

Circulating regulatory B-cell subsets in type 2 diabetic nephropathy

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Abstract

Diabetic nephropathy (DN) is a major microvascular complication of diabetes mellitus (DM) driven by immune activation and chronic inflammatory cascades. Human regulatory B (Breg) cells modulate immune homeostasis and are downregulated in glomerulonephritis. We aim to evaluate B cell subsets counts in type 2 DM patients with and without nephropathy in comparison to normal control individuals. Sixty participants comprising twenty-four patients with type 2 DM without kidney injury, twenty-four patients with DN diagnosed via KDIGO guidelines, and twelve matched healthy controls underwent clinical, biochemical, and flow cytometric evaluations. CD19⁺ B cell subsets count, including Breg cells (CD19⁺CD24^{hi}CD38^{hi}) and memory B cell counts (CD19⁺CD24⁺CD38⁻) were quantified using multi-parametric flow cytometry. B cell count was significantly lower in patients with DN compared to DM and healthy control groups. Moreover, Breg and memory B cell counts were significantly lower in the DN group compared to the DM group. However, no significant difference was observed regarding B cell subsets between the DM and healthy control groups. Breg cells could predict DN (AUC = 0.720, $p = 0.017$) at a cut-off of $\leq 13.5\%$ with 95% sensitivity and 95% specificity. In conclusion, Breg cells serve a vital role in suppressing aberrant immune reactivity, meaning that their insufficiency can trigger heightened autoimmune processes. Ultimately, the Breg subpopulation holds substantial promise as a significant biomarker for predicting both the onset and progression of DN with no correlation with the severity of DN.

Keywords: Type- 2 DM; DN; Immune response; Breg cells; B cell subsets; Memory B cells; Flowcytometry; ACR.

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Introduction

Diabetic nephropathy (DN) represents the most prevalent microvascular complication associated with diabetes mellitus (DM) and has emerged as the primary etiology of chronic kidney disease (CKD) in developed nations.^{1,2} This complex clinical syndrome is defined by persistent albuminuria alongside a

progressive deterioration of renal function, reflecting a characteristic pattern of structural glomerular pathology that damages the filtration barrier.³ Chronic immune system activation and sustained inflammatory processes are fundamentally implicated in the progression of DM and its subsequent transition into DN, driving tissue injury over time.⁴ While the precise molecular and pathophysiological

mechanisms driving DN progression remain incompletely understood.^{5,6} The development of this renal complication in type 2 diabetes generally mirrors the cellular trajectory observed in type 1 DM.⁷

The immune system is essential for preserving systemic homeostasis and neutralizing pathogenic threats. Within this intricate network, B cells have been recognized as vital players in immunological modulation, functioning well outside their traditional involvement in humoral immunity to encompass diverse immunomodulatory capabilities.⁸ In healthy populations, acute inflammatory responses are self-limiting, with resolution governed by the timely secretion of anti-inflammatory mediators and cytokines, including IL-10, derived from specialized regulatory cells. Conversely, chronic inflammatory states feature persistent, unmitigated immune activation, often characterized by a quantitative and functional deficiency of these suppressor cells within both the systemic circulation and localized inflammatory sites.⁹ Over the past decade, a distinct population of suppressor B lymphocytes, designated as Breg cells, has been identified as a critical physiological mechanism for restraining excessive inflammation and preventing tissue damage.¹⁰

B regulatory (Breg) cells serve as vital modulators of the immune response that actively promote and maintain immunological tolerance. Consequently, clinical strategies aimed at isolating, expanding, infusing, or depleting these cells offer therapeutic possibilities across various immune-mediated disorders; while autoimmune pathologies and organ transplantation benefit from Breg cells expansion, oncological conditions and chronic infections may require targeted Breg cells depletion. These regulatory phenotypes are primarily characterized by their capacity to synthesize and secrete IL-10.¹¹ Breg cells exert their suppressive effects through the secretion of inhibitory cytokines, including IL-10, IL-35, and TGF-beta, or via contact-dependent molecular pathways such as PD1/PDL1 and Fas/FasL. Through these complex pathways, Breg cells facilitate Treg cell function, impair the

inflammatory activity of CD4⁺ T cells, CD8⁺ T cells, and dendritic cells, and suppress Th1/Th17 differentiation and IgG production.

Phenotypic surface markers for Breg cells vary significantly between species. In humans, this regulatory population is typically identified within the CD19⁺CD24^{hi}CD38^{hi} fraction, and its quantitative or functional loss is linked to exacerbated autoimmune activity. Key mechanisms of action include the release of immunomodulatory cytokines, the induction of tolerogenic antigen presentation, and the direct suppression of effector immune cells¹²⁻¹⁵. Prior research demonstrates that within the peripheral blood of healthy individuals, CD40 stimulation drives the generation of IL-10-producing B cells, with the highest concentration residing in the CD19⁺CD24^{hi}CD38^{hi} subset.¹⁶⁻¹⁸

These CD19⁺CD24^{hi}CD38^{hi} Breg cells effectively suppress inflammatory cascades, thereby modulating the expression of multiple molecules, including CD5, IgM, CD138, and IL-10 itself, which remains a principal inhibitor of T cell-mediated inflammation and a cornerstone of immunological tolerance.¹⁹ Following *in vitro* CD40 engagement, healthy peripheral blood CD19⁺CD24^{hi}CD38^{hi} Breg cells exhibit the highest capacity for IL-10 production among B-cell fractions and uniquely suppress Th1 differentiation. Beyond inhibiting Th1 and Th17 responses, these regulatory cells can induce the conversion of naive CD4⁺ T cells into Treg and Tr1 cells, enhancing the anti-inflammatory network. While a downregulation of Breg cells has been reported in primary glomerulonephritis,²⁰ data regarding the specific alterations, proportions, and clinical relevance of distinct Breg populations in patients with DM remain limited.

