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Regulatory B and T Cells and Their Association With Clinical Response in New-Onset Lupus Nephritis Patients

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Niveditha Girimaji[1,5], Urvashi Kaundal[2,5], Manish Rathi[1], Aman Sharma[3], Ritambhra Nada[4], Raja Ramachandran[1], Aruna Rakha[2] and Krishan Lal Gupta[1]

[1]Department of Nephrology, Post Graduate Institute of Medical Education and Research, Chandigarh, India; [2]Department of Translational and Regenerative Medicine, Post Graduate Institute of Medical Education and Research, Chandigarh, India; [3]Department of Medicine, Post Graduate Institute of Medical Education and Research, Chandigarh, India; and [4]Department of Histopathology, Post Graduate Institute of Medical Education and Research, Chandigarh, India

Correspondence: Krishan Lal Gupta, Department of Nephrology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India. E-mail: drklgupta@gmail.com; or Aruna Rakha, Department of Translational and Regenerative Medicine Department, Post Graduate Institute of Medical Education and Research, Chandigarh, India. E-mail: aruna_pgi@yahoo.com

[5]These authors contributed equally to this work.

Systemic lupus erythematosus (SLE) is a multi-system disease characterized by a global loss of self-tolerance with activation of autoreactive T and B cells.[S1] Hyperactive B cells produce a variety of antibodies that form immune complexes leading to the effector phase of the disease, and T cells contribute to tissue injury through proinflammatory cytokines.[S1−S3] The imbalance between these autoreactive T-helper cells (Th1/Th2/Th17) and regulatory T and B cells (Tregs and Bregs, respectively) is among the many immune-mediated responses involved in SLE.[S4] Tregs suppress immune responses by modulation of antigen-presenting cell maturation and function, killing of target cells and production of anti-inflammatory cytokines.[S5] Bregs exert suppressive effects by secretion of anti-inflammatory cytokines such as interleukin (IL)−10 and engaging in cell-to-cell contact via activation of cell death markers or co-stimulatory molecules that can also influence T-helper cell plasticity.[1,S6]

Lupus nephritis (LN) is a serious potential feature of SLE. Studies have shown a quantitative and functional deficiency in Tregs in SLE and LN patients.[2,3,S7,S8]

Similarly, abnormalities in Bregs have been reported in SLE.[4−6,S9−S11] However, at present, there is no systematic study reporting the role Bregs in new-onset LN. Therefore, in our study, we aimed to monitor the baseline levels of Breg and Treg populations in new-onset LN patients and changes in their profile in response to immunosuppressive (IS) drugs. We also analyzed the association of regulatory cells with clinical response in LN patients. Unlike Tregs, which are uniformly identified as $CD3^+CD4^+CD25^{hi}FoxP3^+ CD127^{lo}$,[S5] Bregs have been reported to have varying phenotypes, the secretion of IL-10 being characteristic, regardless of phenotype. We studied $CD19^+CD5^+CD1d^{hi}IL-10^+$ Bregs, which have been reported to have potent regulatory function in both murine and human studies.[7,8,S13,S14]

RESULTS

Demographic and Clinical Parameters

During the study period, a total of 25 patients with new-onset LN were recruited. The mean age of the patients was 29.35 ± 9.783 years. There was a female preponderance, with a female:male ratio of

2.33:1. Female individuals were younger than male individuals (female mean age, 26.57 ± 9.8 years; male mean age, 35.83 ± 6.178 years). The median duration of the disease at presentation was 4.5 months (2−10.5 months). A total of 24 patients (96%) were antinuclear antibody−positive, 15 (60%) had elevated anti-double stranded DNA (dsDNA) levels, and 21 had (84%) low complement (C3 or C4). The median (interquartile range [IQR]) SLEDAI-2K scoring at baseline was 16 (12.25−19.00).

On renal biopsy, 6 patients (24%) had class III, 12 (48%) had class 1V, 6 (24%) had class V, and 1 patient (4%) had class IV+V. In all, 12 patients received National Institutes of Health regimen, 4 received Euro-Lupus Nephritis Trial regimen, 6 received steroids with mycophenolate mofetil (MMF), and 3 received multi-target therapy. Seven patients (28%) were non-responders and 18 were responders; of the latter, 15 (60%) had complete remission and 3 (12%) had partial remission. Five of 7 nonresponders had class IV on renal biopsy, 1 had class V, and 1 had class III, who, however, progressed to class IV on repeat biopsy. Of 7 nonresponders, 3 patients (15%) progressed to end-stage renal disease. Demographic and clinical characteristics are described in Table 1 and Supplementary Table S1.

