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Lymphocyte Phenotype during Therapy for Acute Graft-versus-Host Disease: A Brief Report from BMT-CTN 0302

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A B S T R A C T

Although significant strides have been made in understanding the biology of graft-versus-host disease (GVHD) and its prevention over the last 4 decades, little is known about the different populations of lymphocytes and the changes in response to treatment for this condition. BMT-CTN 0302 was a randomized phase II clinical trial in the Blood and Marrow Transplant Clinical Trials Network that assessed the efficacy of combination therapy with steroids plus pentostatin, mycophenolate mofetil, etanercept, or denileukin diftitox in patients with acute GVHD. Patients enrolled in the study underwent blood analysis by flow cytometry on days 0, 14, and 28 of therapy to enumerate the number of total lymphocytes, T cells, B cells, and lymphocytes expressing activation markers. Baseline total lymphocyte counts and subpopulations were similar in the 4 treatment arms. Responding patients had a smaller decrease in total $CD45^+$ cell count ($P = .005$) compared with nonresponding patients at day 28. On univariate analysis, those who developed chronic GVHD had significantly higher $CD8^+$ cell counts at day 14 compared with those without it ($P = .005$). There was no significant association between baseline lymphocyte count and survival. On univariate analysis, among the patients with higher lymphocyte counts at days 14 and 28, there was a trend toward better survival at day 180, although this trend did not reach the predetermined threshold for significance. We found no significant differences in lymphocyte total or subpopulation counts among the 4 treatment arms, and no notable influence on outcomes.

INTRODUCTION

Acute graft-versus-host disease (aGVHD) is a common complication of allogeneic blood and marrow transplantation [1], characterized by an immune attack of donor cells against host tissues, typically leading to skin rash, diarrhea, and/or hyperbilirubinemia. The usual treatment involves the administration of immunosuppressive agents, particularly steroids. This treatment induces frequent clinical responses, but flares are common as steroids are tapered.

The balance between effector and regulatory T cells plays a major role in the development of aGVHD and its resolution. The impact of aGVHD treatment on these defined lymphocyte populations and the association of baseline and post-therapy phenotyping and response to this treatment are uncertain. To better understand the most promising pharmacologic strategies for treatment of aGVHD, knowledge of changes in the lymphocyte regulatory and effector T cell compartments may be important [2]. CD25 and CD69 expression on $CD4^+$ and $CD8^+$ T cells reflects their cellular activation status and may be a useful marker for identifying the association with aGVHD [3,4]. Clearly, $CD4^+CD25^+$ and $CD8^+CD25^+$ T cell phenotypes do not fully characterize effector/regulatory phenotypes, but understanding their relative dynamics (percentage and total numbers) in the setting of aGVHD before and after therapy may provide important insight into T cell immune recovery after aGVHD. We hypothesized that agents tested in this trial (pentostatin, denileukin diftitox, etanercept, and mycophenolate mofetil) could have different effects on lymphocyte populations, and that these changes could correlate with clinical outcomes. We anticipated better response to therapy and potentially less infection in patients who maintained higher $CD4^+$ T cell counts after completion of therapy. In addition, whether counts of B cells ($CD20^+$) are correlated with or contribute to the response to aGVHD therapy in these patients is unknown.

Despite the long-term use of lymphocytes in primary therapy for aGVHD, there is little information on the changes in different lymphocyte populations in response to steroid-based or combination-agent aGVHD therapy. BMT-CTN 0302 was a randomized phase II clinical trial in the Blood and Marrow Transplant Clinical Trials Network that assessed the efficacy of combination therapy with steroids plus 1 of 4 other agents—pentostatin, mycophenolate mofetil (MMF), etanercept, or denileukin diftitox—based on previous studies demonstrating the activity of these agents in steroid-refractory aGVHD [5-9]. Within the BMT-CTN 0302 trial, serial blood samples were collected from study participants and assessed for lymphocyte subsets by flow cytometry. We

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Table 1
Day 0 Descriptive Statistics of Immunophenotyping Data

