Short-Term Immunopathological Changes Associated with Pulse Steroids/IVIG/Rituximab Therapy in Late Kidney Allograft Antibody Mediated Rejection

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Abstract

Background B cell depletion is a common treatment of antibody-mediated rejection (ABMR). We sought to determine the specific immunopathologic effects of this therapeutic approach in kidney transplantation.

Methods This was a prospective observational study of recipients of kidney transplants diagnosed with late ABMR (>3 months after transplant). Patients received treatment with pulse steroids, IVIG, and rituximab. Donor-specific HLA antibodies (DSA), kidney allograft pathology, renal function, immune cell phenotypes, and 47 circulating cytokines were assessed at baseline and at 3 months.

Results We enrolled 23 patients in this study between April 2015 and March 2019. The majority of patients were male (74%) and white (78%) with an average age of 45.6±13.8 years. ABMR was diagnosed at 6.8±5.9 years (4 months to 25 years) post-transplant. Treatment was associated with a significant decline in circulating HLA class I (P=0.003) and class II DSA (P=0.002) and peritubular capillaritis (ptc; P=0.04) compared to baseline. Serum creatinine, BUN, eGFR, and proteinuria (UPC) remained stable. Circulating B cells were depleted to barely detectable levels (P≤0.001), whereas BAFF (P=0.0001), APRIL (P<0.001), and IL-10 (P=0.02) levels increased significantly post-treatment. Notably, there was a significant rise in circulating CD4+ (P=0.02) and CD8+ T cells (P=0.003). We also noted a significant correlation between circulating cytotoxic CD8+ T cells and BAFF (P=0.05), regulatory T cells and IL-10 (P=0.002), and regulatory T cells and HLA class I DSA (P=0.005).

Conclusions Short-term pulse steroids/IVIG/rituximab therapy was associated with inhibition of ABMR (DSA and ptc), stabilization of kidney function, and increased regulatory B cell and T cell survival cytokines. Additional studies are needed to understand the implications of B cell depletion on the crosstalk between T cells and B cells, and humoral components that regulate ABMR.


Introduction

Current immunosuppressive therapies have greatly reduced the incidence of acute rejection and improved short-term allograft survival. Unfortunately, long-term graft survival has not improved at an equal rate, primarily because of challenges associated with detecting and managing late antibody-mediated rejection (ABMR) (1,2). This stark contrast was highlighted by a recent study that showed approximately 75% of patients diagnosed with chronic active ABMR lost their grafts within 2 years (3). There is currently no Food and Drug Administration (FDA)–approved treatment for ABMR (4), but depletion of B cells and removal of donor-specific antibodies (DSA) (5–8), inhibition of plasma cells (9), and IL-6 blockade (10) constitute different aspects of the current standard of care.

Rituximab is a chimeric, murine/human mAb directed against the CD20 antigen found on the surface of normal and malignant pre-B and mature B cells. In chronic ABMR, rituximab has been reported to increase median graft survival (11), reduce the rate of eGFR loss (12), and reduce microcirculation inflammation and DSA (8,13). However, a recent randomized study suggested that the combination of intravenous immunoglobulin (IVIG) and rituximab is not useful in patients displaying transplant glomerulopathy and DSA (6). Unfortunately, this study recruited only half of the planned patient enrollment because of budgetary constraints and slow patient accrual. The effectiveness of B cell depletion in ABMR therefore remains an area of debate and controversy.

Herein, we present findings from a cohort of recipients of kidney transplants who were treated for late...
ABMR with rituximab, IVIG, and pulse steroids. All patients underwent a follow-up biopsy at 3 months and additional blood work to assess the effect of treatment on B and T cell populations and circulating cytokines.

