Regulatory B Cells Are Reduced and Correlate With Disease Activity in Primary Membranous Nephropathy

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Introduction: Primary membranous nephropathy (PMN) is an autoimmune disease. Both T-regulatory cells (TREGs) and B-regulatory cells (BREGs) are decreased in patients with autoimmune disease. We evaluated the TREG and BREG population in patients of PMN treated with cyclical cyclophosphamide and steroid therapy (cCYC/GC).

Methods: Twenty-four patients with PMN resistant to a restrictive strategy and treated with cCYC/GC therapy and 10 healthy controls were enrolled. The proteinuria, serum creatinine, and serum albumin were tested at monthly intervals and blood samples were collected before starting cCYC/GC and at 6 and 8 (2 months wash out) months of therapy. The peripheral blood mononuclear cells (PBMCs) after staining with fluorochrome-conjugated antibodies were then subjected to flow cytometric analysis for detection of TREGs (CD3+CD4+CD25hiCD127loFoxP3+) and BREGs (CD19+CD5+CD1dhiIL10+). TREGs and BREGs are presented as the percentage of T and B cells, respectively. Cases with remission at month 18 were classified as responders, and those without any remission as nonresponders.

Results: Patients with PMN had a lower percentage of TREGs (P = 0.07) and BREGs compared with healthy controls (P = 0.0007). As compared with baseline, there was a significant increase in both BREGs (P = 0.001) and TREGs (P = 0.02) with the treatment (8 months). Patients who responded to therapy by 18 months had an increase in TREG (P = 0.05) and BREG (P = 0.0001) at month 8 compared with baseline.

Conclusion: As compared with healthy controls, patients with PMN displayed a lower percentage of BREGs. Both TREGs and BREGs significantly improved with disease-specific therapy. BREGs had an association with clinical activity.

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Primary membranous nephropathy (PMN) is an autoimmune disease, characterized by the production of autoantibodies that bind to podocyte antigens such as M-type phospholipase A2 receptor (PLA2R)1,2 or thrombospondin 7A.3,4 Over 70% of the patients with PMN have antibodies to either PLA2R or thrombospondin 7A.

Given the central role of antibodies, a role can be envisaged for cells involved in antibody production (i.e., lymphocytes); however, there is a paucity of data on lymphocyte subpopulation in patients with this condition. Regulatory T cells (TREGs) inhibit the activation of B-lymphocytes5–7 and subsequent production of autoantibodies by impeding T-helper cells. During active disease, patients with PMN have a lower number and percentage of CD4+CD25+Foxp3+ TREG compared with healthy controls.8 Patients who responded to rituximab therapy with an increase in TREG on day 8 achieved clinical remission.9 There are no data on TREG in patients treated with cyclophosphamide (CYC) and steroids (GC) (CYC/GC), the first-line regimen for this condition.

Regulatory B cells (BREGs) are formidable suppressors of the immune system in humans. The immunosuppressive effect of BREG is through induction of

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TREG\textsuperscript{3} and suppression of Th1/Th17 response.\textsuperscript{10} BREG can be identified using combinations of different surface markers, such as CD19, CD5, and CD1d.\textsuperscript{11,12} The trademark cytokine produced by BREG is interleukin-10 (IL-10), which interferes with T-cell co-stimulation and contributes to the suppression of the immune system. The strong immunosuppressive role of CD5\textsuperscript{+}CD1d\textsuperscript{hi} BREG has been reported in inflammatory and infectious conditions.\textsuperscript{13,14}

Most of the literature on BREGs in patients with autoimmune diseases is in vasculitis. Patients with active vasculitis had lower BREG than those in clinical remission,\textsuperscript{15} and a decline in BREG was associated with clinical relapse.\textsuperscript{15} There are no data on BREG in PMN. The present study was undertaken to evaluate the TREG and BREG population in PMN and its association with clinical activity in patients treated with CYC/GC.

**METHODS**

**Study Population**

This prospective observational study was carried out at the Postgraduate Institute of Medical Education and Research, Chandigarh. The study included 24 adult patients with biopsy-proven PMN who were treated with cCYC/GC according to standard clinical indications and 10 healthy controls (HCs) (voluntary kidney donors, before donation and matched for age and sex). We excluded patients with prior exposure to immunosuppressive therapy, chronic viral disease, diabetes, and those receiving non–cCYC/GC-based immunosuppression. The Institute Ethics Committee approved the study. This study was conducted as per the Declaration of Helsinki, October 2008. Written informed consent was obtained from all the eligible patients.

