interferon [IFN]- $\gamma$ , interleukin [IL]-8, IL-1 $\beta$ , 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- $\alpha$ , IFN- $\gamma$ , IL-8, IL-1 $\beta$ , IL-6, and IL-17. This relationship remained significant even after controlling for relevant clinical factors and was consistent across all time points. In contrast to HLA DSA, AT1R-Ab was associated with elevations in vascular inflammatory cytokines in the first 2 years posttransplant.

**Conclusions:** This profile of vascular cytokines may be informative for clinical monitoring and designing future studies to delineate the distinct pathophysiology of AT1R-Ab-mediated allograft injury in kidney transplantation.

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**KEYWORDS:** angiotensin II type 1 receptor antibody; cytokine; human leukocyte antigen donor-specific antibody; pediatric nephrology; transplantation

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## See Commentary on Page 510

Antibody-mediated rejection (AMR) remains a leading cause of allograft failure and subsequently contributes to the ongoing organ shortage in renal transplantation.<sup>1–3</sup> Both HLA DSAs and non-HLA autoantibodies have been implicated in AMR, allograft dysfunction, and failure.<sup>2,4–7</sup> AT1R-Ab is a non-HLA antibody that has gained recognition for its detrimental effects on the renal allograft, although its actions may be mediated through a different mechanistic pathway than HLA DSA.<sup>8,9</sup>

AT1R-Ab activates the AT1R, a G-protein-coupled receptor located on endothelial and vascular smooth

muscle cells, which mediates the vasoconstrictive and salt retention actions of angiotensin II.<sup>7</sup> In addition to these classical effects, activation of the AT1R also triggers inflammatory and profibrotic pathways.<sup>10,11</sup> HLA DSA alloantibodies bind to Class I and II HLA receptors on the allograft endothelium. This interaction stimulates endothelial cell activation, proliferation, and migration, leading to the histological changes associated with acute and chronic AMR.<sup>12–15</sup> 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.<sup>16,17</sup>

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<sup>18–23</sup> and distinguish them from those that associate with HLA DSA. Identifying cytokine signatures associated with these antibodies

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may help delineate mechanistic pathways to examine HLA and non-HLA antibody-mediated injury, lead to novel clinical monitoring tools, and identify new therapeutic targets in kidney transplantation.

## METHODS

### Patients and Study Design

In this retrospective study, 65 pediatric kidney transplant patients were monitored for 2 years posttransplant. From August 2005 to November 2014, 83 patients were enrolled in the University of California Los Angeles 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 University of California Los Angeles 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 posttransplant (within the first 3 months), at 6, 12, and 24 months posttransplant, and during suspected episodes of kidney allograft 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 Research Electronic Data Capture tool hosted at UCLA.<sup>24</sup> 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.<sup>9</sup> A total of 233 blood samples at the previously described time points were analyzed. Sample data were complete with the exception of 1 sample missing HLA DSA results.

### Clinical Protocols and Biopsy Evaluation

Immunosuppressive strategies at our institution included induction with either antithymocyte globulin for panel reactive antibodies  $\geq 30\%$ , delayed graft function, or rapid-steroid withdrawal protocol or anti-CD25 monoclonal antibody for those with panel reactive antibodies  $< 30\%$ .<sup>9</sup> Maintenance immunosuppression consisted of steroid-free or steroid-based immunosuppression, a calcineurin inhibitor, and an antimetabolite. Acute and chronic rejection were treated with previously described protocols.<sup>25</sup> Cytomegalovirus, Epstein-Barr

virus, and BK virus were monitored monthly for the first 6 months posttransplant, then at 9, 12, 18, and 24 months posttransplant. Cytomegalovirus and Epstein-Barr virus viremia were treated with a combination of i.v. ganciclovir, oral valganciclovir, and reduction in immunosuppression. BK viremia was treated using previously described protocols.<sup>26</sup>

Patients underwent biopsies at 6, 12, and 24 months posttransplantation per protocol or for clinical indication. Biopsies were evaluated using 2013 Banff Criteria by a blinded pathologist.<sup>27</sup> Our primary analysis included all 233 blood samples and evaluated cytokine profiles, AT1R-Ab, and HLA DSA. To evaluate cytokine profiles in blood samples taken at the time of biopsies with and without biopsy-proven rejection, a subanalysis of 63 blood samples collected 7 days before or 3 days after the time of biopsy was conducted. This window was chosen to account for rapid changes in serum cytokines both in general and in response to immunomodulatory therapy. An additional subanalysis of 129 blood samples collected within 6 weeks of the time of biopsy was used to evaluate cytokine profiles in biopsies with and without elevated vascular inflammation scores (arteritis or glomerulitis). A wider window was chosen for this analysis given that vascular inflammation scores may be less likely to change rapidly.<sup>28</sup>