The objective of this investigation was to quantify and evaluate distinct subsets of B cells in individuals diagnosed with type 2 DM, both in the presence and absence of secondary DN. As well as the assessment of the potential link between the quantitative or functional alterations of the Breg and memory cell populations and the clinical severity of renal impairment.

Materials and Methods

Study Design and Participants

The procedures were conducted on patients admitted to the Nephrology Department, Theodor Bilharz Research Institute. Sixty individuals were enrolled in this investigation. Forty-eight patients suffer from type 2 DM with and without DN, in addition to twelve healthy individuals serving as the control group.

Patients with type1 DM, chronic inflammatory or autoimmune diseases, other causes of proteinuria (uncontrolled hypertension, fever, heart failure), CKD from causes other than DM, acute kidney injury within the 3 months before screening, rapid declines in eGFR (>5 mL/min/year) or sudden onset of albuminuria, active urinary sediment. Family history of non-diabetic forms of kidney disease and medication history (particularly immunosuppressive agents, corticosteroids, or immunomodulatory drugs) were excluded from the study.

A total of 60 participants were enrolled and categorized into three groups: 24 patients with type 2 diabetes mellitus (T2DM) without diabetic kidney injury (Group A), 24 patients with diabetic nephropathy (DN) diagnosed according to KDIGO guidelines²¹ (Group B), and 12 age- and sex-matched healthy controls with normal oral glucose tolerance test parameters and no evidence of diabetes (Group C). Diabetic nephropathy was defined by an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m² and/or a persistent urinary albumin-to-creatinine ratio >30 mg/g for more than 3 months, in the absence of other primary renal diseases, with eGFR calculated using the revised formula²¹.

All participants signed an informed consent form before any procedure. The following data was obtained: full medical history followed by thorough clinical examination for any diabetic complications, fundus examination.

Blood Sampling

A fasting venous blood sample (5 mL) was collected for all subjects. Two Vacutainer tubes were withdrawn for each patient. One lavender-

capped EDTA tube for CBC, HbA1C, and flowcytometric analysis, and one yellow-capped serum separator tube for blood chemistry. A urine sample was collected for ACR.

Routine Investigations

CBC was performed on a DXH 500 Beckman Coulter automated hematology analyzer, Beckman Coulter, USA. Blood chemistry, including FBS, PPBS, HbA1C, Lipid profile, Kidney functions, eGFR, and 24-hour urinary protein were performed on an AU 480 Beckman Coulter automated chemistry analyzer, Beckman Coulter, USA. Finally, Urine analysis, abdominal ultrasound, and ECG were performed by a technician blinded to the research design.

Flow Cytometric Analysis of B Cell Subsets

Surface expressions of CD19, CD24, and CD38 on peripheral blood lymphocytes were assessed by multiparameter flow cytometry. Briefly, 100 μ L of whole blood was incubated with 20 μ L each of PC5-anti-CD19 (Ref A66328), FITC-anti-CD38 (Ref IM0775U), PE-anti-CD24 (Ref IM1428U), IOTest[®] Immunotech, Beckman Coulter, France, for 20 minutes at room temperature in the dark. An isotype-matched control tube containing IgG1-PC5 (Ref. A07798), IgG1-FITC (Ref A07795), and IgG1-PE (Ref A07796) IOTest[®], Immunotech, Beckman Coulter, France was processed in parallel for each sample. Red blood cell lysis was performed using 0.5 mL OptiLyse C lysis solution (Ref A11895), Immunotech, Beckman Coulter, France, for 10 minutes at room temperature, followed by washing with PBS and centrifugation at 1000 rpm for 5 minutes. The cell pellet was resuspended in 1 mL PBS containing 0.1% formaldehyde (prepared by ten-fold dilution of IOTest[®] 3 Fixative Solution (Ref A07800), Immunotech, Beckman Coulter, France and stored until acquisition.

Flow cytometric acquisition was performed on a BD[®] LSR Fortessa, BD Biosciences[™], USA flow cytometer. The argon laser was allowed to warm up for 30 minutes, and instrument alignment was verified using Flow-Set[™] Fluorospheres (Immunotech, Beckman Coulter). A minimum of 20,000 gated lymphocyte events were acquired per sample, and data were

analyzed using BD FACSDiva™ software. The broad gating strategy was applied as follows: lymphocytes were first identified based on forward scatter (FS) and side scatter (SS) characteristics, then CD19⁺ B lymphocytes were selected. Within the CD19⁺ gate, cells co-expressing CD24 and CD38 (CD19⁺CD24^{hi}CD38^{hi}) were identified as regulatory B (Breg) cells, while (CD19⁺CD24⁺CD38⁻) cells were classified as memory B cells. A broad gating strategy was deployed to quantify Breg cells instead of the specific gating of high intensity expression to enhance reproducibility.