**Materials and Methods**

**Study Population and Design**

We conducted a single-center, prospective, observational study of adult (age 18–75 years) recipients of kidney transplants diagnosed with biopsy-proven ABMR between April 2015 and March 2019. All patients received a single dose of rituximab (375 mg/m² BSA), IVIG (200 mg/kg every 2 weeks for 3 months), and dexamethasone (100 mg and taper). Biopsies and blood draws were repeated approximately 3 months after treatment (Figure 1). Demographic and clinical data were collected for all patients including age, race, sex, body mass index, date of kidney transplantation, induction immunosuppression, history of previous transplants, graft loss, and death (Table 1). At the time of index biopsy, biopsy specimens were assessed for features of ABMR according to the Banff 2017 criteria (14). Peripheral blood samples were assessed for MHC I and II DSA, circulating immune cell populations, and cytokines. This study was approved by the University of Wisconsin (UW) School of Medicine and Public Health Institutional Review Board.

**Anti-HLA Antibody Screening by Luminex**

Donor-specific HLA class I and class II antibodies were assessed at the time of index biopsy and again at the follow-up biopsy 3 months later using Luminex single antigen beads (One Lambda, Canoga Park, CA) as reported previously (8,13,15,16). This assay was performed according to manufacturer’s instructions. The strength of DSA was represented as the sum of the mean fluorescence intensity (MFI) of positive individual beads selected to represent each donor antigen. Our virtual crossmatch uses an MFI signal >500 and/or a clear epitope reactivity pattern to assign a positive reactivity. Some transplants performed after 2015 would have crossed positive preexisting DSA levels but these would have been restricted to DSAs detectable at low MFI values.

**PBMC Isolation**

Peripheral blood samples were drawn at the time of index biopsy and again 3 months later. Whole blood was collected in sterile heparinized Vacutainers and processed immediately. PBMCs and plasma were isolated using a Ficoll (cat #17-1440-02; GE Healthcare, Uppsala, Sweden) density-centrifugation gradient as per manufacturer’s instructions. Plasma was removed and stored at −80°C. PBMCs were washed, counted, live frozen, and placed in liquid nitrogen for long-term storage.

**Flow Cytometry**

Single-cell suspensions of PBMCs were prepared from thawed samples and stained for B and T lymphocyte subsets. B lymphocyte subsets included naive B cells (CD3–CD19+CD20+CD27–CD38lo), transitional B cells (CD3–CD19+CD20+CD24+CD38+), memory B cells (CD3–CD19+CD20+CD27+CD38–), mature B cells (CD3–CD19+CD20+CD27+CD38–), and plasmablasts (CD3–CD19+CD20+CD27+CD38hi). T lymphocyte subsets included T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), and T regulatory (Treg) cells (CD3+CD4+CD25+CD127–).

**Antibodies.** The antibodies used for B lymphocyte subsets were as follows: anti-CD3 (clone SK7; Biolegend), anti-CD4 (clone 12H7; Biolegend), anti-CD8 (clone RPA-T8; Biolegend), anti-CD19 (clone HIB19; Biolegend), anti-CD20 (clone L243; Biolegend), anti-CD27 (clone OJ24; BD Biosciences), anti-CD28 (clone BD3; BD Biosciences), anti-CD44 (clone IM7; eBioscience), anti-CD62L (clone MEL-14; BD Biosciences), anti-CD161 (clone M492; BD Biosciences), anti-CD127 (clone HP-127; Biolegend), anti-CD138 (clone 82C11; BD Biosciences), anti-CD24 (clone TS2/16; BD Biosciences), and anti-CD38 (clone HIT2; BD Biosciences).  

![Figure 1](image-url) | Sample collection and analysis were performed at index and follow up biopsy in patients with active ABMR treated with pulse steroids/IVIG/rituximab. Patients with biopsy-confirmed active antibody-mediated rejection (ABMR; *n* = 23) were included in the study. Peripheral blood samples and renal graft biopsies were assessed at the time of index biopsy and again approximately 3 months (3M) after treatment. Donor-specific HLA antibodies (DSA), graft pathology, graft function, B and T cell populations, and peripheral blood cytokines were assessed at both time points. All patients received the same combination treatment including a single dose of rituximab (375 mg/m² BSA) in conjunction with intravenous immunoglobulin (IVIG; 200 mg/kg per 2 weeks for 3 months) and dexamethasone (100 mg and taper). APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor; SAB, single antigen beads.
anti-CD19 (clone SJ25C1; BD Biosciences), anti-CD20 (clone 2H7; BD Biosciences), anti-CD27 (clone M-T271; BD Biosciences), anti-CD38 (clone HIT2; BD Biosciences), anti-CD24 (clone ML5; BD Biosciences), and anti-CD45 (clone H130; BioLegend). For T cell subsets, the antibodies were as follows: anti-CD4 (clone RPA-T4; BioLegend), anti-CD24 (clone ML5; BD Biosciences), and anti-CD45 (clone M-A251; BD Biosciences), and anti-CD127 (clone A019D5; Biolegend).