### AT1R and HLA Antibodies and Cytokine Testing

HLA typing of recipient and donor was performed using molecular methods as previously described.<sup>25</sup> HLA antibodies were detected using a Luminex single antigen bead assay (Immucor, Stanford, CT) and quantified by mean fluorescence intensity (MFI). Antibodies were considered positive when MFI was  $\geq 1000$  for HLA-A, -B, -DR, -DQ, and  $\geq 2000$  for HLA-C and -DP.<sup>29</sup> AT1R-Ab was measured by enzyme-linked immunosorbent-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.<sup>9,30,31</sup> Cytokines were selected based on a literature review of cytokines that have been associated with activation of the AT1R<sup>18-23</sup> and measured in serial posttransplant samples to avoid effects of dialysis and end-stage renal disease. A custom magnetic bead kit including TNF- $\alpha$ , IFN- $\gamma$ , IL-8, IL-1 $\beta$ , IL-6, and IL-17 (EMD Millipore, Darmstadt, Germany) was used per manufacturer's instructions. Fluorescence was quantified using a Luminex (Austin, TX) 200TM instrument.

### Statistical Methods

Before statistical analysis, cytokines were transformed using the  $\log(x+1)$  transformation due to skew in their

distributions. The value of the lowest lower limit of detection (LLD) per cytokine was used for values below the limit of detection. For cytokines in which <50% of the samples were below the LLD (IL-8, TNF- $\alpha$ , IFN- $\gamma$ ), data were analyzed as a continuous outcome. For cytokines in which >50% of the samples were below the LLD (IL-1 $\beta$ , IL-6, IL-17) data were analyzed as a categorical variable ( $\leq$  LLD vs. >LLD). Kruskal-Wallis and  $\chi^2$  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. Model results 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 versus 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). We also evaluated interaction effects (time by AT1R-Ab and HLA DSA by AT1R-Ab) in separate mixed effect linear/logistic regression models. Time was modeled as a categorical variable to ease interpretation of regression coefficients. Wilcoxon rank sum tests were used to compare cytokine levels in samples associated with biopsies with and without arteritis or glomerulitis. *P* values below 0.05 were considered statistically significant and all tests were 2-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.<sup>9</sup> The rate of rejection during the follow-up period (including borderline rejection) was 45%. The rate of viremia with cytomegalovirus, Epstein-Barr virus, or BK virus was 49%, which is consistent with other studies that have shown high rates of viremia in pediatric renal transplant recipients (Table 1).<sup>32</sup> AT1R-Ab >17 units/ml was present in 92 (39%) of 233 blood samples from 38 (58%) of 65 patients, and HLA DSA >1000 MFI was present in 25 (11%) of 233 blood samples from 19 (29%) of 65 patients (Table 1). Most patients with HLA DSA developed DSA to class II antigens and median MFIs were <3000. The median AT1R-Ab level in AT1R-Ab-positive patients was 24