Statistical Methods

All statistical analyses were performed using R software (version 4.1.1). Continuous variables were expressed as mean $\pm$ standard deviation or median (interquartile range), according to their distribution, while categorical variables were presented as frequencies and percentages. Group comparisons were performed using one-way ANOVA or Kruskal–Wallis tests with appropriate post-hoc analyses, and categorical variables were compared using the chi-square test. The Mann–Whitney U test was used for comparisons between the DM and DN groups, and the Jonckheere–Terpstra test assessed trends across the study groups. Associations between B-cell subsets and clinical parameters were evaluated using Spearman's and HbA1c-adjusted partial correlations. Receiver operating characteristic (ROC) curve analysis and univariable logistic regression were performed to assess the diagnostic performance and predictive value of B-cell subsets. For the

primary comparison (diabetes vs diabetic nephropathy), a sensitivity analysis was additionally performed using multivariable logistic regression adjusting for duration of diabetes, the only baseline covariate that differed significantly between these groups; renal-function parameters were not entered as covariates because they define the nephropathy group. A two-sided P value <0.05 was considered statistically significant.

Results

Baseline Clinical and Laboratory Characteristics

The demographic and clinical characteristics of the study population are summarized in Table 1. The three study groups were well matched in age and gender distribution. As expected, diabetes duration is significantly longer in the DN group compared to the DM group (median age 8.0 years vs. 6.0 years, $p = 0.021$). The glycated hemoglobin (HbA1c) levels in the DM and DN cohorts were significantly higher than in healthy controls, with the DN group significantly higher than DM patients ($p < 0.001$). Kidney function tests showed severe impairment in DN patients. The average albumin-creatinine ratio (ACR), serum urea, and creatinine levels were significantly higher in the DN group, but the estimated glomerular filtration rate (eGFR) and hemoglobin concentrations were significantly lower compared to healthy controls and DM patients ($p < 0.001$). In the DN group, the total number of white blood cells and absolute lymphocytes tends to be lower, but did not reach statistical significance.

Table 1. Demographic, clinical, and laboratory characteristics of the study groups.

Characteristic	Control (n=12)	DM (n=24)	DN (n=24)	p -value
Male sex, n (%)	5 (42%)	13 (54%)	11 (46%)	NS
Age, years	46.4 $\pm$ 4.7	44.0 $\pm$ 5.5	47.3 $\pm$ 4.9	NS
Duration of diabetes, years	0 (by definition)	6.0 (4.0–8.0)	8.0 (7.0–8.5) ^b	0.021
HbA1c, %	4.86 $\pm$ 0.26	6.55 $\pm$ 0.39 ^a	6.90 $\pm$ 0.39 ^{ab}	<0.001
ACR, mg/g	10 (9–14)	24 (17–27)	1755 (1100–1950) ^{ab}	<0.001
Urea, mg/dL	29 (25–32)	34 (33–35)	170 (147–180) ^{ab}	<0.001

Table 1. Continued.

Characteristic	Control (n=12)	DM (n=24)	DN (n=24)	<i>p</i> -value
Creatinine, mg/dL	0.95 (0.70–1.00)	0.95 (0.80–1.00)	3.75 (3.15–4.50) ^{ab}	<0.001
eGFR, mL/min/1.73m ²	97.6 (87.1–106.3)	91.5 (86.9–97.3)	17.0 (13.1–19.1) ^{ab}	<0.001
Hemoglobin, g/dL	13.0 (11.3–13.6)	12.8 (11.0–14.0)	11.0 (10.0–12.0) ^{ab}	0.001
WBC, cells/ μ L	9478 (8306–9875)	9265 (8538–9775)	8950 (5175–9912)	NS
Absolute lymphocyte count, cells/ μ L	2204 (1425–2822)	1984 (1426–2565)	1518 (558–2056)	NS

Data are median [IQR] except age and HbA1c (mean $\pm$ SD). *p* value from one-way ANOVA (age, HbA1c) or Kruskal–Wallis (others); sex by chi-square; duration of diabetes by Mann–Whitney (DM vs DN). ^a*p* < 0.05 vs Control; ^b*p* < 0.05 vs DM (post-hoc). ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate (CKD-EPI 2021); DN, diabetic nephropathy. *p* > 0.05 is not significant (NS).

Flow Cytometric Analysis of B Cell Subsets

A quantitative investigation demonstrated a significant reduction in total B cells, Breg cells CD19+CD24hiCD38hi, and memory B cells CD19+CD24+CD38- in patients with DN

compared with diabetic patients without nephropathy and healthy controls. On the contrary, there were no significant differences between the control and the diabetic group. (Fig.1)