**Gating and Analysis.** All experiments were analyzed on a BD LSR Fortessa at the UW Carbone Cancer Center Flow Cytometry core facility. All gating and flow analysis was conducted using FlowJo version 10.5.3 (Treestar Inc., Ashland, OR). Cells were gated for singlets, live cells, and then sorted into white blood cell lineages with CD45. B cells were gated in a similar manner as B cells described above through the lymphocyte gate, and then gated for CD3+ B cells.

**Cytokine Measurements**

Plasma levels of B cell activating factor (BAFF), a proliferation-inducing ligand (APRIL), and 45 cytokines were assessed on cryopreserved samples collected at the time of index and follow-up biopsy. ELISAs were used to measure BAFF (DY124-05; R&D) and APRIL (BMS2008; Invitrogen). The additional 45 cytokines were measured using the Human Magnetic Luminex Assay kit from R&D (LSXAHM). These cytokines included CD40L, EGF, elastin, basic fibroblast growth factor, fms-like tyrosine kinase receptor-3 (Flt-3) ligand, fractalkine, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granzyme B, growth-regulated oncogene-α, growth-regulated oncogene-β, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1 receptor agonist, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17a, IL-17f, IL-33, IL-1β, interferon-γ-induced protein 10 (C-X-C motif chemokine 10), monocytic chemoattractant protein-1, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, MIP-3α, MIP-3β, programmed death-ligand 1, platelet derived growth factor -AA (PDGF-AA), platelet derived growth factor-BB (PDGF-BB), regulated on activation normal T cell expressed and secreted (RANTES), TGF-α, TNF-α, tumor necrosis factor-related apoptosis-inducing ligand, and vascular endothelial growth factor (VEGF). All assays were conducted according to manufacturer’s instructions. Briefly, ELISA assays were conducted on diluted patient plasma (1:2) and applied to plates coated with human BAFF or APRIL capture antibody. Color development was achieved through the sequential addition of Streptavidin–horseradish peroxidase and substrate solution. The OD was read at 450 nm within 15 minutes. All samples were performed in duplicate. For IL-10, plasma was diluted (1:2) and incubated with antibody-coated MagPlex beads according to manufacturer’s instructions. Antibody binding was detected using biotinylated primary antibody and streptavidin-phycocerythrin and read on the Luminex 200 analyzer within 90 minutes.

**Statistical Analysis**

The D’Agostino–Pearson (omnibus K2) normality test was used for all data sets. Comparisons between pre- and post-treatment were achieved using a paired t test or Wilcoxon signed-ranks test as appropriate. The Pearson correlation test was performed to determine the association between circulating cytokines, DSA, and lymphocyte subpopulations. P values ≤0.05 were considered statistically significant. Tables are presented as mean ± SD and figures are presented as mean ±SEM. Analyses were performed using GraphPad Prism version 8.

**Results**

**Patient Demographics and Characteristics**

A total of 23 patients were enrolled in this study. ABMR was diagnosed at 6.8 ± 5.9 years (minimum, 4 months; maximum, 25.2 years) post-transplant. Demographic information is summarized in Table 1. The majority of patients were male (74%) and white (78%) with a mean age of 45.6 ± 13.8 years. At the time of ABMR diagnosis, mean serum creatinine, eGFR, and proteinuria were 1.6 ± 0.45 mg/dl, 50.5 ± 16.9 ml/min, and 1.1 ± 1.5 g/g, respectively (Table 2).