**Table 1.** Demographic and clinical characteristics

Variable	n (%) / mean (SD) / median (IQR)
Age, median (IQR)	15.7 (12.9–17.7)
Sex, male, n (%)	39 (60)
Race, n (%)	
White	47 (72.3)
Asian	4 (6.2)
Black	4 (6.2)
Other	10 (15.4)
Etiology of ESRD, n (%)	
Obstructive uropathy	16 (24.6)
Dysplasia	9 (13.8)
FSGS	9 (13.8)
Glomerulonephritis	9 (13.8)
PKD	2 (5.3)
Other or unknown	20 (30.8)
Hispanic ethnicity, n (%)	36 (55.4)
Deceased donor, n (%)	40 (61.5)
Mean HLA mismatch	1.2 (0.5)
Time on dialysis, years, median (IQR)	2.2 (1–2.9)
Preemptive transplant, n (%)	14 (21.5)
ATG induction (vs. IL-2 inhibitor), n (%)	6 (9.2)
Steroids-based immunosuppression, n (%)	31 (47.7)
EBV, CMV, or BK viremia, n (%)	29 (49.2)
Biopsy-proven rejection, n (%)	29 (44.6)
CMV PCR (copies/ml) peak in patients with CMV viremia, median (IQR)	12,725 (5010–40,936)
EBV PCR (copies/PCR) peak in patients with EBV viremia, median (IQR)	109 (35–191)
BK PCR (copies/ml) peak in patients with BK viremia, median (IQR)	9865 (6225–60,375)
Posttransplant HLA DSA positive, n (%)	19 (29.2)
HLA class I positive, n (%)	5 (7.7)
HLA class II positive, n (%)	12 (18.5)
HLA class I and II positive, n (%)	2 (3.1)
HLA DSA-positive samples Class I, MFI, median (IQR)	2258 (1838–3006)
HLA DSA positive samples class II, MFI, median (IQR)	2229 (1902–5500)
AT1R-Ab-positive patients (at any time point), n (%)	38 (58.5)
Preformed AT1R-Ab, n (%)	15 (23.1)
<i>de novo</i> AT1R-Ab, n (%)	17 (26.2)
Positive posttransplant, pretransplant status unknown, n (%)	6 (9.2)
AT1R-Ab positive samples level (Units/ml), median (IQR)	24 (20–31)
Number of samples per patient, mean (SD)	3.7 (1.1)
Number of samples per time point per patient, mean (SD)	1.2 (0.5)
Number of samples per time point (months posttransplantation), n (%)	
0 (1 d–3 mo)	36 (15.5)
6 (3–9 mo)	70 (30.0)
12 (9–15 mo)	68 (29.2)
24 (16–24 mo)	59 (25.3)

AT1R-Ab, angiotensin II type 1 receptor antibody; ATG, antithymocyte globulin; BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; HLA DSA, human leukocyte antigen donor-specific antibodies; IQR, interquartile range; MFI, mean fluorescence intensity; PCR, polymerase chain reaction; PKD, polycystic kidney disease. Blood samples analyzed were from a cohort of 65 pediatric kidney transplant recipients. These 65 patients yielded 233 blood samples for analysis.

units/ml (Table 1). In our previous work, we found that patients in this cohort who were positive for AT1R-Ab during the first 2 years posttransplantation had

significantly higher median levels of TNF- $\alpha$ , IL-8, and IL-1 $\beta$  across time-points during the follow-up period.<sup>9</sup> This patient-level data prompted our investigation into the sample-level data for the current study and to compare cytokine profiles of HLA DSA.

### 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 Figure 1a and b, respectively. AT1R-Ab was a predictor of higher levels of serum inflammatory vascular cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-8, IL-1 $\beta$ , IL-6, and IL-17, which mirrored similar trends in our patient-level data.<sup>9</sup> In comparison, HLA DSA was not associated with elevations in any of the 6 cytokines. IL-1 $\beta$ , 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 ( $\leq$  LLD = negative vs.  $>$  LLD = positive). In contrast, only 5%, 32%, and 14% of samples were outside the limits of detection for the assay for TNF- $\alpha$ , IFN- $\gamma$ , and IL-8, respectively. Therefore, these cytokines were analyzed as continuous outcomes.

Given we had more samples positive for AT1R-Ab than HLA DSA, we conducted a 4-group subanalysis to preliminarily examine if AT1R-Ab was associated with increased serum cytokines in samples both with and without HLA DSA. This also allowed us to assess the data for any trends suggesting possible synergism. Overall, the effect of AT1R-Ab on elevations of serum cytokines remained consistent in samples with and without HLA DSA (Table 2). Notably, IL-8 and IL-1 $\beta$

