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Double-Negative T Cell Levels Correlate with Chronic Graft-versus-Host Disease Severity

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Chronic graft-versus-host disease (cGVHD) is a major complication, affecting 50% to 80% of long-term survivors of allogeneic hematopoietic stem cell transplantation. Current cGVHD therapies are neither specific nor curative, and patients are typically maintained for several months to years under immunosuppressive regimens that are associated with important side effects and increased susceptibility to life-threatening infections. As a result, continued investigation into the pathology of the disease and the search for novel diagnostic and therapeutic strategies to treat cGVHD remains a high priority. We report that the cellular dynamics of various immune cell subsets are related to cGVHD onset and severity in a cohort of allogeneic hematopoietic stem cell transplantation recipients. We document a decrease in the proportion of $CD45RO^+$ $CD4^-CD8^-$ (double-negative [DN]) T cells at the onset of cGVHD, a time at which serum levels of B cell activating factor and B cells are increased. We also find that DN T cell levels are correlated with cGVHD severity. Our present findings are in line with the view that activated DN T cells exhibit their immunoregulatory potential by eliminating B cells in vivo. Taken together, these findings suggest that maintaining elevated DN T cell numbers before the onset of cGVHD may prevent pathological B cell responses.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can eradicate several blood cancers that are otherwise incurable by chemotherapy alone, due in large part to the graft-versus-tumor effect [1]. Despite indisputable successes due to the graft-versus-tumour effect, the efficacy of allo-HSCT is hampered by cancer recurrence and the development of graft-versus-host disease (GVHD) [2,3]. Notably, reduced-intensity conditioning in lieu of myeloablative conditioning has improved patient survival following allo-HSCT by attenuating the acute toxic effects [4]. However, a corollary of this has been an increase in the number of patients at risk for the development of GVHD. GVHD is divided into acute (aGVHD) and chronic (cGVHD) forms, with cGVHD affecting 50% to 80% of long-term survivors following allo-HSCT [5]. cGVHD is classified based on the severity of symptoms as well as the tissues affected [6].

Chronic tissue inflammation can ultimately lead to irreversible fibrosis and organ insufficiency. In addition, current cGVHD therapies, which rely heavily on nonspecific immunosuppression, are neither specific nor curative. Patients are typically maintained for an extended period on immunosuppressive regimens, which are associated with significant side effects and predispose to life-threatening infections. Consequently, cGVHD remains a substantial cause of morbidity and mortality in allo-HSCT recipients [7-9].

Whereas aGVHD is triggered by direct T cell recognition of histocompatibility antigens, the pathophysiology of cGVHD is reminiscent of classical autoimmune disorders, involving both alloreactive T cells and B cells [10-15]. More specifically, B cells participate in antigen presentation to T cells, germinal center formation, and alloantibody and autoantibody production, all of which contribute to disease pathophysiology [10-12]. As a result, both T cell- and B cell-targeted therapies have been used to treat cGVHD. Indeed, T cell-depleted stem cell transplantation, as well as the addition of antithymocyte globulins

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to conventional GVHD prophylaxis before transplantation, are associated with decreased risk of cGVHD [16,17]. Moreover, clinical trials have demonstrated that B cell elimination using rituximab, an anti-CD20 monoclonal antibody, improves the outcome of cGVHD in steroid-refractory patients [11,18,19]. However, plasmocytes and activated B cells present within the affected tissue are known to down-regulate CD20 and to become refractory to rituximab depletion [20]. Thus, alternative therapeutic approaches are needed.

In recent studies, B cell activating factor (BAFF) plasma levels were also correlated with the development and severity of cGVHD [11,21-23]. BAFF, a member of the tumor necrosis factor family, is involved in the survival and maturation of peripheral B cells [24]. However, excess production of BAFF is correlated with the survival of autoreactive and alloreactive B cells and the loss of peripheral tolerance [25,26], which is associated with antibody-mediated autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and Sjögren's syndrome [27-29]. Consequently, an interest in BAFF-targeted therapies for the treatment of various autoimmune diseases and cGVHD has emerged [14,30-32].