Day 0 Immunophenotyping, Cells/μL	Treatment Arm				All (n = 120)	*P* Value[†]
	Etanercept (n = 30)	MMF (n = 29)	Denileukin Diftitox (n = 33)	Pentostatin (n = 28)		
Lymphocyte $CD45^+$						.69
Number	20	23	27	19	89	
Mean (SD)	2.1 (1.4)	2.4 (1.3)	2.3 (1.2)	2.0 (1.3)	2.2 (1.2)	
Median (range)	2.6 (0-4.3)	2.8 (0-4.1)	2.5 (0-3.8)	2.5 (0-3.8)	2.6 (0-4.3)	
$CD3^+$						.75
Number	30	29	33	28	120	
Mean (SD)	2.3 (0.6)	2.5 (0.5)	2.4 (0.6)	2.4 (0.8)	2.4 (0.6)	
Median (range)	2.4 (1.2-3.5)	2.5 (1.3-3.3)	2.4 (1.4-3.6)	2.3 (0.8-3.9)	2.4 (0.8-3.9)	
$CD4^+$						.92
Number	30	29	33	28	120	
Mean (SD)	1.9 (0.8)	2.1 (0.5)	2.0 (0.6)	2.0 (0.7)	2.0 (0.6)	
Median (range)	2.0 (0-3.8)	2.0 (1.0-2.8)	2.1 (0.5-3.3)	2.1 (0.7-3.5)	2.1 (0-3.8)	
$CD8^+$						.90
Number	30	29	33	28	120	
Mean (SD)	2.0 (0.7)	2.1 (0.6)	2.0 (0.7)	2.0 (0.9)	2.1 (0.7)	
Median (range)	2.1 (0.7-3.8)	2.0 (0.5-3.1)	2.0 (0.6-3.5)	2.1 (0-3.7)	2.1 (0-3.8)	
$CD25^+$						.73
Number	23	22	32	26	103	
Mean (SD)	1.5 (0.9)	1.5 (0.6)	1.6 (0.6)	1.4 (0.7)	1.5 (0.7)	
Median (range)	1.4 (0-3.9)	1.5 (0.7-2.5)	1.4 (0-2.6)	1.5 (0-2.8)	1.4 (0-3.9)	
$CD69^+$						.70
Number	21	19	24	25	89	
Mean (SD)	1.5 (0.6)	1.5 (0.6)	1.6 (0.6)	1.3 (0.8)	1.5 (0.7)	
Median (range)	1.6 (0-2.4)	1.5 (0.7-2.6)	1.6 (0.5-2.9)	1.5 (0-2.9)	1.6 (0-2.9)	
$CD20^+$						.48
Number	25	25	31	24	105	
Mean (SD)	0.8 (0.8)	1.1 (1.0)	0.8 (0.8)	0.7 (0.9)	0.8 (0.9)	
Median (range)	0.7 (0-3.1)	0.7 (0-3.3)	0.5 (0-3.3)	0.5 (0-3.4)	0.7 (0-3.4)	
$CD4^+/CD25^+$						.86
Number	23	21	30	26	100	
Mean (SD)	1.4 (0.8)	1.4 (0.6)	1.3 (0.6)	1.3 (0.7)	1.3 (0.7)	
Median (range)	1.3 (0-3.8)	1.5 (0-2.3)	1.3 (0-2.3)	1.4 (0-2.8)	1.4 (0-3.8)	
$CD4^+/CD25^-$*						.88
Number	23	21	30	26	100	
Mean (SD)	1.7 (0.9)	1.8 (0.6)	1.9 (0.7)	1.9 (0.9)	1.8 (0.8)	
Median (range)	1.8 (0-3.0)	1.9 (0-2.8)	2.0 (0-3.2)	2.0 (0-3.4)	1.9 (0-3.4)	

* Computed as $CD4^+/CD25^- = CD4 - CD4^+/CD25^+$.
† *P* values from the Kruskal-Wallis test.

examined differences after each treatment and assessed whether changes in lymphocyte populations were correlated with aGVHD response, development of chronic graft-versus-host disease (cGVHD), and survival.

MATERIALS AND METHODS

BMT-CTN 0302 and Lymphocyte Phenotyping

A total of 180 patients (median age, 50 years) were randomized to receive methylprednisolone at 2 mg/kg/day plus etanercept, MMF, denileukin diftitox, or pentostatin, as reported by Alousi et al. [5]. Two-thirds underwent myeloablative bone marrow transplantation. The graft source was peripheral blood in 61% of patients, bone marrow in 25%, and umbilical cord blood in 14%; 53% of the grafts were from unrelated donors. Patients who received MMF for prophylaxis (24%) were randomized to a non-MMF arm. At randomization, aGVHD was grade I-II in 68% and grade III-IV in 32%. One-hundred and forty subjects had immunophenotyping data from at least 1 time point.