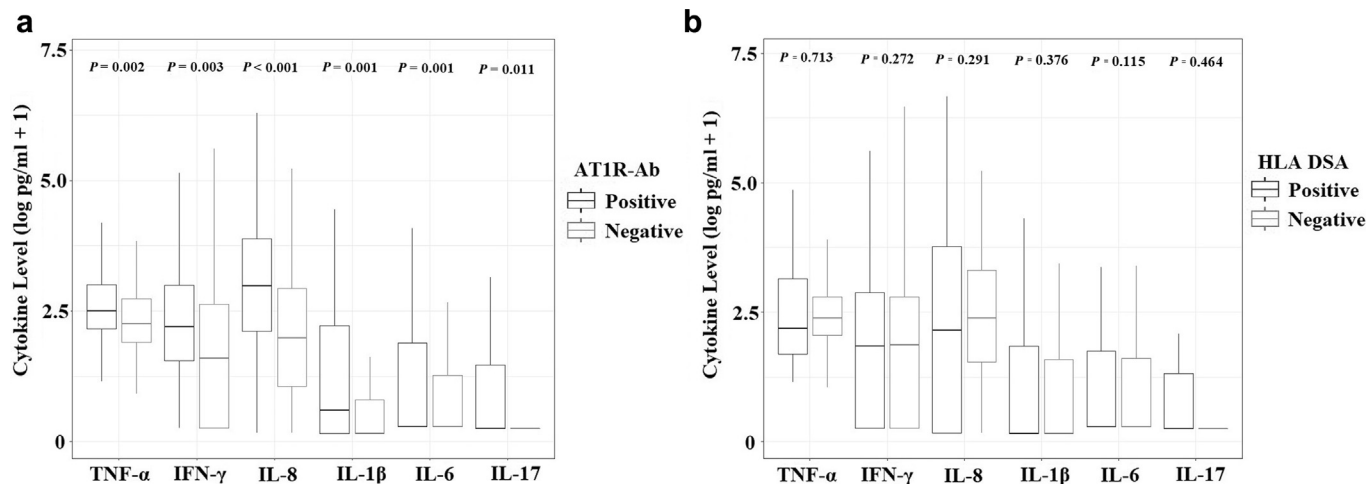
levels appeared higher in AT1R-Ab-positive and HLA DSA-negative samples when compared with all other groups. In contrast, TNF- $\alpha$  and IL-17 were higher in samples positive for both AT1R-Ab and HLA DSA when compared with all other groups.

### Adjusted Analysis for Clinical Factors Associated With Increased Cytokine Levels

As serum cytokines can be influenced by multiple factors, we examined models that controlled for relevant clinical variables, including age, sex, mean HLA mismatch, living versus deceased donor, time post-transplant, viremia, and rejection. After controlling for these factors, the relationship between AT1R-Ab and all 6 cytokines remained significant (Table 3). Time posttransplant was associated with cytokine levels for all cytokines except IL-6. In the multivariable model, living donor ( $P = 0.013$ ) and mean HLA mismatch ( $P = 0.04$ ) were also associated with detectable IL-6 levels (data not shown).

To follow up our preliminary examination of the data by both AT1R-Ab and HLA DSA status as noted previously, we assessed the interaction effects between HLA DSA and AT1R-Ab on cytokine levels. Although there appeared to be a potential trend in the data toward synergism for TNF- $\alpha$  and IL-17 in the initial analysis, a regression model showed there were no statistically significant interaction effects (data not shown).

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 subanalysis of samples that



**Figure 1.** Distribution of cytokine data in (a) AT1R-Ab positive ( $>17$  units/ml) versus negative and (b) HLA DSA positive (MFI  $>1000$ ) versus 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 are controlled for patient-level random effect. The median levels of IL-1 $\beta$  (in all groups except AT1R-Ab positive), IL-6, and IL-17 were equal to the lower limit of detection for the respective cytokine. AT1R-Ab, angiotensin II type 1 receptor antibody; HLA DSA, human leukocyte antigen donor-specific antibodies; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.

**Table 2.** Comparison of cytokine levels in blood samples with and without AT1R-Ab and HLA DSA

Cytokine	HLA DSA- AT1R-Ab- (n = 126)	HLA DSA- AT1R-Ab+ (n = 81)	HLA DSA+ AT1R-Ab- (n = 14)	HLA DSA+ AT1R-Ab+ (n = 11)	P
pg/ml	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	
TNF- $\alpha$	8.8 (5.76–14.4)	11.11 (7.83–16.56)	7.8 (5.15–8.48)	20.44 (5.33–43.05)	0.023
IFN- $\gamma$	3.57 (0.29–11.37)	8.52 (4.07–19.84)	5.88 (0.92–17.5)	4.13 (0.58–13.35)	0.006
IL-8	6.11 (2.06–16.72)	19.94 (7.67–46.56)	7.03 (0.18–41.75)	10.22 (4.46–65.69)	<0.001
% >LLD	n (%)	n (%)	n (%)	n (%)	
IL-1 $\beta$	34 (26.98)	44 (54.32)	5 (35.71)	3 (27.27)	0.001
IL-6	32 (25.4)	34 (41.98)	4 (28.57)	5 (45.45)	0.069
IL-17	26 (20.63)	21 (25.93)	2 (14.29)	5 (45.45)	0.249

AT1R-Ab, angiotensin II type 1 receptor antibody; HLA DSA, human leukocyte antigen donor-specific antibodies; IFN, interferon; IL, interleukin; IQR, interquartile range; TNF, tumor necrosis factor.

