Biol Blood Marrow Transplant 25 (2019) 451–458

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Biomarkers

Comprehensive B Cell Phenotyping Profile for Chronic Graft-versus-Host Disease Diagnosis

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Article history:
Received 11 July 2018
Accepted 8 November 2018

Key Words:
Chronic graft-versus-host disease
B cell
Biomarker
BAFF
Steroid use

A B S T R A C T

Previous studies have reported single B cell-related chronic graft-versus-host disease diagnostic (cGVHD) biomarkers, such as B cell-activating factor (BAFF), CD21^{low}, and immature B cells, but research on the performance of biomarker combinations and the covariate effect of steroids is lacking. The primary objective of this study was to determine the most accurate combination of B cell populations using cell surface staining flow cytometry in an independent cohort of patients with cGVHD. Secondary objectives included assessing the effect of corticosteroid use at sample collection on the makeup and accuracy of the diagnostic panel and identifying the mechanism underlying low surface expression of BAFF receptor (BAFF-R) on B cells in cGVHD. Flow cytometry analysis was performed in an adult cohort of post-HCT patients with cGVHD onset (n = 44) and time-matched recipients without cGVHD (n = 63). We confirmed that the onset of cGVHD was associated with higher soluble BAFF (sBAFF) levels, elevated CD27^{-}CD10^{-}CD21^{low} CD19^{+} B cell and classical switched memory B cell counts, and reduced transitional and naïve B cell counts. The highest single B cell population area under the receiver operating characteristic (ROC) curve (AUC) was .72 for transitional type 1 CD21^{low} B cells. We also showed a significant inverse relationship between sBAFF and surface BAFF-R expression caused by sBAFF modulation of BAFF-R. Steroid use at sample collection influenced the significance of the sBAFF:B cell ratio, naïve and marginal zone-like B cells. The optimal combination of B cell subsets most significantly associated with cGVHD onset with or without concurrent corticosteroid use resulted in ROC AUCs of .87 and .84, respectively. Transitional and CD21^{low} B cells were the only populations present in both panels; however, analyzing only these populations resulted in ROC AUCs of .79 and .78, respectively. This suggests that the inclusion of other populations and use of different panels depending on steroid use is necessary to achieve better accuracy. sBAFF was not a component of either panel. These novel B cell profiles could be tested prospectively in patients post-HSCT and could lead to focused mechanistic studies.

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is now the leading cause of late nonrelapse mortality and morbidity following allogeneic hematopoietic cell transplantation (HCT) [1]. The pathogenic role of B cells in cGVHD was first identified in murine models in 1995 [2]. The roles of abnormalities in B cell function have been confirmed in humans owing to the successful use of B cell- directed therapies such as rituximab [3] and approval of ibrutinib, the first Food and Drug Administration-approved drug for cGVHD therapy [4].

One of the most consistently identified B cell-associated abnormalities in cGVHD is a high level of soluble B cell activating factor (BAFF), which is inversely correlated with expression of BAFF-R, its principal receptor, on B cells [5,6]. BAFF is a critical soluble factor necessary for normal peripheral B cell maturation and survival [7]. The importance of BAFF and its principal receptor, BAFF-R, to peripheral B cell maturation and survival is demonstrated by the presence of arrested B cell

Financial disclosure: See Acknowledgments on page 457.
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https://doi.org/10.1016/j.bbmt.2018.11.007
1083-8791/

development at the stage of transitional B cells and severe reduction of all subsequent B cell stages in mice lacking BAFF [8] or BAFF-R [9] and humans lacking BAFF-R [10]. In contrast, transgenic mice overexpressing BAFF develop lymphocytic disorders with autoimmune manifestations and have vastly increased numbers of mature B cells [11].

It has been previously shown that in patients post-HCT without cGVHD, B cells express lower levels of surface BAFF-R compared with patients without cGVHD who have supranormal B cell numbers with higher levels of surface BAFF-R that could act as a sink for excessive soluble BAFF (sBAFF). It has been hypothesized that this leads to higher levels of circulating sBAFF in cGVHD, which rescues autoreactive B cell clones [12]. It has been shown that increased BAFF occupancy of BAFF-R accounts for low BAFF-R detection by antibodies on $CD19^+$ B cells in cGVHD [13].

cGVHD is also associated with defects in B cell development, including decreased numbers of naïve B cells and increased numbers of antigen-experienced $CD27^+$ B cells in the setting of total B cell lymphopenia [14]. A B cell subpopulation that has received considerable attention is $CD21^{low}$ B cells [15,16]. In normal B cell development, the differential expression of CD21 identifies 2 subsets of $CD10^+$ transitional B cells. $CD21^{low}$ transitional B cells (T1 B cells) are less mature than the $CD21^{+/hi}$ subset (T2 B cells) [17]. Low CD21 expression also defines $CD19^{low}CD27^-$ plasmablasts. In contrast, a recent report characterized the expanded $CD21^{low}$ population in cGVHD as $CD10^-$, $CD27^-$, and $CD20^{hi}$ with features of exhaustion including increased expression of inhibitory receptors, altered expression of chemokine and adhesion molecules, and poor proliferative response and calcium flux in response to B cell receptor triggering [18].

