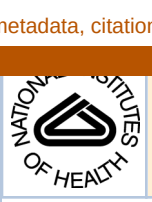

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Bone Marrow B cell Precursor Number after Allogeneic Stem Cell Transplantation and GVHD Development

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Abstract

Patients without chronic graft-versus-host disease (cGVHD) have robust B cell reconstitution and are able to maintain B cell homeostasis after allogeneic hematopoietic stem cell transplantation (HSCT). To determine whether B lymphopoiesis differs before cGVHD develops, we examined bone marrow (BM) biopsies for terminal deoxynucleotidyl transferase (TdT) and PAX5 immunostaining early post-HSCTat day 30 when all patients have been shown to have high B cell activating factor (BAFF) levels. We found significantly greater numbers of BM B cell precursors in patients who did not develop cGVHD compared with those who developed cGVHD (median = 44 vs 2 cells/high powered field [hpf]; respectively; $P < .001$). Importantly, a significant increase in precursor B cells was maintained when patients receiving high-dose steroid therapy were excluded (median = 49 vs 20 cells/hpf; $P = .017$). Thus, we demonstrate the association of BM B cell production capacity in human GVHD development. Increased BM precursor B cell number may serve to predict good clinical outcome after HSCT.

Keywords

Immunology; Graft-versus-host disease; B-cell development; Autoimmunity

INTRODUCTION

In the absence of B cell activating factor (BAFF), normal B cell homeostasis in murine models is not possible [1]. Early after hematopoietic stem cell transplantation (HSCT), patients have high BAFF levels, suggesting its vital role in human B cell reconstitution [2]. Patients who have undergone HSCT have decreased total B cells, including memory B cells, associated with immune deficiency and increased infection [3–6]. Studies of excess BAFF in

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chronic graft-versushost disease (cGVHD) revealed that a previously described supernormal "surge" in naïve B cell number after HSCT [7–9] accounted for significantly lower BAFF/B cell ratios and decreased B cell auto-reactivity [2, 10]. Thus, akin to what has been demonstrated in mouse models of autoimmunity, a peripheral naïve B cell compartment is critical for the maintenance of B cell tolerance [11–13]. Furthermore, only those patients with cGVHD able to robustly recover naïve B cell compartments after targeted B cell depletion demonstrate clinical improvement [10, 14]. Whether recovery of B cell homeostasis after HSCT is due to B lymphopoiesis in the bone marrow (BM) remains unclear [15, 16].

Increased BM precursor B cell numbers have been found after HSCT [17], but examinations of BM aspirates in GVHD have been inconclusive [15]. Decreased frequency of B cell precursors in BM aspirates using flow cytometry before, during, and after development of acute GVHD (aGVHD) and cGVHD have been observed, but given the variability of sample cellularity and composition, these studies are difficult to interpret [15, 18, 19]. Additionally, in a murine model, the hematopoietic niche that was vital for B lymphopoiesis was altered when GVHD was present, further suggesting that examination of BM core biopsies from patients before GVHD development was warranted [20].Wereport a B cell-specific BM difference after HSCT that will instruct future studies of GVHD pathophysiology. If further studies confirm our findings, increased BM precursor B cell number may serve as a predictive marker of good clinical outcome after HSCT.