For cytokines with <50% of samples above the lower limit of detection (LLD) (TNF- $\alpha$ , IFN- $\gamma$ , IL-8) data are summarized as median and IQR and compared using the Kruskal-Wallis test. For cytokines in which  $\geq$ 50% of samples were below the LLD (IL-1 $\beta$ , IL-6, IL-17) data are summarized as percent positive where positive is >LLD and compared using  $\chi^2$  test. The comparison of all 4 groups was statistically significant for all cytokines except IL-6 and IL-17.

were collected within 7 days before and 3 days after a biopsy (see Methods). Of the 233 samples, 63 met this criterion. 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. On this subanalysis, there was also no association between any of these cytokines and the presence of any acute or chronic vascular findings. However, on analysis of all biopsy-matched samples ( $\pm$  6 weeks) in the cohort, we found blood samples with arteritis and glomerulitis had significantly higher levels of IL-8, IL-1 $\beta$ , and IL-6 ( $P = 0.010$ ,  $P = 0.038$ ,  $P = 0.010$ ), consistent with our patient-level data in patients who were AT1R-Ab positive (Table 4).<sup>9</sup>

### The Relationship Between Time Posttransplant and Cytokine Levels

Time posttransplant was significantly associated with cytokine levels for all cytokines except for IL-6

(Figure 2a–f). Given the significant individual associations of time and AT1R-Ab on cytokine levels, we assessed for any interaction effect between these 2 variables. We found no significant interaction suggesting that the impact of AT1R-Ab on cytokine levels was consistent across all time points posttransplant (data not shown).

## DISCUSSION

We demonstrate that AT1R-Ab has significant associations with vascular inflammatory cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-8, IL-1 $\beta$ , IL-6, and IL-17 and renal vascular biopsy findings distinct from HLA DSA in pediatric renal transplantation. The association between AT1R-Ab and these serum cytokines remained significant even when controlling for HLA DSA, in addition to time posttransplant, age, sex, mean HLA mismatch, donor type, presence of rejection, and viremia. Moreover, our longitudinal samples allowed us to demonstrate the consistency of the relationship between AT1R-Ab and cytokine elevations over time. This unique cytokine profile suggests that AT1R-Ab may activate different pathways from HLA DSA and potentially exert renal allograft damage through a separate mechanism.

Since the original paper describing AT1R-Ab in patients with acute AMR and hypertension,<sup>8</sup> multiple studies<sup>9,16,17,33–40</sup> have indicated an association

**Table 3.** Multivariable models for effect of AT1R-Ab or HLA DSA positivity on cytokine levels

Cytokine	AT1R-Ab+ (n = 92)	P	HLA DSA+ (n = 25)	P
Linear models <sup>a</sup>	Coefficient (95% CI)		Coefficient (95% CI)	
TNF- $\alpha$	0.38 (0.14–0.63)	0.002	−0.01 (−0.42 to 0.39)	0.883
IFN- $\gamma$	0.71 (0.25–1.18)	0.002	0.35 (−0.38 to 1.08)	0.360
IL-8	1.04 (0.61–1.47)	<0.001	0.45 (−0.24 to 1.14)	0.221
Logistic models <sup>b</sup>	OR (95% CI)		OR (95% CI)	
IL-1 $\beta$	3.03 (1.32–6.96)	0.008	0.86 (0.25–2.96)	0.817
IL-6	3.89 (1.34–11.36)	0.012	2.74 (0.55–13.58)	0.155
IL-17	2.56 (0.99–6.62)	0.049	1.26 (0.32–4.89)	0.714

AT1R-Ab, angiotensin II type 1 receptor antibody; CI, confidence interval; HLA DSA, human leukocyte antigen donor-specific antibodies; IFN, interferon; IL, interleukin; LLD, lower limit of detection; OR, odds ratio; TNF, tumor necrosis factor.

