Circulating B Cells, Plasma Cells, and Treg Associate with ANCA Levels in ANCA-associated Vasculitis

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RESULTS

Patient Characteristics

We enrolled 33 patients with new-onset AAV with rapidly progressive glomerulonephritis, 31 patients with CKD, and 12 healthy controls (HCs) (Table 1). None of the CKD patients had immune-mediated kidney disease or diabetic nephropathy secondary to type 1 diabetes mellitus, and none were taking immunosuppression at sampling. The AAV group patients were slightly older compared with the other 2 groups and had a higher percentage of females compared with the...
Table 1. Baseline characteristics of the study population

|                        | AAV group, n = 33 | CKD group, n = 31 | HC group, n = 12 | P value |
|------------------------|-------------------|------------------|-----------------|---------|
| Age [yr]               | 68.2 ± 14.9       | 58.3 ± 16.7      | 57.0 ± 8.8      | 0.006^{CKD,HC} |
| Males                  | 15 (45.4%)        | 23 (74.1%)       | 5 (41.7%)       | 0.038^{CKD} |
| Renal biopsy           | 24 (72.7%)        | —                | —               | —       |
| Serum creatinine [mg/dl]| 3.0 (1.4–16.2)    | 3.2 (0.5–6.5)    | —               | 0.23    |
| eGFR [ml/min per 1.73 m²]| 15.6 (2.6–49.9)  | 19.2 (9.0–135.6) | —               | 0.008   |
| Urinary protein [mg/dl]| 74 (16–578)       | —                | —               | —       |
| Urinary RBC [number per field]| 50 (2–100)     | —                | —               | —       |
| C-reactive protein [mg/L]| 16.2 (0.8–308.4) | —                | —               | —       |
| ANCA [U/ml]           | 483.5 (0.9–3671.9)| —                | —               | —       |
| Anti-PR3 Ab           | 7 (21.2%)         | —                | —               | —       |
| Anti-MPO Ab           | 26 (78.8%)        | —                | —               | —       |
| Induction therapy     |                   |                  |                 |         |
| Initial steroid dose [mg]b| 500 (0–1000)   | —                | —               | —       |
| CPX                    | 23 (69.7%)        | —                | —               | —       |
| AZA                    | 2 (6.1%)          | —                | —               | —       |
| Maintenance therapy   |                   |                  |                 |         |
| AZA                    | 2 (6.1%)          | —                | —               | —       |
| RTX                    | 9 (27.3%)         | —                | —               | —       |
| Steroids only         | 10 (30.3%)        | —                | —               | —       |
| Plasmapheresis         | 3 (9.7%)          | —                | —               | —       |
| Outcomes              |                   |                  |                 | —       |
| ESKD                   | 11 (33.3%)        | —                | —               | —       |
| Death                  | 8 (24.2%)         | —                | —               | —       |

Ab, antibody; AAV, anti-neutrophil cytoplasmic antibody–associated vasculitis; AZA, azathioprine; CKD, chronic kidney disease; CPX, cyclophosphamide; eGFR, estimated glomerular filtration rate; ESKD, end-stage renal disease; HC, healthy control; MPO, myeloperoxidase; PR3, proteinase 3; RBC, red blood cells; RTX, rituximab.

bDose reported is for methylprednisolone.

Continuous data are presented as mean ± SD or as median (range), and categorical data are presented as number (percent). The P values with superscripts indicate which groups had a statistically significant difference versus AAV in a pairwise comparison (P < 0.025). The Kruskal-Wallis test was used for overall comparison between the 3 groups. The Mann-Whitney U test was used for pairwise comparisons of continuous variables and Fisher’s exact test was used for categorical variables.

CKD group. About 75% of those in the AAV group underwent a kidney biopsy. One third of the AAV patients required renal replacement therapy and one fourth eventually died (Table 1). All samples for AAV and CKD patients were collected before the initiation of immunosuppressive therapies, including steroids.

Percentages of Circulating Plasma Cells, B Cells, and Tregs Increased Selectively in AAV Patients

We measured the percentages of 68 circulating B- and T-cell subsets. The Kruskal-Wallis test was used to identify those that were statistically significant with at least one comparison between AAV, CKD, and HCs (Supplementary Table S1). P values were adjusted for multiple testing using the Holm-Bonferroni procedure. Among the jointly significant subsets, by using the Mann-Whitney U test with a significance level of 0.025, we identified those that were statistically significant in the 2 relevant pairwise comparisons (i.e., between AAV and CKD and between AAV and HCs), namely plasma cells, B cells, and Tregs (Figure 1a, Supplementary Table S1, and Supplementary Figures S1 and S2).

Percentages of total B cells, plasma cells, and Tregs were also the subsets with the highest importance for distinguishing AAV group from the other groups, according to random forest analysis (i.e., even after accounting for the information provided by the other subsets; Figure 1b). In contrast, naive CD8⁺ T cells were significantly lower in AAV patients compared with CKD patients and HCs (Figure 1a). As naive CD8⁺ T cells did not help in discriminating the AAV group patients from the other 2 groups in random forest analysis (Figure 1b), we did not include them in further analyses.

