Biol Blood Marrow Transplant 25 (2019) 2143–2151

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Allogeneic – Adult

Effect of Sirolimus on Immune Reconstitution Following Myeloablative Allogeneic Stem Cell Transplantation: An Ancillary Analysis of a Randomized Controlled Trial Comparing Tacrolimus/Sirolimus and Tacrolimus/Methotrexate (Blood and Marrow Transplant Clinical Trials Network/BMT CTN 0402)

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Article history:
Received 10 January 2019
Accepted 26 June 2019

Keywords:
Sirolimus
Immune reconstitution
Myeloablative
Tregs
B lymphocytes

A B S T R A C T

Although allogeneic hematopoietic cell transplantation (HCT) is a potentially curative therapy for hematologic neoplasms, one of its limiting toxicities continues to be graft-versus-host disease, both acute (aGVHD) and chronic (cGVHD). Sirolimus is a mammalian target of rapamycin inhibitor that has proven effective in GVHD prophylaxis in combination with a calcineurin inhibitor, such as tacrolimus. The impact of sirolimus on immune reconstitution has not been comprehensively investigated in vivo thus far, however. Here we present an ancillary analysis of the randomized study BMT-CTN 0402 that examined the effect of sirolimus on immune subsets post-transplantation. We further examine the association between different lymphocyte subsets and outcomes post-transplantation in each arm. BMT-CTN 0402 was a randomized trial (n = 304) comparing 2 GVHD prophylaxis regimens, tacrolimus/sirolimus (Tac/Sir) and tacrolimus/methotrexate (Tac/MTX), in patients with acute myelogenous leukemia, acute lymphoblastic leukemia, or myelodysplastic syndrome undergoing myeloablative HLA-matched HCT. There were no differences in 114-day GVHD-free survival (primary endpoint), aGVHD, cGVHD, relapse, or overall survival (OS) between the 2 arms. Of the 304 patients, 264 had available samples for the current immune reconstitution analysis. Blood samples were collected at 1, 3, 6, 12, and 24 months post-HCT. Multiparameter flow cytometry was performed at the project laboratory (Esoterix Clinical Trials Services) in a blinded fashion, and results for the 2 arms were compared. Multivariable Cox regression models, treating each phenotypic parameter as a time-dependent variable, were constructed to study the impact of reconstitution on clinical outcomes. There were no significant differences in patient and transplantation characteristics between the Tac/Sir and Tac/MTX arms in this analysis. Absolute lymphocyte count and $CD3^+$ cell, $CD4^+$ cell, and conventional T cell (Tcon) counts were significantly decreased in the Tac/Sir arm for up to 3 months post-HCT, whereas $CD8^+$ cells recovered even more slowly (up to 6 months) in this arm. Interestingly, there was no clear difference in the absolute number of regulatory T cells (Tregs, defined as $CD4^+CD25^+$ cells) between the 2 arms at any point post-HCT; however, the Treg:Tcon ratio was significantly greater in the Tac/Sir arm in the first 3 months after HCT. B lymphocyte recovery was significantly compromised in the Tac/Sir arm from 1 month to 6 months after HCT, whereas natural killer cell reconstitution was not affected in the Tac/Sir arm. In the outcomes analysis, higher numbers of $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, and Tregs were associated with better OS. Neither Treg numbers nor the Treg:Tcon ratio was correlated with GVHD. Our findings indicate that Tac/Sir has a more profound T cell suppressive effect than the combination of Tac/MTX in the early post-transplantation period, and particularly compromises the recovery of $CD8^+$ T cells, which have been implicated in aGVHD. Sirolimus used in vivo with tacrolimus does not appear to result in increased absolute numbers of Tregs, but might have a beneficial effect on the Treg:Tcon balance in the first 3

Financial disclosure: See Acknowledgments on page 2151.

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https://doi.org/10.1016/j.bbmt.2019.06.029

months after transplantation. Nonetheless, no differences in aGVHD or cGVHD between the 2 arms were observed in the parent randomized trial. Calcineurin-inhibitor free, sirolimus-containing GVHD prophylaxis strategies, incorporating other novel agents, should be investigated further to maximize the potential favorable effect of sirolimus on Treg:Tcon balance in the post-transplantation immune repertoire. Sirolimus significantly compromises B cell recovery in the first 6 months post-HCT, with potential complex effects on cGVHD that merit further study.

INTRODUCTION

Acute graft-versus-host disease (aGVHD) occurs in 30% to 35% of HLA-matched hematopoietic stem cell transplantation (HCT) recipients, whereas the estimated rate of chronic GVHD (cGVHD) in this population ranges from 30% to 70% [1]. The use of calcineurin inhibitor (eg, tacrolimus, cyclosporine)-based prophylaxis has lowered the incidence of aGVHD, but achieving control of cGVHD with this regimen has proven more challenging [2]. Calcineurin inhibitors are typically used in combination with methotrexate (MTX), with the potential downstream toxicities of nephrotoxicity, myelosuppression, and mucositis. Thus, effective agents with better adverse effect profiles continue to be an area of active research interest.

Sirolimus is a mammalian target of rapamycin (mTOR) inhibitor with potent immunosuppressive properties that was originally developed for use in solid organ transplantation. It binds to the same immunophilin as tacrolimus (FKBP12); however, it acts at a later stage of T cell cycle progression and blocks cytokine-mediated signal transduction pathways [3], thereby preventing T cell activation and proliferation in a synergistic manner with tacrolimus [4]. Consequently, sirolimus has been used in combination with tacrolimus (Tac/Sir) for GVHD prophylaxis, with promising results. In the early 2000s, sirolimus was shown to be safe for use in GVHD prophylaxis regimens along with tacrolimus and low-dose MTX [5]. It was later shown to be effective in HLA-matched related and unrelated donor transplantation in combination with tacrolimus only [6], as well as in double umbilical cord blood transplantation [7]. Thrombotic microangiopathy and hepatic veno-occlusive disease were found to be associated with the use of sirolimus in this context, particularly when used in concert with a myeloablative regimen, such as busulfan/cyclophosphamide (Cy) or total body irradiation (TBI) [8].

A Phase II randomized controlled trial (RCT) comparing Tac/Sir with tacrolimus and MTX (Tac/MTX) as GVHD prophylaxis in 74 patients found significantly less grade II-IV aGVHD and moderate/severe cGVHD in the Tac/Sir arm, but similar overall survival (OS) and patient-reported quality of life in the 2 arms [9]. The largest RCT comparing the combination of Tac/Sir with Tac/MTX (standard of care) as GVHD prophylaxis (BMT-CTN 0402; n = 304) in matched related donor HCT used 114-day grade II-IV aGVHD-free survival as its primary endpoint in an intention-to-treat analysis [10]. Interestingly, there were no significant differences between the 2 arms in the primary endpoint or in grade II-IV aGVHD, cGVHD, relapse-free survival, or OS. In a pointwise post hoc analysis, severe (grade III-IV) aGVHD was reduced in the Tac/Sir arm, and oropharyngeal mucositis was significantly less frequent in the Tac/Sir arm. Thus, Tac/Sir was considered an acceptable alternative to the standard of care as a GVHD prophylaxis regimen but was not superior to Tac/MTX.