MATERIALS AND METHODS

BM samples were collected on a protocol approved by the Institutional Review Board at The University of North Carolina. From January 2010 to March 2011, we identified 30 patients actively being followed in the BM Transplant Clinic who were at least 12 months post-peripheral blood allogeneic HSCT (median = 29 months), had no evidence of active or relapsing hematologic malignancy, and for whom routine "day 30" post-transplant biopsies were available for evaluation. No patients receiving BM grafts were identified in this group. Five hundred cell differential counts were performed on Wright-Geimsa stained BM aspirate smears, and the corresponding hematoxylin and eosin stained, formalin fixed, and paraffin-embedded biopsy sections were reviewed for marrow cellularity and distribution of hematopoietic elements. To identify precursor B cells, tissue sections were stained by immunohisto-chemistry for terminal deoxynucleotidyl transferase (TdT), a marker of primarily early lymphoid progenitors, PAX5, a B cell lineage specific antigen, and CD3, a pan-T cell marker (all antibodies: Leica-Microsystems, Wetzler, Germany). Positive-staining cells were enumerated based on a method previously published [17]. Briefly, after antibody validation, two independent observers blinded to clinical history counted the number of positive-stained cells per 600× high powered field (hpf) in areas representative of BM cellularity and distribution. The frequency of positive-stained cells per 500-total nucleated cells was also determined. Eleven patients with untreated lymphoma without marrow involvement or other abnormalities and who had not undergone HSCT ("No HSCT") served as reference samples. Descriptive statistics are reported as medians, with ranges or percentages where appropriate. Comparisons between groups were made for continuous variables using Wilcoxon rank sum tests and Fisher Exact tests for categorical variables.

RESULTS

Thirty patients being actively followed in the clinic had been followed for a median of 898 days after HSCT. Fifteen of the 30 patients developed cGVHD (a median of 233 days [range, 127–799 days] after HSCT), and 15 never developed cGVHD (a median follow-up

of 853 days [range, 370–2051 days] after HSCT). A thorough secondary pathology review was performed to confirm cancer remission in all patients. With respect to the 8 cases of B cell lymphoma/leukemia, all of 8 patients had confirmation of normal and/or donor karyotype by cytogenetic analysis and 6 of 8 patients underwent flow cytometry of BM aspirate to confirm the B cells measured by PAX5 were not due to relapsing disease. Clinical characteristics are presented in Table 1. All patients were receiving tacrolimus or tacrolimus plus “mini-dose” methotrexate; some also received anti-thymocyte globulin (ATG) or alemtuzumab for aGVHD prophylaxis. Five of the study patients were treated with alemtuzumab before, or at the time of BM evaluation. These patients are distributed approximately equally in all groups analyzed (3 without cGVHD, 2 with cGVHD). Although the numbers of treated individuals is not sufficient to draw independent and significant conclusions with respect to alemtuzumab effect, the median number of TdT + cells per hpf was 42 with a range of 2 to 62 that was notably not different from the patients not treated with alemtuzumab (P =.60). Nine of the patients who developed cGVHD and 3 of the patients who never developed cGVHD were receiving high-dose steroids for aGVHDat the time of BM evaluation (median days of steroid therapy before biopsy = 12.5 vs 11, respectively). All patients who developed cGVHD had at least grade II aGVHD, but importantly, at day 30, there were 6 patients who later developed cGVHD, but at day 30, BM biopsy showed no evidence of aGVHD and the patients were not receiving steroids. “Day 30” posttransplant peripheral blood and BM findings are summarized in Table 2. Unfractionated and $CD3^+$ T cell donor chimerism and overall BM cellularity were not different between cGVHD and no cGVHD groups. The median time to peripheral neutrophil engraftment was not different, and the day 30 peripheral blood lymphocyte, monocyte, and platelet counts did not differ significantly between cGVHD and the steroid-treated groups. The total peripheral WBC count was higher in the cGVHD group, owing primarily to the increased neutrophil counts (median = 5.6×10^3/μL vs 3.5×10^3/μL; P =.02). However, the difference was not significant after exclusion of steroid therapy (WBC = 6.5×10^3/μL vs 4.8×10^3/μL; P =.40; neutrophils =4.9×10^3/μL vs 3.3×10^3/μL; P =.20). Total marrow cellularity and cellular composition in associated BM aspirates did not differ between patients who developed or did not develop cGVHD. Thus, cells in the BM aspirates at day 30 did not differ significantly in patients who later developed cGVHD.