Individual linear and logistic regression models were created for each cytokine. Linear models were used when <50% of samples were below the LLD (TNF- $\alpha$ , IFN- $\gamma$ , IL-8) and logistic models were used otherwise (IL-1 $\beta$ , IL-6, IL-17). Viremia and rejection were assessed on the patient level as any viremia or rejection during the follow-up period. Time posttransplantation was significant for all cytokines except IL-6.

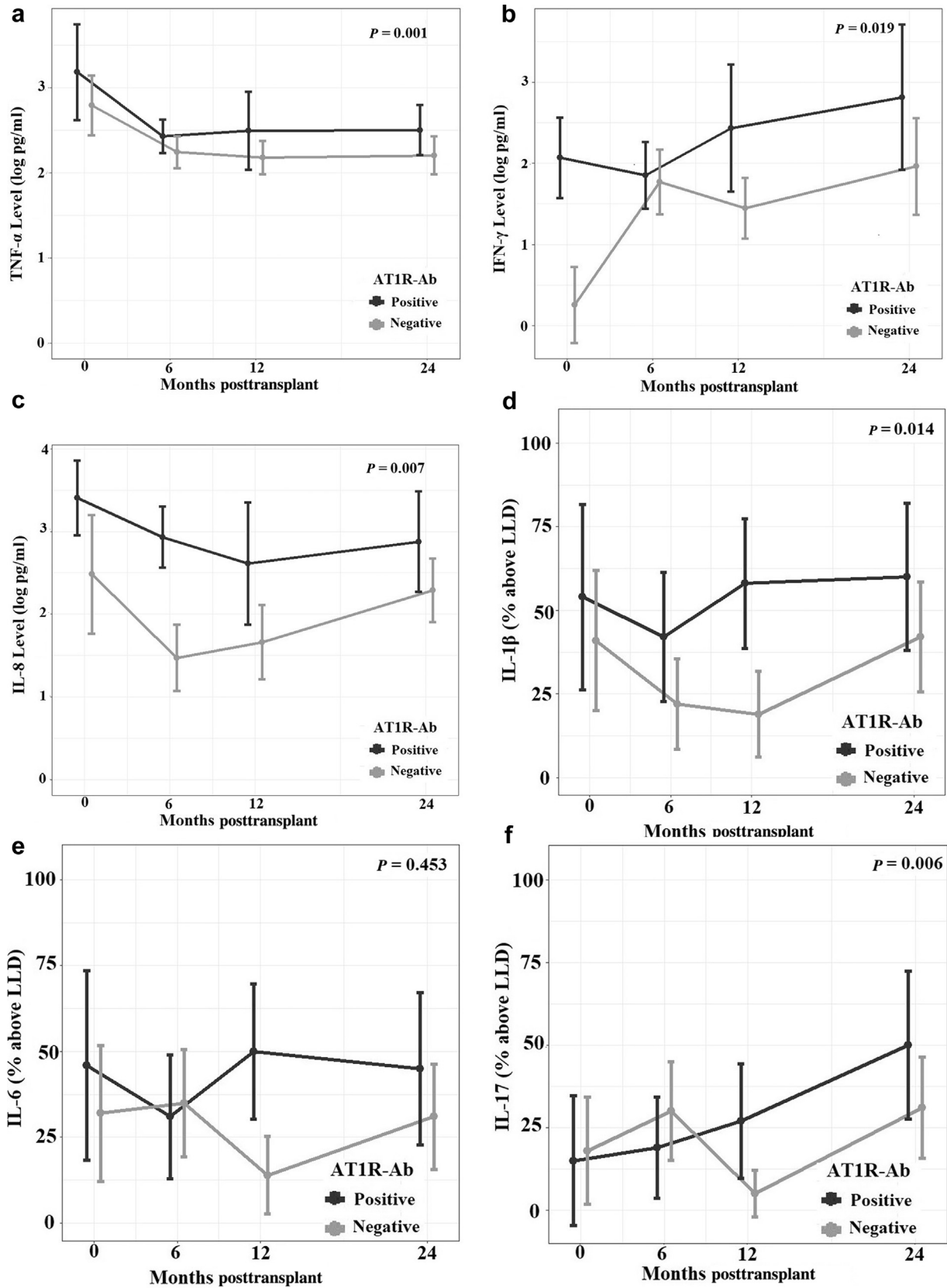
<sup>a</sup>Linear regression models adjusted for patient level random effect, time, age, sex, mean HLA mismatch, living versus deceased donor, viremia, and presence of rejection. Cytokines log transformed for analysis.