Results from several studies have suggested that immunoregulatory immune cells play important roles in cGVHD prevention, as the alloreactivity of newly transplanted T and B cells toward host antigens is counterbalanced by mechanisms of immune tolerance. Indeed, both $CD4^+CD25^+FoxP3^+$ T regulatory cells (Tregs) and natural killer T (NKT) cells have been suggested as major contributors of allotolerance following allo-HSCT [33-36], and a reduction in Treg frequency has been reported in cGVHD [37,38]. Other regulatory cells besides Tregs and NKT cells have been defined. Our group has a longstanding interest in a distinct subset of regulatory T cells, $CD4^-CD8^-TCR\alpha\beta^+$ double-negative (DN) T cells [39-42]. DN T cells compose 1% to 3% of the peripheral T cell population and inhibit immune responses in an antigen-specific manner [42-49]. Specifically, we and others have shown that DN T cells target B cells [39,42]. Moreover, infusion of DN T cells reduces autoantibody levels and decreases the onset of type 1 diabetes in mice [40,50]. Considering that DN T cells demonstrate an immunoregulatory potential toward B cells and that B cells are a relevant target for treating cGVHD, we aimed to examine the cellular dynamics of various immune cell subsets, including DN T cells, in relation to cGVHD onset and severity in a cohort of allo-HSCT recipients.

PATIENTS AND METHODS

Study Population

Serial blood samples for analysis of BAFF levels and immune cell frequencies were obtained from patients undergoing allo-HSCT at Maisonneuve-Rosemont Hospital between 2011 and 2014. Written informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Maisonneuve-Rosemont Hospital Ethics Committee. cGVHD status at time of sample collection was determined according to documented clinical examination and laboratory testing in accordance with the Maisonneuve-Rosemont Hospital Criteria and National Institutes of Health cGVHD consensus criteria [6]. The date of cGVHD diagnosis was defined as the time when these consensus criteria were first met. Clinical characteristics of the 29 patients with plasma BAFF data and the 43 patients with immune cell population data (26 of whom are also included in the BAFF analysis) are presented in Table 1. Our analysis included samples collected at the diagnosis of cGVHD and at 90 ± 30 days before and after diagnosis.

Flow Cytometry Analysis of Peripheral Blood Mononuclear Cells

Patient blood was collected into EDTA-containing tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation (Ficoll-Hypaque; Amersham Pharmacia Biotech, Piscataway, NJ) and resuspended at a concentration of 10^6 cells/mL in X-VIVO 15 medium without phenol red (BioWhittaker; Lonza, Basel Switzerland), supplemented with 2.5% heat-inactivated fetal bovine serum (HyClone, ThermoFisher Scientific, USA). Fluorochrome-conjugated monoclonal antibodies specific for TCRαβ (FITC), CD4 (PerCP-Cy5.5), CD8 (APC-H7), CD19 (V450), CD45RA (V450), and CD45RO (PE) (BD Biosciences, San Jose, CA) were used to stain PBMCs for various immune cell populations. A representative gating strategy for DN T cells is shown in Supplementary Figure 1. All data were collected using an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Table 1
Clinical Characteristics of the Study Population