Complete response (CR) required resolution of all signs and symptoms of aGVHD in all organs without intervening salvage therapies. A partial response (PR) was defined as an improvement of 1 stage in 1 or more organs without progression in any organ. Day 28 CR rates were 26% for etanercept, 60% for MMF, 53% for denileukin diftitox, and 38% for pentostatin. Corresponding 9-month overall survival (OS) rates were 47%, 64%, 49%, and 47%. Blood was obtained at baseline on day 0 (time of study enrollment), day 14, and day 28 of GVHD treatment; blood was collected irrespective of response to GVHD therapy. Lymphocyte phenotyping by flow cytometry gated $CD45^+$ cells (total lymphocytes) and enumeration of $CD3^+$, 4^+, 8^+, 20^+, 25^+, 69^+, and $CD4^+25^+$cells was performed at the transplantation center and recorded as number of cells of each subset phenotype per microliter of blood. These values were collected by the BMT-CTN data coordinating center and analyzed in conjunction with the clinical outcomes of response and the 4 randomly assigned treatments. Immunophenotyping and outcome data were analyzed in conjunction with a previously reported clinical study [5] that included 180 subjects, 140 of whom had immunophenotyping data from at least 1 time point. The reasons for missing data included samples not obtained (54%), participant refused (33%), participant missed clinical visits (1%), participant died or too ill (5%), subset not assessed/performed/calculated by laboratory (5%), and not part of the flow cytometry assessment (2%). The protocol team reviewed the clinical endpoints of response (CR and PR) at day 28 and cGVHD in all patients while still blinded to the treatment assignment and without knowledge of the immunophenotyping data.

Statistical Analyses

The primary objective of this study was to examine how the 4 randomly assigned agents influenced circulating lymphocyte populations, and how these changes correlated with clinical outcomes. Clinical outcomes analyzed were aGVHD response at day 28, survival at 6 months, and cGVHD by 9 months. Lymphocyte data at each time point were compared among the treatments using the Kruskal-Wallis test, after $\log_{10}(x + 1)$ transformation to induce normality. Linear mixed models were used to examine changes in lymphocyte populations over time and evaluate their relationship to treatment.

Univariate case-control comparisons for each lymphocyte subpopulation were performed at each time point, along with a comparison of changes in lymphocytes from baseline using the Mann-Whitney *U* test. Cases were defined as patients experiencing an event (day 28 CR or CR + PR, death within 6 months, or cGVHD by 9 months), whereas controls were those alive at the same time points without the event. Logistic regression was used for the day 28 GVHD response in multivariate analyses. Cox regression modeling was used to analyze OS in landmark analyses by treating lymphocyte counts at day 14 or day 28 as covariates in multivariate analyses. The Cox regression model was used in landmark analysis for cGVHD by treating day 28 lymphocyte subpopulations as covariates in multivariate analyses. Lymphocyte populations were explored using 2 approaches: as a continuous measurement or as binary covariates using the median as a cutoff. The assigned treatment arm and patient characteristics that could possibly affect outcomes, including graft type, donor type, and aGVHD grade at onset, were considered in the multivariate analyses. Correlations among the lymphocyte population were explored using the Pearson correlation test. All *P* values were 2-tailed and

were considered significant at $P < .01$ owing to the large number of comparisons. Data were analyzed using SAS version 9 (SAS Institute, Cary, NC).

RESULTS

Lymphocyte Population Changes

Baseline lymphocyte total count and subpopulation counts were similar in the 4 treatment arms (Table 1). At days 14 and 28 after treatment, modest differences in subpopulations of $CD3^+$, $CD4^+$, $CD8^+$, and $CD25^+$ were seen, whereas total lymphocyte count ($CD45^+$) was similar among the treatment arms, although no differences were statistically significant at $P < .01$ (Tables 2 and 3). Linear mixed models produced similar modest differences in these lymphocyte subpopulations, which were not statistically significant at $P < .01$. No significant changes in lymphocytes with activation markers ($CD25^+$ and $CD69^+$), or in lymphocytes associated with regulatory T cell subsets ($CD4^+25^+$), or total B cells ($CD20^+$) were noted after therapy or among the 4 treatment cohorts.