The effect of sirolimus on various T cell subsets, such as conventional T cells (Tcons) and regulatory T cells (Tregs) has been studied in mice. The addition of sirolimus led to reduced expansion of alloreactive Tcons and aGVHD lethality in mice [11]. Concomitantly, expansion of polyclonal Tregs was observed with conserved high FOXP3 expression. This differential effect on 2 major T cell subsets was attributed to the reduced use of the mTOR pathway in Tregs compared with Tcons. Limited analyses of the in vivo effect of sirolimus on post-transplantation immune reconstitution been performed in smaller numbers of patients have suggested that Treg reconstitution is better preserved with Tac/Sir than with Tac/MTX [9,12]. However, a comprehensive analysis of the effect of sirolimus on immune reconstitution after HCT has not been reported and is critical to understand how sirolimus affects T cell and B cell subsets as well as natural killer (NK) cells in vivo.

BMT-CTN 0402, the largest RCT to date comparing sirolimus-based GVHD prophylaxis (Tac/Sir) with MTX-based prophylaxis (Tac/MTX), provides the ideal platform for studying the effect of sirolimus on post-transplantation immune reconstitution. Here we present the results of an analysis comparing immune reconstitution in the Tac/Sir and Tac/MTX arms. We also analyze the association between different immune subsets and post-transplantation outcomes in each arm.

METHODS

Study Design

An open-label, Phase III, multicenter RCT was performed by the Blood and Marrow Transplant Clinical Trials Network (BMT-CTN 0402) comparing Tac/Sir with Tac/MTX as a GVHD prophylaxis regimen following matched related donor peripheral blood stem cell HCT. The primary endpoint for this RCT was 114-day grade II-IV aGVHD-free survival in an intention-to-treat analysis. Subjects age <60 years with acute leukemia in remission, myelodysplastic syndrome, or chronic myelogenous leukemia in chronic or accelerated phase were eligible. All patients (n = 304) received TBI-based myeloablative conditioning (1200 cGy) along with Cy or etoposide. Patients receiving a busulfan/Cy-containing conditioning regimen were excluded from the analysis due to excessive toxicity when combined with sirolimus for GVHD prophylaxis and were not part of either the parent RCT or the present analysis. Tacrolimus was started on day -3 (.02 mg/kg/day i.v., with a trough level maintained at 5 to 10 ng/mL), and sirolimus was also started on day -3 (loading dose of 12 mg, followed by 4 mg/day to maintain a trough level of 3 to 12 ng/mL). MTX was administered i.v. on day +1 (15 mg/m^2) and on days +3, +6, and +11 (10 mg/m^2).

Of the 304 subjects, 264 had available samples for this analysis (Tac/Sir, n = 132; Tac/MTX, n = 132). Randomization was maintained for the flow cytometry analysis for this immune reconstitution analysis.

Flow Cytometry

Written informed consent was obtained for this immune reconstitution analysis before sample collection in accordance with the Declaration of Helsinki. Protocol approval was obtained from the Institutional Review Board of each participating institution. In all subjects, blood samples were collected at months 1, 3, 6, 12, and 24 post-HCT and included a 3-mL EDTA peripheral blood sample (to calculate absolute cell population counts by flow cytometry) and a 10-mL ACD peripheral blood sample (for immunophenotypic analyses by multiparameter flow cytometry), which were shipped to the project laboratory (Esoterix Clinical Trials Services) for immediate analysis. Protocol-specified immunophenotypic analyses were performed with a panel of fluorophore-conjugated monoclonal antibodies specific for cell surface determinants. Flow cytometry was performed in a blinded fashion without knowledge of patient treatment.

A panel of monoclonal antibodies were used to identify the following T-cell subsets as follows: $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, Tregs, $CD3^+CD4^+CD25^+$, and Tcons ($CD3^+CD4^+$ minus $CD3^+CD4^+CD25^+$) cells. B cells were defined as $CD19^+$cells as well as a B cell subset, $CD19^+CD27^+$ cells. NK cells were defined as $CD3^-CD56^+CD16^+$ as well as $CD3^-CD56^+CD16^-$ and NK T cells were defined as $CD3^+CD56^+$ cells. A subcompartment analysis of T cells included naive $CD4^+$ T cells ($CD4^+CD45RA^+CD62L^+$), effector $CD4^+$ T cells ($CD4^+CD45RA^+CD62L^-$), naive $CD8^+$ cells ($CD8^+CD45RA^+CD62L^+$), and effector $CD8^+$ cells ($CD8^+CD45RA^+CD62L^-$). Proliferating naïve and effector cells in the $CD4^+$ and CD8+ compartments were designated by $Ki67^+$. The Treg:Tcon ratio

was defined as Treg ($CD4^+CD25^+$)/Tcon ($CD4^+$ minus $CD4^+CD25^+$), and the Treg:CD8 ratio was defined as Treg ($CD4^+CD25^+$)/$CD8^+$.

Statistical Analysis

Analysis included participants who were randomized and underwent transplant and had available samples only. Baseline characteristics were compared using Fisher's exact test, the χ^2test, or the Wilcoxon rank-sum test, as appropriate. The Wilcoxon rank-sum test was also used to compare immune reconstitution data between the 2 treatment arms at each time point. Multivariable Cox regression models treating each phenotypic parameter as a time-dependent variable were constructed to study the impact of reconstitution on clinical outcomes. Clinical endpoints considered in this study included OS, nonrelapse mortality (NRM), relapse, grade II-IV aGVHD, grade III-IV aGVHD, cGVHD, and grade II-IV aGVHD-free survival. These endpoints have been defined previously [10]. For GVHD, NRM, and relapse, cause-specific Cox regression analysis was performed treating each phenotypic parameter as a time-dependent variable. Potential prognostic factors considered in the regression analysis included GVHD prophylaxis, age, recipient and donor sex, disease, disease risk, Karnofsky Performance Status score, conditioning regimen, cytogenetic risk, and cytomegalovirus (CMV) serostatus of recipient and donor at HCT. All immune-phenotypic data were natural log-transformed for regression analysis. Correlation analysis was performed using Spearman's rank-order correlation. Because the primary focus of this ancillary immune reconstitution study was the effect of sirolimus on T cell subsets, a nominal *P* value of .01 was preset as an ad hoc adjustment for multiple comparisons of these subsets and their ratios (Tregs, Tcons, CD8 cells, Treg:Tcon, Treg:CD8). All tests were 2-sided. Analyses were performed using SAS 9.3 (SAS Institute, Cary, NC) and R version 3.2.2 (CRAN Project; www.cran.r-project.org).