<table>
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<th>Characteristics</th>
<th>Value</th>
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<td>Average age, mean (SD)</td>
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<tr>
<td>Female gender, n (%)</td>
<td>6 (26%)</td>
</tr>
<tr>
<td>White race, n (%)</td>
<td>18 (78%)</td>
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<tr>
<td>BMI, mean (SD)</td>
<td>29.5 (8.1)</td>
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<tr>
<td>Diabetes, n (%)</td>
<td>7 (30%)</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>19 (83%)</td>
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<tr>
<td>Retransplant, n (%)</td>
<td>7 (30%)</td>
</tr>
<tr>
<td>Induction rATG/IL2(−)/alemuzumab, n/n/n</td>
<td>5/16/2</td>
</tr>
<tr>
<td>Transplant to ABMR interval, mean (SD), yr</td>
<td>6.8 (5.9)</td>
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</tbody>
</table>

BMI, body mass index; rATG, rabbit anti-thymocyte globin; ABMR, antibody-mediated rejection.
Pulse Steroids/IVIG/Rituximab Therapy Was Associated with Reduction of ABMR

To determine if treatment with rituximab, IVIG, and dexamethasone inhibited ABMR, we compared baseline and 3-month DSA and renal pathology (Figure 2). We noted a significant reduction in class I (1640±529 versus 564±301 MFI, P = 0.003), class II (13,121±3443 versus 7292±2091 MFI, P = 0.002), and total DSA (14,607±3863 versus 7831±2183 MFI, P = 0.001) (Figure 2A). There was also a significant decline in the Banff peritubular capillaris (ptc) score (1.6±0.16 versus 1.2±0.17, P = 0.04) (Figure 2B) and a trend toward decreased sum microvascular inflammation (mvi) score (3.6±0.27 versus 2.9±0.37, P = 0.09). The remaining acute Banff inflammation scores (ia, it, iv, g) and C4d declined without reaching statistical significance. There was no change in chronicity scores (ci, ct, cv, cg, ah, mm, i-IFTA) and kidney graft function remained stable (Table 2).

Pulse Steroids/IVIG/Rituximab Therapy Resulted in Reduced Circulating B Cells and Increased Circulating T Cells

We assessed the effects of pulse steroids/IVIG/rituximab therapy on peripheral blood lymphocytes. B cell and T cell phenotypes at baseline and approximately 3 months after treatment using flow cytometry analyses are shown in Figure 3. Total lymphocyte counts decreased from 68,300±18,300 cells/mm$^3$ to 62,600±17,200 cells/mm$^3$, P = 0.04. As expected, total B cells were significantly reduced in patients undergoing pulse steroids/IVIG/rituximab therapy (7969±1286 versus 565±414 per 100,000 lymphocytes, P<0.001) (Figure 3A). This overall reduction was reflected in multiple subsets of B cells including transition B cells (492±234 versus 95±79, P = 0.001), naive B cells (3433±695 versus 285±231, P<0.001), mature B cells (145±30.7 versus 5.3±3.3, P<0.001), memory B cells (1126±212 versus 49±30, P<0.001), and plasmablasts (5±1 versus 0.3±0.3, P = 0.0001). In contrast to B cells, there was a significant increase in circulating T cell populations after treatment (Figure 3B). We observed an increase in total T cells (63,924±4125 versus 74,530±4773, P = 0.006), CD4+ T cells (36,328±3483 versus 43,154±3813, P = 0.01), and CD8+ T cells (22,823±2432 versus 27,124±2441, P = 0.003) after treatment. The expression of Treg cells trended higher in post-treatment samples, but was not statistically significant (822±162 versus 1154±243, P = 0.08).

Pulse Steroids/IVIG/Rituximab Therapy Was Associated with Increased Systemic Levels of B Cell Survival Cytokines and IL-10

To determine the effect of B cell depletion on circulating cytokines including those associated with B cell survival and function, we examined changes in 47 cytokines using ELISA and a magnetic multiplex Luminex-based assay. We only noted significant upregulations in circulating levels of BAFF (891.5±144.5 pg/ml, P = 0.0001), APRIL (8.9±3.7 ng/ml versus 18±3.6 ng/ml, P = 0.001), and IL-10 (2.6±0.94 pg/ml versus 11.8±4.5 pg/ml, P = 0.02) (Figure 4).