Cell Populations and GVHD Response

Univariate analysis identified a moderately strong association between declining lymphocyte count and GVHD response. Patients responding (CR/PR) at day 28 had a smaller decrease in total $CD45^+$ cell count at day 28 compared with nonresponding patients ($P = .005$). On multivariate logistic regression modeling of GVHD response, after adjusting for day 14 GVHD response, there was a significant effect of changes in total lymphocyte count from baseline to day 28 (odds ratio, 4.63; 95% confidence interval [CI], 1.41-15.20; $P = .012$) with greater drops in lymphocyte count associated with significantly lower likelihood of response. Figure 1 presents a boxplot of $CD45^+$ cell counts in day 28 GVHD responders versus nonresponders. No other lymphoid subsets were associated with GVHD response (Supplemental Table 1).

Cell Populations and Chronic GVHD

On univariate analysis, patients with a higher $CD8^+$ count at day 14 had a greater frequency of cGVHD, and patients who developed cGVHD had significantly higher $CD8^+$ cell counts at day 14 compared with those without cGVHD ($P = .005$). In Cox regression modeling of cGVHD, the hazard ratio for risk of cGVHD for patients with $CD8^+$ values above the median ($\geq$100 cells/μL) compared with those with lower $CD8^+$ values was 1.88 (95% CI, 0.97-3.66; $P = .06$). No other baseline risk factor demonstrated a significant association with cGVHD. The cumulative incidence of cGVHD by $CD8^+$ value is shown in Figure 2. No other lymphoid subsets were associated with risk of cGVHD (Supplemental Table 2).

Lymphocyte Populations and Survival

There was no significant association between baseline lymphocyte count and survival, relapse, or CMV infection. Univariate analysis detected a trend toward better survival at day 180 in patients with elevated lymphocyte counts at days 14 and 28, although this did not reach the predetermined threshold for significance ($P < .01$). Cox regression modeling of OS, after adjusting for GVHD response, revealed a significant favorable effect of total lymphocyte count above versus below the median at day 14 (hazard ratio, 2.90; 95% CI, 1.42-5.92; $P = .004$), but not at day 28 ($P = .53$). Kaplan-Meier

Table 2
Day 14 Descriptive Statistics of Immunophenotyping Data

Day 14 Immunophenotyping, Cells/μL	Treatment Arm				All (n = 97)	*P* Value
	Etanercept (n = 23)	MMF (n = 25)	Denileukin Diftitox (n = 25)	Pentostatin (n = 24)		
Lymphocyte $CD45^+$						.32
Number	13	22	19	21	75	
Mean (SD)	2.1 (1.5)	2.5 (1.3)	1.9 (1.3)	1.9 (1.2)	21 (1.3)	
Median (range)	24 (0-4.3)	2.6 (0-4.6)	2.3 (0-3.3)	2.2 (0-3.7)	2.4 (0-4.6)	
$CD3^+$						.04
Number	23	25	25	23	96	
Mean (SD)	2.0 (0.9)	2.6 (0.5)	2.2 (0.8)	2.1 (0.7)	2.2 (0.7)	
Median (range)	2.1 (0-3.8)	2.5 (1.8-4.0)	2.2 (0-3.5)	2.1 (0.3-2.9)	2.3 (0-4.0)	
$CD4^+$						.13
Number	22	25	25	24	96	
Mean (SD)	1.6 (0.8)	2.0 (0.7)	1.9 (0.7)	1.7 (0.7)	1.8 (0.7)	
Median (range)	1.7 (0-3.2)	2.2 (0.7-3.1)	2.0 (0-3.1)	1.6 (0-3.5)	1.8 (0-3.5)	
$CD8^+$						.22
Number	22	25	25	24	96	
Mean (SD)	1.8 (0.8)	2.2 (0.7)	1.9 (0.8)	1.9 (0.9)	1.9 (0.8)	
Median (range)	2.0 (0-3.4)	2.3 (0.3-3.7)	1.8 (0.3-3.3)	2.0 (0-3.6)	2.0 (0-3.7)	
$CD25^+$						.02
Number	19	18	21	20	78	
Mean (SD)	1.1 (0.6)	1.4 (0.7)	1.4 (0.6)	1.0 (0.5)	1.2 (0.6)	
Median (range)	1.3 (0-2.0)	1.6 (0-2.4)	1.6 (0.3-2.2)	1.1 (0-1.9)	1.3 (0-2.4)	
$CD69^+$						.12
Number	19	17	18	22	76	
Mean (SD)	1.0 (0.8)	1.4 (0.7)	1.4 (0.6)	1.1 (0.6)	1.2 (0.7)	
Median (range)	1.0 (0-3.0)	1.4 (0-2.9)	1.3 (0.3-2.4)	1.2 (0-2.3)	1.2 (0-3.0)	
$CD20^+$						.39
Number	19	19	22	22	82	
Mean (SD)	0.9 (0.8)	0.9 (0.9)	1.0 (0.8)	0.6 (0.5)	0.8 (0.8)	
Median (range)	0.8 (0-2.8)	0.8 (0-2.6)	0.9 (0-2.8)	0.5 (0-1.6)	0.8 (0-2.8)	
$CD4^+/CD25^+$						.12
Number	19	18	21	21	79	
Mean (SD)	1.1 (0.6)	1.4 (0.7)	1.2 (0.7)	1.0 (0.6)	1.2 (0.7)	
Median (range)	1.2 (0-1.9)	1.6 (0-2.5)	1.3 (0-2.2)	1.0 (0-2.3)	1.2 (0-2.5)	
$CD4^+/CD25^-$						.37
Number	19	18	21	21	79	
Mean (SD)	1.5 (0.8)	1.7 (0.8)	1.9 (0.7)	1.6 (0.7)	1.7 (0.8)	
Median (range)	1.4 (0-3.2)	1.9 (0-2.9)	2.0 (0-3.1)	1.6 (0-3.5)	1.7 (0-3.5)	