This report is submitted on behalf of the Blood and Marrow Transplant Clinical Trials Network (BMT-CTN).

RESULTS

Patient and Transplantation Characteristics

Patient and transplantation characteristics for 264 subjects are summarized in Table 1. The median recipient age was 44 years (range, 18 to 59 years) in the Tac/Sir group and 42 years (range, 12 to 55 years) in the Tac/MTX group. Myeloid malignancies composed the majority (65.9%) of diseases in the Tac/Sir group as well as the Tac/MTX group (55%); however, the proportion of patients with acute lymphoblastic leukemia was disproportionately higher in the Tac/MTX group compared with the Tac/Sir group (45% versus 33.3%). Karnofsky Performance Status, donor-recipient sex match and conditioning regimen (Cy/TBI, VP-16/TBI) were comparable in the 2 arms. The distribution of donor-recipient CMV serostatus is detailed in Table 1.

Immune Reconstitution

Total WBC Count and Absolute Lymphocyte Count after HCT

The recovery of total WBC count and absolute lymphocyte count (ALC) in each arm is summarized in Figure 1. Total WBC count recovery was similar in the 2 arms at all time points post-HCT except at 3 months, when it was significantly lower in the Tac/Sir arm (median, $3.71 \times 10^3/\mu L$ for Tac/Sir versus $4.6 \times 10^3/\mu L$ for Tac/MTX; $P = .0053$). The ALC was significantly lower in the Tac/Sir arm for up to 3 months after HCT ($P < .0001$), but was not significantly different in the 2 arms at the .01 level thereafter. $CD3^+$ cell recovery was significantly delayed in the Tac/Sir arm for up to 3 months (median absolute CD3 count at 3 months, $315 \times 10^3/\mu L$ for Tac/Sir versus $565 \times 10^3/\mu L$ for Tac/MTX; $P < .0001$), but absolute numbers were similar at later time points (Figure 2A).

Reconstitution of Major T Cell Populations

The reconstitution of $CD4^+$ T cells, $CD8^+$ T cells, Tcons, and Tregs ($CD4^+CD25^+$) is detailed in Figure 2. $CD4^+$ cell numbers followed the same trajectory as $CD3^+$ T cell numbers and were significantly lower in the Tac/Sir arm in the first 3 months only (median absolute CD4 count at 3 months, $162 \times 10^3/\mu L$ for Tac/Sir versus $246 \times 10^3/\mu L$ for Tac/MTX; $P < .001$) (Figure 2A). Absolute numbers of $CD8^+$ T cells were significantly lower in the Tac/Sir arm for up to 6 months (ie, at 1, 3, and 6 months). The median absolute $CD8^+$ cell count was $121 \times 10^3/\mu L$ for Tac/Sir versus $304 \times 10^3/\mu L$ for Tac/MTX ($P < .0001$) at 3 months and $195.5 \times 10^3/\mu L$ for Tac/Sir versus $287 \times 10^3/\mu L$ for Tac/MTX ($P = .009$) at 6 months. Recovery of $CD8^+$ cells was similar in the 2 arms at 12 and 24 months (Figure 2B).

The absolute numbers of Tcons were derived by subtracting $CD4^+CD25^+$ cells from total $CD4^+$ cells. Tcons followed the same trajectory as $CD3^+$ T cells and were significantly lower in the Tac/Sir arm in the first 3 months only (median absolute Tcon count at 3 months, $66 \times 10^3/\mu L$ for Tac/Sir versus $109 \times 10^3/\mu L$ for Tac/MTX; $P < .001$) (Figure 2C). Tregs were defined as $CD4^+CD25^+$ cells in this analysis. Absolute numbers of $CD4^+$ Tregs were not significantly different at the .01 level in the Tac/Sir and Tac/MTX arms at any point (1, 3, 6, 12, or 24 months) (Figure 2D). In addition, absolute numbers of $CD3^+CD8^+CD25^+$ cells were similar in the 2 arms (data not shown).

Overall, the absolute number of $CD3^+$ T cells was lower in the Tac/Sir arm in the early post-transplantation period only (up to 3 months), driven by delayed Tcon, $CD4^+$, and $CD8^+$ cell recovery, with $CD8^+$ cells recovering the slowest. Treg ($CD3^+CD4^+CD25^+$) reconstitution was somewhat lower at 1 month and 3 months in the Tac/Sir arm compared with the Tac/MTX arm; however, the significance did not reach the .01 level (median level, $98.5 \times 10^3/\mu L$ for Tac/Sir versus $124.5 \times 10^3/\mu L$ for Tac/MTX [$P = .02$] at 1 month and $81 \times 10^3/\mu L$ for Tac/Sir vs $111 \times 10^3/\mu L$ for Tac/MTX [$P = .029$] at 3 months).

Treg:Tcon and Treg:CD8 Ratios

The Treg:Tcon ratio was defined as $CD4^+CD25^+$/($CD4^+$ minus $CD4^+CD25^+$). These ratios were then compared in both arms at each time point. The Treg:Tcon ratio was significantly higher in the Tac/Sir arm compared with the Tac/MTX arm at 1 month and 3 months, due largely to the delayed recovery of Tcons in the Tac/Sir arm, but similar in the 2 arms at 6, 12, and 24 months (Figure 2E). The Treg:CD8 ratio followed a similar trajectory and was significantly higher in the Tac/Sir arm at 1 month and 3 months, again reflecting the delayed recovery of $CD8^+$cells in the Tac/Sir arm. Thereafter, the Treg:CD8 ratio was similar in the 2 arms (Figure 2F).

Reconstitution of Naive and Effector T Cells

Absolute numbers of naive CD4+ T cells ($CD4^+CD45RA^+CD62L^+$) were significantly lower at 1 month and 3 months in the Tac/Sir arm but were similar in the 2 arms thereafter (Figure 3A). Proliferating naive $CD4^+$ T cells ($CD4^+CD45RA^+CD62L^+Ki67^+$) were significantly lower only at 1 month after transplantation (data not shown). Thus, recovery of naive T cells was more compromised than recovery of mature T cells in the sirolimus arm. Recovery of effector CD4+ T-cells ($CD4^+CD45RA^+CD62L^-$) was lower at 3 and 6 months in the Tac/Sir arm (Figure 3B). Proliferating $CD4^+$ effector cells ($CD4^+CD45RA^+CD62L^-Ki67^+$) recovered at the same rate in both arms (data not shown).