**Sample Collection, Treatment, and Monitoring**

We collected data on proteinuria, serum creatinine, liver function tests, lipid profile, and anti-PLA2R levels (Euroimmun AG, Lubeck, Germany) at baseline. Proteinuria, serum creatinine, and serum albumin were repeated every month for the 6 months and then quarterly until month 12. Venous blood samples were drawn at baseline and at the end of 6 and 8 months (i.e., 2 months after completion of the immunosuppressive therapy) for analysis of TREG and BREG populations. Samples were subjected to density gradient centrifugation for isolation of PBMCs, which were then cryopreserved and stored in liquid nitrogen until analysis.

**Immune Cell Profiling**

Immunophenotyping was performed on PBMCs using fluorochrome-labeled monoclonal antibodies. Briefly, for determination of TREG (CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{hi}CD127\textsuperscript{lo}FoxP3\textsuperscript{+}), PBMCs were stained for cell surface markers using BV711-labeled anti-CD3, APC-labeled anti-CD4, PE-Cy7 labeled anti-CD25, and BV421-labeled CD127. After surface staining, cells were fixed using BD Cytofix/CytopermTM (BD Biosciences, Gurugram, India) and then stained for intracellular FoxP3 using PE-labeled anti-FoxP3 antibody as per manufacturer’s protocol. The gating strategy for TREG is indicated in Figure 1. For BREG subsets, PBMCs were stained with fluorescein isothiocyanate–labeled anti-CD19, APC Cy7-labeled anti-CD5, and BV421-labeled anti-CD1d antibody, fixed using BD Cytofix/CytopermTM, stained for intracellular IL-10 using APC-labeled anti–IL-10 antibody as per manufacturer’s protocol. Samples were acquired on FACS ARIA II (BD Biosciences, San Jose, CA), unstained sample was used to set the negative peak, and analysis was performed on FlowJo software (Ashland, OR). BREGs were identified as CD19\textsuperscript{+}CD5\textsuperscript{+}CD1d\textsuperscript{hi}IL-10\textsuperscript{+}, and the gating strategy is indicated in Figure 2.

**Statistical Analysis**

Depending on the distribution, data are expressed as mean ± SD (range) and median (interquartile range). The TREG and BREG population was expressed as percentage of T and B cells, respectively. Patients were stratified as responders (either partial or complete remission) or nonresponders based on the proteinuria, serum albumin, and creatinine at 18 months using standard criteria.\textsuperscript{16} Groups were compared using 2-tailed Mann-Whitney or Wilcoxon matched pairs signed rank test as appropriate. The analysis was performed using GraphPad Prism Software (GraphPad, La Jolla, CA). Multivariate analysis was performed using SPSS software (version 24.0; IBM Corp., Armonk, NY). A P value <0.05 was considered significant.

**RESULTS**

The age of the study subjects was 39.29 ± 12.80 (range, 15–66) years, and the duration of illness before starting immunosuppressive treatment was 10.25 ± 1.75 (range, 8–15) months. PMN was PLA2R-related in 19 (79%) patients. Baseline data for all parameters are indicated in Table 1. The median anti-PLA2R antibody titer was 165 (IQR 110.0–249.9) RU/ml. The median proteinuria, serum albumin, and serum creatinine were 7.69 (5.05,10.00) g/d, 2.10 (1.96,2.57) g/dl, and 0.88 (0.71,1.17), respectively.
T-Regulatory Cells and B-Regulatory Cells
At baseline, the proportion of TREG as to the total T cells was lower in patients with PMN compared with HCs, but this difference was not statistically significant ($P = 0.07$). Patients with PMN had significantly lower proportion of BREG as to the total B cells at baseline in comparison with HCs ($P = 0.0007$). The details of TREG and BREG are mentioned in Table 2. There was no association of anti-PLA2R levels with TREG ($P = 0.13$) and BREG ($P = 0.80$).

**DISCUSSION**
We highlight the temporal trends in BREG and TREG populations in patients with PMN and their association with disease activity following treatment. Compared with HCs, the before treatment BREG proportion was lower in patients with PMN. Patients who responded to therapy showed a recovery in the BREG population.

The individual details of both the BREG and TREG with clinical activity is mentioned in Table 2. There was no difference in the proteinuria, duration of proteinuria, and serum anti-PLA2R levels between responders and nonresponders (Supplementary Table S1). On multivariate analysis, gender, age, proteinuria, serum creatinine, TREG, BREG, and PLA2R levels at baseline did not have any association with outcome, although the BREG showed a trend toward such an association ($P = 0.09$).