Whereas manual aspirate differential counts irrespective of concurrent steroid therapy were not different, B and T cell-specific markers, along with TdT staining, highlighted significant differences in the absolute numbers of precursor B cells (Figure 1A). Although TdT expression is not lineage-specific, B cell precursors represent the vast majority of the TdT^+ pool in the BM [21], and analysis of sequential sections also demonstrated correlation of PAX5 and TdT staining distribution. TdT^+ cells were increased in all patients early after HSCT. Consistent with previous analyses of healthy BM, the reference group had similarly rare lymphoid precursors [17]. Notably, patients who did not develop cGVHD had significantly higher precursor B cell numbers relative to patients who later developed cGVHD (median = 44 vs 2 cells/hpf; P =.0007). Patients with prior receipt of ATG or alemtuzemab for aGVHD prophylaxis were equally distributed between the no cGVHD and cGVHD groups (Table 1) and these patients did not have lower precursor B cell numbers compared to untreated patients (data not shown). Patients receiving high-dose steroids at the time of biopsy had low absolute numbers of precursor B cells (Figure 1B), likely due to steroid-induced apoptosis [22]. Six of the 15 patients with cGVHD, shown in the ‘no steroid’ portion of Figure 1B, had no a GVHD or steroid treatment before the BM biopsy. Importantly, the group with a significantly decreased B cell precursor number without aGVHD or steroid treatment at the time of analysis (n = 6), later developed cGVHD (Figure 1B). Patients who never developed cGVHD had significantly higher BM precursor B cell numbers compared with those who later developed cGVHD(median = 49 vs 20 cells/hpf; P

=.0170; Figure 1B). Thus, the presence of B cell precursors early after HSCT correlated with decreased incidence of GVHD after HSCT.

A similar pattern of PAX5 to TdT staining was found in patients who underwent HSCT (Figure 1C). Patients who did not develop cGVHD, had higher numbers of B cells relative to those who developed cGVHD, but no significant differences in total B cell numbers were appreciated after accounting for steroid effect (Figure 1D). The absolute numbers of $CD3^+$ T cells was not different with respect to future cGVHD status (Figure 1E). Exclusion of steroid therapy had no impact on BM T cell numbers in either group (Figure 1F). Thus, taken together, we have found that the BM difference between patients who later develop cGVHD was B cell specific.

DISCUSSION

Having previously demonstrated the importance of B cell homeostasis and BAFF in GVHD [2, 10], we aimed to determine whether BM B lymphopoiesis contributed to cGVHD development. Because high plasma BAFF levels are found in all patients 30 days post-HSCT (at day 30), we studied the BM at this time point, when B cell recovery, not yet apparent by peripheral blood testing, was likely occurring in the BM. We found that B cell precursors were increased in patients who never developed cGVHD. Although steroid therapy had a dramatic effect on B lymphopoiesis, significant difference in precursor numbers was evident after the exclusion of steroid therapy. Thus, although steroid therapy is associated with quantifiable and dramatic suppression of B lymphopoiesis in the BM, failure to robustly produce pre-B cells in the marrow in cGVHD could not be explained by steroid effect alone. This finding is consistent with previous work showing that patients without altered B cell homeostasis avert B cell autoimmunity [2, 10].

All patients in our cohort who subsequently developed cGVHD had previous aGVHD, although aGVHD had not yet developed in 6 of these cases by the time of BM evaluation. The data from our study cannot entirely confirm the loss of the B precursor pool as an independent consequence of cGVHD alone. However, aGVHD is a risk factor for cGVHD [23–25] and our data, taken together with previous findings, suggest diminished B lymphopoiesis is related to cGVHD development. The systemic inflammatory T cell response typifying aGVHD may promote an environment ineffective for B lymphopoiesis and permissive for subsequent development of human cGVHD [26].

Because B lymphopoiesis after HSCT, in the context of active transitioning into the periphery and secondary lymphoid organs, is highly relevant to B cell autoimmunity, total BM B cells of all maturational stages, stained with PAX5, were also enumerated. Total B cell number was increased in patients who never developed cGVHD relative to those who did (median = 35 vs 8 cells/hpf; P =.008). However, as the pattern of PAX5 staining was similar compared to TdT, largely owing to the TdT^+ pool, the difference was not significant after exclusion of steroid therapy (41.5 vs 16 cells/hpf; P =.10). Together, these data corroborate a more dynamic process of B cell development and transition after HSCT. Importantly, differences in BM production were B cell specific, as the T cell number was not associated with the development of aGVHD or cGVHD and irrespective of steroid therapy.