**Correlations between Humoral and Cellular Arms of the Immune Response**

We next examined the association between circulating DSA, cytokines, T cells, and B cells to test the hypothesis of a tight network of regulatory components during ABMR. We noted a statistically significant positive correlation between total B cells and MHC I DSA (P = 0.02) and MHC II DSA (P = 0.05) (Table 4). There was also a possible correlation between memory B cells and MHC II DSA (P = 0.05), but not MHC I DSA. Treg cells showed a statistically significant positive correlation with IL-10 (P = 0.002) and MHC I DSA levels (P = 0.005). BAFF levels were negatively correlated with total B cell (P = 0.05) and memory B cell (P = 0.005) populations. Similarly, APRIL levels were negatively correlated with both total (P = 0.02) and memory (P = 0.03) B cell.

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**Table 2. Pre- and post-treatment kidney function**

<table>
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<tr>
<th>Measure</th>
<th>Pretreatment (mean±SD)</th>
<th>Post-treatment (mean±SD)</th>
<th>P Value</th>
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<td>Serum creatinine (mg/dl)</td>
<td>1.6±0.45</td>
<td>1.7±0.56</td>
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<tr>
<td>BUN (mg/dl)</td>
<td>30.7±11.4</td>
<td>29.9±12.8</td>
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<tr>
<td>eGFR (ml/min per 1.73 m$^2$)</td>
<td>50.5±16.9</td>
<td>49.8±17.9</td>
<td>0.78</td>
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<tr>
<td>UPC</td>
<td>1.1±1.5</td>
<td>1.2±0.26</td>
<td>0.78</td>
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UPC, urine protein-creatinine ratio.

**Table 3. Banff and HLA values at the time of index biopsy**

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<th>t</th>
<th>v</th>
<th>g</th>
<th>ptc</th>
<th>mvi</th>
<th>c4d</th>
<th>ci</th>
<th>ct</th>
<th>cv</th>
<th>cg</th>
<th>ah</th>
<th>mm</th>
<th>i-IFTA</th>
<th>Class I HLA</th>
<th>Class II HLA</th>
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<td>0.3</td>
<td>0.0</td>
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<td>1.6</td>
<td>3.6</td>
<td>1.0</td>
<td>1.2</td>
<td>1.3</td>
<td>1.0</td>
<td>1.2</td>
<td>0.9</td>
<td>1.1</td>
<td>1.6</td>
<td>1640</td>
<td>13,121</td>
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<td>SD</td>
<td>0.7</td>
<td>0.6</td>
<td>0.0</td>
<td>0.8</td>
<td>0.5</td>
<td>0.7</td>
<td>1.4</td>
<td>0.7</td>
<td>1.3</td>
<td>1.0</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>2424</td>
<td>16,149</td>
</tr>
</tbody>
</table>

i, interstitial inflammation; t, tubulitis; v, intimal arteritis; g, glomerulitis; ptc, peritubular capillaris; mvi, sum microvascular inflammation; C4d, complement component C4d; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular fibrosis intimal thickening; cg, glomerular basement membrane double contours; ah, arteriolar hyalinosis; mm, mesangial matrix expansion; i-IFTA, scarred cortical inflammation.
populations. CD8+ T cells were positively correlated with BAFF levels ($P \leq 0.05$).

**Discussion**

Our study demonstrates that short-term depletion of B cells using a pulse steroids/IVIG/rituximab regimen was associated with a modest reduction of ABMR, stabilization of kidney function, upregulation of T cells, and increased levels of B cell and regulatory T cell survival cytokines. Rituximab depletes CD20+ B cells and may therefore prevent B cell and antibody-mediated injury. Although this concept makes immunologic sense, the role of rituximab in the treatment of ABMR remains controversial. The debate arises from a number of uncontrolled observational studies and underpowered randomized trials (3,6–8,11–13,17–19).