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

**Table 4.** Comparison of cytokines in blood samples associated with biopsies with and without arteritis or glomerulitis

Cytokine	g+v = 0 (n = 125)	g+v > 0 (n = 4)	P
pg/ml	Median (IQR)	Median (IQR)	
TNF- $\alpha$	9.2 (6.6–14.5)	9.1 (7.5–107.9)	0.704
IFN- $\gamma$	7.2 (0.6–25.2)	10.1 (7–1689.7)	0.608
IL-8	7.8 (3.5–31.1)	61.9 (44.3–770.8)	0.010
IL-1 $\beta$	0.2 (0.2–2.1)	8.3 (5.6–68.8)	0.038
IL-6	0.3 (0.3–4.7)	6.1 (4.9–95.1)	0.010
IL-17	0.3 (0.3–2.7)	1.1 (0.3–225.5)	0.323

IFN, interferon; IL, interleukin; IQR, interquartile range; TNF, tumor necrosis factor. Cytokine levels in blood samples collected within 6 weeks of biopsies with and without glomerulitis (g) or arteritis (v) were compared using the Wilcoxon rank sum test. IL-8, IL-1 $\beta$ , and IL-6 were elevated in samples associated with biopsies with arteritis or glomerulitis.



**Figure 2.** Cytokine levels by AT1R-Ab status over time. Relationship of (a) TNF- $\alpha$ , (b) IFN- $\gamma$ , (c) IL-8, (d) IL-1 $\beta$ , (e) IL-6, and (f) IL-17 and AT1R-Ab status in blood samples by time posttransplantation. Time posttransplant (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. This may indicate that the effect of AT1R-Ab status on each cytokine was constant over time. AT1R-Ab, angiotensin II type 1 receptor antibody; IFN, interferon; IL, interleukin; LLD, lower limit of detection; TNF, tumor necrosis factor.

between AT1R-Ab with vascular inflammation and poor clinical outcomes. Studies with longitudinal measurements of AT1R-Ab<sup>9,16,36</sup> remain limited. Furthermore, although AT1R-Ab has been reported to potentially synergize with HLA DSA,<sup>16,40</sup> their mechanistic interaction remains unclear. Our initial work revealed an association between AT1R-Ab and IL-8, IL-1 $\beta$ , and TNF- $\alpha$  in patients with AT1R-Ab at any time during the first 2 years posttransplant.<sup>9</sup> As cytokines may fluctuate rapidly, we undertook this additional analysis to examine the relationship between AT1R-Ab, HLA DSA, and cytokines at the sample level with the aim of elucidating differences in inflammatory profiles.

Because AT1R-Ab has been shown to be an allosteric agonist<sup>7,8</sup> of the AT1R, we measured cytokines associated with activation of the AT1R and vascular inflammation. Angiotensin II has been shown to stimulate TNF- $\alpha$  production in glomerular endothelial cells in rats,<sup>41</sup> and both IL-17 and IFN- $\gamma$  are involved in angiotensin II-mediated increases in blood pressure and salt retention in mice.<sup>19</sup> Angiotensin II-treated renal proximal tubular cells and mouse macrophages secrete IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ .<sup>20,42</sup> In addition, IL-8 and IL-6 have been implicated in the pathophysiology of other vascular inflammatory conditions associated with AT1R-Ab, such as preeclampsia and scleroderma.<sup>18,21,22,43</sup> Based on these *in vitro* and animal model data, we examined these specific cytokines in patients expected to have elevated AT1R signaling. Accordingly, we found that AT1R-Ab was associated with significant elevations of these cytokines in renal transplant recipients *in vivo*. In the multivariable models, the relationship between cytokines and AT1R-Ab remained consistent when controlling for other clinical variables, and only IL-6 had other significant predictors (aside from time). The additional association of IL-6 with living donation may be reflective of regenerative processes occurring in the allograft,<sup>44</sup> but this relationship requires further investigation. In addition, the cytokines had varying levels during the early posttransplant time point, which may be partially reflective of a proinflammatory state associated with end-stage renal disease. Although the potential immune effects of AT1R signaling are complex,<sup>45–47</sup> activation of AT1R is considered to be proinflammatory, which is consistent with our data.