Characteristics	Flow Cytometry (N = 43)	BAFF Level (N = 29)
Patients		
Age, yr, median (range)	51 (24-63)	51 (24-65)
Female sex, n (%)	13 (30)	8 (28)
Male sex	30 (70)	21 (72)
Conditioning regimen, n (%)		
Myeloablative	35 (81)	23 (79)
Reduced intensity	8 (19)	6 (21)
Stem cell source, n (%)		
Bone marrow	8 (19)	6 (21)
Peripheral blood	35 (81)	23 (79)
Disease, n (%)		
Acute myelogenous leukemia	13 (30)	11 (38)
Chronic myelogenous leukemia	1 (2)	1 (3)
Acute lymphoblastic leukemia	5 (12)	2 (7)
Chronic lymphocytic leukemia	2 (5)	2 (7)
Multiple myeloma	3 (7)	3 (10)
Myelodysplastic syndrome	9 (21)	6 (21)
Non-Hodgkin lymphoma	8 (19)	4 (14)
Acute leukemia (unclassified)	1 (2)	0
Aplastic anemia	1 (2)	0
GVHD prophylaxis, n (%)		
MMF/CsA	8 (19)	5 (17)
MMF/tacrolimus	6 (14)	5 (17)
MTX/CsA	25 (58)	16 (55)
MTX/tacrolimus	1 (2)	1 (3)
MTX/CsA/ATG	3 (7)	2 (7)
Interval from transplantation to cGVHD diagnosis, d, median (range)	175 (105-420)	154 (99-322)
cGVHD severity, n (%)		
None	9 (21)	0
Mild	4 (9)	4 (14)
Moderate	20 (47)	18 (62)
Severe	10 (23)	7 (24)
cGVHD onset presentation, n (%)		
None	9 (21)	0
De novo	16 (37)	17 (59)
Quiescent	16 (37)	11 (38)
Progressive	2 (5)	1 (3)

MMF indicates mycophenolate mofetil; CsA, cyclosporin A; MTX, methotrexate; ATG, antithymocyte globulin.

Processing of Patient Plasma

Blood was drawn into standard EDTA-containing collection tubes. Plasma was separated from whole blood cells by centrifugation at 600 × *g* and then stored in aliquots at -80°C. Plasma was then used for BAFF measurements after a first thaw.

BAFF ELISA

Soluble BAFF in patient plasma samples was measured with a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommended procedures.

Statistical Methods

The Wilcoxon matched-pairs signed-rank statistical test was applied where appropriate, and linear regression analysis was used to investigate association between the severity of GVHD and the frequency of immune cell subsets. A P value < 0.05 was considered statistically significant.

RESULTS

We first examined prospectively the changes in plasma BAFF levels and immune cell subset frequencies in a cohort of patients with hematologic malignancies who underwent allo-HSCT. Plasma was collected from patients during their routine

monthly visits following allo-HSCT beginning at 30 days post-transplantation, and PBMCs were collected monthly up to the third month and then every second month thereafter. Clinical characteristics of these patients are presented in Table 1. Notably, the demographic data are similar in the patients included in the flow cytometry analysis and those included in the BAFF analysis. The patients included in the study cohort in which the frequency of immune cell subsets (n = 43) and plasma BAFF levels (n = 29) were analyzed were predominately male (70% for flow cytometry and 72% for and BAFF level) and had a median age of 51 years (range, 24 to 63 years and 24 to 65 years, respectively). The majority of patients received a myeloablative conditioning (MAC) regimen (81% and 79%, respectively), and the others received reduced-intensity conditioning (RIC; 19% and 21%, respectively). Patients received granulocyte colony stimulating factor-mobilized peripheral blood (81% and 79%, respectively) or bone marrow (19% and 21%, respectively) stem cells (Table 1). In terms of cGVHD severity, patients were categorized as mild (4 patients in both the flow cytometry and BAFF groups), moderate (20 and 18 patients, respectively), or severe (10 and 7 patients, respectively). Although very few patients developed the progressive form of cGVHD with persistent acute clinical features (2 patients and 1 patient), the majority of patients were classified with de novo (16 and 17 patients, respectively) or quiescent (16 and 11 patients, respectively) cGVHD (Table 1).

