A randomized phase II study to evaluate tacrolimus in combination with sirolimus or methotrexate after allogeneic hematopoietic cell transplantation

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ABSTRACT

Background
There is evidence suggesting that sirolimus, in combination with tacrolimus, is active in the prevention of graft-*versus*-host disease. Sirolimus-based immune suppression may suppress alloreactive T cells, while sparing the survival and function of regulatory T cells.

Design and Methods
We conducted a randomized trial to compare the impact of sirolimus/tacrolimus against that of methotrexate/tacrolimus on the prevention of graft-*versus*-host disease and regulatory T-cell reconstitution.

Results
Seventy-four patients were randomized 1:1 to sirolimus/tacrolimus or methotrexate/tacrolimus, stratified for type of donor (sibling or unrelated) and the patients' age. The rate of grade II-IV acute graft-*versus*-host disease at 100 days was 43% (95% CI: 27-59%) in the sirolimus/tacrolimus group and 89% (95% CI: 72-96%) in the methotrexate/tacrolimus group (P<0.001). The rate of moderate/severe chronic graft-*versus*-host disease was 24% (95% CI: 7-47%) in the sirolimus/tacrolimus group and 64% (95% CI: 41-79%) in the methotrexate/tacrolimus group (P=0.008). Overall survival and patient-reported quality of life did not differ between the two groups. On days 30 and 90 post-transplant, sirolimus-treated patients had a significantly greater proportion of regulatory T cells among the $CD4^+$ cells in the peripheral blood, and isolated regulatory T cells were functional.

Conclusions
These data demonstrate that sirolimus/tacrolimus prevents grade II-IV acute graft-*versus*-host disease and moderate-severe chronic graft-*versus*-host disease more effectively than does methotrexate/tacrolimus, and supports regulatory T-cell reconstitution following allogeneic hematopoietic cell transplantation. *Trial registration: (NCT00803010)*

Key words: tacrolimus, sirolimus, methotrexate, combination therapy, GVHD prophylaxis.

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The online version of this article has a Supplementary Appendix.

Introduction

Current pharmacological strategies do not prevent acute graft-*versus*-host disease (GVHD) adequately following allogeneic hematopoietic cell transplantation (HCT).[1,2] Investigators have demonstrated in single-arm phase II trials that the combination of sirolimus (SIR) and tacrolimus (TAC) has activity in the prevention of acute GVHD.[3-7] Antin *et al.* originally reported a low incidence of grade II-IV acute GVHD following a triple combination of TAC/SIR/methotrexate (MTX).[3] However, Furlong *et al.* reported serious toxicity and a greater risk of GVHD using the combination of a calcineurin inhibitor/SIR/MTX.[6] Cutler *et al.* found a low incidence of grade II-IV acute GVHD following the two-drug combination of SIR/TAC,[4,5] while these findings were not reproduced by Rodriguez *et al.*[7] A phase III trial (CTN 0402) has compared SIR/TAC to MTX/TAC in adults under the age of 60 with myelodysplasia, or acute or chronic myeloid leukemia, treated with cyclophosphamide, total body irradiation and sibling donor HCT; the results from this trial are awaited. We aimed to study the activity of SIR/TAC in GVHD prevention among patients with a broader range of ages, diseases, conditioning therapies and donor types. Acknowledging the inter-institutional variation in observed rates of acute GVHD, due in large part to the identification of gastrointestinal involvement,[8] we performed a randomized controlled trial comparing SIR/TAC to MTX/TAC.

The ability of regulatory T cells (Treg) to prevent acute GVHD in pre-clinical studies provides the rationale for translation to humans, but common immunosuppressive regimens inhibit Treg after HCT.[3-7] SIR influences several key processes relevant to the pathogenesis of GVHD and allows $CD4^+CD25^+$ Treg expansion, proliferation, and survival in pre-clinical models.[9-14] While effector T cells are sensitive to the inhibitory effect of SIR, Treg expand in the presence of SIR.[10,12,15,16] Coenen *et al.* demonstrated that SIR preserved the potently suppressive $CD27^+$ Treg subset and that Treg cultured in the presence of SIR had greater suppressive capacity than Treg cultured with cyclosporine.[17] SIR also inhibits the differentiation of naïve CD4 T cells into Th17 cells and promotes the generation of Treg.[18] We hypothesized that SIR-based immune suppression would suppress alloreactive T cells, support selective recovery of Treg, and thus prevent GVHD more effectively.