Together, our data reveal that the difference in peripheral B cell composition found in GVHD relates to BM production capacity. Although pathologic production of BAFF is found in cGVHD [27], physiologic BAFF, as it relates to BM B lymphopoiesis early after myeloablation or B cell depletion, is crucial for B cell homeostasis [1, 9, 28]. Thus, future

research in GVHD prophylaxis and pathophysiology should focus on B lymphopoiesis and the BM niche.

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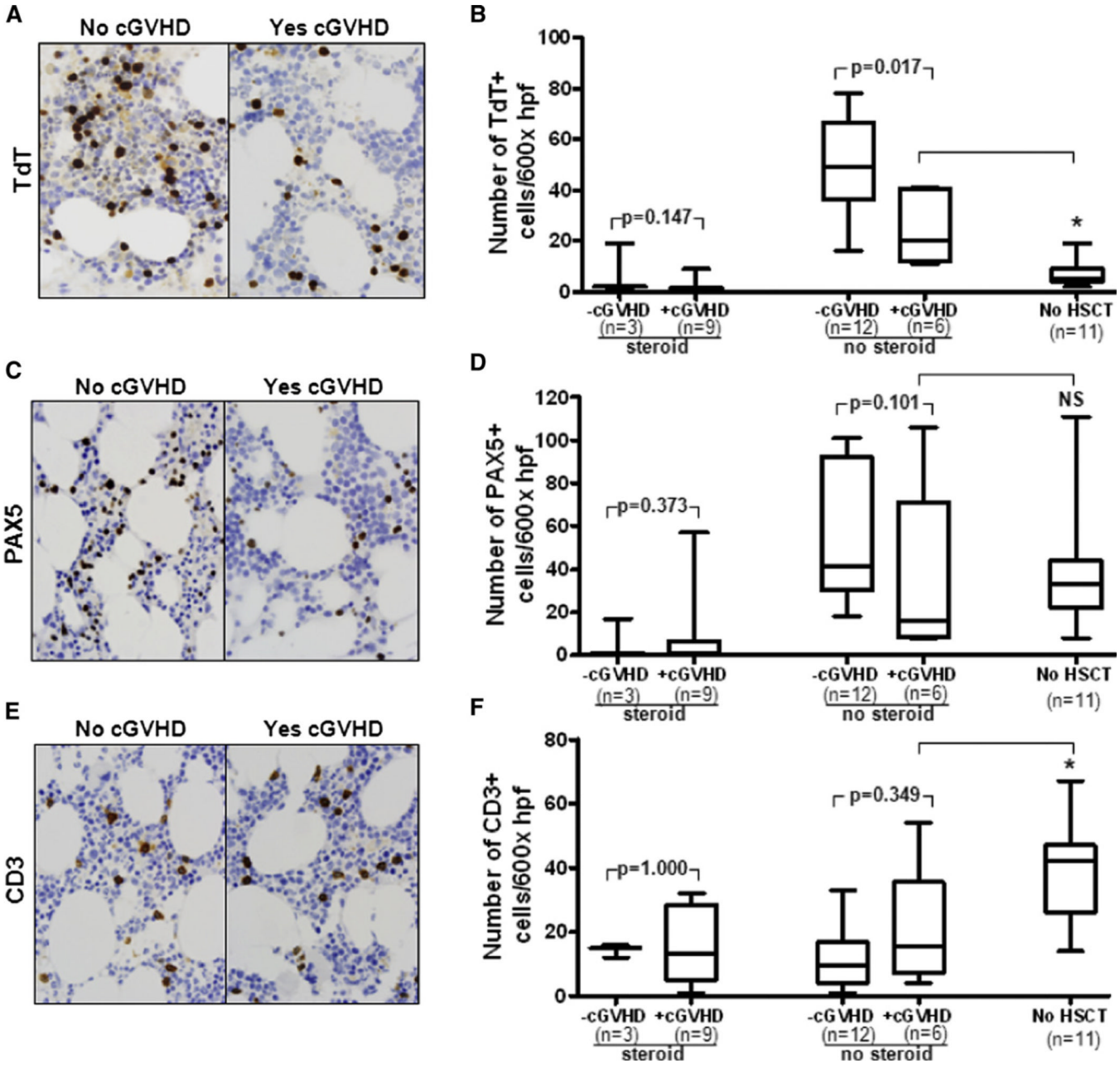

Figure 1.