Pathogenic T cells are major participants in the pathophysiology of cGVHD [16,51]. We analyzed the proportion of conventional T cell subsets at 3 distinct timepoints—3 months before diagnosis, at the time of diagnosis, and 3 months after diagnosis—and found that among total T cells, the number of $CD4^+$ T cells ($CD4^+CD8^-CD3^+TCR_{\alpha\beta}^+$) decreased (Figure 1A), whereas the number of $CD8^+$ T cells ($CD4^-CD8^+CD3^+TCR_{\alpha\beta}^+$) increased (Figure 1B), before the onset of cGVHD. Absolute lymphocyte numbers showed a similar trend (Supplementary Table 1). These findings may be explained by the dynamics of T cell reconstitution following allo-HSCT; previous reports have shown that $CD8^+$ T cell numbers recover rapidly after transplantation, whereas $CD4^+$ T cell reconstitution is significantly slower [2,52,53].

B cells also play important roles in the immune reaction leading to cGVHD [10-12]. Interestingly, BAFF plasma levels have been shown to correlate with cGVHD development [22,54], with excess BAFF promoting survival of self-reactive B cells [25,26]. Consequently, we also analyzed B cells and BAFF plasma levels. Our longitudinal analysis of B cells revealed a significant increase in B cells ($CD19^+CD3^-$) among circulating mononuclear cells at the time of cGVHD diagnosis