In the CD8 compartment, the number of naive cells ($CD8^+CD45RA^+CD62L^+$) were significantly lower in the Tac/Sir arm in the first 3 months and at 12 months after transplantation (Figure 3C) and proliferating naïve $CD8^+$ cells ($CD8^+CD45RA^+CD62L^+Ki67^+$) followed the same trajectory (data not shown). The number of $CD8^+$ effector cells ($CD8^+CD45RA^+CD62L^-$) was also significantly lower in the Tac/Sir arm in the first 6 months after transplantation (Figure 3D). Of these, the number of proliferating CD8 effector cells

Table 1
Baseline Patient Characteristics

Characteristic	Tac/Sir (N = 132)	Tac/MTX (N = 132)	All (N = 264)
Recipient age, yr, median (range)	44 (18-59)	42 (12-58)	44 (12-59)
Recipient sex, n (%)			
Male	64 (48.5)	73 (55)	137 (51.9)
Female	68 (51.5)	59 (45)	127 (48.1)
Donor sex, n (%)			
Female	56 (42.4)	48 (36)	104 (39.4)
Male	76 (57.6)	84 (64)	160 (60.6)
Donor age, yr, median (range)	45 (14-66)	41 (13-64)	44 (13-66)
Primary diagnosis, n (%)*			
AML	60 (45.5)	56 (42)	116 (43.9)
CR1	49	49	98
CR2	11	7	18
ALL	44 (33.3)	59 (45)	103 (39.0)
CR1	37	48	85
CR2	7	11	18
CML	9 (6.8)	11 (8)	20 (7.6)
AP	2	2	4
CP	7	9	16
MDS	18 (13.6)	6 (5)	24 (9.1)
RA	1		1
RARS	2		2
RAEB 1	3	3	6
RAEB 2	5		5
CMML	3	2	5
Other	4	1	5
ABL	1 (.8)		1 (.4)
CR1	1		1
Donor-recipient sex match, n (%)			
F/F	29 (22.0)	21 (16)	50 (18.9)
F/M	27 (20.5)	27 (20)	54 (20.5)
M/F	39 (29.6)	38 (29)	77 (29.2)
M/M	37 (28.0)	46 (35)	83 (31.4)
Karnofsky Performance Status score, n (%)			
≥90%	89 (67.4)	98 (74.3)	189 (70.8)
<90%	43 (32.6)	34 (25.7)	77 (29.2)
Donor-recipient CMV serostatus, n (%)†			
+/+	54 (40.9)	43 (33)	97 (36.7)
+/-	10 (7.6)	24 (18)	34 (12.9)
-/+	27 (20.5)	37 (28)	64 (24.2)
-/-	35 (26.5)	23 (17)	58 (22.0)
Unknown	6 (4.6)	5 (4)	11 (4.2)
Conditioning regimen, n (%)			
Cy-TBI	107 (81.1)	105 (79.6)	212 (80.3)
VP16-TBI	25 (18.9)	27 (20.5)	52 (19.7)

AML indicates acute myelogenous leukemia; CR1, first complete response; CR2, second complete response; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; AP, acute phase; CP, chronic phase; MDS, myelodysplastic syndrome; RA, refractory anemia; RARS, refractory anemia with ring sideroblasts; RAEB 1, refractory anemia with excess blasts 1; RAEB 2, refractory anemia with excess blasts 2; CMML, chronic myelomonocytic leukemia; ABL, acute biphenotypic leukemia.

* $P = .05$.

† $P = .01$.$P > .05$ for all other comparisons.

($CD8^+CD45RA^+CD62L^-Ki67^+$) was significantly lower in the Tac/Sir arm at 1 month and 6 months after HCT (data not shown). Thus, the effect of sirolimus on CD8 cell recovery was driven primarily by its effect on effector cells.

Recovery of B Lymphocytes, NK Cells, and NK T Cells Post-HCT

Immune reconstitution of $CD19^+$ B lymphocytes is shown Figure 4A. Absolute numbers of $CD19^+$ cells were similar very early after HCT (1 month) but were significantly lower at 3 and 6 months in the Tac/Sir arm. However, B cell recovery normalized thereafter and was similar to that in the control arm at 12 and 24 months.

NK cell ($CD3^-CD16^+CD56^+$ and $CD3^-CD16^-CD56^+$) recovery in both arms is described in Figure 4B. Absolute numbers of all NK cells ($CD3^-CD56^+$) were significantly lower in the Tac/Sir arm only at 1 month. Subsequently, absolute numbers of NK cells were similar in the 2 arms at all time points.