Morphologic and immunohistochemical analysis of day-30 post-hematopoietic stem cell transplantation (HSCT) bone marrow (BM) biopsies in patients who never developed or who developed chronic graft-versus-host disease (cGVHD). (A) Representative micrograph of terminal deoxynucleotidyl transferase (TdT) immunohistochemical staining in patients who never developed ("No cGVHD") or who subsequently developed cGVHD ("Yes cGVHD"). (B) Absolute numbers of TdT^+ BM B cell precursors in patients who never developed (−) vs those who later developed (+) cGVHD, in relation to steroid therapy at the time of BM evaluation (+ vs − steroid). Reference BM ("No HSCT") showed significantly fewer precursors compared to the + or − cGHVD group after steroid effect was excluded (*P =.03 and P=.01, respectively). (C) Representative micrograph or PAX5 immunohistochemical staining in patients who never developed ("No cGVHD") or who

develop cGVHD ("Yes cGVHD"). (D) Total BM B cell number as determined by PAX5 staining in patients who never developed (−) vs those who developed (+) cGVHD, currently (+), or never treated with (−) steroids. Compared to the reference samples ("No HSCT"), total B cell numbers were similar between patients who had undergone HSCT; NS indicating not significant. (E) Representative micrograph of CD3 immunohistochemical staining in patients who never developed ("No cGVHD") or who develop cGVHD ("Yes cGVHD"). (F) Absolute $CD3^+$ T cell number in patients who never developed (−) or developed (+) cGVHD, either treated with (+) steroids or never treated with (−) steroids. Reference BM ("No HSCT") showed significantly more $CD3^+$ T cells compared to the + or − cGVHD group after steroid therapy was excluded (*P =.04 and P < .01, respectively). Description of patient groups: *−cGVHD (total n = 15)*: patients who never developed cGVHD including those who received steroid treatment for acute GVHD (aGVHD; n =3) and patients who were not receiving steroid treatment at the time of BM biopsy (n = 12), followed for a total median of 792 and 980 days, respectively. + *cGVHD (total n = 15)*: patients who developed GVHD (n =15) receiving high-dose steroids (≥0.5 mg/kg; n =9) vs those without steroid therapy from time of HSCT to time of BM biopsy (n = 6), followed for a total median of 630 and 1100 days, respectively. *No HSCT (total n=11)*: Age and gender-matched reference biopsies from untreated patients with non-Hodgkin lymphoma (NHL; diffuse large B cell lymphoma [n = 7]; low-grade NHL [n = 2], follicular lymphoma, grade 3 [n = 1], classic Hodgkin lymphoma [n =1]) who underwent staging BM evaluation. For all micrographs, the original magnification was 600×. Box and whisker plots express the median, 25th and 75th percentile at the ends of the box, and 5th and 95th percentile at the ends of the whiskers.

Table 1

Clinical Characteristics of Patients at Day 30 with (+) or without (−) Future cGVHD