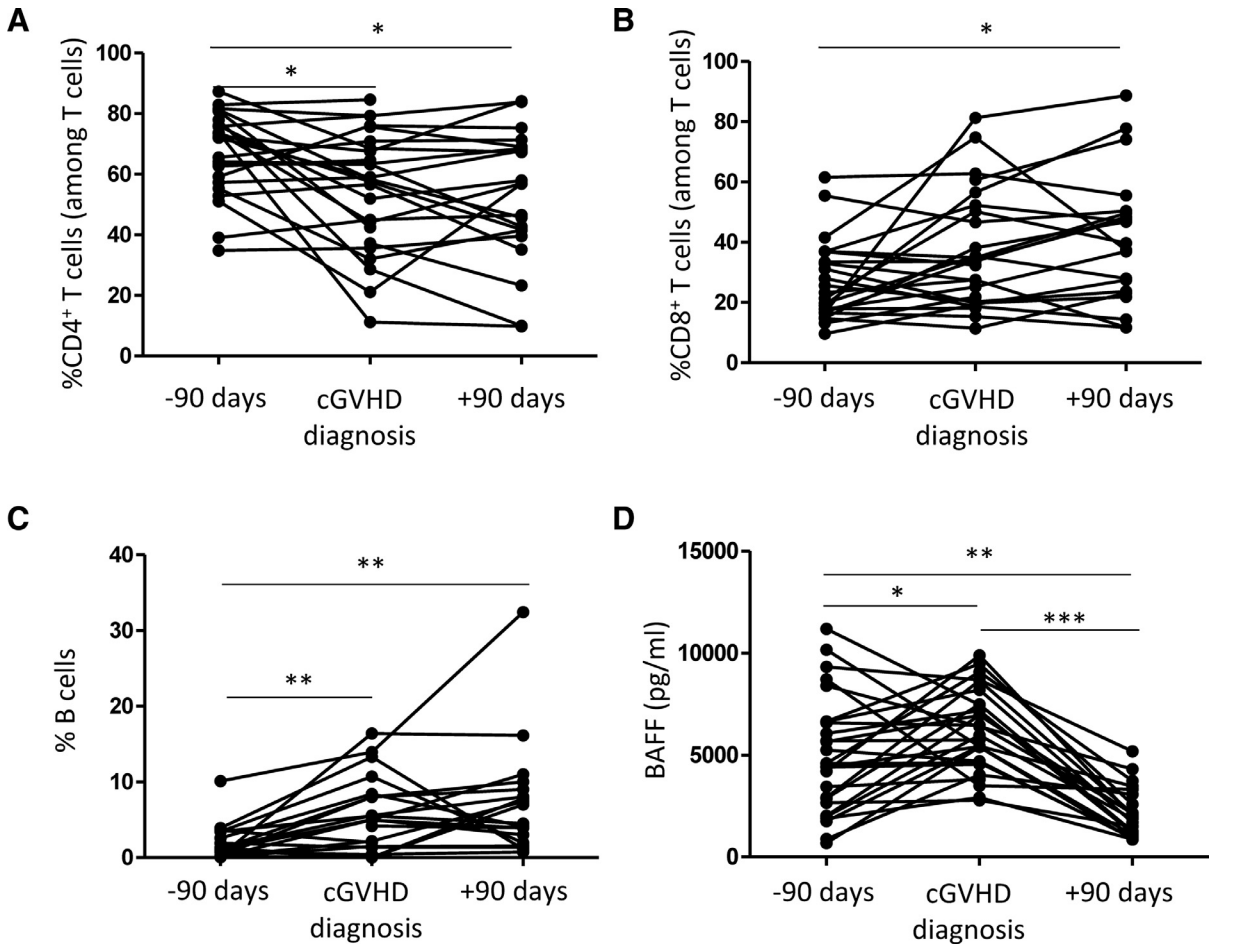

Figure 1. Longitudinal assessment of the frequency of conventional T cell subsets, B cells, and plasma BAFF levels before, at, and following cGVHD diagnosis. Shown are $CD4^+$ T cell ($CD4^+CD8^-TCR\alpha\beta^+$) (A) and $CD8^+$ T cell ($CD4^-CD8^+TCR\alpha\beta^+$) (B) frequency among total T cells, B cell frequency ($CD19^+CD3^-$) (C), and plasma BAFF concentration (D) in allo-HSCT recipients at 90 days before (n = 24, 24, 20, and 29, respectively), at the time of (n = 26, 26, 22, and 29, respectively), and 90 days after (n = 21, 21, 19, and 22, respectively) the diagnosis of cGVHD with the number of samples available at each time point. The lines link data points from a given patient. See Supplementary Table 1 for lymphocyte numbers and data for each patient. $^*P < .05$; $^{**}P \leq .01$; $^{***}P \leq .001$.

(Figure 1C, Supplementary Table 1). In parallel, BAFF plasma levels were significantly increased at the onset of clinical cGVHD (Figure 1D). Interestingly, BAFF plasma levels decreased significantly by 3 months post-diagnosis (Figure 1D). These findings are in agreement with previous studies reporting similar increases in both B cell counts and BAFF levels at cGVHD diagnosis, followed by a decrease in BAFF plasma levels [22,54,55].

DN T cells have been shown to regulate B cell activity in various mouse models [40,41,50]; however, this T cell subset has not yet been investigated in the context of cGVHD. Consequently, we performed a longitudinal assessment of DN T cells ($CD4^-CD8^-CD3^+TCR_{\alpha\beta}^+$) in our cohort. We found a significantly lower proportion of antigen-experienced DN T cells (ie, $CD45RO^+CD45RA^-$) at the time of cGVHD diagnosis (Figure 2A). Moreover, at 3 months post-diagnosis, the proportion of $CD45RO^+$ DN T cells rose significantly from the level at the time of diagnosis (Figure 2A). Of note, we did not observe any significant differences in the proportions of $CD45RO^+CD4^+$ T cells or $CD8^+$ T cells during the longitudinal assessment (Figure 2B and C, Supplementary Table 2). Our results suggest an inverse correlation between the proportion of antigen-experienced DN T cells and both the expansion of B cells and associated BAFF plasma levels.

To investigate whether elevated proportions of antigen-experienced DN T cells can help predict cGVHD severity, we assessed $CD45RO^+$ DN T cells before the onset of clinical manifestations of cGVHD in patient blood samples. We analyzed $CD45RO^+$ DN T cells at 3 months post-transplantation, a time point preceding the onset of cGVHD. We found that patients who eventually developed severe cGVHD presented with a significantly lower ($P = .04$) percentage of $CD45RO^+$ DN T cells at 3 months post-transplantation compared with those who did not develop the disease (Figure 3A, Supplementary Table 3). Moreover, we noted an inverse relationship between $CD45RO^+$ DN T cells and cGVHD severity (none, mild, moderate, or severe) (Figure 3A).