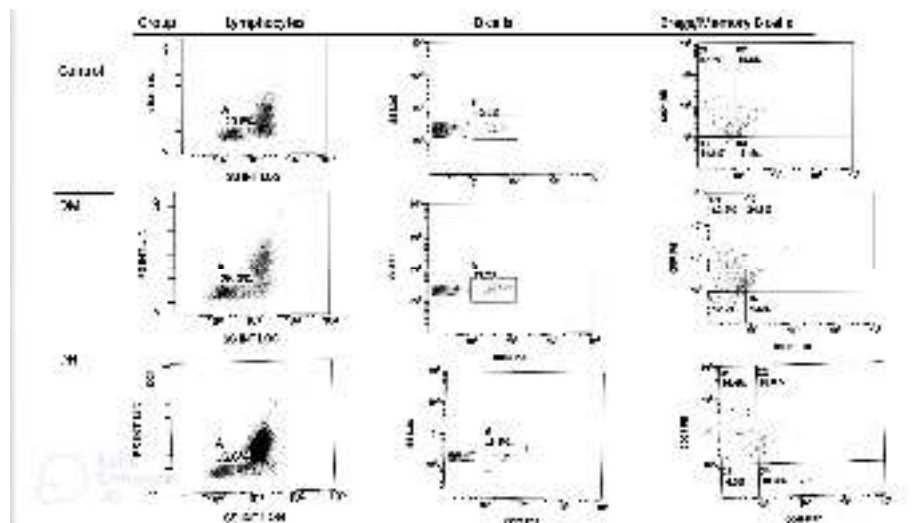


Figure 1. Flow cytometry gating technique for detection of peripheral B-cell subsets. Peripheral blood cells were first gated based on forward scatter (FSC) and side scatter (SSC) properties to exclude debris and non-lymphocyte populations. Next, CD19+ B cells were detected in the lymphocyte gate. The CD19+ population was then divided into B cell subsets according to CD24 and CD38 expression. Regulatory B (Breg) cells were identified as CD19+CD24hiCD38hi cells; memory B cells as CD19+CD24+CD38- cells. Dot plots are shown that depict gating from research participants, highlighting the distribution of various subsets. The B-cell subsets were quantified and compared in healthy controls, diabetic patients, and patients with diabetic nephropathy. All samples were gated using the same approach.

Peripheral B-cell Subsets are Markedly Depleted in DN

The distribution of peripheral B cell subsets in the study groups was evaluated in Table 2, Fig.2. The absolute count of B cells in the DN group was significantly reduced compared to the DM

group and the healthy control group (*p* < 0.002). Similarly, Breg cell CD19+CD24hiCD38hi and memory B cell CD19+CD24+CD38- counts were profoundly diminished in DN patients compared to DM patients (*p* < 0.005, *p* < 0.009), respectively. In this study, the B cell subsets

counts were evaluated using a broad gating protocol based on the expressions of CD19, CD24, and CD38. Although several studies used rigorous, high-intensity signals to identify a narrow fraction of Breg cells (CD19⁺CD24^{hi}CD38^{hi}), the broad gating strategy (CD19⁺CD24⁺CD38⁺) was proven to be highly sensitive to the metabolic changes in DN patients, indicating a selective collapse of the embedded Breg subset. This was further supported by the simultaneous depletion of the

memory B cell subset (CD19⁺CD24⁺CD38⁺) of the DN cohort. The fact that the distribution of B cell subsets remained indistinguishable between healthy controls and DM patients confirmed that broad gating provided a very stable, reproducible baseline and underscores that the loss of Breg and memory B cell fractions is a pathological characteristic that is unique to the onset of DN rather than a generalized DM effect.

Table 2. Peripheral B-cell subsets across the study groups.

B-cell subset	Control	DM	DN	<i>p</i>	ε ²	Trend <i>p</i>
B cells, cells/μL	116 (75–182)	132 (80–193)	60 (21–80) ^{ab}	0.002	0.177	0.003
Breg cells/μL	15.0 (9.0–26.2)	23.0 (8.0–62.2)	5.5 (1.0–14.0) ^b	0.005	0.151	0.009
Memory B cells/μL	13.0 (8.0–17.5)	17.0 (6.2–40.5)	4.5 (1.0–10.0) ^b	0.009	0.131	0.012

Data are median [IQR] (cells/μL). *P* from Kruskal–Wallis; ε², epsilon-squared effect size; Trend *P* from Jonckheere–Terpstra (control → DM → DN). ^a *p* < 0.05 vs Control; ^b *p* < 0.05 vs DM (Dunn, Bonferroni). *p* ≤ 0.05 is significant.

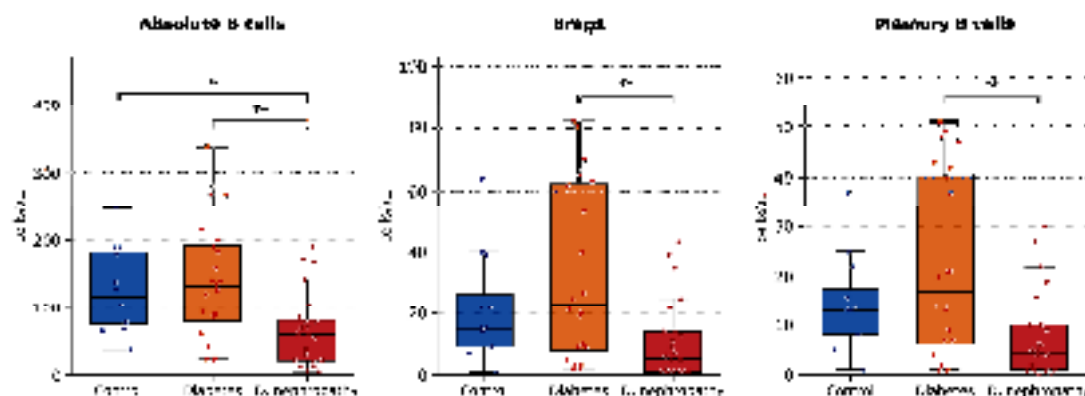


Figure 2. Peripheral B-cell subsets across the study groups. Box plots show the median, IQR, and range with individual points (*n* = 12 control, 24 DM, 24 DN). All three subsets are significantly reduced in diabetic nephropathy versus diabetes; absolute B cells are also reduced versus control. **p* < 0.05, ***p* < 0.01 (Kruskal–Wallis with Dunn–Bonferroni post-hoc).