Table 3
Day 28 Descriptive Statistics of Immunophenotyping Data

Day 28 Immunophenotyping, Cells/μL	Treatment Arm				All (n = 108)	*P* Value
	Etanercept (n = 25)	MMF (n = 31)	Denileukin Diftitox (n = 28)	Pentostatin (n = 24)		
Lymphocyte CD45$^+$						.16
Number	16	23	23	21	83	
Mean (SD)	1.7 (1.3)	2.4 (1.3)	2.0 (1.2)	1.8 (1.2)	2.0 (1.2)	
Median (range)	1.8 (0-4.1)	2.6 (0-4.6)	2.4 (0-2.5)	2.1 (0-3.6)	2.3 (0-4.6)	
CD3$^+$						.20
Number	25	31	28	24	108	
Mean (SD)	2.2 (0.6)	2.3 (0.6)	2.2 (0.7)	1.9 (0.6)	2.2 (0.7)	
Median (range)	2.2 (1.0-3.9)	2.3 (0.9-3.5)	2.2 (0.3-3.5)	1.9 (0-2.7)	2.2 (0-3.9)	
CD4$^+$						.04
Number	24	30	27	24	105	
Mean (SD)	1.8 (0.6)	1.9 (0.6)	1.8 (0.6)	1.4 (0.6)	1.7 (0.6)	
Median (range)	1.6 (0.5-3.1)	2.0 (0.7-3.1)	1.8 (0.5-3.1)	1.5 (0-2.5)	1.7 (0-3.1)	
CD8$^+$						.04
Number	24	30	27	24	105	
Mean (SD)	2.0 (0.7)	2.1 (0.6)	1.9 (0.8)	1.5 (0.8)	1.9 (0.7)	
Median (range)	2.0 (0.6-3.6)	2.0 (0.7-3.8)	1.9 (0.5-3.5)	1.4 (0-2.6)	1.9 (0-3.8)	
CD25$^+$						.37
Number	19	22	24	22	87	
Mean (SD)	1.3 (0.8)	1.3 (0.6)	1.3 (0.6)	1.0 (0.6)	1.2 (0.6)	
Median (range)	1.4 (0-3.6)	1.4 (0-2.6)	1.4 (0.3-2.2)	1.1 (0-2.0)	1.3 (0-3.6)	
CD69$^+$						.25
Number	19	22	19	22	82	
Mean (SD)	1.3 (0.9)	1.3 (0.6)	1.3 (0.6)	1.0 (0.6)	1.2 (0.7)	
Median (range)	1.4 (0-3.6)	1.4 (0-2.6)	1.4 (0-2.5)	1.1 (0-1.9)	1.2 (0-3.6)	
CD20$^+$						.21
Number	20	23	24	21	88	
Mean (SD)	1.0 (0.8)	0.7 (0.8)	0.8 (0.8)	0.5 (0.5)	0.7 (0.8)	
Median (range)	1.0 (0-3.1)	0.5 (0-2.6)	0.5 (0-2.7)	0.3 (0-1.3)	0.5 (0-3.1)	
CD4$^+$/CD25$^+$						.06
Number	19	22	23	23	87	
Mean (SD)	1.4 (0.7)	1.2 (0.7)	1.1 (0.7)	0.8 (0.6)	1.1 (0.7)	
Median (range)	1.4 (0-3.6)	1.2 (0-2.5)	1.4 (0-2.2)	0.7 (0-1.8)	1.1 (0-3.6)	
CD4$^+$/CD25$^-$						.19
Number	19	22	23	23	87	
Mean (SD)	1.5 (0.7)	1.7 (0.7)	1.7 (0.7)	1.1 (0.8)	1.5 (0.8)	
Median (range)	1.4 (0-2.6)	1.6 (0.5-3.0)	1.6 (0-3.1)	1.3 (0-2.4)	1.5 (0-3.1)	