Bregs Are Lower at Baseline in LN Patients and Increase With Immunosuppression

Bregs were expressed as percentage among CD19+ B cells. The gating strategy of Bregs and Tregs are depicted in Figures 1 and 2, respectively. We observed that LN patients had significantly lower Bregs at baseline in comparison to healthy controls (HCs) (median [IQR], 0.93 [0.28−1.66] vs. median [IQR], 2.38 [1.5−3.74], $P = 0.001$). Furthermore, Bregs were compared at baseline and 2 and 6 months after initiation of immunosuppression (median [IQR], 0.93 [0.28−1.66], median [IQR], 0.79 [0.13−2.0], median [IQR], 1.56 [0.33−5.45], respectively). There was a significant increase in Breg from 2 months to 6 months ($P = 0.008$; Figure 3) and from baseline to 6 months ($P = 0.005$; Figure 3). At the end of 6 months, Bregs in LN patients were similar to those in HC (median [IQR], 1.56 [0.33−5.45] vs. median [IQR], 0.93 [0.28−1.66], $P = 0.439$). We further analyzed LN patients as responders and nonresponders. We found that responders had an increase in Bregs from 2 to 6 months ($P = 0.017$; Figure 4) and from baseline to 6 months ($P = 0.02$; Figure 4), whereas there was no significant change in nonresponders from 2 to 6 months ($P = 0.3$; Figure 4) or from baseline to 6 months ($P = 0.47$, Figure 4). The median values of Bregs in LN and HC are presented in Table 2.

Table 1. Demographic and clinical characteristics of subjects (N = 25)

Characteristic	Value
Age, yr, mean ± SD	30.68 ± 19.9
Female: age, yr, mean ± SD	29.89 ± 9.95
Male: age, yr, mean ± SD	33.16 ± 10.28
Female sex, n (%)	19 (76)
Duration of disease, mo	4.5 (2−10.5)
Blood pressure, mean ± SD	128.6 ± 17.98/79.76 ± 10.93
Serum creatinine, mg/dl:	
Baseline	2.424 ± 0.80
2 mo	1.07 (0.81−1.7)
6 mo	0.96 (0.6−1.65)
eGFR, ml/min per 1.73 m², baseline	54.95 (32.2−106)
24-h Urinary protein (mg/d)	
Baseline	2923 (1831−5975)
2 mo	1491 (482.5−3902)
6 mo	450.0 (110.0−1540)
Serum albumin, g/dl	
Baseline	2.424 ± 0.80
2 mo	3.152 ± 0.63
6 mo	3.514 ± 0.75
ANA, n (%)	24 (96)
Positive anti-dsDNA, n (%)	15 (60)
Anti-dsDNA, IU/ml	85.7 (19.5−283.5)
Low complements, n (%)	21 (84)
C3, mg/dl	62.53 (42−100.5)
C4, mg/dl	9.500 (6.9−19)
SLEDAI-2K	
Baseline	16 (12.5−19.5)
6 mo	2 (0−10)

ANA, antinuclear antibody; C3, complement 3; C4, complement 4; dsDNA, double stranded DNA; eGFR, estimated glomerular filtration rate; LN, lupus nephritis; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index−2K.
Data are median (interquartile range) of each group of subjects unless otherwise indicated.

Tregs Are Normal in LN Patients and Do Not Change With Immunosuppression

Tregs were expressed as percentage among CD4+ T cells. There was no significant difference between baseline Tregs in LN patients and HCs (median [IQR], 2.71 [1.04−8.23] vs. median [IQR], 8.49 [1.55−10.8], $P = 0.137$; Figure 3). Furthermore, Tregs were compared at baseline and 2 and 6 months after initiation of immunosuppression (median [IQR], 2.71 [1.04−8.23], median [IQR], 1.7 [0.7−2.99], median [IQR], 2.56 [1.01−5.38], respectively). There was no change in Tregs from baseline to 6 months ($P = 0.737$; Figure 3). However, Tregs increased significantly at 6 months compared to 2 months ($P = 0.025$; Figure 3). In both responders and nonresponders, Tregs remained unchanged from baseline to 6 months ($P = 0.94$ and $P = 0.61$, respectively). The median values of Tregs in LN patients and HCs are presented in Table 2.