In routine clinical practice, the disease manifestations of cGVHD are diverse and result from inflammatory changes in a variety of organs. Sometimes patients with clinically suspected cGVHD begin first-line treatment with corticosteroids before the diagnosis is confirmed and potentially before peak grade is achieved. Furthermore, many patients have already experienced acute GVHD and are continuing corticosteroid therapy. Therefore, it is crucial to better understand how corticosteroid use at the time of potential cGVHD diagnosis influences the significance of B cell-related biomarkers.

We took an integrative approach, using blood samples from patients with cGVHD, to better characterize the relationship between the different B cell factors and populations in cGVHD. We evaluated the relative relationship of $CD21^{low}$B cells, memory and immature B cells, sBAFF, and BAFF-R and found unique combinations of B cell markers with and without concomitant steroid use that were strongly associated with cGVHD. We hypothesized that our study establishes a framework within which further mechanistic studies could be pursued.

METHODS

Sample Collection and Processing Protocol

Patient plasma samples were collected as part of the Chronic GVHD Consortium Protocol 6501 cohort study as described previously, with appropriate consent [19]. The remaining white blood cells were separated by density gradient using Ficoll-Paque PLUS (GE Healthcare, Chicago, Illinois). Following platelet depletion, cells were slowly frozen in 10% dimethyl sulfoxide/fetal bovine serum at -80°C and stored in the vapor phase of liquid nitrogen until analysis. Blood was also collected from healthy donors at BC Children's Hospital as part of a local research study approved by our Institutional Review Board.

Reagents

The recombinant human sBAFF was an unmodified 152-amino acid polypeptide (amino acids 134 to 285 of full-length BAFF) produced in *Escherichia coli* with molecular weight of 17.0 kDa (catalog no. 310-13; PeproTech, Rocky Hill, NJ). Mass spectrometry confirmed the molecular weight. The human 697 relapsed pre-B ALL cell line was purchased from DSMZ (Braunschweig, Germany). Belimumab (GSK Canada) was purchased from our hospital pharmacy.

Isolation of Healthy Donor $CD19^+$ B cells

Human B cells were purified from freshly isolated peripheral blood mononuclear cells using the StemSep Human B Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. The purity of the $CD19^+$ fraction was typically >95% as determined based on CD19 expression by FACS.

BAFF Enzyme-Linked Immunosorbent Assay

Measurements of sBAFF were done in thawed plasma using a quantitative sandwich enzyme immunoassay (Quantikine Human BAFF/BLyS Kit; R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions.

Flow Cytometry Analysis of Peripheral B Cells

Flow cytometry analysis was performed on thawed cells using the following antibodies: IgD-BV510 (clone IA6-2), CD21-FITC (clone Bu32), CD19-Pacific Blue (clone HIB19), CD27-BV785 (clone O323), CD38-PE (clone HIT2), CD10-APC (clone HI10), CD5-PE-Cy7 (clone UCH2), CD24-PE-DAZZLE 594 (clone ML5), BAFF-R-APC-Cy7 (clone 11C1), and 7AAD (all from BioLegend, San Diego, CA). We chose to use the 11C1 clone for the anti-BAFF-R antibody because it has been shown to be insensitive to receptor occupancy with BAFF, allowing proper determination of surface BAFF-R expression [20–22]. The gating strategy is shown in Supplementary Figure 1. The lymphocyte gate was established using forward and side scatter. Only $CD19^+$ B cells were analyzed, to ensure that $CD3^+$ cells were not included in the analysis of CD38 or CD27 expression.

Statistical Methods

Data were analyzed both as actual values and after log-transformation. The latter approach is sensitive to effects associated with proportional changes rather than with absolute changes, and we had no basis on which to assume which one was more appropriate for any given variable. For ease of interpretation, mean differences on the log scale data were reexpressed as fold changes. Differences between cGVHD case and control groups were assessed using the 2-sample *t* test. With cGVHD status as the dependent variable, logistic regression was used with forward selection to define optimal combinations of B cell variables associated with cGVHD. The results adjusted for any steroid use at sample collection are from a multivariate regression model that includes cGVHD status and steroid use. The Spearman rank correlation was used for correlation analysis. All *P* values are 2-sided and unadjusted for multiple comparisons.