Because our longitudinal analysis of $CD45RO^+$ DN T cells and B cells revealed an inverse correlation, we next compared B cells with the severity of cGVHD. Interestingly, we found a positive linear relationship between B cell proportion and cGVHD severity (Figure 3B). Taken together, these findings suggest that low levels of $CD45RO^+$ DN T cells, in addition to elevated B cell and BAFF plasma levels, are correlated with a diagnosis of cGVHD and may potentially serve as a biomarkers of cGVHD severity.

DISCUSSION

cGVHD is a common and morbid complication of allo-HSCT that manifests as a result of a highly complex immune pathology involving B cells, T cells, and other cells [56]. Immunoregulatory cells are believed to play an important role in cGVHD prevention by inhibiting the alloreactivity of newly transplanted T and B cells toward host antigens. In the present study, we performed a longitudinal assessment of various immune cell populations, including DN T cells, as well as of BAFF levels in the context of cGVHD. To our best knowledge, this study is the first to investigate the potential role of DN T cells in the development of cGVHD. We found a lower proportion of antigen-experienced DN T cells along with elevated B cell counts and plasma BAFF levels at the time of cGVHD diagnosis. Note that we focused on the proportion of immune cells rather than on absolute numbers, acknowledging that the proportion of immunoregulatory cells relative to other immune cells is more predictive of regulatory activity [57]. Moreover, we found that the proportion of antigen-experienced DN T cells at 3 months post-transplantation (thus before the onset of cGVHD) correlated with the severity of cGVHD, such that a higher frequency of $CD45RO^+$ DN T cells was seen in patients who did not develop the disease. Taken together, our findings suggest