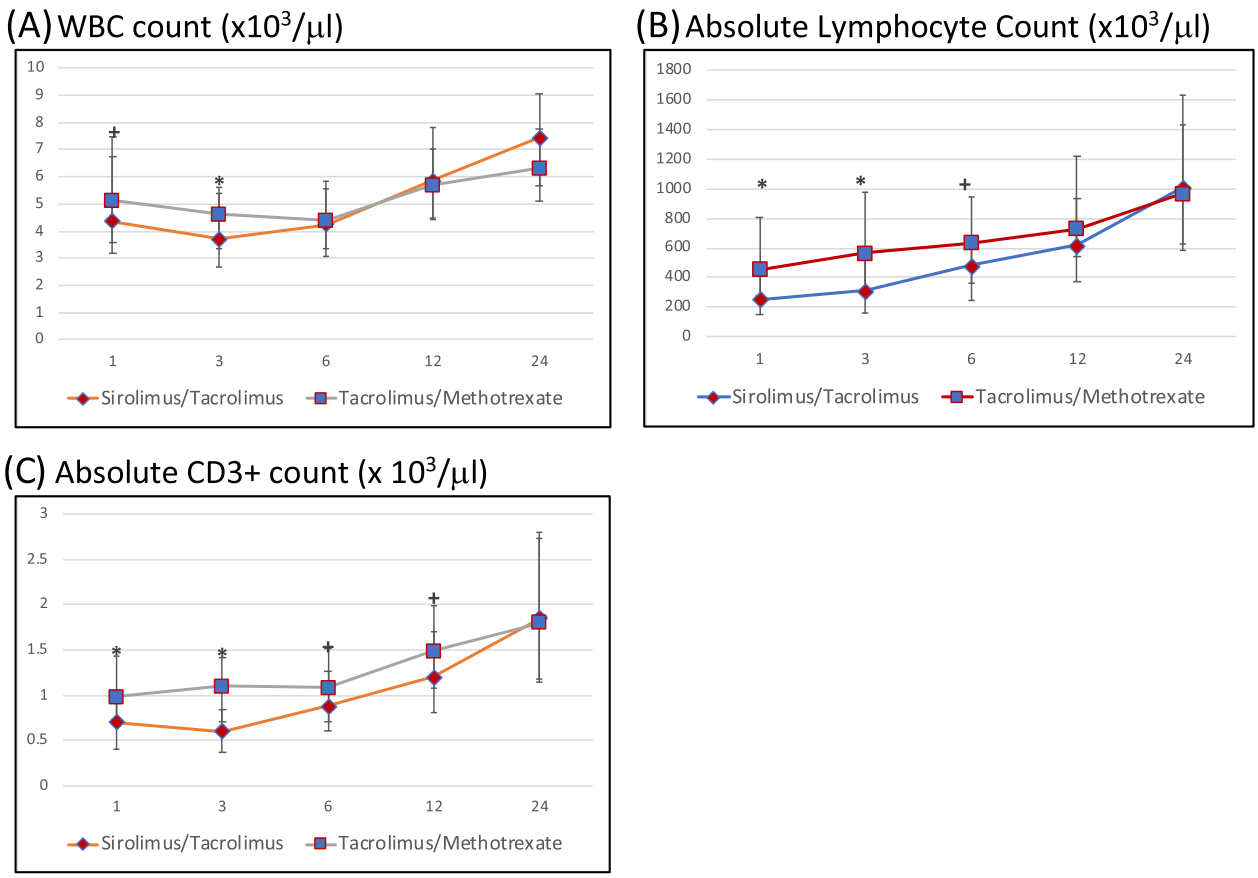

Figure 1. Post-allogeneic HCT reconstitution of total WBC count (A), ALC (B), and absolute $CD3^+$ cell count (/μL) (C) by treatment arm at months 1, 3, 6, 12, and 24. The median cell count for each population is represented at each time point. $^*P < .01$; $^+.01 \leq P$ value $< .05$. The median total WBC count was significantly lower in the Tac/Sir arm at 3 months only. The median ALC was significantly lower in the Tac/Sir arm for up to 3 months post-transplantation but not thereafter.

Impact of Immune Reconstitution on Clinical Outcomes (Using Log-Transformed Absolute Values for Cell Subtypes)

The impacts of various immune subsets on clinical outcomes were analyzed by constructing multivariable Cox regression models using natural log-transformed absolute values of cell counts as time-dependent variables. Higher ALC, $CD3^+$ cell, $CD3^+CD4^+$ cell, Tcon, and Treg ($CD4^+CD25^+$) numbers were all associated with significantly improved OS and NRM at the .01 level (Table 2) but not with relapse (Table 2), aGVHD of any grade, or cGVHD (Table 3). Higher $CD3^+CD8^+$ cell numbers were also associated with improved OS but had no effect on other outcomes, including GVHD. The Treg:Tcon and Treg:CD8 ratios were not correlated with any clinical outcome at the .01 level.

Among other cell subsets, an increased number of B lymphocytes ($CD19^+$) was associated with improved OS and NRM but not with other outcomes; an increased number of NK cells ($CD3^-CD56^+$) was also associated with OS. None of the cell subtypes was associated with relapse except WBC count, with increased count associated with reduced relapse rates (Tables 2 and 3).

We repeated this analysis limiting immune subsets measured at early time points only (1 month and 3 months). The result remained largely similar, except that the effect of $CD19^+$ cells was not significant (Supplementary Table 1), reflecting delayed (after 3 months) $CD19^+$ reconstitution.

DISCUSSION

The recovery of various immune subsets following HCT is a gradual process and can take up to 1 year to approximate levels found in healthy individuals. Typically, the innate immune system (granulocytes, monocytes, and NK cells) recovers in the first few weeks after HCT, followed by T and B lymphocyte recovery over a period of months. Usually, $CD4^+$ T lymphocytes recover slower than $CD8^+$ T cells [13]. The recovery of various T cell subsets has been studied more comprehensively in recent years, and there has been particular interest in Tregs. Tregs normally compose 5% to 10% of circulating T lymphocytes and are instrumental in controlling effector T cell immune responses in sites of inflammation [14]. They have an important role in the immune system, where poor Treg recovery has been significantly associated with both aGVHD and cGVHD [14]. In general, an imbalance between recovery of Tregs and Tcons has been associated with cGVHD [13]. In vitro studies have suggested that sirolimus has a Treg-sparing effect with subsequent beneficial effects on GVHD in murine models [11,15]. However, the effects of sirolimus in combination with a calcineurin inhibitor (tacrolimus) in vivo may or may not reflect the effects seen in murine models.

Here we present a comprehensive analysis of the randomized trial BMT-CTN 0402, comparing immune reconstitution in the Tac/Sir and Tac/MTX arms in an attempt to better delineate the in vivo effect of sirolimus. This was a unique opportunity to explore the effect of sirolimus on recovery of immune subsets without significant confounders and biases, because the arms were randomized, and flow cytometry was performed in a blinded fashion.

Patients who received Tac/Sir had compromised T-cell reconstitution in the early post-transplant period with