RESULTS

Study Population

We analyzed B cell-related parameters in samples from 107 patients, including 44 patients with cGVHD (median time of onset, 207 days post-HCT; range, 83 to 424 days) and 63 patients without cGVHD with a median time of sample collection 194 days post-HCT (range, 153 to 430 days). Onset cGVHD samples were obtained at a median of 6 days after diagnosis of cGVHD (range, 12 days before diagnosis to 29 days after diagnosis). There were no significant differences between the patients with cGVHD and those without cGVHD in patient or transplantation characteristics except for stem cell source and steroid use at the time of sample collection (Table 1). Compared with the no-cGVHD cohort, the cGVHD cohort had a significantly higher frequency of use of peripheral blood stem cells as a graft source (84% versus 51%), significantly less use of cord blood grafts (9% versus 43%), and a significantly higher percentage of patients receiving steroids at the time of sample collection (43% versus 10%). Our initial analyses were performed in a subcohort of patients who were not receiving steroids at the time of sample collection (25 with cGVHD and 57 controls). A second analysis was performed in the total cohort after adjusting for the effect of steroids. There were no statistically significant differences in absolute lymphocyte count (ALC), % $CD19^+$ of ALC, or absolute $CD19^+$ count between patients with cGVHD and those without cGVHD post-HCT in

Table 1
Patient and Transplantation Characteristics

Characteristics	cGVHD (N = 44)	No cGVHD (N = 63)
Time from HCT to sample collection, n (%)		
Early (<9 mo)	29 (66)	36 (57)
Late (>9 mo)	15 (34)	27 (43)
Previous acute GVHD, n (%)		
No	22 (50)	27 (43)
Yes	22 (50)	36 (57)
Age, yr, n (%)		
<50	16 (36)	25 (40)
≥50	28 (64)	38 (60)
Donor type, n(%)		
Matched related	19 (43)	17 (27)
Matched unrelated	14 (32)	17 (27)
Mismatched	11 (25)	29 (46)
Stem cell source, n (%)§		
Peripheral blood stem cells	37 (84)	32 (51)
Bone marrow	3 (7)	4 (6)
Cord blood	4 (9)	27 (43)
Conditioning regimen, n (%)		
Myeloablative without total body irradiation	8 (18)	8 (13)
Myeloablative with total body irradiation	10 (23)	19 (30)
Nonmyeloablative	26 (59)	36 (57)
GVHD prophylaxis, n (%)		
CNI + MTX/MMF ± sirolimus	38 (86)	48 (76)
CNI ± sirolimus	6 (14)	10 (16)
Other	0	5 (8)
Sex, n (%)		
Male	26 (59)	34 (54)
Female	18 (41)	29 (46)
Female donor to male patient, n (%)		
No	34 (77)	49 (78)
Yes	10 (23)	14 (22)
Cytomegalovirus serostatus, n (%)		
Negative	20 (45)	26 (41)
Positive	24 (55)	37 (59)
Disease diagnosis, n (%)		
Acute myelogenous leukemia	11 (25)	25 (40)
Acute lymphoblastic leukemia	7 (16)	9 (14)
Chronic myelogenous leukemia	1 (2)	4 (6)
Chronic lymphocytic leukemia	2 (5)	2 (3)
Myelodysplastic syndrome	12 (27)	9 (14)
Hodgkin lymphoma/non-Hodgkin lymphoma	7 (16)	10 (16)
Multiple myeloma	3 (7)	2 (3)
Other	1 (2)	2 (3)
Receipt of steroids at sample collection, n (%)*		
No	25 (57)	57 (90)
Yes	19 (43)	6 (10)
Days from HCT to cGVHD, median (range)	207 (83 to 424)	
Days from cGVHD to sampling, median (range)	6 (-12 to 29)	
Skin involvement, %	61	
Oral involvement, %	66	
Gastrointestinal involvement, %	23	
Eye involvement, %	45	
Joint involvement, %	15	
Lung involvement, %	47	
Liver involvement, %	51	
Genital involvement, %	16	
NIH overall severity: mild/moderate/severe, %	16/29/25	

CNI indicates calcineurin inhibitor; MTX, methotrexate; MMF, mycophenolate mofetil; NIH, National Institutes of Health.
*$P < .01$; the difference between groups is considered statistically significant.

both the no-steroid and total (including those who received steroids) cohorts.

BAFF-R Surface Expression Is Associated with sBAFF Levels in cGVHD in the Absence of Steroids

Because steroids have been shown to have a significant effect on B cell-associated factors after HCT [6], our initial analyses were limited to patients who were not receiving steroids at the onset of cGVHD compared with a cohort without cGVHD and not receiving steroids. Similar to a previous study [19], we found a strong inverse relationship between sBAFF and $CD19^+$ cell count (cells/μL; $r = -.65$; $P < .0001$) (Figure 1A) and significantly higher sBAFF level (1.5-fold; $P = .009$) (Figure 1B) in patients with cGVHD. In addition, we found significantly lower (.7-fold; $P = .002$) mean fluorescence intensity (MFI) of BAFF-R surface expression on $CD19^+$ B cells from patients with cGVHD (Figure 1C). There were also a significantly higher sBAFF:$CD19^+$ B cell ratio (2.9-fold; $P = .03$) (Figure 1D) and BAFF-R surface expression (2.2-fold; $P = .003$) (Figure 1E) in patients with cGVHD. Soluble BAFF levels had a strong inverse relationship with BAFF-R MFI ($r = -.78$; $P < .0001$) (Figure 1F), but not because of competition between sBAFF and the monoclonal antibody (11C1) detecting surface BAFF-R (Figure 2A). Plasma from patients post-HCT decreased the surface expression of BAFF-R on the pre-B acute lymphoblastic cell line 697, an effect mediated by sBAFF, as demonstrated by increased expression of BAFF-R when sBAFF was neutralized with belimumab. The effect of blocking sBAFF with belimumab on surface BAFF-R expression was strongly correlated with sBAFF concentration ($r = .72$) (Figure 2B).