A recent randomized study indicated that the combination of IVIG and rituximab was not useful for the treatment of chronic ABMR (6). However, as noted by Moreso et al. (6), the trial was underpowered because it recruited only half (25 patients) of the planned enrollment due to budgetary constraints and slow accrual. Similarly, Sautenet et al. (7) outlined how the small sample size in their randomized

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**Figure 2.** | **Pulse steroids/IVIG/rituximab treatment is associated with a reduction in ABMR.** Peripheral blood samples were collected at the time of index biopsy (i.e., pretreatment, white bars) and again after treatment at a 3 month follow-up biopsy (i.e., post-treatment, gray bars). Blood samples were assayed for HLA class I and class II DSA by Luminex. Biopsy pathology was assessed using the Banff 2017 criteria. Patients demonstrated (A) a reduction in circulating HLA class I and class II DSA and (B) a reduction in the ptc score after treatment. Data were compared using a paired t test or Wilcoxon signed-ranks sum test and are expressed as mean±SEM. Statistical significance is designated as *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.  

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**Figure 3.** | **Circulating T cell subsets are elevated after rituximab treatment.** PBMCs were isolated from whole blood and assessed for B and T cell populations via flow cytometry before (i.e., pretreatment, white bars) and after (post-treatment, gray bars) intervention. Treatment resulted in (A) near complete ablation of circulating B cell populations, (B) whereas total circulating T cells, as well as CD4+ and CD8+ subsets, were significantly increased. T regulatory (Treg) cell expression trended higher post-treatment, but did not reach statistical significance. Data were compared using a paired t test or Wilcoxon signed-ranks sum test and are expressed as mean±SEM. Statistical significance is designated as **$P \leq 0.01$ and ***$P \leq 0.001$.  

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study could have overlooked important differences between treatment and control groups. Conversely, observational studies have favored a role for rituximab in the management of ABMR, especially if the drug is used early in the process \( (3,6–8,11–13,17–19) \). Because of these inconsistencies and a lack of appropriately powered rigorous studies, there are currently no FDA-approved therapies for ABMR \( (4) \).

An important observation from our study was the rise in circulating CD4\(^+\) and CD8\(^+\) T cells after B cell depletion. Although we noted no evidence of T cell–mediated rejection (TCMR) during follow-up, this finding is consistent with previous studies demonstrating that induction therapy with B cell depletion in patients with low immunologic risk was associated with increased rates of TCMR \( (20) \). Conversely, induction therapy with alemtuzumab, a T and B cell–depleting agent, was associated with increased incidence of ABMR \( (21,22) \). These studies are suggestive of off-target consequences of treatment on regulatory

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Table 4. Correlations between immune cell populations, cytokines, and DSA

<table>
<thead>
<tr>
<th>Measure</th>
<th>B Cells</th>
<th>T Cells</th>
<th></th>
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<td></td>
<td>Total</td>
<td>Memory</td>
<td>CD3+</td>
<td>CD4+</td>
<td>CD8+</td>
<td>Treg</td>
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<tr>
<td>MHC I DSA</td>
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<tr>
<td>CC</td>
<td>0.383</td>
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<td>( P^a )</td>
<td>0.02(^b)</td>
<td>0.13</td>
<td>0.71</td>
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<td>( P^a )</td>
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<td>42</td>
<td>42</td>
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<tr>
<td>IL-10</td>
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<tr>
<td>CC</td>
<td>−0.109</td>
<td>−0.213</td>
<td>0.07</td>
<td>−0.121</td>
<td>0.245</td>
<td>0.705</td>
</tr>
<tr>
<td>( P^a )</td>
<td>0.69</td>
<td>0.43</td>
<td>0.80</td>
<td>0.66</td>
<td>0.36</td>
<td>0.002(^b)</td>
</tr>
<tr>
<td>( N )</td>
<td>16</td>
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</tbody>
</table>

**Notes:** DSA, donor-specific HLA antibody; Treg, T regulatory cell; CC, correlation coefficient; BAFF, B cell activating factor; APRIL, A proliferation-inducing ligand.

\(^b\)Pearson correlation computation was conducted.