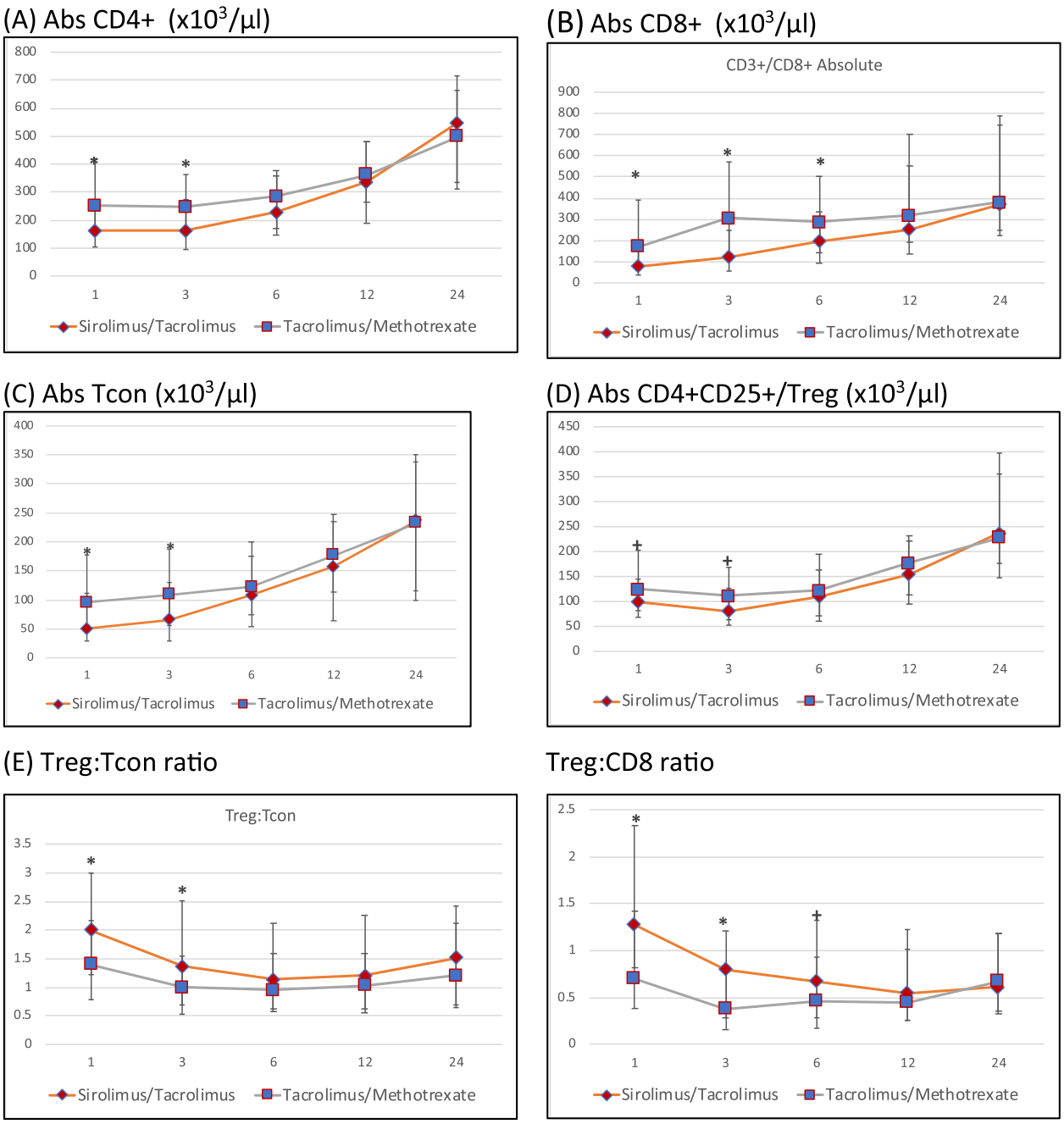

Figure 2. Post-allogeneic HCT reconstitution of major T cell populations ($CD4^+$, $CD8^+$, $CD4^+CD25^+$/Tregs, $CD4^+CD25^-$/Tcons), Treg:Tcon ratio, and Treg:CD8 ratio by treatment arm at post-HCT months 1, 3, 6, 12, and 24. The median cell count/μL for each population is represented at each time point. $^*P < .01$; $^+.01 \leq P$ value $< .05$. Absolute $CD3^+$ and $CD4^+$cell counts were significantly lower in the Tac/Sir arm at 1 month and 3 months post-transplantation. Absolute $CD8^+$cell counts were significantly lower in the Tac/Sir arm from 1 to 6 months post-transplantation but not thereafter. Treg counts were similar in the 2 arms at all time points. Treg:Tcon and Treg:CD8 ratios were significantly higher in the Tac/Sir arm for up to 3 months post-transplantation.

significantly lower CD3+ cells, CD4+ cells, Tcons, and ALC in the first 3 months after transplantation compared with the Tac/MTX arm. Sirolimus specifically blocks T cell proliferation via mTOR inhibition by acting at a different point in the cell cycle than tacrolimus [4]; thus, this synergistic effect on T cell suppression is not unexpected. Interestingly, the T cell subset most profoundly affected in the Tac/Sir arm was $CD8^+$ T lymphocytes, which were present in significantly lower numbers in this arm up for to 6 months after transplantation.

We found no significant difference at the .01 level in Treg reconstitution between the Tac/Sir and Tac/MTX arms at any time point. Although the Treg level was somewhat lower in the Tac/Sir arm early after HCT, the relative difference in Treg level was much smaller than the significant differences seen in Tcons and $CD3^+CD8^+$ cells. This is consistent with previous studies in murine models suggesting that sirolimus spares Tregs [11,15,16]. In humans, a calcineurin inhibitor-free transplantation platform (fludarabine/treosulfan/ATG-Fresenius conditioning with post-transplantation Cy and sirolimus for GVHD prophylaxis) in the haploidentical setting, showed that Treg numbers were significantly higher while patients were receiving sirolimus (day +30) compared to when they had

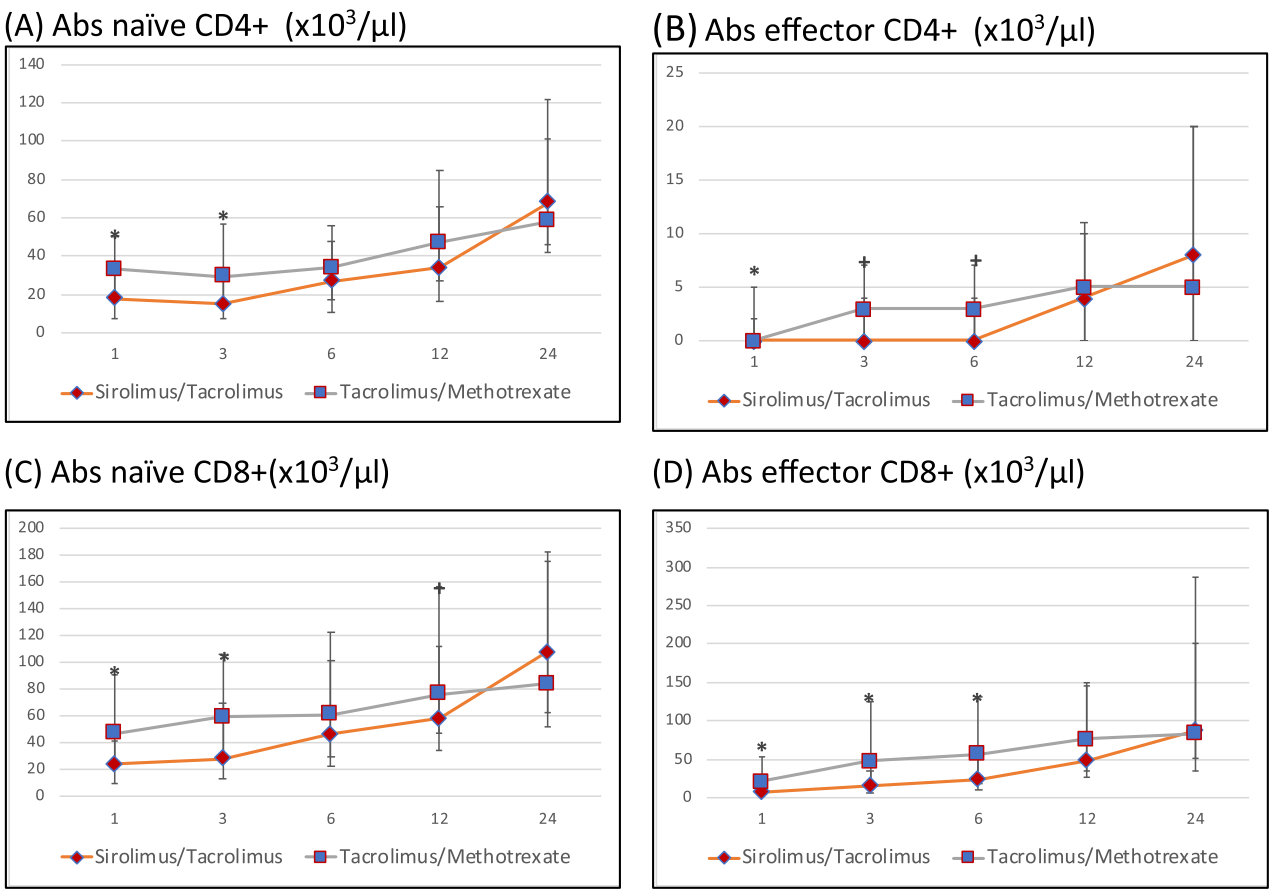

Figure 3. Post-allogeneic HCT reconstitution of T cell subsets (naive $CD4^+$and effector $CD4^+$ T cells and naive $CD8^+$ and effector $CD8^+$ T cells) by treatment arm at post-HCT months 1, 3, 6, 12, and 24. The median cell count/μL for each population is represented at each time point. *$P < .01$; +$.01 \leq P$ value $< .05$. Absolute naive $CD4^+$ cell count was significantly lower in the Tac/Sir arm at 1 month and 3 months post-HCT, whereas absolute effector T cells count was significantly lower at 3 and 6 months. Absolute naive $CD8^+$ cell count was significantly lower for up to 3 months and then again at 12 months post-transplantation, whereas effector $CD8^+$ cell count was significantly lower in a sustained manner at 1, 3, and 6 months post-transplantation.