DISCUSSION

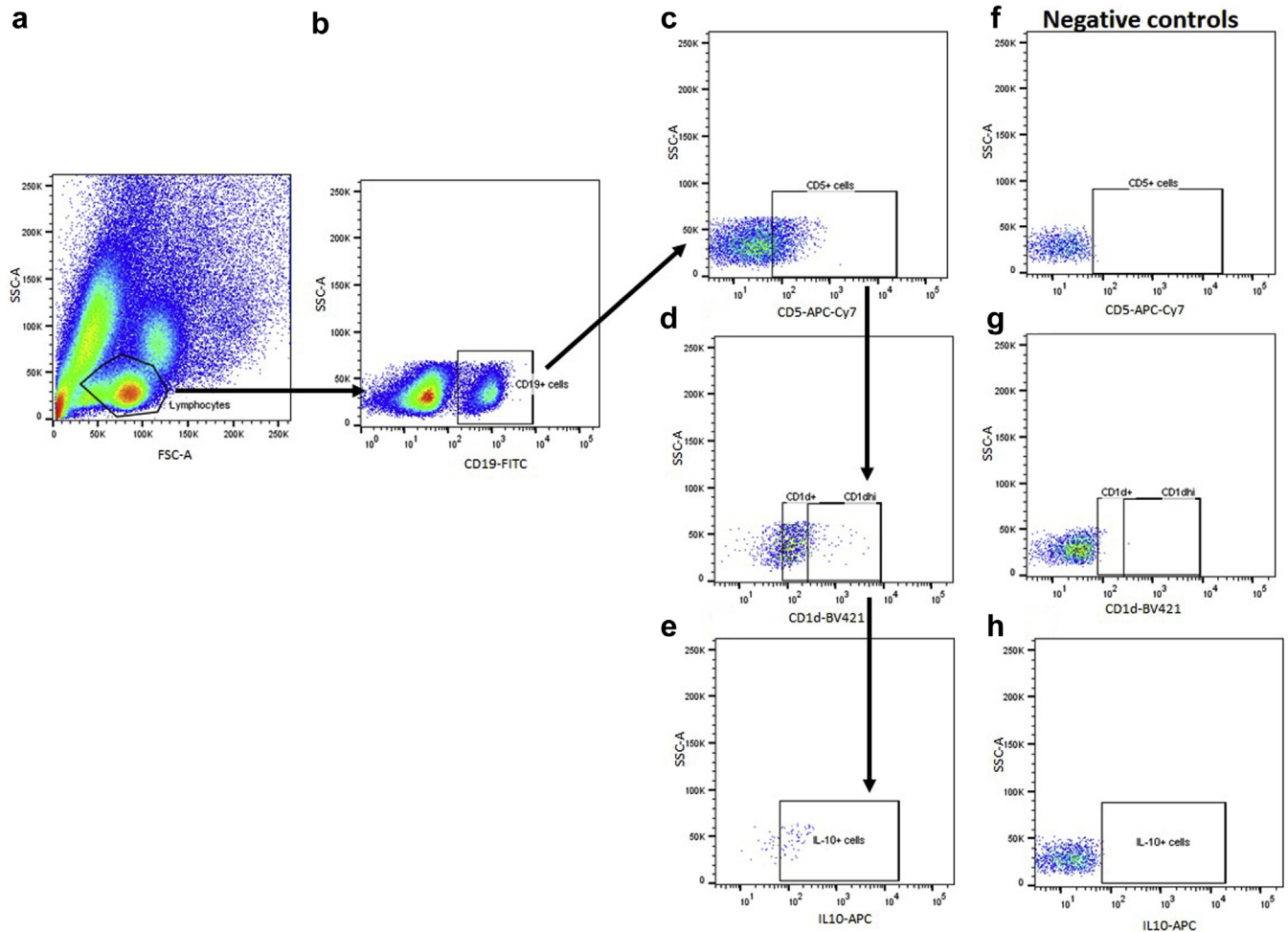In the prospective controlled study of 25 newly diagnosed LN patients, we found that Bregs were decreased