Characteristic	−cGVHD	+cGVHD	*P* Value
No. of patients	15	15	
Median age, years (range)	56 (18–67)	39 (20–67)	.70
Sex, no. of men (%)	9 (60)	9 (60)	1
Female donor to male recipient (%)	1 (7)	2 (13)	1
Conditioning regimen (%)			
Myeloablative	6 (40)	7 (47)	1
Nonmyeloablative	9 (60)	8 (53)	
Source of graft (%) Peripheral blood	15 (100)	15 (100)	1
HLA matching (%)			
Matched, unrelated	8 (53)	8 (53)	1
Matched, related	7 (47)	7 (47)	
GVHD prophylaxis (%)			
Tac vs Tac + MTX	6 (40)	6 (40)	1
ATG	6 (40)	7 (47)	1
Alemtuzumab	3 (20)	2 (13)	1
Follow-up, days (range)	853 (370–2051)	910 (365–2178)	.90
Disease (%)			
AML	3 (20)	5 (33)	.50
AML from MDS	2 (13)	0 (0)	
MDS	1 (7)	2 (13)	
CML	1 (7)	2 (13)	
CMML	1 (7)	0 (0)	
PV	1 (7)	0 (0)	
Primary myelofibrosis	0 (0)	1 (7)	
AA	0 (0)	2 (13)	
ALL	2 (13)	1 (7)	
MCL	0 (0)	1 (7)	
DLBCL	1 (7)	0 (0)	
FL	2 (13)	0 (0)	
CLL/SLL	0 (0)	1 (7)	
Hodgkin lymphoma	1 (7)	0 (0)	
Time to neutrophil engraftment, days	15 (12–22)	14 (11–20)	.40
Grades II-IV aGVHD * (%)	2 (13)	9 (60)	<.0001

cGVHD indicates chronic graft-versus-host disease; Tac, tacrolimus; MTX, methotrexate; ATG, anti-thymocyte globulin; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; CMML, chronic myelomonocytic leukemia; PV, polycythemia vera; AA, aplastic anemia; ALL, acute lymphoblastic leukemia; MCL, mantle cell lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; aGVHD, acute graft-versus-host disease.

* At the time of bone marrow evaluation.

Table 2

BM and Peripheral Blood Findings in Patient 30 Days after HSCT with (+) or without (−) Future cGVHD Development

Characteristic	−cGVHD	+cGVHD	*P* value
No. of patients	15	15	
Day of BM evaluation after transplantation	30 (28–44)	31 (30–39)	.30
Peripheral blood			
WBC ($\times 10^3/\mu L$)	5.4 (2.7–13.1)	6.7 (2.9–27.6)	04 *
Neutrophils	3.5 (1.5–12.1)	5.6 (2.2–25.7)	.02 *
Lymphocytes	0.6 (0.3–1.2)	0.5 (0.1–1.3)	.09
Monocytes			
Hemoglobin (g/dL)	11.6 (8.9–13.5)	11.4 (8.5–13.4)	.50
Platelets ($\times 10^3/\mu L$)	124 (29–282)	111 (23–231)	.30
BM Cellularity (%)	45 (30–90)	50 (30–80)	.60
Chimerism studies			
>95% of total cells donor (%)	15 (100)	14 (93)	1
>95% of T cells donor (%)	4 (31)	4 (31)	1
BM cellularity (%)	50 (30–80)	45 (30–90)	.60
IHC counts (per 600×hpf)			
TdT	44 (1–78)	2 (0.5–41)	.0007
PAX5	35 (1–101)	8 (0.5–106)	.008 *
CD3	11 (1–33)	15 (1–54)	.40
N †	14	14	
BM aspirate differential (%)			
Blasts	2 (1–9)	2 (0–3)	.50
Promyelocytes	1 (1–4)	2 (0–7)	.40
Myelocytes	7 (2–12)	8 (5–15)	.60
Maturing granulocytes	43 (21–59)	43 (11–53)	.40
Erythrocytes	33 (19–49)	28 (7–56)	.90
Lymphocytes	8 (1–25)	6 (1–20)	.40
Monocytes	3 (0–6)	2 (0–5)	.20
Eosinophils	5 (0–13)	5 (0–14)	.90
Plasma cells	1 (0–3)	1 (0–5)	.80

BM indicates bone marrow; HSCT, hematopoietic stem cell transplantation; cGVHD, chronic graft-versus-host disease; IHC, immunohistochemical; hpf, high powered field; TdT, terminal deoxynucleotidyl transferase.

IHC counts indicate enumeration by immunohistochemical analysis described in the methods section.

* Not significant after exclusion of steroid treated group.

† One case from each of the groups did not have a bone marrow aspirate available for evaluation.