Evaluation of $CD19^+21^{low}$ B Cell Subsets Associated with cGVHD Onset in the Absence of Steroids

We evaluated whether the $CD27^-CD10^-$ subpopulation of $CD21^{low}CD19^+$ B cells was associated with cGVHD in our study population. We found a significantly higher percentage of $CD21^{low}$ cells within the total $CD19^+$ B cell pool (+14%; $P = .008$) in patients with cGVHD compared with post-HCT no-cGVHD controls (Figure 3A). sBAFF level had a strong positive relationship with %$CD21^{low}$ B cells ($r = 0.67$; $P < .0001$) (Figure 3B). The absolute number of $CD27^-CD10^-CD21^{low}$ $CD19^+$ B cells was significantly higher in the patients with cGVHD (+48 cells/μL; $P = .03$) (Figure 3C). Further analysis of this subpopulation showed that intermediate to high expression of IgD and CD38. $CD21^{low}$ $CD19^+$ B cells also contained significantly lower percentages of T1 transitional $CD27^-CD10^+$ B cells in patients with cGVHD (-9%; $P = .02$) (Figure 3D).

Evaluation for Other B Cell Subsets Associated with cGVHD Onset in the Absence of Steroids

We further evaluated other B cell subsets associated with cGVHD, including naïve B cells ($CD27^-IgD^+$), classical switched memory B cells ($CD27^+IgD^-$), nonswitched memory or marginal zone-like B cells ($CD27^+IgD^+$), double-negative memory B cells ($CD27^-IgD^-$), and transitional B cells ($CD19^+CD24^{high}CD38^{high}$). We found a significantly lower percentage of naïve B cells (-9%; $P = .04$) (Figure 4A) and a higher percentage of classical switched memory B cells (+7%; $P = .009$) (Figure 4B) in the patients with cGVHD. Finally, cGVHD was associated with lower percentages and absolute numbers of transitional cells (-11%; $P = .02$ and -93 cells/μL; $P = .04$, respectively) (Figure 4C and D).

The Impact of Steroids on B Cell Populations at cGVHD Onset

The significant B cell populations identified in the no steroid subcohort were analyzed in the entire cohort after adjusting for the effect of steroid use (Table 2). There were no longer any significant differences in the sBAFF:B cell ratio or in naïve B cells between patients with cGVHD and no-cGVHD controls. All other differences remained significant.

Combinations of B Cell Subsets as cGVHD Diagnostic Profiles Inclusive of Corticosteroid Use

We identified various B cell populations and sBAFF concentrations that were either higher or lower at the onset of

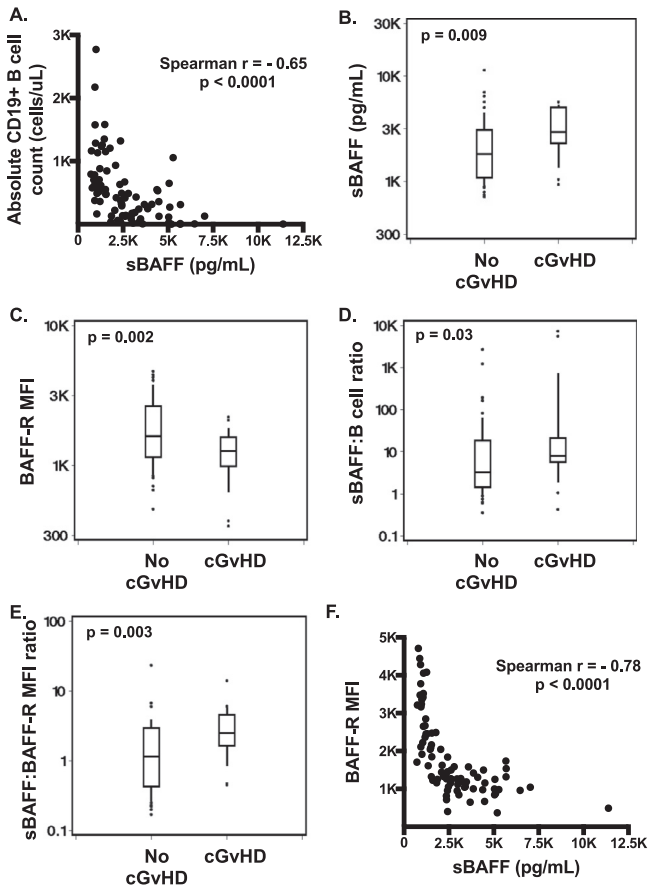

Figure 1. Relationships between sBAFF and BAFF-R expression. (A) Correlation between CD19$^+$ B cell count and sBAFF. (B-E) Differences in sBAFF (B), BAFF-R MFI (C), sBAFF:B cell ratio (D), and sBAFF:BAFF-R ratio (E) between patients with cGVHD and those without cGVHD post-HCT. (F) Correlation between BAFF-R MFI and sBAFF.