The 6 cytokines measured in our study are involved in vascular inflammatory pathways<sup>23</sup> and may provide insights into AT1R-Ab pathogenesis. Endothelial cells can produce IL-1 $\beta$  and IL-8, which are known to promote leukocyte chemotaxis and arterogenesis.<sup>23,48–50</sup> Elevations in IL-6<sup>51–56</sup> and TNF- $\alpha$ <sup>54,57,58</sup> also have been associated with rejection, mortality, and allograft loss in renal transplantation. Interestingly, in our analysis, the association between AT1R-Ab and these

cytokines remained significant even when controlling for rejection. Taken together, these data suggest AT1R-Ab may cause vascular inflammation that is not necessarily exclusively associated with acute rejection. This is consistent with our patient-level clinical findings in this cohort of (i) decline in estimated glomerular filtration rate in patients with AT1R-Ab both with and without rejection, and (ii) the association between AT1R-Ab and arteritis or glomerulitis on biopsy.<sup>9</sup> Furthermore, arteritis or glomerulitis on biopsy was associated with elevations in IL-8, IL-1 $\beta$ , and IL-6 at the sample level. Therefore, our data may provide guidance in investigating novel therapeutic targets. The IL-6 inhibitor tocilizumab has been used in a small number of cases to treat AT1R-Ab-associated AMR.<sup>28</sup> TNF- $\alpha$  inhibition is currently being trialed in the prevention of ischemia reperfusion injury ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02495077) Identifier: NCT02495077) in renal transplantation. Furthermore, there is the potential to use IL-1 and IL-17 blockers, currently approved for the treatment of autoimmune conditions, in the context of transplantation.<sup>59,60</sup> Additional study is required to understand the roles of these pathways and potential for pharmacologic intervention.

By contrast, there were no statistically significant associations between HLA DSA and increases in serum cytokines in our cohort. Although we chose cytokines based on described AT1R pathways, this was still somewhat unexpected, given elevations in cytokines in our panel also have been associated with HLA Class I and II signaling *in vitro*. Activation of endothelial cells by HLA Class I has been associated with increases in IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ .<sup>61,62</sup> HLA class II binding has been associated with increased IL-6 production in endothelial cells and promotion of TH17 lymphocyte expansion.<sup>63</sup> In peripheral blood mononuclear cells isolated from transplant patients, some studies have shown HLA antibodies increase cytokines (TNF- $\alpha$ , IFN- $\gamma$ , and IL-6<sup>64</sup>) whereas others have not.<sup>65</sup> We found an association between IL-6 and HLA mismatch, but not HLA DSA. Several factors may have affected the analysis of these relationships in our cohort. HLA DSAs were less prevalent in our samples than AT1R-Ab, which may have reduced our power to detect differences. We mitigated this by using a model that simultaneously included both HLA DSA and AT1R-Ab. Furthermore, most of the HLA DSAs in our cohort were low to moderate MFI and our samples were drawn relatively early posttransplantation. It is possible that we may have observed an effect of HLA DSAs on these cytokines, particularly IL-6, in samples with higher MFIs or from time points later posttransplantation.

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 limited our ability to do subanalyses by HLA Class or MFI. In addition, we examined cytokines previously associated with AT1R activation, which would not have accounted for all cytokines that could be associated with HLA DSA. We also acknowledge other potential study limitations. Most blood samples did not have time-matched biopsy samples per our previously described criteria. This limited our ability to effectively analyze direct temporal relationships between cytokine profiles and biopsy findings. Cytokine levels fluctuate quickly and may be rapidly affected by treatment for rejection. Therefore, we limited our primary analyses to relating antibodies with cytokines because they were all tested in the same blood samples. The availability of longitudinal samples, however, allowed us to assess relationships at multiple time points posttransplantation. We also were 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 patients with renal transplantation. Currently, the differences in non-HLA and HLA antibody-mediated allograft injury are poorly understood. This profile of vascular cytokines may inform the design of future studies to elucidate the distinct pathophysiology of AT1R-Ab-mediated injury in kidney transplantation, develop adjunct clinical monitoring tools, and help identify potential therapeutics, specifically targeting cytokines, for the treatment of non-HLA antibody-mediated allograft injury.

## DISCLOSURE

MHP has an industry grant from Veloxis Pharmaceuticals that was not used to fund this project and has served on an advisory board for Bristol-Myers Squibb. EFR has received consulting fees for Genentech, travel support from One Lambda, and has a grant from Immucor. None of these funds were used to fund this project. All the other authors declared no competing interests.

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