been weaned off (day +180) [17]. This further suggests that the Treg-sparing effect of sirolimus might have been more pronounced had tacrolimus not been used concomitantly for GVHD prophylaxis, as it was in BMT-CTN 0402. To investigate this issue more comprehensively, we examined the Treg:Tcon ratio in the 2 arms and found that it was significantly higher in

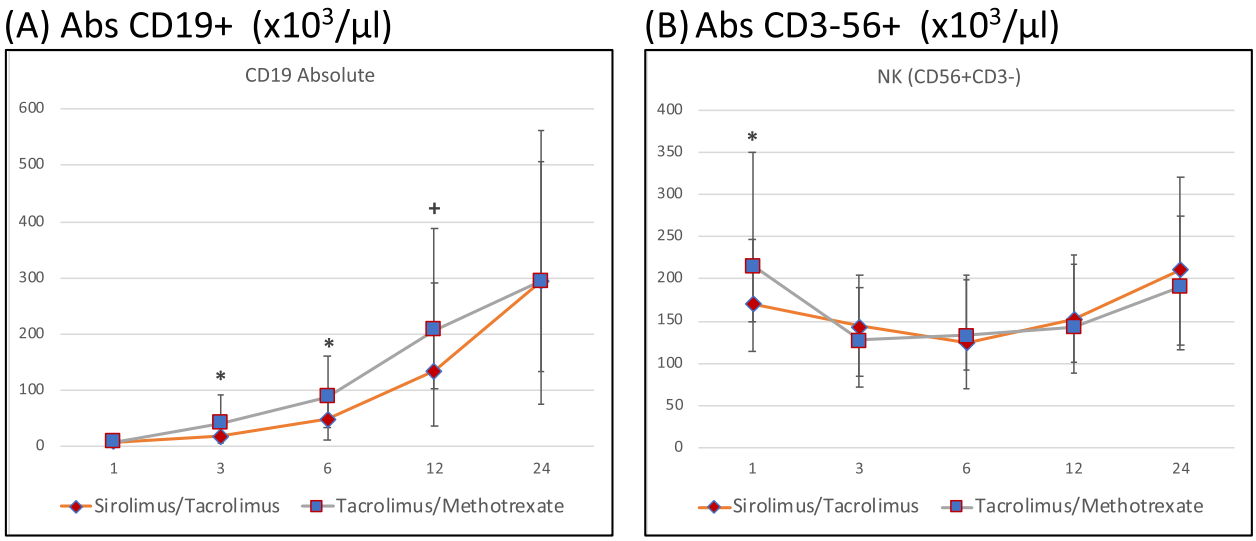

Figure 4. Post-allogeneic HCT reconstitution of B cells and NK cells by treatment arm at months 1, 3, 6, 12, and 24. The median cell count/μL for each population is represented at each time point. *$P < .01$; +$.01 \leq P$ value $< .05$. Absolute $CD19^+$ (B lymphocyte) counts are significantly lower in the Tac/Sir arm at 3 and 6 months post-transplantation. Absolute NK cell count was significantly lower in the Tac/Sir arm only at 1 month post-transplantation but not thereafter.

Table 2
Cox Multivariable Model for Relapse, NRM, and OS by Immunophenotype

Cell Type	Relapse			NRM			OS		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
$CD3^+$	.73	.56-.97	.03	.56	.40-.80	**.0015**	.55	.44-.69	<**.0001**
$CD3^+CD4^+$	.76	.57-1.02	.06	.46	.34-.64	<**.0001**	.49	.40-.62	<**.0001**
$CD3^+CD8^+$	.79	.63-.99	.04	.72	.53-.98	.03	.66	.55-.80	<**.0001**
$CD3^+CD4^+CD25^+$	.89	.69-1.13	.33	.68	.55-.84	**.0004**	.67	.57-.78	<**.0001**
Tcon	.82	.65-1.04	.10	.55	.41-.72	<**.0001**	.57	.47-.69	<**.0001**
Treg:Tcon	1.13	.69-1.84	.63	1.38	.76-2.51	.29	1.54	1.03-2.28	.03
Treg:CD8	1.29	.72-2.32	.39	.80	.35-1.82	.60	1.12	.66-1.91	.68
$CD19^+$	1.27	1.05-1.53	.0144	.71	.58-.86	**.0006**	.74	.65-.84	<**.0001**
$CD3^-CD56^+$	.73	.56-.96	.02	.65	.46-.91	.012	.66	.53-.81	**.0001**
Total WBC	.56	.39-.82	.0029	1.79	.93-3.47	.08	.81	.56-1.18	.28
ALC	.83	.57-1.20	.33	.64	.38-1.07	.09	.57	.42-.76	**.0001**
cDC ($Lin^-/HLADR^+/CD123^-/CD11c^+$)	.991	.875-1.123	.89	.995	.838-1.182	.96	1.012	.903-1.134	.833
pDC ($Lin^-/HLADR^+/CD123^+/CD11c^-$)	1.192	1.008-1.41	.039	.808	.683-.956	.013	1.016	.902-1.146	.79

$P < .01$ is indicated in bold type.
cDC indicates conventional dendritic cells; pDC, plasmacytoid dendritic cells.

the Tac/Sir arm for up to 3 months after transplantation; however, this effect was lost at later time points. We previously showed that a higher Treg:Tcon ratio at 90 days after transplantation is associated with lower rates of cGVHD [13], and thus the effect of sirolimus on this ratio may be indicative of its efficacy as a GVHD prophylaxis agent, at least in the context of cGVHD. The Treg:CD8 ratio was also significantly increased in the Tac/Sir arm for up to 3 months after transplantation. Thus, although the absolute number of Tregs was not higher in the Tac/Sir arm in vivo, sirolimus affects the balance between regulatory and effector cells in favor of Tregs, which has important implications for GVHD prevention. It should be noted that the effects of different rates of immunosuppression tapering have not been accounted for in this analysis, because these data were not available.