Design and Methods

Study design

We conducted a prospective, randomized comparison of SIR/TAC *versus* MTX/TAC (NCT00803010). This trial was approved by the University of South Florida Institutional Review Board. Randomization was stratified for age (≥ 50 *versus* <50 years), and donor type (sibling *versus* unrelated); these two factors were selected for stratification based on existing evidence that they have an impact on the risk of developing GVHD. Other clinical variables were not included in stratification. All patients received peripheral blood mobilized grafts. The primary objective of this trial was to evaluate the efficacy of SIR/TAC *versus* MTX/TAC in the prevention of grade II-IV acute GVHD. The study was powered to detect a difference in the incidence of grade II-IV acute GVHD between patients in the two treatment groups. Among MTX/TAC-treated patients, we anticipated a grade II-IV acute GVHD rate of 80%, based on that observed in MTX/TAC-treated patients in a recent, prospective clinical trial at our center.[19] Based on previously published results of a single-arm phase II SIR/TAC trial results in which the incidence of grade II-IV acute GVHD was approximately 20% in comparison to previously reported incidence of 40-50% following MTX/TAC,[4,5] our *a priori* hypothesis was that we would observe a 50% reduction in this primary endpoint. The trial design included a concurrent comparator to facilitate interpretation, as there is variation in reported baseline incidences of grade II-IV acute GVHD between centers. With 56 evaluable patients without competing risks, a two-sided log-rank test would have 90% power at a level of significance of 0.1. We anticipated that 20% of evaluable patients would develop competing-risk events within 100 days, and adjusted the total sample size to 70. We then increased the sample size to 74 (37 in each arm) to allow for a 5% drop-out.

Patients

The patients included in this study were aged 16 – 70 years with an ejection fraction ≥45%, lung function tests (forced expiratory volume in 1 sec, forced vital capacity and carbon monoxide diffusing capacity) ≥ 50% predicted values, aspartate and alanine aminotransferase levels <3 times the upper limit of normal, creatinine clearance ≥50 cc/min, and Karnofsky Performance Status ≥60%. The patients had the following diseases: acute myelogenous leukemia of intermediate/high risk in first complete remission or beyond; myelodysplastic syndrome with an International Prognostic Scoring System score of ≥1.5; myeloproliferative disorders; chronic myelogenous leukemia; acute lymphoblastic leukemia; chronic lymphocytic leukemia; severe aplastic anemia; multiple myeloma; and Hodgkin's or non-Hodgkin's lymphoma. Patients with hepatitis B, hepatitis C or human immunodeficiency virus, uncontrolled systemic infection, or an HCT-comorbidity index ≥ 3 were excluded.[20]

Treatment protocol

Eligible donors were sibling or unrelated donors matched for HLA-A, B, C, and DRB1 by high-resolution typing. Peripheral blood products, mobilized with granulocyte colony-stimulating factor, were targeted to a $CD34^+$ cell dose of $5\text{-}10\text{x}10^6$/kg. Use of anti-lymphocyte antibodies and cyclophosphamide-containing regimens was prohibited, but the conditioning regimen was otherwise not mandated. Institutional standards for prophylaxis and monitoring of bacterial, viral, and fungal infections were followed.

Graft-versus-host disease prophylaxis

TAC was administered intravenously from day -3 at a dose of 0.02 mg/kg/day, before conversion to the oral formulation prior to hospital discharge. The serum TAC target for patients receiving MTX was 5-15 ng/mL, whereas for patients given SIR, the target TAC was 3-7 ng/mL. According to the protocol, patients without evidence of acute GVHD and not on therapy with systemic glucocorticoids were eligible for TAC tapering at day 50 following HCT. SIR was administered as a 9 mg oral loading dose on day -1, followed by maintenance to a target of 5-14 ng/mL. The protocol mandated that SIR should be continued for at least 1 year post-HCT. MTX was administered on day +1 at a dose of 15 mg/m^2, and then at a dose of 10 mg/m^2 on days 3, 6, and 11. Beyond the above specifications, the protocol did not mandate a particular tapering schedule for TAC, SIR, systemic glucocorticoids, or other immune suppressive agents; these schedules were decided by the treating physicians.