Figure 1. Gating strategy of regulatory B cells (Bregs). Gating has been applied to identify lymphocytes and to exclude debris. (a) Lymphocytes were located by their size and granularity. (b) B cells were then identified by CD19$^+$ expression. For Bregs, CD19$^+$ cells were further gated for (c) CD5$^+$, (d) CD1d^{hi}, and (e) IL-10$^+$ expression. (f−h) Negative gating strategy for identifying Bregs. FSC-A, forward scatter-area; IL, interleukin; SSC-A, side scatter-area.

in LN patients as compared to HCs. Moreover, we found an association of Breg with clinical activity (renal).

In the majority of autoimmune diseases, B cells are pathogenic, as they secrete autoantibodies.[S15] However, Bregs are vital in suppressing autoimmune responses and their impairment, both functional and quantitative, have been described in autoimmune diseases.[S16−S18] Bregs exert regulatory properties mainly by producing the immunosuppressive cytokine IL-10, which suppresses the differentiation of immune cells into effector/memory subsets.[S2,S3] Human Bregs are of varying phenotypes such as CD19$^+$CD24^{high}CD38^{high}, CD24^{high}CD27$^+$, CD19$^+$CD25^{high}CD86^{high}CD1d^{high}, CD19$^+$TIM1$^+$ B cells, and CD19$^+$CD5$^+$CD1d^{high} B cells, but their regulation depends on IL-10 regardless of phenotype.[9] Studies have shown that CD19$^+$CD5$^+$CD1d^{hi} B cells secrete IL-10 and negatively regulate immune responses and autoimmune diseases in mice and humans.[7,8,S13,S14] In our study, we investigated this lymphocyte subgroup as Bregs.