The reduction in B cells indicates the presence of DN, but not its severity

To determine whether the B-cell count is consistent with the severity of renal disease, Spearman correlation analysis was performed within the group in Table 3 and Fig. 3. In the DM and DN groups, no significant correlations

between B cell subsets and renal parameters (eGFR, creatinine, ACR, urea) were observed. The total absence of significant correlations in disease cohorts shows that the lack of B cells is not a continuous marker of renal functional decline, but a feature of the DN disease state itself.

Table 3. Within-group Spearman correlations between B-cell subsets and renal parameters.

Parameter	Control (n=12)†	DM (n=24)	DN (n=24)
<i>B cells</i>			
eGFR	+0.57	+0.11	-0.03
Creatinine	-0.23	-0.20	+0.12
ACR	+0.03	+0.36	-0.01
Urea	+0.31	-0.06	-0.23
<i>Breg cells</i>			
eGFR	+0.76*	-0.07	-0.11
Creatinine	-0.57	-0.12	+0.10
ACR	-0.14	+0.21	-0.03
Urea	+0.58*	+0.19	+0.07
<i>Memory B cells</i>			
eGFR	+0.80*	-0.10	-0.10
Creatinine	-0.61*	-0.04	+0.10
ACR	-0.13	+0.24	-0.00
Urea	+0.59*	+0.13	+0.03

Values are Spearman ρ ; * $p < 0.05$ (uncorrected). † Control-group coefficients are exploratory: with $n = 12$, no multiplicity correction, and a normal eGFR range, they are statistically fragile. No coefficient was significant within the DM or DN groups, indicating that B-cell counts do not track renal impairment severity within the disease groups. ACR, albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate (CKD-EPI 2021); DN, diabetic nephropathy.

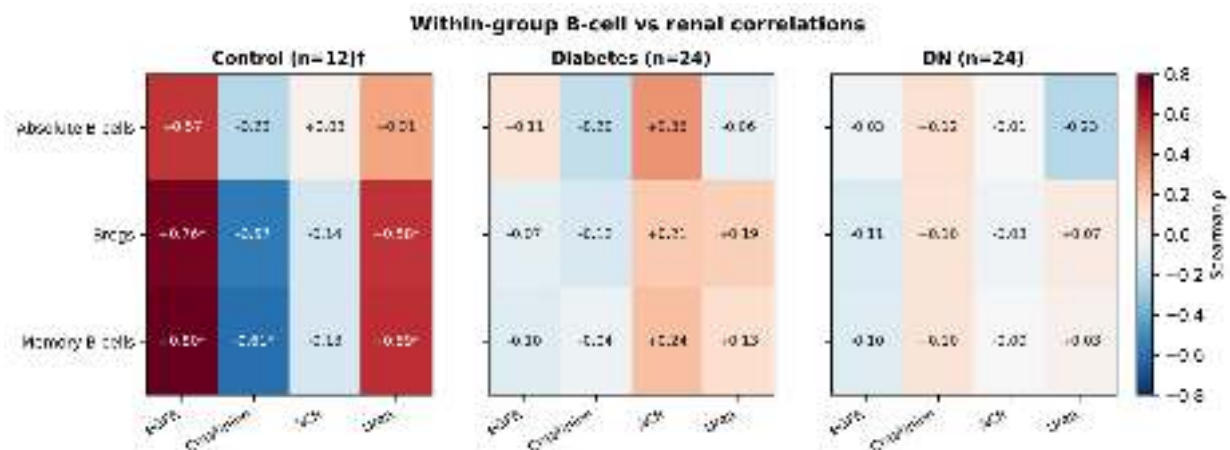


Figure 3. Within-group correlations between B-cell subsets and renal parameters. Each panel shows Spearman ρ computed inside one group. Appreciable coefficients occur only in the small control group († exploratory, $n = 12$); within the DM and DN groups, the correlations are near zero, indicating that B-cell depletion marks the presence rather than the severity of DN. * $p < 0.05$.

Whole-cohort Correlations are Driven by between-Group Separation

When analyzing the entire cohort (N = 60), significant correlations between the B-cell subsets, kidney function tests and HbA1c in Table 4, Fig. 4 were found. For example, B cell counts are positively related to eGFR (+0.46, $p < 0.05$) and negatively to serum creatinine (-0.43,

$p < 0.05$). These correlations remain largely significant after HbA1c adjustments. Given the lack of intra-group correlation observed in disease cohorts, however, the findings of these whole cohorts reflect a clear separation between the DN group suffering from severe illness and the other two groups, rather than a continuous gradient within the individual.

Table 4. Whole-cohort Spearman correlations between B-cell subsets and clinical parameters (N = 60).

Subset	eGFR	Creatinine	ACR	Urea	HbA1c
B cells	+0.46* / +0.42*	-0.43* / -0.38*	-0.30* / -0.20	-0.42* / -0.36*	-0.23
Breg cells	+0.39* / +0.32*	-0.42* / -0.35*	-0.29* / -0.14	-0.28* / -0.17	-0.27*
Memory B cells	+0.37* / +0.31*	-0.40* / -0.32*	-0.26* / -0.10	-0.29* / -0.19	-0.26*

Values are Spearman ρ (raw / HbA1c-adjusted partial). * $p < 0.05$. These pooled coefficients reflect concurrent between-group differences in B-cell counts and renal function and should not be read as continuous within-individual associations; within-group analysis showed no relationship with renal severity (see text). Exploratory; not corrected for multiple comparisons. $p > 0.05$ is not significant (NS).