Study end-points

Neutrophil and platelet engraftment were defined by standard methods. Mucositis was graded according to Common Toxicity Criteria version 4.0. The diagnosis and grading of severity of

thrombotic microangiopathy (TMA) was based on the BMT Clinical Trials Network consensus.[21] Hepatic veno-occlusive disease (VOD) was diagnosed according to standard clinical criteria.[22] Acute GVHD was scored weekly from HCT to day 100. In keeping with established clinical practice, biopsy confirmation of acute GVHD was not required by the protocol,[23] although biopsies were taken if considered necessary by the treating physician. These GVHD biopsies were reviewed by pathologists at our institution, who were blind to the study participation and study arm assignment. Chronic GVHD was scored according to NIH consensus criteria.[24] Peripheral blood sorted (CD3 and CD33) and bone marrow donor chimerism were assessed at days 30, 90, 180, and 360 by polymerase chain reaction. Disease was restaged on days 30, 90, 180, and 360, at 18 months, and 2 years following HCT. Patient-reported quality of life was assessed using the Functional Assessment of Cancer Therapy – Blood and Marrow Transplantation (FACT-BMT) questionnaire at baseline (pre-HCT), and on days 30, 90, 180, 270, 360, 560, and 740 post-HCT.[25]

T regulatory cell repopulation and suppressive function after hematopoietic stem cell transplantation

Treg reconstitution analysis was performed on peripheral blood samples drawn from all the HCT recipients at baseline (prior to beginning the conditioning regimen and HCT) and on days 0, 30, 90, 180, and 360 after HCT. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-hypaque gradient centrifugation and stained with labeled antibodies (CD3PerCp, CD4FITC, CD25PE, CD127Alexa 647 and mouse IgG1 isotype controls from BD Biosciences). Samples were analyzed using a FACS Calibur flow cytometer with CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA). T cells were identified by gating on $CD3^+$ and $CD4^+$ populations, and Treg were defined by a $CD4^+CD25^{bright}CD127^-$ phenotype. The reciprocal relationship between negative surface CD127 and high intracellular FoxP3 expression was confirmed in a subset (n=15) of samples on day 30 (r=0.94).

The suppressive potential of Treg was examined in blood cells obtained between 90 and 180 days after HCT from subsets of patients from the SIR/TAC and MTX/TAC groups. $CD4^+CD25^+CD127^-$ Treg were isolated on a BD FACSAria II high-speed cell sorter (BD Biosciences, San Jose, CA, USA). Treg were added in different ratios to $1x10^{1884}$ self $CD4^+CD25^-$ T responder cells in the presence of 1:1 CD3/CD28 beads (Invitrogen Corporation, Carlsbad, CA, USA) in 96-well round-bottomed plates. Proliferation was analyzed by [^{3}H] thymidine incorporation using a gas scintillation counter (Matrix 96 β-counter, Canberra Packard, Meriden, CT, USA). Cells were pulsed with 1 μCi/well ^{3}H-thymidine for the last 18 h in culture and harvested on day 5 to measure proliferation. Results are expressed in counts per minute (cpm) for at least triplicate measurements.

Statistical methods

Analyses of all end-points were conducted on the intent-to-treat population. The cumulative incidence of grade II-IV acute GVHD was estimated and compared by Gray's test.[26] Survival was analyzed using the Kaplan-Meier method and compared using the log-rank test. Cumulative incidences of non-relapse mortality and relapse were estimated and compared. Pointwise 95% confidence intervals for survival curves and cumulative incidence curves were computed using log-log transformation. Associations between GVHD outcomes and time-dependent measures (serial TAC and SIR levels, serial measures of Treg) were analyzed using a Cox regression model with time-varying covariates. A two-sided Wilcoxon's rank-sum test was employed to test differences in percent Treg (% Treg/total $CD4^+$ cells) on days 30, 90, 180 and 360 at a significance level of 0.05 (α=0.025 at each time point using the Bonferroni-Holm adjustment).

Results

Patients' characteristics and compliance with therapy

From September, 2008 to May, 2011, 175 patients were assessed for eligibility of whom 101 were excluded for the following reasons: not meeting inclusion criteria (n=72), declined to participate (n=16), no insurance coverage for trial (n=8), and disease progression (n=5). Thus, 74 patients were randomized 1:1 to SIR/TAC or MTX/TAC. No patients were lost to follow-up and all were included in the reported analyses (*Online Supplementary Figure S1*). Baseline characteristics were well matched (Table 1). There were differences in represented diseases across study arms, but these did not reach statistical significance. There was no difference in conditioning regimens by study arm. Of note, the predominant conditioning regimen used was pharmacokinetic targeted IV busulfan in combination with fludarabine, which represents our institutional standard for myeloablative conditioning. In both the SIR/TAC and MTX/TAC groups, there was one case of prior single autologous HCT, and one case of tandem autologous HCT for multiple myeloma. Among 37 patients treated with MTX/TAC, 34 completed all doses of MTX; three received three doses of MTX, followed in two cases by initiation of mycophenolate mofetil as substitute prophylaxis. The final dose of MTX was not given because of grade 4 mucositis (n=2) or liver dysfunction (n=1). Overall compliance with SIR was excellent: of the 37 patients treated with SIR/TAC, only two discontinued SIR (both because of grade I TMA, at days 77 and 150 post-HCT). Among the 17 alive who had been followed up for more than 1 year at the time of analysis, 16 were receiving SIR as planned per protocol.