cGVHD in patients who were not receiving steroid treatment. We evaluated the associations of each of these cell populations and sBAFF with the onset of cGVHD through multivariate analyses to identify the strongest associations. The strongest markers were a combination of the BAFF-R surface expression, CD27$^-$CD10$^-$CD21^{low} CD19$^+$ B cell count, CD19$^+$CD24^{high}CD38^{high} cell count, and percentage of classical switched memory B cells (CD27$^+$IgD$^-$) (AUC, .85; Table 3).

Analysis of the entire cohort, including patients receiving corticosteroids at sample collection, revealed a different B cell panel consisting of %CD27$^-$CD10$^+$ of CD21^{low}CD19$^+$ B cells, CD27$^-$CD10$^-$CD21^{low} CD19$^+$ B cell count, CD19$^+$CD24^{high}CD38^{high} cell count and percentage, and percentage of naïve and marginal zone-like B cells (AUC, .87; Table 4). Measurements related to sBAFF concentrations did not add information to either multivariate analysis.

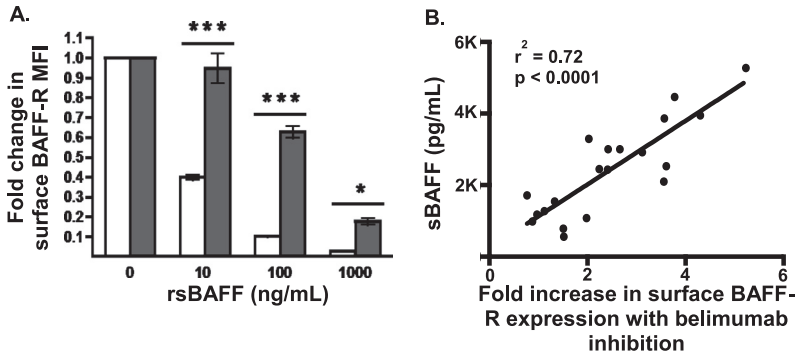

Figure 2. Effect of sBAFF on surface BAFF-R expression. (A) Healthy donor isolated CD19$^+$ B cells (n = 5) were incubated overnight in complete medium (10% FCS in 1640 RPMI) at 37°C with stated concentrations of recombinant sBAFF (rsBAFF), and surface expression of BAFF-R was measured by flow cytometry and compared with that in complete medium alone (white bars). To measure the effect of receptor occupancy on flow cytometry antibody binding caused by rsBAFF binding to BAFF-R, the same concentrations of rsBAFF were incubated with the same healthy donor CD19$^+$ B cells at 4°C (gray bars). The bars represent mean ± SEM compared using a paired t test. $^*P < .05$; $^{***}P < .001$. (B) Plasma from patients post-HCT down-modulates surface expression of BAFF-R on pre-B ALL 697 cells. The 697 cells were incubated with a 1:1 mix of complete medium and patient plasma (n = 20) with or without belimumab (10 ug/mL) overnight, and surface expression of BAFF-R was measured by flow cytometry. The MFI was compared between both conditions for each sample and expressed as fold increase in BAFF-R due to sBAFF inhibition by belimumab. This was then correlated to sBAFF levels.