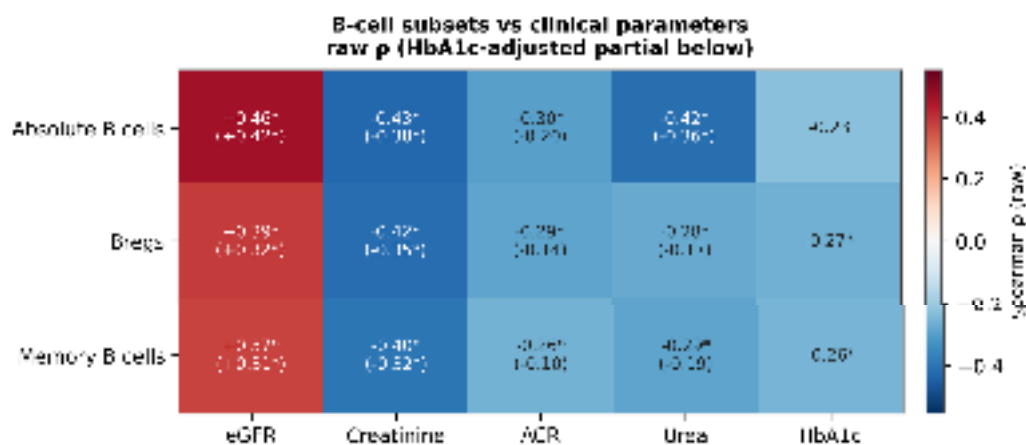


Figure 4. Whole-cohort Spearman correlations between B-cell subsets and clinical parameters. Cell color encodes the raw Spearman ρ ; the HbA1c-adjusted partial coefficient is shown in parentheses. The pattern reflects between-group separation rather than a continuous within-individual relationship (see text). * $p < 0.05$.

B-cell Subgroups Effectively Distinguish DN from DM and Healthy Control

Pairwise receiver operating characteristics (ROC) analysis was performed to evaluate the discriminatory capabilities of B cell subsets in Table 5, Fig. 5. None of the subsets can distinguish between healthy control patients and DM patients (AUC 0.60 and $p < 0.05$). On the contrary, all three subsets significantly

distinguish DN from control and DM patients. For critical comparisons between DN and DM groups, B cell count was the most important (0.768, 95% CI 0.63–0.89, $p = 0.001$), followed by Breg cells (AUC 0.752, $p = 0.003$) and memory B cells (AUC 0.730, $p = 0.006$). The optimal B cell count cut-off value of 78 cells/ μ L that distinguishes DM from DN with a sensitivity of 75% and a specificity of 75%.

Table 5. Pairwise ROC analysis of B-cell subsets for group discrimination.

Comparison/subset	AUC	95% CI	Cut-off	Sens.	Spec.	P
<i>Control vs DM (n = 36)</i>						
B cells	0.542	0.35–0.74	—	—	—	NS
Breg cells	0.582	0.38–0.76	—	—	—	NS
Memory B cells	0.569	0.38–0.74	—	—	—	NS
<i>Control vs DN (n = 36)</i>						
B cells	0.760	0.59–0.90	≤60	54%	92%	0.012
Breg cells	0.733	0.54–0.89	≤6	54%	92%	0.024
Memory B cells	0.740	0.55–0.89	≤6	67%	83%	0.021
<i>DM vs DN (n = 48)</i>						
B cells	0.768	0.63–0.89	≤78	75%	75%	0.001
Breg cells	0.752	0.61–0.87	≤14	79%	62%	0.003
Memory B cells	0.730	0.58–0.86	≤6	67%	75%	0.006

AUC, area under the curve; 95% CI by bootstrap (2000 resamples) with marker orientation fixed by group means, so the CI may include 0.5 for non-discriminating comparisons; cut-off at the Youden index (cells/ μ L), shown only where the AUC was significant; P by Mann–Whitney (equivalent to AUC $\neq$ 0.5). Comparisons are exploratory and not corrected for multiple testing. Sens., sensitivity; Spec., specificity. $p > 0.05$ is not significant (NS).

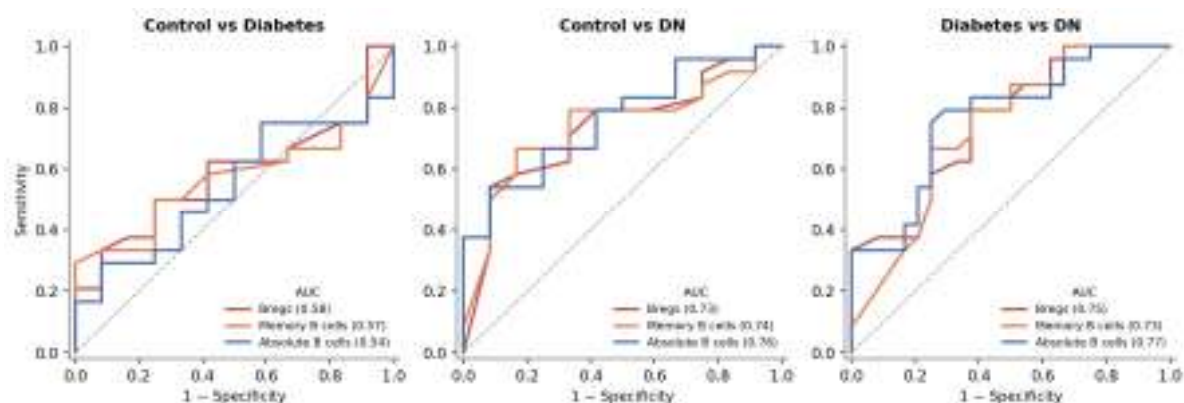


Figure 5. ROC curves of B-cell subsets for each pairwise comparison. B-cell counts discriminate DN from both control and DM but not control from DM (curves near the diagonal).