We found that the baseline Breg population in LN were significantly reduced. Similarly, a study by Wang *et al.* showed decreased numbers of Bregs and serum IL-10 in patients with new-onset SLE as compared to HC.[5] Heinmann *et al.* analyzed both CD19$^+$CD24^{hi}CD38^{hi}Breg population and IL-10$^+$ Bregs in stimulated peripheral blood mononuclear cells (PBMCs) of 34 SLE patients and 21 healthy controls. The percentage of Bregs was not different in the 2 groups, but percentages of IL-10$^+$ Bregs were significantly decreased in SLE patients, in particular those with LN.[6] Few studies have reported a functional defect in CD19$^+$CD24^{hi}CD38^{hi}Breg population in SLE.[4,S9−S11] We studied Bregs after initiation of immunosuppression and found a significant increase from 2 to 6 months and from baseline to 6 months. After IS therapy, LN patients had Bregs similar to those of HCs. This implies that Breg deficiency could contribute to the onset of LN, and that IS therapy contributes to improving the Breg population. This expansion occurred as a result of all forms of
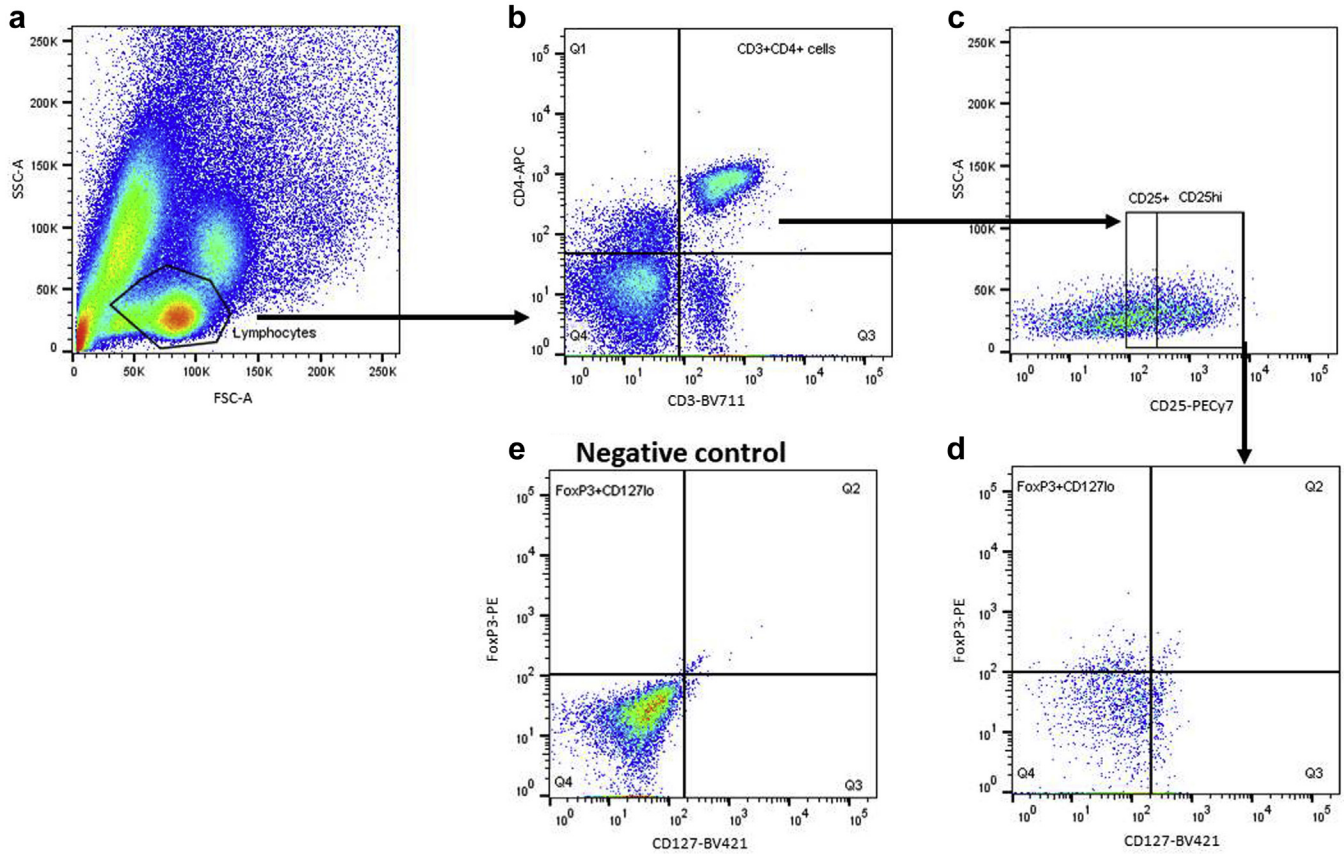
Figure 2. Gating strategy of regulatory T cells (Tregs). Gating has been applied to identify lymphocytes and to exclude debris. (a) Lymphocytes were located by their size and granularity. (b) T cells were then identified by $CD3^+CD4^+$ expression. For Tregs, $CD3^+CD4^+$ cells were further gated for (c) $CD25^{hi}$ and (d) $FoxP3^+CD127^{lo}$ expression. (e) Negative gating strategy for identifying Tregs. FSC-A, forward scatter-area; SSC-A, side scatter-area.

immunosuppression and could represent a pan–treatment effect not specific to the particular immuno-suppression used. A larger sample size would be required to effectively study the impact of specific IS regimens on Bregs. These drugs might affect activation and proliferation of B cells or, alternatively, might affect
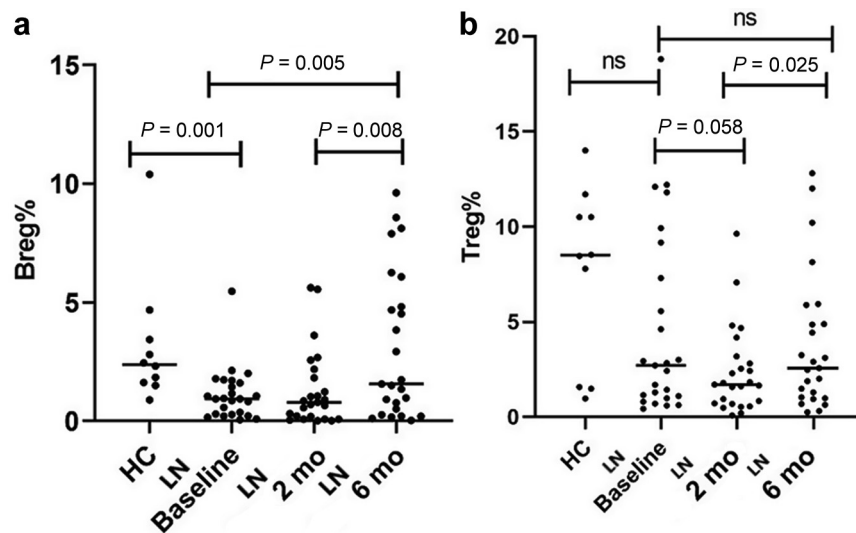