Exhausted T cells have been shown to be increased in AAV patients. Although cells expressing exhaustion markers were also increased in AAV patients compared with HCs in our study, they were not significantly different than CKD controls, suggesting that such difference is shared across individuals with kidney impairment. We did not observe significant differences in cytokine production in phorbol myristate acetate plus ionomycin-stimulated T and B cells across the 3 study groups (data not shown). However, we did not focus on autoreactive cells, and therefore potential differences across groups may have been diluted in our analyses.

Interaction Between Immune Cell Percentages and ANCA Levels

We next aimed at assessing the pattern of interaction among percentages of B cells, plasma cells, and Tregs and disease activity. We used ANCA levels as the primary marker of disease activity and fitted linear
Figure 1. Cell subsets that differ significantly between anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) and control subjects. (a) Cell subsets showing a significant difference between AAV patients and both chronic kidney disease (CKD) patients and healthy control (HC) subjects. Significant pairwise differences (AAV vs. CKD and AAV vs. HC; $P < 0.025$) are seen after adjusting for multiple testing (Holm-Bonferroni procedure). (b) Variable importance among subsets in identifying AAV patients using random forest analysis. (Continued)
regression models of log(ANCA) on the selected cell subsets (Supplementary Table S2). These analyses revealed a significant 3-way interaction between B cells, plasma cells, and Tregs ($P = 0.008$). In other words, the type of relationship between each of these subsets with log(ANCA) depended on the value of the other 2 subsets. The results and the coefficients of the regression model are reported in Supplementary Table S2, whereas interpretation of this interaction is exemplified in Supplementary Figure S3. Plasma cells had a positive correlation with log(ANCA), provided that the B-cell levels were also high, whereas Treg levels were relatively low (Supplementary Figure S3). Similarly, B cells had a positive correlation with log(ANCA) provided that the plasma cells were also high and Tregs were low (not shown). Neither the cell subsets nor ANCA levels predicted remission after treatment. We did not find any significant relation between B cells, plasma cells, and Tregs and rate of end-stage kidney disease and patient death (data not shown).

**DISCUSSION**

In our comprehensive immunophenotypic study we have shown that rapidly progressive glomerulonephritis due to AAV is associated with a higher percentage of B cells, plasma cells, and Tregs and decreased naïve CD8$^+$ T cells compared with CKD patients and HCs. Importantly, we have analyzed a large cohort of AAV patients and included patients with severe renal involvement, which are usually not included in AAV studies. The inclusion of control groups with normal or impaired kidney function but no active immune processes is another strength of our investigation.

Our results confirm and extend previous findings that percentages of B cells and plasma cells are increased in patients with AAV and provide a rationale for using B- and plasma-cell–depleting agents such as anti-CD20 (rituximab and ofatumumab) in AAV treatment. $^7$ Consistently, data from a murine AAV model showed that bortezomib, a proteasome inhibitor that depletes short- and long-living plasma cells, is able to deplete myeloperoxidase-specific plasma cells and prevent anti-myeloperoxidase IgG-mediated necrotizing crescentic glomerulonephritis. $^6$

Of note, conventional therapy such as cyclophosphamide, not only targets B cells but also plasma cells. Despite a recent retrospective, single-center study demonstrating equal efficacy for rituximab and cyclophosphamide as induction therapy, $^3$ patients presenting with more severe AAV tended to receive cyclophosphamide to reach rapid suppression of disease activities. Our data on plasma cells may in part explain the importance of plasma-cell depletion in the induction phase.

Previous studies have shown increased levels of circulating Tregs in AAV patients, likely due to a compensatory response to the chronic inflammatory milieu. Intriguingly, our model supports the concept that large numbers of B cells and plasma cells are associated with ANCA levels only when the percentage of circulating Tregs is not increased enough to potentially control the autoimmune response. This may implicate the importance of cell interaction and supports the concept that poorly functional or relatively low Treg levels unleash autoreactive B cells and plasma cells to produce pathogenic autoantibodies. $^8$

In conclusion, our data indicate that increased percentages of circulating B cells and plasma cells represent the main immune phenotypic abnormality in AAV patients. Levels of both cell types correlate with ANCA levels, especially if Treg levels are not proportionally increased. Overall, the results provide a rationale for therapies aimed at inhibiting autoreactive B cells and plasma cells while increasing Treg number or function, a feature that may at least partially explain the success of anti-CD20 therapies. $^9$

**DISCLOSURE**

All the authors declared no competing interests.

**SUPPLEMENTARY MATERIAL**

Supplementary File (PDF)

Supplementary Methods.

Table S1. List of analyzed cell subsets.

Table S2. Multiple regression model on log(ANCA).

Table S3. Flow cytometry panels.

Table S4. Flow cytometry antibodies.

Figure S1. Gating strategy for lymphocytes and singlets.

Figure S2. Representative flow cytometry plots for significantly different subsets.

Figure S3. Predicted log(ANCA) as a function of circulating plasma cells.
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