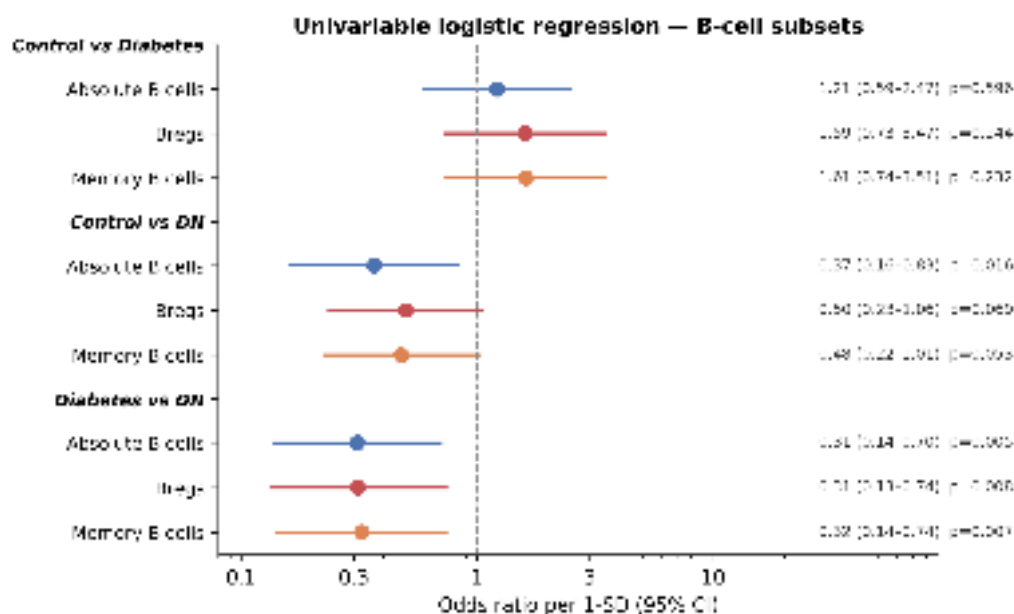
The univariable logistic regression further confirmed these results in Table 6 and Fig. 6. A lower number of B cell count is highly related to DN, and the likelihood of DN (in contrast to DM alone) decreases by about 69 % for each 1

standard deviation increase (OR = 0.31, 95 % CI = 0.14–0.70, $p < 0.01$). This indicates that the reduction of the B cell count is strongly associated with DN.

Table 6. Univariable logistic regression of B-cell subsets for group discrimination (odds ratio per 1-SD increase).

Comparison / subset	Univariable			Duration-adjusted [†]		
	OR per SD	95% CI	<i>p</i>	OR per SD	95% CI	<i>p</i>
<i>Control vs Diabetes (n = 36)</i>						
Absolute B cells	1.21	0.59–2.47	NS	—	—	—
Breg	1.59	0.73–3.47	NS	—	—	—
Memory B cells	1.61	0.74–3.51	NS	—	—	—
<i>Control vs Diabetic nephropathy (n = 36)</i>						
Absolute B cells	0.37	0.16–0.83	0.016	—	—	—
Breg	0.50	0.23–1.06	NS	—	—	—
Memory B cells	0.48	0.22–1.01	NS	—	—	—
<i>Diabetes vs Diabetic nephropathy (n = 48)</i>						
Absolute B cells	0.31	0.14–0.70	0.005	0.30	0.13–0.70	0.005
Breg	0.31	0.13–0.74	0.008	0.29	0.12–0.73	0.009
Memory B cells	0.32	0.14–0.74	0.007	0.30	0.12–0.73	0.008

OR per SD, odds ratio for the more advanced group per 1-SD increase in the subset (Wald test); OR < 1 indicates fewer B cells associated with the more advanced group. Each subset modelled separately; *p* values are not corrected for multiple testing and may differ from the Mann–Whitney *p* in Table 4 (different tests). CI, confidence interval. Duration-adjusted OR, odds ratio additionally adjusted for duration of diabetes; adjustment was performed for the primary comparison (diabetes vs diabetic nephropathy) only, for which duration of diabetes differed between groups (*p* = 0.021). [†] Adjusted estimates shown; —, not adjusted. *p* > 0.05 is not significant (NS).

**Figure 6.** Univariable logistic regression of B-cell subsets for pairwise group discrimination. Odds ratios (per 1-SD increase) with 95% CI for membership in the more advanced group; the dashed line marks OR = 1. B-cell counts do not distinguish control from DM (CI cross 1), but lower counts are associated with DN versus both control and DM.

Discussion

The current study evaluated B cell subsets counts in type 2 DM patients with and without DN in comparison to healthy control individuals. It was found that the B cell count was significantly lower in DN patients compared to both the control & DM groups (p value <0.002). Moreover, Breg and memory B cell counts were significantly lower in DN compared to both control & DM groups (p value <0.005 , P value <0.009), respectively. However, no significant differences were observed in any B cell subsets between DM patients and healthy controls, suggesting that the decline of B cell count is directly related to the development of renal complications, rather than DM itself.