Figure 3. Percentage of regulatory B cells (Bregs) and regulatory T cells (Tregs) in healthy controls (HCs) (baseline), lupus nephritis (LN) patients (baseline, 2 months, and 6 months). (a) Data represent changes in Bregs as percentages among CD19+ B cells and (b) Tregs as percentages among CD4+ T cells for HCs and LN patients at baseline, 2 months, and 6 months. Data are represented as individual results (dots) and median (interquartile range). Comparison between HCs and LN patients was performed using Mann−Whitney test and between patient groups at different points of time using paired Wilcoxon signed-rank test.
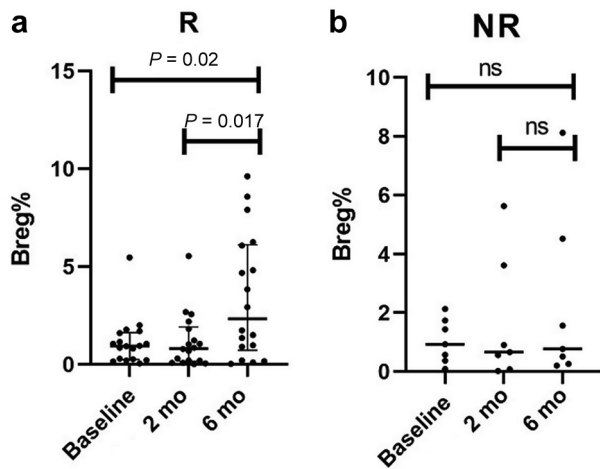
Figure 4. Percentage of regulatory B cells (Bregs) in responder (R) and nonresponder (NR) lupus nephritis (LN) patients (baseline, 2 months, and 6 months). Data represent changes in Bregs as percentages among CD19+ B cells for R (a) and NR (b) LN patients at baseline, 2 months, and 6 months. Data are represented as individual results (dots) and median (interquartile range). Comparison between patient groups at different points of time was performed using paired Wilcoxon signed-rank test.

the bone marrow and thereby the circulating B cells.[S19–S21]

We further analyzed Bregs in responders and nonresponders. Breg populations increased after immunosuppression in responders. In contrast, in nonresponders, no significant change was observed. These data suggest that clinical response to immunosuppression parallels improvement in Breg population. This observed impairment in Bregs in LN and their increase with immunosuppression could be a cause or consequence of the disease activity. In either case, this depletion could contribute to the extent of tissue damage. Wang et al. evaluated the effect of immunosuppression on Breg in SLE and showed an increase in $CD19^+CD5^+CD1d^{hi}IL-10^+$ regulatory B cells with treatment.[5] Heinmann et al. showed a negative correlation between daily steroid dose and proportion of Bregs in LN patients.[6] However, studies of Bregs specific to LN and studies that have

systematically examined the effect of immunosuppression over a follow-up period of 6 months are lacking. To our knowledge, our study would be 1 of the first such studies.

Bregs are also known to induce Tregs, thus playing a significant role in T-cell plasticity and promoting Treg expansion.[S22] Foxp3, a transcription factor that closely defines Tregs, controls the expression of genes involved in determining the suppressive phenotype.[S5] Autoimmune diseases may develop as a consequence of altered balance between Tregs and self-reactive conventional T cells.