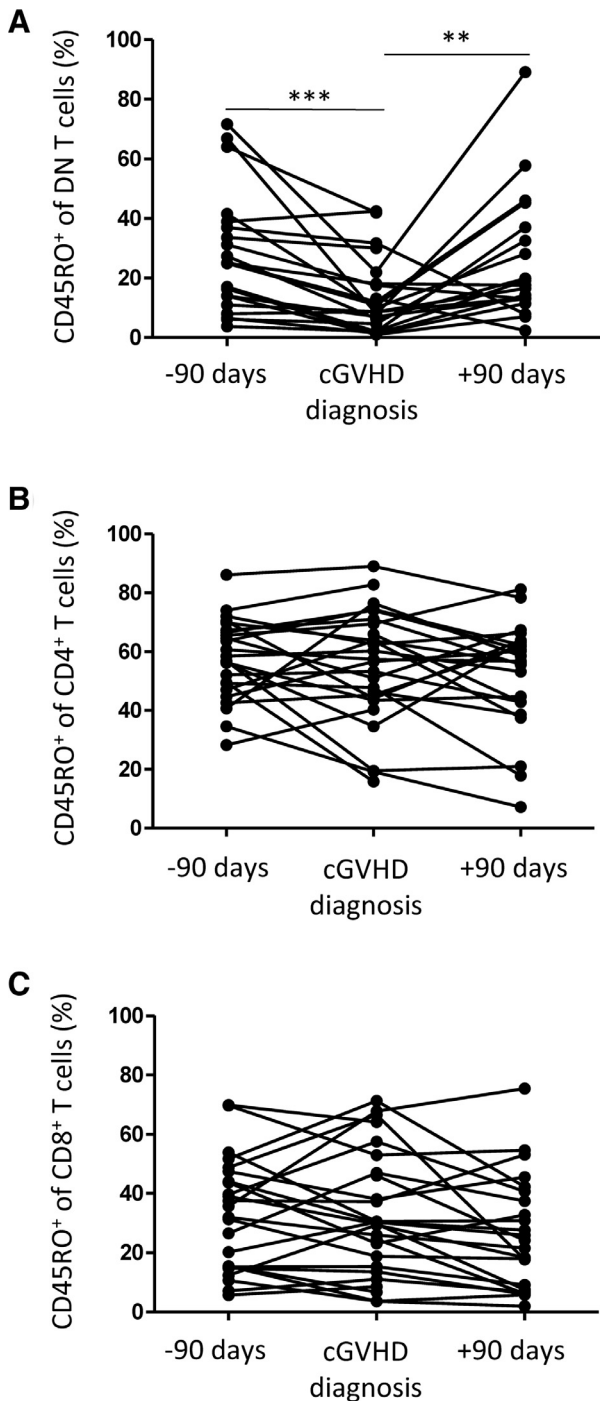

Figure 2. Longitudinal assessment of the frequency of CD45RO-expressing T cell subsets before, at, and following cGVHD diagnosis. Shown are CD45RO-expressing ($CD45RO^+CD45RA^-$) DN T cells ($CD4^-CD8^-TCR\alpha\beta^+$; gating strategy shown in Supplementary Figure 1) (A), $CD4^+$ T cells ($CD4^+CD8^-TCR\alpha\beta^+$) (B), and $CD8^+$ T cells ($CD4^-CD8^+TCR\alpha\beta^+$) (C) in allo-HSCT recipients 90 days before, at the time of, and 90 days after the diagnosis of cGVHD, with n = 21, 23, and 19 (A) and n = 24, 26, and 21 (B and C) at each time point, respectively. The lines link data points from a given patient. See Supplementary Table 2 for lymphocyte numbers and data for each patient. $^{**}P \leq .01$; $^{***}P \leq .001$.

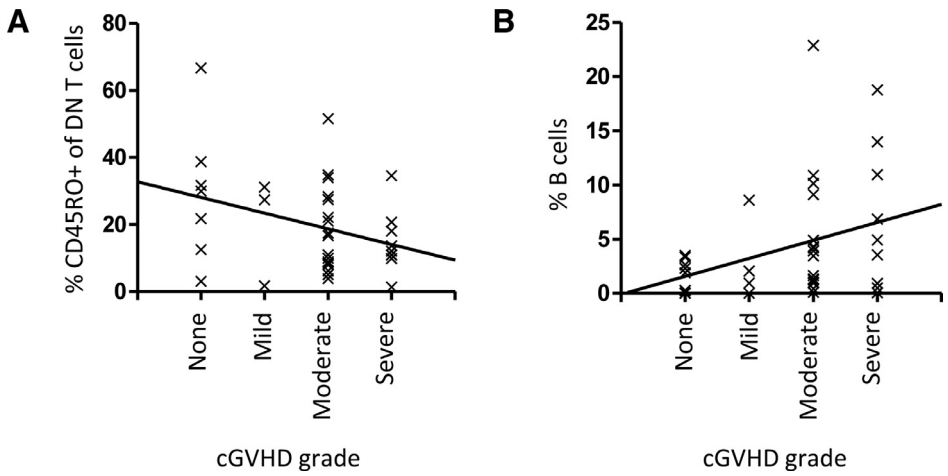

Figure 3. Correlation of CD45RO$^+$ DN T cells and B cells with cGVHD severity. (A) Percentage of CD45RO-expressing DN T cells among total DN T cells plotted against cGVHD severity, revealing an inverse correlation (n = 34; $P = .013$; $r^2 = .1776$). (B) Percentage of B cells plotted against cGVHD severity, revealing a positive correlation (n = 38; P = .035; $r^2 = .1171$). See Supplementary Table 3 for absolute numbers and data for each patient.

that CD45RO$^+$ DN T cells are negatively correlated with B cell frequency and BAFF level, known markers of cGVHD severity [11,21-23].

Interestingly, DN T cells also play protective roles in aGVHD [41], which is triggered by direct T cell recognition of histocompatibility antigens. Indeed, infusion of murine MHC-mismatched DN T cells or DN T cell clones was found to promote allograft tolerance without inducing GVHD [58-61] and to reduce the severity of aGVHD [60]. Studies in humans also have reported that a deficiency in DN T cell frequency is associated with an increased risk of aGVHD [62]. Furthermore, the proportion of DN T cells is inversely correlated with aGVHD severity [53,62]. These data are in line with the broad antigen-specific immunoregulatory role of DN T cells in the context of aGVHD, where they cause immune suppression, likely by dampening pathogenic T cell responses [42]. However, until the present study, the dynamics of DN T cell frequency and its impact on cGVHD had not been investigated.