Nevertheless, these findings are consistent with increasing evidence indicating that Breg cells loss accompanies the progression of DN. These study findings matched a study which reported a significant decrease in the circulation of Breg cells in DN patients compared to healthy controls ($p < 0.0001$) and DM ($p = 0.0419$).²²

Although this study could not detect any significant individual group correlations in the studied groups regarding B cell subsets and kidney functions. However, it demonstrated that in the entire cohort ($N = 60$), B cell count showed a notable inverse relationship with elevated serum creatinine (-0.43 , $p < 0.05$), while a positive relation was found regarding eGFR ($+0.46$, $p < 0.05$). These observations partially align with prior data from Li et al.²², who found that the Breg cells' frequencies demonstrated a significant positive correlation with both eGFR and systemic IL-10 levels, alongside a pronounced negative correlation with 24-hour urinary protein excretion. Taken together, these parallel phenotypic and clinical correlations strongly imply that the quantitative depletion and functional impairment of B cell subsets are actively involved in the pathogenesis and progression of DN.

The results of this study indicated that B cell count could significantly detect DN (0.768, 95% CI 0.63–0.89, $p = 0.001$), at a cut-off value of 78 cells/ μ L, with 75% sensitivity, 75% specificity, while Breg cells and memory B cell counts can detect DN with lower sensitivity and specificity.

The current investigation demonstrated that circulating CD19% B cell frequencies were significantly reduced in patients with DN, presenting a notable inverse relationship with elevated ACR values. These observations align with prior data from reference,²³ where Breg frequencies demonstrated a significant positive correlation with both eGFR and systemic IL-10 levels, alongside a pronounced negative correlation with 24-hour urinary protein excretion. Taking together, these parallel phenotypic and clinical correlations strongly imply that the quantitative depletion and functional impairment of Breg subsets are actively involved in the pathogenesis and progression of DN.

In alignment with our findings, some studies reported that although the regulatory role of IL-10, IL-10 might have a pathogenic role in DN. A study reported that individuals with long-standing T1DM and DN, IL-10 levels were a better predictor of albuminuria than are glycaemic control, blood pressure control or diabetes duration.²⁴ Another study reported that polymorphisms in IL-10 genes might confer susceptibility to DN.²⁵ These results might explain why the level of B reg was higher in patients with DN than DM only and suggest that there might be role for genetic polymorphism in the development of DN even in type 2 D.M.

Many studies linked between the high level of memory B cells and the deterioration of kidney function in DN as the highest level of memory B cells leads to rapid immune inflammatory flare up²⁶ as upon exposure re-exposure to diabetes-related antigens (such as advanced glycation end-products or altered podocyte proteins), memory B cells rapidly differentiate into antibody-secreting plasma cells. These local antibodies and cytokines directly injure the glomerular filtration barrier and amplify tubulointerstitial fibrosis.²⁷

Although the previous findings were against our results, a study conducted on adult patients with nephrotic syndrome detecting the level of memory cells found that memory cells were low during active disease and tend to normalize after treatment the same as B reg²⁸.

These observations collectively suggest that harnessing the immunomodulatory potential of

Breg cells could represent a novel therapeutic strategy and a promising target for future clinical exploration in the management of DN but with no correlation with DN severity.

Limitations of the Study

The study was limited by a cross-sectional design and relatively small sample sizes, especially in control groups (n=12). Also, the limited number of patients with early stages of type 2 DM before kidney damage. The various subtypes of Breg cells should be studied for their role in DN. The lack of functional test (e.g., production of IL-10) limits our ability to evaluate the functionality and not just the quantity of Breg cells.

Recommendations

Concurrently, additional longitudinal investigations focused on Breg cells are warranted to fully elucidate their precise mechanistic involvement in the initiation, pathogenesis, and clinical progression of DN. A deeper molecular exploration of these Breg lymphocyte subpopulation dynamics could unveil novel targeted therapeutic pathways. Furthermore, there remains a critical need for large-scale, prospective studies dedicated to identifying novel, highly sensitive biomarkers capable of predicting renal impairment in diabetic cohorts at the earliest possible stage for optimizing early preventative measures of DN. More studies are needed to assess role of Breg cells in the progression of other autoimmune diseases.

Conclusion

Diabetes mellitus represents the primary etiology driving the progression toward long-term renal impairment and advanced kidney failure, concurrently compounding cardiovascular co-morbidity and mortality rates. Cumulative scientific evidence underscores the critical contribution of both native and acquired immunological pathways to the exacerbation of DN. Breg cells comprise a distinct B lymphocyte subpopulation capable of downregulating inflammatory cascades, and their functional or quantitative deficiency is directly linked to unmitigated immune-mediated damage,

thereby elucidating their pathogenetic role in the development of this metabolic disease. Circulating frequencies of B cell subsets, particularly the specific Breg cell subset, were found to be significantly diminished in patients suffering from secondary DN. Breg cells subset can significantly predict DN as well as its progression.

Author Contributions

AMO: Conceptualization, Methodology, Validation, Supervision, Writing review & editing. MMA: Methodology, Validation, Supervision, Writing review & editing. MFM: Methodology, Validation, Supervision, Writing review & editing. OMS: Methodology, Investigation, Supervision, Writing review & editing. KET: Formal Analysis, Writing review & editing. AAI: Methodology, Investigation, Writing review & editing. GKS: Methodology, Validation, Supervision, Writing original draft, Writing review & editing.

Declaration of Conflicting Interests

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Ethical approval

The study's protocol was designed according to the Helsinki Declaration and was approved by Ain-Shams University Research Ethics Committee (ASU-REC) (Approval No. MD 36/ 2021),

Informed consent

All participants provided written informed consent before enrollment and before any study-related procedures.

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