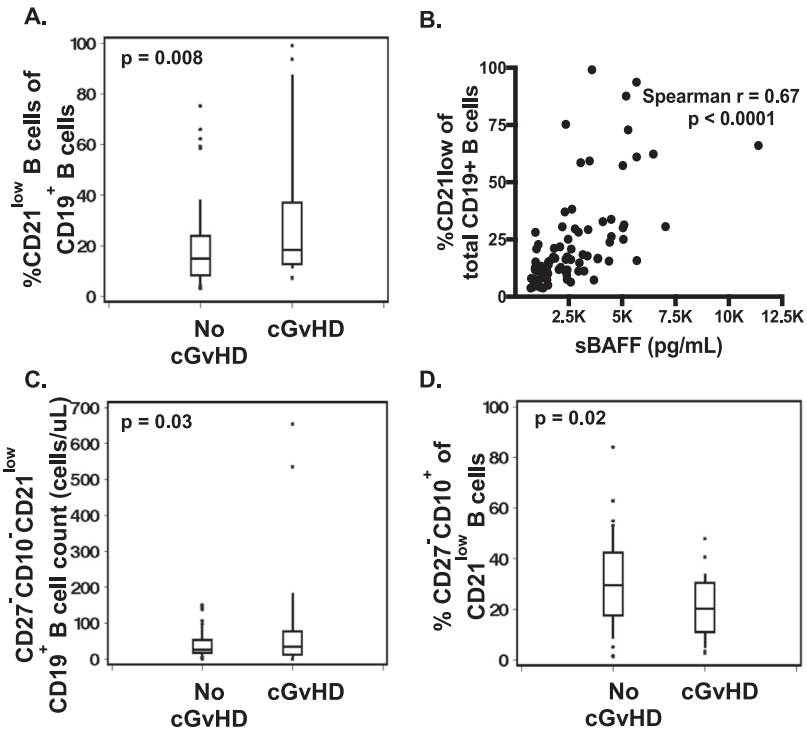

Figure 3. $CD21^{low}$ B cell populations. (A, C, and D) Differences in $\%CD21^{low}$ B cells (A), $CD27^{-}CD10^{-}CD21^{low}CD19^{+}$ cells (C), and $\%CD27^{-}CD10^{+}$ of $CD19^{+}CD21^{low}$ B cells (D) between patients with cGVHD and those without cGVHD. (B) Correlation between $\%CD21^{low}$ of total $CD19^{+}$ B cells and sBAFF.

DISCUSSION

In our cohort of adult HCT recipients, we have confirmed that the onset of cGVHD is associated with significantly higher plasma concentrations of sBAFF. It initially appears that sBAFF levels are higher in patients with cGVHD than in controls owing to lower numbers of $CD19^{+}$ B cells and associated BAFF-Rs, given the inverse correlations seen between sBAFF levels and $CD19^{+}$ B cell counts and BAFF-R surface expression. However, our data also suggest that the decreased surface expression of the primary BAFF receptor, BAFF-R, results from higher circulating sBAFF concentrations rather than from an intrinsic B cell defect. Consistent with previous studies in other

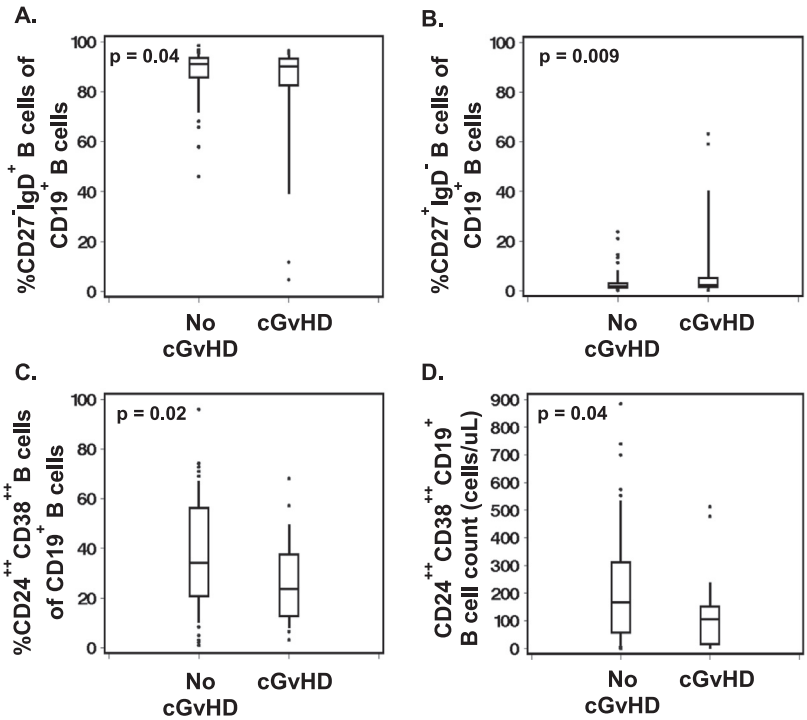

Figure 4. Other B cell subsets associated with cGVHD onset. Differences in naïve B cells ($CD27^{-}IgD^{+}$), classical switched memory B cells ($CD27^{+}IgD^{-}$), and transitional B cells ($CD19^{+}CD24^{high}CD38^{high}$) between patients with cGVHD and those without cGVHD.