The pathogenesis of cGVHD is multifactorial and is mediated by several immune cell types, including B cells, which can participate in pathogenic antibody secretion and antigen presentation associated with disease pathophysiology [11,63]. Here we found low levels of CD45RO$^+$ DN T cells and elevated levels of both B cells and BAFF at the time of cGVHD diagnosis. Interestingly, we and others have shown that DN T cells exhibit immunoregulatory potential by eliminating autoreactive activated B cells [39,50,64]. Indeed, in mouse models, activated DN T cells target pathogenic B cells in an antigen-specific manner [39,50]. Moreover, the adoptive transfer of DN T cells results in a significant antigen-specific reduction in antibody levels in both a mouse model of type 1 diabetes and in rat heart xenotransplantation [40,50]. Taken together, these data point to a prominent immunoregulatory function of DN T cells, where the antigen-specific elimination of activated B cells results in decreased antibody levels. Consequently, the injection of DN T cells in patients could prove to be a potentially relevant therapeutic approach for cGVHD.

Based on findings in rodents, we propose that the inverse relationship between antigen-experienced DN T cells and both B cell and BAFF levels at the time of cGVHD diagnosis may be related to the immunoregulatory function of DN T cells. Indeed, as B cells and antibodies contribute to cGVHD [11,63], the antigen-specific elimination of activated B cells by DN T cells could lead to a decrease in disease severity. This is in line with our documentation of a negative correlation between the frequency of CD45RO$^+$ DN T cells and cGVHD severity. Thus, our findings suggest that antigen-experienced DN T cells could eliminate pathogenic B cells, thereby contributing to both decreased cGVHD severity and onset. This result will need to be replicated in independent cohorts to determine the potential use of CD45RO$^+$ DN T cells in predicting cGVHD severity. Future cohorts should include a larger number of patients to allow assessment of potential confounding factors, such as medication, influencing immune cell proportion. In addition, it would be of interest to validate the immunoregulatory function of DN T cells toward activated B cells in CD45RO$^+$ DN T cells isolated from allo-HSCT recipients and patients with cGVHD.

We observed elevated levels of both B cells and BAFF and low levels of CD45RO$^+$ DN T cells at the time of cGVHD diagnosis, which reverted to low levels of both B cells and BAFF and elevated levels of CD45RO$^+$ DN T cells by 90 days post-diagnosis. This finding suggests that cGVHD treatment alleviates the immune-inflammatory setting, allowing restoration of immune homeostasis. Alternatively, it may suggest that at the time of cGVHD diagnosis, antigen-experienced DN T cells migrate from the blood to the site of inflammation (ie, the affected tissues) to mediate their immunoregulatory function. At this site, they eliminate self-reactive B cells, allowing for restoration of immune homeostasis. Although this matter remains elusive, mouse models or analysis of biopsy specimens of the affected tissues collected in future patient cohorts would help unravel this matter.

Taken together, our data suggest links among increasing BAFF levels, growing B cell frequency, and decreasing antigen-experienced DN T cell proportion leading up to the onset of disease in cGVHD patients. Moreover, our correlative analyses reveal that DN T cell proportion, in combination with other biomarkers, may improve sensitivity for predicting disease severity. As a result, we will continue to assess the correlation between CD45RO$^+$ DN T cells and cGVHD severity in all future prospective allo-HSCT cohorts. Given that DN T cells target pathogenic B cells and lead to the decrease in antibody levels in vivo [39,50,64], our findings suggest that DN T cells merit further investigation in the context of cGVHD pathology, both to improve biomarker sensitivity and as a potential therapeutic approach.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online https://doi.org/10.1016/j.bbmt.2018.09.008.

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