We performed a further analysis of $CD4^+$ and $CD8^+$ T cell recovery to determine whether sirolimus preferentially affected naïve or mature cell subsets. Within the $CD4^+$ compartment, proliferation of naïve T cells (Ki-67^+) was lower in the first month after transplantation, and the number of naïve cells was significantly lower for up to 3 months after transplantation in the Tac/Sir arm. $CD4^+$ effector cells were significantly decreased in the sirolimus arm for only 1 month, suggesting that naive CD4 T cells were preferentially affected by sirolimus. In contrast, $CD8^+$ effector cells were compromised for up to 6 months, indicating that sirolimus affected CD8 cell recovery more than CD4 T cell recovery.

We found that the absolute numbers of $CD19^+$ B lymphocytes were similar in both arms at 1 month, but recovery was slower in the Tac/Sir arm at 3 and 6 months after transplantation. The effects of sirolimus on B lymphocytes have been investigated in vitro but has never been analyzed in vivo in the context of HCT. Using purified human B lymphocytes from healthy volunteers, sirolimus profoundly inhibited B cell proliferation at clinically relevant concentrations in vitro. In contrast, tacrolimus had a minimal effect on B cells [18,19]. Thus, the effect of B lymphocytes seen in the Tac/Sir arm is likely a direct effect of sirolimus. The implications of this in the context of cGVHD are likely complex. The role of B cells in the pathogenesis of cGVHD has been highlighted in recent years. A large, diverse mature B cell pool contains B lymphocytes, which can sequester B cell activating factor (BAFF); subsequently, autoreactive B lymphocytes, which require BAFF to survive, are not able to proliferate and mediate cGVHD [20]. It is possible that

Table 3
Cox Multivariable Model for aGVHD and cGVHD by Immunophenotype

Cell Type	aGVHD Grade II-IV			aGVHD Grade III-IV			cGVHD		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
$CD3^+$	.96	.70-1.31	.78	.90	.56-1.43	.65	1.22	.98-1.51	.08
$CD3^+CD4^+$	.87	.62-1.21	.40	.80	.50-1.28	.35	1.22	.96-1.54	.11
$CD3^+CD8^+$	.99	.77-1.27	.93	.87	.59-1.28	.48	1.16	.98-1.38	.08
$CD3^+CD4^+CD25^+$	.84	.59-1.17	.30	.75	.49-1.16	.20	1.08	.86-1.35	.51
Tcon	.91	.69-1.20	.52	.82	.55-1.22	.33	1.21	1.01-1.46	.04
Treg:Tcon	.99	.56-1.73	.96	1.19	.51-2.78	.69	.71	.49-1.04	.08
Treg:CD8	.71	.37-1.37	.30	.92	.34-2.53	.87	.73	.46-1.15	.17
$CD19^+$	.84	.67-1.06	.14	.77	.56-1.05	.10	1.03	.92-1.15	.66
$CD3^-CD56^+$	.99	.71-1.37	.94	1.02	.61-1.69	.95	.90	.74-1.11	.32
Total WBC	1.74	1.08-2.81	.02	.90	.45-1.79	.76	.98	.70-1.36	.89
ALC	.93	.62-1.39	.71	.60	.32-1.12	.11	1.15	.88-1.50	.30
cDC ($Lin^-/HLADR^+/CD123^-/CD11c^+$)	.992	.881-.117	.89	.995	.821-1.206	.96	1.009	.92-1.107	.84
pDC ($Lin^-/HLADR^+/CD123^+/CD11c^-$)	.956	.803-1.138	.61	.985	.756-1.284	.91	.956	.866-1.056	.37

sirolimus depletes both alloreactive and autoreactive B lymphocytes and thus eventually does not have a significant effect on the incidence of cGVHD. Further studies with concomitant measurement of BAFF levels in patients who receive sirolimus may inform this issue further.

In the outcomes analysis performed on this subset, no cell subtype was associated with aGVHD or cGVHD, including Tregs and Treg:Tcon ratio. However, higher absolute numbers of all T cell subtypes as well as B lymphocytes were associated with better OS. This likely reflects more robust immune reconstitution. Relapse was not affected by any cell subtype at the .01 level, although $CD3^+$, $CD8^+$, $CD19^+$, and NK cells were borderline significantly associated with relapse ($.01 < P$ value $< .05$).

We acknowledge a limitation of our study in that we did not correlate infectious complications—specifically CMV and other viral reactivations—with immune reconstitution, and this should be studied further in the future. It should be noted that in the parent RCT, there was no difference between arms in terms of infectious complications or infectious dates [10]. We acknowledge that the definition used for Tregs ($CD3^+CD4^+CD25^+$) could be further refined by current standards by the addition of Foxp3 or CD127 to the phenotypic definition, and we will pursue this in follow-up studies. We further acknowledge that because this is a retrospective analysis of an existing dataset with multiple unplanned analyses, there is an increased possibility of a type 1 error.

In conclusion, we describe the effect of sirolimus used as GVHD prophylaxis on immune reconstitution post-transplantation in the context of a large RCT. Sirolimus in combination with tacrolimus has a more profound T cell suppressive effect than the combination of Tac/MTX in the early post-transplantation period, and particularly compromises recovery of $CD8^+$ T cells, with potential implications in the prevention of aGVHD. Sirolimus when used with tacrolimus does not appear to increase absolute numbers of Tregs (defined as $CD4^+CD25^+$ T cells) but might have a beneficial effect on the Treg:Tcon balance in the first 3 months after transplantation. This also suggests that calcineurin inhibitor-free, sirolimus containing GVHD prophylaxis strategies, incorporating other novel agents (for, eg, OX40L blocking antibody KY1005 as shown by Kean et al [21]) should be investigated further to maximize the potential favorable effect of sirolimus on Treg:Tcon balance in the post-transplantation immune repertoire. Tregs should be defined more rigorously (preferably as $CD4^+CD25^+Foxp3^+$ cells) to more comprehensively validate these results. Finally, sirolimus significantly compromises B cell recovery in the first 6 months after HCT, with potential complex effects on cGVHD that merit further study.

ACKNOWLEDGMENTS

The authors thank all the participating centers and principal investigators at each participating center and the patients who participated in BMT CTN 0402.

Financial disclosure: Support for this study was provided by Grant U10HL069294 to the Blood and Marrow Transplant Clinical Trials Network from the National Heart, Lung, and Blood Institute (NHLBI) and the National Cancer Institute, along with contributions by Wyeth Pharmaceuticals. BMT CTN 0402 biospecimens were obtained from the NHLBI's Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC). The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the aforementioned parties. Additional support was provided by the Division of Allergy, Immunology, and Transplantation, National Institute of Allergy and Infectious Diseases for the ancillary study, "GVHD Prophylaxis—Immune Reconstitution."

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

SUPPLEMENTARY MATERIALS

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2019.06.029.

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