Engraftment and early toxicity

Time to neutrophil engraftment did not differ between patients treated with SIR/TAC (median 16 days; range, 11-22) or MTX/TAC (median 16 days; range, 12-28) (*P*=0.57). Time to platelet engraftment was also similar in the SIR/TAC (median 12 days; range, 6-20) and MTX/TAC (median 16 days; range, 10-33) groups (*P*=0.6). No significant differences were observed in donor chimerism at any of the time points studied (days 30, 90, and 360 post-HCT). Peak mucositis did not differ significantly between the two treatment groups (Table 2). The cumulative incidence of hepatic VOD did not differ significantly [SIR/TAC 5% (95% CI: 1-21%) *versus* MTX/TAC 3% (95% CI: 0.4-19%)] (*P*=0.56). The VOD severity is presented in Table 2. Notably, the incidence of VOD observed in this study is lower than that previously reported.[27] The cumulative incidence of TMA did not differ significantly between the two groups [SIR/TAC 25% (95% CI: 14-44%) *versus* MTX/TAC 20% (95% CI: 10-38%)] (*P*=0.48). TMA occurred in nine SIR/TAC-treated patients and seven MTX/TAC-treated patients (*P*=0.57). Maximal TMA grades in both groups are presented in Table 2.

Acute graft-versus-host disease

The cumulative incidence of grade II-IV acute GVHD at 100 days was 43% (95% CI: 27-59%) in the SIR/TAC group, and 89% (95% CI 72-96%) in the MTX/TAC group (*P*<0.001) (Figure 1). Adjusting for age >50 *versus* ≤50 years

and donor type in a multivariable model, SIR/TAC was associated with a lower hazard for grade II-IV acute GVHD (HR 0.28, 95% CI 0.15-0.52; $P<0.001$) compared to MTX/TAC. Significant reductions in grade II-IV acute GVHD were observed both for patients with matched sibling donors (41% *versus* 78%; $P=0.02$) and those with matched unrelated donors (45% *versus* 100%; $P=0.001$). The cumulative incidence of grade III-IV acute GVHD did not differ significantly (14% *versus* 11%; $P=0.71$). While the incidence of grade II-IV acute GVHD in the MTX/TAC arm was higher than that reported in some publications, it is consistent with the incidence observed at our center in a previous randomized comparative trial.[19] Inter-institution variation in the observed incidence of acute GVHD is largely due to how aggressively diagnostic endoscopy is pursued to assess the etiology of gastrointestinal (GI) disturbances.[8] As differences between the two arms of the study did not emerge until nearly 28 days after transplantation, it is unlikely that the higher incidence of grades II-IV GVHD in the MTX/TAC arm was related to toxic effects of MTX on the GI epithelium. The distribution of the overall acute GVHD grades differed significantly between the two treatment groups, largely because of a reduction of grade II disease in the SIR/TAC group (Table 3). As regards individual target organs, we only observed significant differences in acute GVHD stage between treatment groups for GI disease (Table 3). Considering the site of GI involvement, SIR/TAC-treated patients had reductions in both isolated upper GI (SIR n=3, MTX n=10) and combined upper/lower GI involvement (SIR n=5, MTX n=12), but not isolated lower GI involvement (SIR n=7, MTX n=7). Using time-dependent Cox modeling, we could not detect significant relationships between drug (TAC, SIR) levels and grade II-IV or grade III-IV acute GVHD.

Table 1. Baseline characteristics of the study sample.