Patients were divided into 2 groups based on their responsiveness to the cCyc/GC therapy. Of 24 patients with PMN, 14 showed either partial ($n = 8$) or complete remission ($n = 6$), whereas 10 were nonresponders. Two of the 10 patients with resistant disease developed severe sepsis and died between 12 and 18 months. Patients who responded to therapy had an increase in TREG at the eighth month compared with baseline ($P = 0.05$). On the other hand, compared with baseline, there was an increase in BREG at the eighth month ($P < 0.001$) and sixth month ($P < 0.001$) in responders, whereas those with resistant disease did not show any significant increase in the BREG. The individual details of both the BREG and TREG with clinical activity is mentioned in Table 2. There was no difference in the proteinuria, duration of proteinuria, and serum anti-PLA2R levels between responders and nonresponders (Supplementary Table S1). On multivariate analysis, gender, age, proteinuria, serum creatinine, TREG, BREG, and PLA2R levels at baseline did not have any association with outcome, although the BREG showed a trend toward such an association ($P = 0.09$).

**T-Regulatory Cells and B-Regulatory Cells**
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This phenomenon was not observed in the treatment-resistant cases. Although there are no data on BREG in PMN, there have been studies in other autoimmune diseases, like antineutrophil cytoplasmic antibody–associated vasculitis (AAV) and systemic lupus erythematosus. BREGs negatively correlate with disease activity in AAV. Todd et al.17 evaluated BREGs in 46 patients with AAV. As in our study, patients with AAV exhibited a reduction in BREGs compared with HCs. The reduction was greater in patients with proteinase-3 AAV compared with myeloperoxidase-AAV. Patients with the relapsing disease had a trend toward lower BREG frequency compared with patients with stable remission. Wilde et al.15 evaluated BREGs in 30 patients with AAV, and also found the frequency to be lower as compared with HCs. They found a positive correlation between BREGs and TREGs in patients in stable remission.

It is evident from animal models that BREG deficiency is associated with autoimmune disease. Although there are no data on BREG in PMN, there have been studies in other autoimmune diseases, like antineutrophil cytoplasmic antibody–associated vasculitis (AAV) and systemic lupus erythematosus. BREGs negatively correlate with disease activity in AAV. Todd et al.17 evaluated BREGs in 46 patients with AAV. As in our study, patients with AAV exhibited a reduction in BREGs compared with HCs. The reduction was greater in patients with proteinase-3 AAV compared with myeloperoxidase-AAV. Patients with the relapsing disease had a trend toward lower BREG frequency compared with patients with stable remission. Wilde et al.15 evaluated BREGs in 30 patients with AAV, and also found the frequency to be lower as compared with HCs. They found a positive correlation between BREGs and TREGs in patients in stable remission.

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Table 1. Clinical parameters of patients with primary membranous nephropathy at baseline and follow-up

| Parameters                  | Value                      |
|-----------------------------|----------------------------|
| Age (yr)                    | 39 (32.75–48.00)           |
| Sex (F:M)                   | 07:17                      |
| Duration (mo)               | 10 (9–11)                  |
| Anti-PLA2R titers (n = 19)  |                            |
| Baseline                    | 165.7 (110.00–249.90)      |
| 6 mo                        | 7.75 (1.86–37.13)          |
| 8 mo                        | 4.80 (0.80–71.59)          |
| Proteinuria (g/d) (n = 24)  |                            |
| Baseline                    | 7.69 (5.05–10.00)          |
| 6 mo                        | 1.29 (0.71–3.10)           |
| 12 mo                       | 1.55 (0.26–4.31)           |
| 18 mo                       | 1.34 (0.25–4.02)           |
| Serum albumin (g/dl) (n = 22)|                          |
| Baseline                    | 2.10 (1.96–2.57)           |
| 6 mo                        | 3.86 (3.50–4.27)           |
| 12 mo                       | 4.04 (3.28–4.38)           |
| 18 mo                       | 4.40 (3.66–4.65)           |
| Serum creatinine (mg/dl) (n = 22)|            |
| Baseline                    | 0.88 (0.71–1.17)           |
| 6 mo                        | 0.80 (0.72–1.01)           |
| 12 mo                       | 0.87 (0.78–1.21)           |
| 18 mo                       | 0.90 (0.74–1.07)           |

Values are expressed as median (interquartile range).
and multiple sclerosis as well. Taken together, these findings are consistent with observation that BREGs restrain the immune system.

The present study is the first to evaluate the BREG and its association with clinical activity in PMN. BREGs inhibit immune responses directly by inducing secretion of immunoregulatory factors, including IL-35 and transforming growth factor β1. By inducing transforming growth factor β1 secretion, BREGs can cause CD4+ T-cell apoptosis and CD8 T-cell anergy. Some studies have shown that BREGs also suppress the immune system by inducing TREGs, which mediate anti-inflammatory effects and inhibit the proinflammatory Th1/Th17 response.