\(^*\)\( P < 0.05 \).
elements of the immune system. Beyond the production of antibodies, B cells serve as active regulators of T cell-mediated responses through functions including antigen presentation, costimulation, and cytokine production (23). In autoimmune diseases, B cell depletion can affect T cell populations and often correlates with a reduction in disease activity (24–27); however, less is known in the context of ABMR. In particular, the use of B cell–depleting drugs indiscriminately targets all CD20+ cells, including B regulatory (Breg) cells, which may play a role in promoting graft tolerance (28). This concept was illustrated by a clinical trial comparing induction therapy with rituximab and daclizumab in patients who were nonsensitized, low-risk kidney transplant recipients (20). The study was halted early because of high rates of acute TCMR rejection in the rituximab-treated group (20). In addition, Kamburova et al. (29) demonstrated that B cells exposed to rituximab in vitro enhance CD4+ T cell proliferation compared with controls. However, the same authors later examined T cell populations in patients receiving rituximab versus standard of care for induction therapy after transplantation and did not observe significant differences during the follow-up period (30). These discrepancies suggest that the timing of B cell depletion and patients’ sensitization status might have a strong effect on the risk for subsequent TCMR. In particular, patients who are sensitized or those with poor immunosuppressive drug compliance could be susceptible to TCMR. It is also possible that potential cytotoxic effects of CD4+ and CD8+ T cells are suppressed by Treg cells. Studies in both autoimmune and renal diseases have reported significant elevation in circulating Treg cells after B cell depletion with rituximab (24,26,31,32).

Vigna-Perez et al. (26) described both an increase in circulating Treg cells and enhanced suppressive function in patients with lupus after rituximab treatment. Further analysis into the functional capacity of Treg cells was beyond the scope of our study but, given the association of Treg cells with allograft tolerance, this avenue presents an exciting area for further study. In summary, evidence suggests that elevated T cell levels may be associated with poor clinical response after rituximab treatment. However, this depends on the timing (induction therapy versus rejection), dose (single dose versus repeated doses), and context (autoimmunity versus alloimmunity, sensitized versus not sensitized host). It is possible that factors such as baseline immunosuppression, alterations in B cell costimulatory signals, and increased Treg populations could explain an elevation in T cells without a corresponding change in T cell–mediated injury.

The effects of rituximab therapy on circulating cytokines was limited to a small number of B cell growth factors and regulatory cytokines. Only BAFF, APRIL, and IL-10 showed significant changes after B cell depletion in our study. BAFF and APRIL were significantly elevated after pulse steroids/IVIG/rituximab treatment and showed a negative correlation with circulating total and memory B cells as well as DSA. BAFF and APRIL are cytokines involved in B cell development, proliferation, maturation, and differentiation (33,34). Increased BAFF levels are associated with DSA and ABMR in clinical and experimental kidney transplantation (33,34). BAFF/APRIL blockade using atacicept (TACI-Ig) reduced the humoral portion of rejection in a nonhuman primate model of ABMR (35). In agreement with our findings, high levels of BAFF after B cell depletion have been reported and may be related to the survival, function, and specificity of remaining or repopulating B cells (36–38). High levels of BAFF are associated with autoimmune diseases and B cell alloreactivity; however, it remains unclear if a similar response occurs in transplant patients after B cell depletion (38,39). Kamburova et al. (30) demonstrated that BAFF receptor (BAFF-R) expression on circulating B cells was reduced up to 24 months after rituximab induction, suggesting the regulation of BAFF signaling post-treatment extends beyond ligand availability. We did not observe the development of autoimmune manifestations after treatment, but more work is needed to determine if pulse steroids/IVIG/rituximab in patients with late ABMR induces autoreactive B cells. We also noted a positive correlation between CD8+ T cells and BAFF. T cells have been reported to express BAFF as well as BAFF-R and TACI (40,41). Previous work has demonstrated that BAFF stimulation of activated T cells enhances cellular proliferation and cytokine production (40,42,43). BAFF exposure has also been shown to increase Bcl2 expression in stimulated splenic T cells, suggesting BAFF-BAFF-R signaling may facilitate T cell survival (40). This response may partially explain our observations, although further mechanistic studies are required.