estimates of OS based on day 14 CD45$^+$ cell count above or below the median value (≥200 cells/μL) are shown in Figure 3. It appears that higher CD69$^+$ cell counts at day 14 (P = .009) and day 28 (P = .05) may be correlated with better survival. No other lymphocyte subpopulations were associated with survival (Supplemental Table 3).

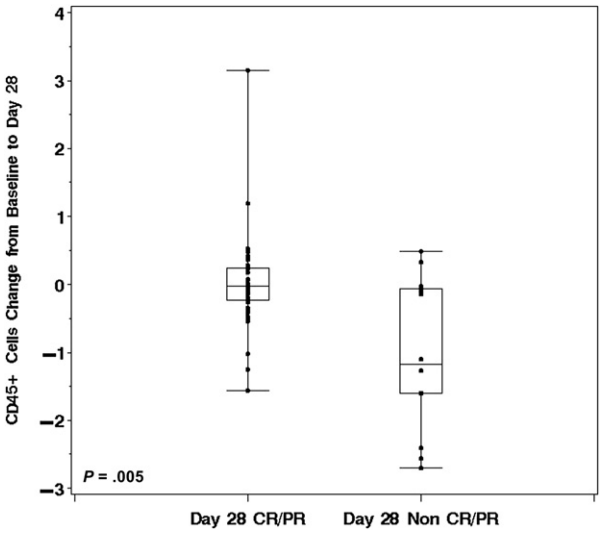

Figure 1. Boxplot of CD45$^+$ cell counts for day 28 GVHD responders versus nonresponders. The *P* value reflects the CD45$^+$ cell count change from baseline to day 28 posttreatment for day 28 responders versus nonresponders. Data are on a log scale, with a 1-unit change equal to +10 and a 2-unit change equal to +100.

DISCUSSION

We examined the influence of 4 different, randomly assigned GVHD therapies to cause distinct changes in lymphocyte populations that would help understand and potentially predict the observed differences in therapeutic effects of each treatment. The 4 treatment arms demonstrated no significant differences in total lymphocyte or lymphocyte subpopulation counts, and treatment had no notable influence on outcomes.

We performed a detailed and prospective characterization of lymphoid populations through the initial course of aGVHD therapy with steroids plus 1 of the 4 novel agents. Although we found no significant differences in lymphocyte populations during the first 28 days among the 4 study groups, we did find an association between higher CD45$^+$ lymphocyte counts at day 28 after initiation of therapy and response, and an apparent trend toward an association between higher CD8$^+$ counts early after treatment and the subsequent development of cGVHD [10]. The results of Grogan et al. [10] suggest that CD8$^+$ T cells in patients with cGVHD are characterized by increased activation and proliferation, and this cell population may be present early during an episode of aGVHD.

We also observed a trend toward better survival in patients with elevated CD45$^+$ lymphocyte populations at day 14 and day 28. These results are intriguing, and suggest that preservation of lymphocyte populations after aGVHD therapy is crucial for immune recovery and protection against subsequent infections.