Table 2
B Cell Variables Considering Steroid Use at Sample Collection

B cell variable	25 Cases and 57 Controls, No Steroid-Using Patients Included (Univariate AUC)	44 Cases and 63 Controls, Including Steroid-Using Patients (Univariate AUC)	Steroid Effect*
sBAFF/BAFF-R pathway			
Fold change in sBAFF, pg/mL	1.5×, $P = .009$ (.69)	1.3×, $P = .02$ (.63)	NS
Fold change in BAFF-R MFI on $CD19^+$ B cells	.7×, $P = .002$ (.68)	.7×, $P = .004$ (.68)	NS
Fold change in sBAFF:B cell ratio	2.9×, $P = .03$ (.64)	NS	8.2×, $P = .0003$
Fold change in sBAFF:BAFF-R MFI ratio	2.2×, $P = .003$ (.70)	1.9×, $P = .004$ (.66)	NS
$CD21^{low}$			
Absolute change in %$CD21^{low}$ of $CD19^+$	+14%, $P = .008$ (.66)	+13%, $P = .01$ (.66)	+25%, $P < .0001$
$CD21^{low}CD19^+$ (cells/μL)	NS	NS	-52, $P = .05$
cGVHD- associated $CD21^{low}$			
Absolute change in %$CD27^-CD10^-$ of $CD21^{low}CD19^+$	NS	+8%, $P = .05$ (.65)	NS
$CD27^-CD10^-CD21^{low}CD19^+$ (cells/μL)	+48, $P = .03$ (.53)	+37, $P = .04$ (.50)	NS
Transitional type 1(T1) $CD21^{low}$			
Absolute change in %$CD27^-CD10^+$ of $CD21^{low}CD19^+$	-9%, $P = .02$ (.65)	-13%, $P < .0001$ (.72)	-9%, $P = .02$
$CD27^-CD10^+$ $CD21^{low}CD19^+$ (cells/μL)	NS	NS	-15, $P = .003$
Naïve			
Absolute change in %$CD27^-$ IgD^+ of $CD19^+$	-9%, $P = .04$ (.58)	NS	-21%, $P = .0003$
$CD27^-IgD^+CD19^+$ (cells/μL)	NS	NS	-285, $P = .01$
Classical switched memory			
Absolute change in %$CD27^+IgD^-$ of $CD19^+$	+7%, $P = .009$ (.58)	+6%, $P = .05$ (.59)	+10%, $P = .007$
$CD27^+IgD^-CD19^+$ (cells/μL)	NS	NS	-5, $P = .02$
Marginal zone-like			
Absolute change in %$CD27^+IgD^+$ of $CD19^+$	NS	-1%, $P = .04$ (.59)	+1%, $P = .004$
$CD27^+IgD^+CD19^+$ (cells/μL)	-4, $P = .04$ (.63)	-5, $P = .004$ (.64)	NS
Transitional			
Absolute change in %$CD24^{++}CD38^{++}$ of $CD19^+$	-11%, $P = .02$ (.65)	-14%, $P = .0004$ (.69)	-15%, $P = .002$
$CD24^{++}CD38^{++}CD19^+$ (cells/μL)	-93, $P = .04$ (.64)	-110, $P = .002$ (.67)	-128, $P = .003$

*This column shows the difference between patients on steroids at sample collection and patients without steroid use at sample collection regardless of GVHD status.

Table 3
Optimal Combination of B Cell Subsets Diagnostic of cGVHD Onset Without Steroid Use

Cell Variable	Phenotyping	Multivariate ROC AUC
Increased cGVHD-associated $CD21^{low}$*	$CD27^-CD10^-CD21^{low}CD19^+$ (cells/μL)	.84
Decreased transitional*	$CD24^{hi}CD38^{hi}CD19^+$ (cells/μL)	
BAFF-R surface expression	BAFF-R MFI	
Classical switched memory	%$CD27^+IgD^-$ of $CD19^+$	

*Combining only these 2 populations yields an ROC AUC of .79.

Table 4
Optimal Combination of B Cell Subsets Diagnostic of cGVHD Onset Irrespective of Steroid Use

Cell Variable	Phenotyping	Multivariate ROC AUC
Increased cGVHD-associated $CD21^{low}$*	%$CD27^-CD10^+$ of $CD21^{low}CD19^+$	.87
	$CD27^-CD10^-CD21^{low}CD19^+$ (cells/μL)	
Decreased transitional*	%$CD24^{hi}CD38^{hi}$ of $CD19^+$	
	$CD24^{hi}CD38^{hi}CD19^+$ (cells/μL)	
Naïve	%$CD27^-IgD^+$ of $CD19^+$	
Marginal-zone like	%$CD27^+IgD^+$ of $CD19^+$	

*Combining only these 2 populations yields an ROC AUC of .78.

disorders [20–24], binding of sBAFF from post-HCT plasma was found to decrease surface BAFF-R expression, and this effect was inhibited with belimumab, a fully human IgG1γ recombinant monoclonal antibody against sBAFF. Therefore, the reduced surface expression of BAFF-R could be considered a surrogate marker of BAFF activation. This idea is supported by work showing that B cells from patients with cGVHD are activated and primed for survival via BAFF-mediated pathways [25]. Our data also support the hypothesis that BAFF regulates surface BAFF-R expression through BAFF-R internalization in response to ligand binding.

The addition of sBAFF to our multivariate analysis did not improve the model, suggesting that changes in the numbers of B cells or their subsets represent the primary driver associated with cGVHD, and that the higher sBAFF concentrations may represent a secondary effect.