We also observed an increase in circulating APRIL levels. APRIL is a critical survival factor for mature B lymphocytes and plasma cells, the primary source of alloantibody. We recently demonstrated that targeting of APRIL and BAFF with atacicept can significantly deplete mature B lymphocytes and antibody-secreting cells, and effectively decrease ABMR when given post-transplant in a sensitized animal model (44). Compared to BAFF, there is more limited information on circulating APRIL levels in response to B cell depletion in clinical transplantation; studies of patients receiving rituximab therapy for lupus, Sjögren disease, and lymphoma have reported conflicting results, with APRIL levels either decreasing or remaining unchanged in the post-treatment period (45–47). In addition to its effects on plasma cell survival and B cell class switching, APRIL can also promote T cell activation and priming through binding of the TACI receptor (48). In a study of APRIL transgenic mice, the authors also noted enhanced survival of CD3+ cells was associated with elevated Bcl2 levels (49). As suggested previously, enhanced survival of T cells may present one mechanism by which T cell populations increase after treatment in our subjects. Although APRIL alone has not demonstrated utility as a biomarker for rejection, a study in patients with lupus demonstrated that peripheral APRIL levels and TACI expression on CD3+ cells correlated with organ damage and disease activity (50). More detailed investigations of APRIL expression patterns and their potential effect on T cells in patients who are transplant recipients may provide exciting new avenues for research.

Finally, we observed a significant increase in IL-10 in the post-treatment period which was positively correlated with Treg cells. IL-10 can be produced by most immune cells, but is most often associated with immunomodulatory T helper type 2 cell responses (28,51). Elevated levels of IL-10 have been noted in the blood and urine of patients with active kidney rejection, but IL-10 production by Breg and Treg cells...
is also an important mechanism for promoting immune tolerance (51–53). Because we processed frozen samples only, we were unable to accurately assess Breg cells using intracytoplasmic staining of IL-10 although we would expect a downregulation of Breg cells after rituximab therapy. As a result, the elevation in circulating IL-10 may be more related to changes in Treg cells (54,55). IL-10 has been shown to drive differentiation and suppressive function of Treg cells (54–56). In the absence of TCMR in follow-up biopsies, it is possible that pulse steroids/IVIG/rituximab facilitated the expansion or function of Treg cells, hence providing a regulatory milieu after treatment.

Our study is limited by its single-center, observational nature; the absence of a control group; and specific analyses of Breg cells. However, our short-term observations provide proof of concept that pulse steroids/IVIG/rituximab treatment strategies weaken ABMR progression and stabilize renal allograft function, while upregulating T cells and increasing B cell and T cell regulatory cytokines. These observations suggest that treatment interventions that target ABMR disrupt a complex and continuous regulatory network between humoral and cellular immunity. Prospective, mechanistic, patient-centered, multifaceted treatment strategies will be required to avoid compensatory responses that could negatively affect long-term outcomes.

Acknowledgments
The authors would like to thank the UW Carbone Cancer Center Flow Cytometry Shared Instrumentation Core staff for their assistance and expertise.

Author Contributions
F. Aziz, A. Djamali, N. Garg, L. Hidalgo, D. Mandelbrot, M. Mohamed, S. Panzer, S. Parajuli, R. Redfield, T. Singh, K. Van Hyfte, and W. Zhong were responsible for investigation and resources; F. Aziz, N. Garg, L. Hidalgo, M. Mohamed, S. Panzer, S. Parajuli, R. Redfield, S. Reese, K. Van Hyfte, N. Wilson, and W. Zhong were responsible for methodology; K. Degner and A. Djamali wrote the original draft of the manuscript; K. Degner, A. Djamali, S. Reese, and N. Wilson were responsible for data curation and formal analysis; A. Djamali was responsible for funding acquisition, project administration, and validation; A. Djamali and T. Singh conceptualized the study; A. Djamali and N. Wilson were responsible for supervision and visualization; all authors reviewed and edited the manuscript.

Disclosures
All authors have nothing to disclose.

Funding
This work was supported by internal research funds provided by the University of Wisconsin School of Medicine and Public Health Department of Medicine and National Institutes of Health, UW Transplant Research grant T32 AI125623. The BD LSR Fortessa was purchased by the UW Carbone Cancer Center Flow Cytometry Shared Instrumentation Core with the support of UW Carbone Cancer Center grant 1S100OD018202-01.

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Received: December 20, 2019 Accepted: March 11, 2020