Studies that have evaluated Tregs in SLE and LN have shown a quantitative and functional deficiency. Tregs have also been shown to correlate with LN disease activity, response, and treatment given.[2,3,S7,S8] However, we observed no significant difference in Treg populations in LN patients and HCs. Tregs did not differ between responders and nonresponders. Yates et al. in 2008 also demonstrated no differences in Treg numbers and function between LN/active LN patients and controls.[S23] Our study is in contrast with studies that have shown a lower percentage of Tregs in LN patients, particularly in patients with active LN[S24–S26] and newly diagnosed SLE. However, apart from a defect in Treg frequency, studies have shown anomalies in Treg-mediated immunosuppression and their target effector T cells, making them less susceptible to suppression by Treg.[S7,S8] Therefore, functional analysis of Tregs and their target cells would be necessary to elucidate the immunological dysfunction caused by Tregs.

Studies in SLE have shown that IS treatment with steroids, cyclophosphamide, antimetabolites, belimumab, and stem cell transplantation led to improvement in Treg numbers, suggesting immunological remission.[S27–S31] We observed that with immunosuppression, although Tregs did not increase overall at 6 months, after an initial decrease, they did increase dramatically from 2 to 6 months. We hypothesize that the initial fall could be secondary to the effect of

Table 2. Percentage of regulatory B and T cells in LN patients and HC

Parameter analyzed	Bregs (%)			Tregs (%)		
Time point	Baseline	2 mo (M-2)	6 mo (M-6)	Baseline	2 mo (M-2)	6 mo (M-6)
Total	0.93 (0.28–1.66)[a]	0.79 (0.13–2.0)	1.56 (0.33–5.45)[b,c]	2.71 (1.04–8.23)[a]	1.7 (0.7–0.99)	2.56 (1.01–5.38)[c]
Responders	0.94 (0.26–1.63)	0.82 (0.28–1.91)	2.33 (0.16–6.12)[b,c]	2.20 (1.07–5.99)	1.67 (0.67–3.43)	2.52 (1.02–4.86)
Nonresponders	0.93 (0.37–1.74)	0.66 (0.08–3.61)	0.77 (0.26–4.52)	4.61 (0.70–12.20)	1.79 (0.69–2.43)	2.90 (0.94–8.14)
HCs	2.38 (1.5–3.74)			8.49 (1.55–10.8)		

Breg, B regulatory cells; HC, healthy controls; LN, lupus nephritis; Treg, T regulatory cells.
[a]$P < 0.05$ vs. HCs.
[b]$P < 0.05$ vs. baseline.
[c]$P < 0.05$ vs. 2 mo (M-2).
Data are median (interquartile range) of each group of subjects. Bregs are expressed as percentages among CD19+ B cells, and Tregs as percentages among CD4+ T cells for HCs and LN patients at baseline, 2 mo, and 6 mo. Comparison between HC and LN was performed using Mann−Whitney test and between patient groups at different points of time using paired Wilcoxon signed-rank test.

cyclophosphamide, which is known to suppress Tregs, and that the secondary increase could represent Treg repopulation.[S32] Analysis at further time points would reveal additional information.

This, to our knowledge, is the first study to comprehensively analyze Bregs and Tregs in patients with new-onset LN and to systematically follow them after treatment. We recognize that our study has certain limitations, including the small sample size and the lack of functional studies of different subsets of B and T cells. Thus, further validation of these findings in a larger population is warranted.

In conclusion, we found that Bregs were deficient in new-onset LN patients and increased in responders with immunosuppression. The findings from this study provide new insights into the mechanisms underlying the pathogenesis of LN and the mechanism by which IS therapy achieves immunological remission. A better understanding of these processes can aid in the design of new immunotherapies for the intervention of LN.

DISCLOSURE

All the authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)
Supplementary Methods.
Supplementary References.
Table S1. Individual clinical characteristics of subjects.
Table S2. List of antibodies used.

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Clinical and Healthcare Utilization Outcomes of Parathyroidectomy in CKD and Dialysis Patients

Scott A. Jones[1], Katharine Hegerty[1], Carla Scuderi[1,2], Jessica Eglington[1], Ben Green[1,2] and Andrew Mallett[1,2]

[1]Department of Renal Medicine, Royal Brisbane and Women's Hospital, Brisbane, Queensland, Australia; and [2]School of Medicine, The University of Queensland, Brisbane, Queensland, Australia

Correspondence: Scott Jones, Department of Renal Medicine, Level 9 Ned Hanlon Building, Royal Brisbane and Women's Hospital, Butterfield Street, Herston, QLD, 4029. E-mail: scottjones10@hotmail.com