We also independently confirmed an association of an elevated %$CD21^{low}$ B cells in the presence of cGVHD [15,16]. Our data suggest that $CD10^-CD27^-$ B cells represent the majority of this $CD21^{low}$ B cell subset, similar to observations in Sjögren's syndrome, rheumatoid arthritis, and common variable immunodeficiency [26]. A consistent finding in autoimmune and inflammatory diseases is that this population remains responsive to TLR-9 stimulation [27]. Previously, our group showed that cGVHD is associated with a B cell population that is highly responsive to TLR-9 ligation [28]. High SYK expression is a common feature of $CD21^{low}$ B cells, and this

high expression is sufficient to drive constitutive phosphorylation of SYK and its immediate targets, Bruton's tyrosine kinase and phospholipase Cγ2. The high SYK expression is induced by CpG deoxynucleotides (CpG ODN), a TLR9 agonist, but not by B cell antigen receptor stimulation [29]. We hypothesize that the presence of increased CD21^{low} B cells may result from chronic activation caused by inflammation; for example, it has been shown that treatment of chronic hepatitis C infection rescues normal CD21 expression in a CD21^{low} B cell population [30].

We show that cGVHD is associated with a lower frequency of transitional B cells [18]. We hypothesize that the smaller pool of transitional B cells overall in cGVHD could be explained by increased inflammatory effects on B lymphopoiesis. In mice, inflammation diverts lymphoid progenitors away from the B lineage and mobilizes developing B cells to the spleen and away from other secondary lymphoid organs [31–33]. TLR ligation induces the differentiation of common lymphoid progenitors into innate immune cells, such as dendritic cells [34]. Redirected differentiation of common lymphoid progenitors as a result of TLR ligation also could directly reduce the B cell progenitor pool. Decreased numbers of CD19$^+$ B cells were observed in association with osteoblast destruction in the bone marrow biopsy specimens from patients with cGVHD [35], and significantly greater numbers of bone marrow B cell precursors were found in patients who did not develop cGVHD compared with those who developed cGVHD [36,37].

Our study highlights the importance of considering steroid use when studying abnormal B cell populations associated with cGVHD onset as potential biomarkers, because our results indicate that steroid use regardless of cGVHD status has a stronger impact on certain B cell populations. This does not necessary mean that patients on steroids have different cGVHD mechanisms, but instead demonstrates how steroids may secondarily alter the B cell profile generated by cGVHD. The transitional CD27$^-$CD10$^+$ and CD27$^-$CD10$^-$CD21^{low}CD19$^+$ B cell populations remained significantly associated with cGVHD onset after adjustment for steroid use. These findings provide candidate B cell populations that could be potential therapeutic targets in patients with steroid-resistant cGVHD. In patients with systemic lupus erythematosus, it has been previously shown that plasmacytoid dendritic cells drive the differentiation of immature regulatory B cells, and that these interactions are normalized in patients responding to rituximab therapy [38]. After HCT, the increase in plasmacytoid dendritic cells in bone marrow allografts is associated with less cGVHD [39], and rituximab has been successfully used as salvage therapy for steroid refractory cGVHD [40].

Limitations of our study include that our results were not replicated in a separate cohort or applied prospectively to patients post-HCT, and we did not perform any mechanistic investigations on the abnormal B cell subsets to determine whether they are pathogenic and not simply reactive changes. These are important research directions for future studies.

Our comprehensive analysis of B cell subpopulations has provided a clearer picture of the dysregulated B cell lymphopoiesis associated with the onset of cGVHD. We hypothesize that the CD27$^-$CD10$^-$CD21^{low}CD19$^+$ B cell population may be uniquely selected at the expense of normal B cell development and transitional B cell populations in a persistent inflammatory setting, such as cGVHD. Furthermore, the presence of elevated sBAFF in an overall B cell lymphopenic environment may further promote the survival of B cells in the circulation. This profile of B cell dysregulation supports the idea that inflammation is as an important risk factor for cGVHD in HCT recipients. Therefore, therapies could be aimed at dampening proinflammatory signals post-HCT by neutralizing IL-6 or boosting counter-regulatory responses through the expansion of immunomodulating cell populations, such as regulatory natural killer cells. Finally, our comprehensive B cell profile could serve as an improved diagnostic tool for cGVHD in prospective clinical trials.

ACKNOWLEDGMENTS

Financial disclosure: This work was supported by a CIHR project grant (to K.R.S.), a Vanier Canada Graduate Scholarship (to J.R.), a project grant from the Regensburg Institute of Interventional Immunology (to D.W.) and grant CA163438 from the National Cancer Institute (to S.J.L.).

Conflict of interest statement: There are no conflicts of interest to report.

Authorship statement: J.R. designed and performed research, analyzed and interpreted data, and wrote the manuscript; A.K. performed research, analyzed and interpreted data, and wrote the manuscript; S.A. performed research; B.S. analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; P.J.M., S.J.L., D.W., M.A., and C.C. collected data and wrote the manuscript; and K.R.S. designed the research, analyzed and interpreted data, and wrote the manuscript.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.11.007.

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