This study has some limitations. The number of patients was modest, and our data were incomplete at some time

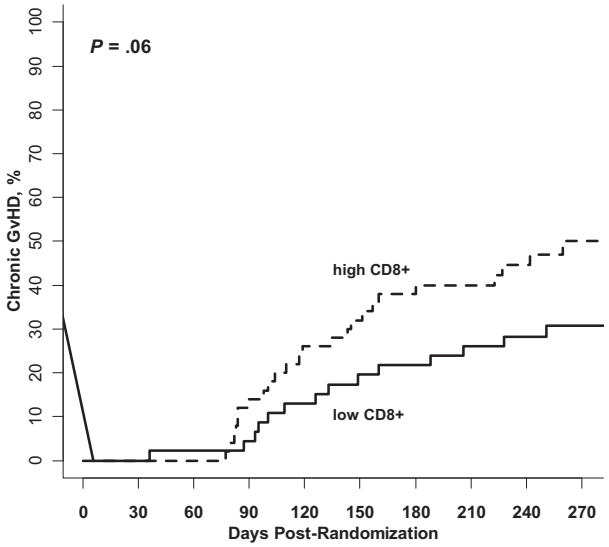

Figure 2. Cumulative incidence of cGVHD by day 14 $CD8^+$ lymphocyte count. The *P* value reflects a comparison of the cumulative incidence of cGVHD for patients with higher $CD8^+$ counts (cells $\geq$100/μL; n = 50) at day 14 versus patients with lower $CD8^+$ counts (cells <100/μL; n = 46) at day 14.

points. Given the different mechanisms of action of the study drugs (MMF and pentostatin as lymphoid metabolic inhibitors, etanercept as a TNF-α blocker, and denileukin diftitox as a $CD25^+$ cell–directed lytic immunotoxin), differing effects on circulating lymphoid subsets might have been predicted. Somewhat surprisingly, all the 4 study drugs caused similar changes in counts of both total lymphocytes and lymphocyte subpopulations. This is likely influenced and confounded by the fact that all 4 of our study cohorts were treated concurrently with high-dose corticosteroids. In addition, our phenotypic analysis was limited and insufficient to accurately characterize the functionality of T cell subsets, reflecting the feasibility of conducting multicolor flow cytometry analysis of specimens in the multi-institutional setting.

Although $CD4^+CD25^+$ and $CD8^+CD25^+$ T cell phenotypes are useful in identifying the association with GVHD [3,4], they do not fully characterize effector/regulatory phenotypes. The addition of intracellular staining to forkhead box P3 (FoxP3) might have aided the assessment of regulatory T cells; however, some studies have reported decreased numbers of $CD4^+CD25^+Foxp3^+$ T cells in patients with GVHD, whereas others have not [11,12]. On the other hand, our findings are consistent with those reported by Przepiorka et al. [13], which also showed that phenotypic changes in T cell subpopulations do not predict response to daclizumab in patients with active GVHD.

Although this detailed examination of lymphocyte subpopulations did not clarify the mechanism of response or why the 4 study agents led to different clinical outcomes, we suggest that future studies of GVHD therapy include ongoing evaluation of lymphocyte, serum, or tissue biologic markers to uncover important details of the immunobiology that can help further refine clinical treatment approaches.

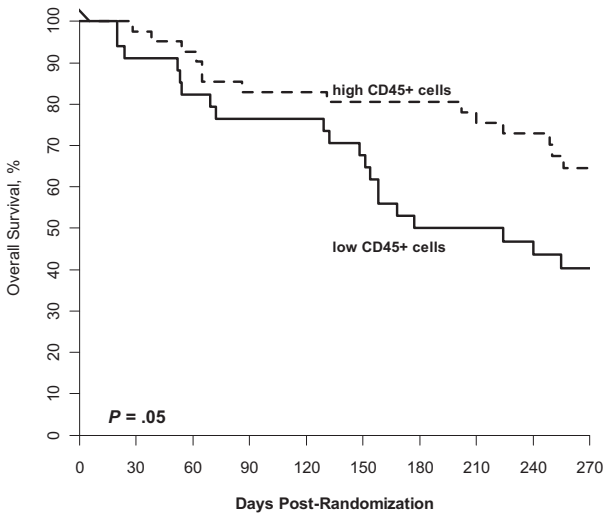

Figure 3. OS by day 14 $CD45^+$ lymphocyte count. The *P* value reflects a comparison of OS probability for patients with higher $CD45^+$ cell counts (cells $\geq$200/μL; n = 41) at day 14 versus patients with lower $CD45^+$ cell counts (cells <200/μL; n = 34) at day 14.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.12.003.

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