The TREG population was also lower in patients with PMN, but the difference did not reach statistical significance, possibly because of small patient numbers. TREGs suppress immune responses by modulation of antigen-presenting cell maturation and function, killing of target cells, and production of anti-inflammatory cytokines. Through these mechanisms, they suppress the activity of CD4+ T cells, CD8+ T cells, dendritic cells, and B cells. Foxp3, a transcription factor that strictly defines TREG, controls the expression of several genes involved in determining the suppressive phenotype. Autoimmune disease may develop because of an altered balance between TREG cells and self-reactive conventional T cells. Studies of TREG in PMN have shown varied results; with a few showing the reduced number and the others have found no quantitative differences. Wang et al. studied the role of B lymphocytes and T lymphocyte subsets, including TREG cells, in the pathogenesis of PMN and found that patients had a lower TREG and higher CD4+/CD8+ ratio compared with HCs. They, however, did not study BREGs nor did they evaluate the therapeutic response. Roccatello et al. showed that in 17 patients with PMN treated with rituximab, there was an increase in the TREG population and IL-35 at month 12, which positively correlated with clinical response. Rosenzwajg et al. evaluated 33 lymphocyte subpopulations and 27 cytokine types in 25 patients

### Table 2. Proportion of B- and T-regulatory cells in patients with primary membranous nephropathy and healthy controls

| Parameter         | TREG (%)           | BREG (%)           |
|-------------------|--------------------|--------------------|
|                   | Baseline 6 mo 8 mo | Baseline 6 mo 8 mo |
| Patients with PMN |                    |                    |
| Responders        | 4.01 (3.20–4.98)   | 4.01 (3.12–5.19)   |
| Nonresponders     | 3.72 (2.67–5.32)   | 3.72 (2.67–5.32)   |
| Healthy subjects  | 8.49 (1.55–10.80)  | 8.49 (1.55–10.80)  |

Values are provided as median (interquartile range). Wilcoxon test was used to test the differences between and among the groups.

Figure 3. T-regulatory (TREG) cell percentage at various time points in patients with primary membranous nephropathy (PMN) and healthy controls (HCs). *P < 0.05. Circle, median value in healthy control; square, median value at baseline; upward triangle, median value at 6 months; downward triangle, median value at 8 month. ns, not significant.
with PMN treated with rituximab. They observed that patients who responded had a significantly lower percentage of TREGs at baseline compared with non-responders, which increased post-treatment. In non-responders and those on supportive treatment alone, TREGs remained unchanged. They concluded that analysis of TREGs might help predict early response to rituximab. Fervenza et al. evaluated B- and T-cell subpopulations in patients with PMN treated with rituximab therapy and found that none of the TREG subset analyses revealed any significant quantitative difference with treatment. The only persistent change in lymphocyte subsets was a relative increase in natural killer cells that persisted up to 18 months. The baseline CD4+/CD8+ ratio failed to predict response to therapy. In the current study, patients with PMN had a significant increase in the TREG post-treatment (eighth month). Larger sample size would help in confirming the observation. CYC has been shown to suppress TREG and could explain the lower TREG at 6 months compared with baseline. The limitation (CYC effect), as mentioned previously, was addressed by a 2-month wash-out period to negate the effect of oral CYC. The result of the current study is like the report by Roccatello et al.

The findings from our study suggest that an increase in BREG percentage might be a marker for monitoring the efficacy of cCYC/GC therapy in PMN patients. The hypothesis may be tested in a prospective clinical trial. The main limitations of our study include small sample size, short duration of follow-up, and lack of functional assessment of TREG and BREG. However, we believe that the current study is a pilot and lays the foundation for future studies on immunological markers in PMN. To conclude, BREGs are significantly reduced in patients with PMN and have an association with clinical activity.

**DISCLOSURE**

VJ received grants from Baxter Healthcare, GSK, Biocon, Zydus Cadilla, and NephroPlus. All the other authors declared no competing interests.

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**SUPPLEMENTARY MATERIAL**

Supplementary File (PDF)

**Figure S1.** T-regulatory cell percentage at various time points in responders and nonresponders.

**Figure S2.** B-regulatory cell percentage at various time points in patients with primary membranous nephropathy (PMN) and healthy controls (HCs). *P < 0.05; **P < 0.01. Circle, median value of healthy control; square, median value at baseline; upward triangle, median value at 6 months; downward triangle, median value at 8 months. ns, not significant.

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