	Methotrexate	Sirolimus	
Recipient age (median, range)	48 (23-69)	49 (25-68)	$P=0.36$
Gender			
Male	23	28	$P=0.21$
Female	14	9	
Diagnosis			$P=0.08$*
Acute lymphoblastic leukemia	10	5	
First complete remission	10	5	
Acute myelogenous leukemia	8	15	
First complete remission	5	8	
Second complete remission	2	3	
Primary induction failure	1	2	
First relapse	0	1	
No treatment	0	1	
Chronic lymphocytic leukemia	4	3	
Complete remission	2	2	
Partial remission	1	0	
Stable disease	1	1	
Chronic myelogenous leukemia	0	2	
First chronic phase	0	2	
Myelodysplastic syndrome	7	2	
Complete remission	2	0	
Hematologic improvement	4	1	
Stable disease	1	0	
Not treated	0	1	
Multiple myeloma	2	6	
Complete remission	1	4	
Very good partial remission	0	1	
Partial remission	1	1	
Myeloproliferative disease	2	0	
Stable disease	2	0	
Non-Hodgkin's lymphoma	4	1	
Second coplete remission	0	2	
Third complete remission or beyond	1	0	
First partial remission	1	0	
Second partial remission	0	1	
Primary induction failure	1	0	
First relapse (sensitive)	1	0	
Third relapse or beyond (untreated)	0	1	
CIBMTR risk category			$P=0.52$
High	8	7	
Intermediate	7	7	
Low	20	23	
Other	2	0	
Donor			$P=0.82$
Matched sibling donor	18	17	
Matched unrelated donor	19	20	
Recipient:Donor CMV matching			$P=0.06$
Negative: negative	12	10	
Negative: positive	7	1	
Positive: negative	8	16	
Positive: positive	10	10	
Donor gender			$P=0.35$
Female	21	17	
Male	16	20	
Donor age (median, range)	37 (18-65)	37 (22-67)	$P=0.3$
Conditioning regimen			$P=0.22$
FluBu	30	26	
Pento/Bu	5	4	
Flu/Mel	2	7	

**Diagnosis: P=0.08; Remission status: P=0.69; CMV: cytomegalovirus; Bu: busulfan; Flu: fludarabine; pento: pentostatin; Mel: melphalan.*

Acute graft-versus-disease therapy

We collected comprehensive data on prednisone, beclomethasone and budesonide therapy in affected patients. The proportion of living patients on prednisone was not significantly different between groups compared

Table 2. Summary of toxicities.

Variable	Levels	MTX (%)	SIR (%)	P value
Mucositis CTC Grade				
	1	3 (8.1)	8 (21.6)	0.12
	2	9 (24.3)	13 (35.1)	
	3	21 (56.8)	15 (40.5)	
	4	4 (10.8)	1 (2.7)	
TMA				
	No	30 (81.1)	28 (75.7)	0.57
	Yes	7 (18.9)	9 (24.3)	
TMA grade				
	1	4 (10.8)	9 (24.3)	0.17
	2	2 (5.4)	0 (0.0)	
	4	1 (2.7)	0 (0.0)	
	N/A	30 (81.1)	28 (75.7)	
VOD				
	No	36 (97.3)	35 (94.6)	0.56
	Yes	1 (2.7)	2 (5.4)	
VOD grade				
	None	36 (97.3)	35 (94.6)	0.57
	Moderate	1 (2.7)	1 (2.7)	
	Severe	0 (0.0)	1 (2.7)	
	Total	37 (50.0)	37 (50.0)	

CTC: common toxicity criteria; N/A: not available.

weekly up to 100 days and monthly following day 100. There was no significant difference in the proportion of patients receiving systemic glucocorticoids at either 6 months (SIR/TAC 52%, MTX/TAC 59%) or 1 year (SIR/TAC 24%, MTX/TAC 25%) following HCT (*P*=NS). To spare systemic glucocorticoids, patients with acute upper GI GVHD were treated with beclomethasone and those with acute intestinal GVHD with budesonide, either alone or in combination with systemic glucocorticoids. Fewer patients in the SIR/TAC arm were treated with beclomethasone for manifestations of acute GVHD (*P*<0.05 for weeks 5, 6, 9, 10 and *P*<0.01 for weeks 11-14), while point-wise comparisons for budesonide were not significantly different. Ten patients treated with SIR/TAC and six treated with MTX/TAC discontinued TAC after intentional tapering in the absence of primary disease relapse or TAC toxicity, including TMA. The cumulative incidence of intentional TAC discontinuation at 30 months post-HCT did not differ between groups (SIR/TAC 36%, MTX/TAC 30%; *P*=0.16). At the time of analysis, one and three patients treated with SIR/TAC and MTX/TAC, respectively, had successfully discontinued all immune suppressive agents. A longer follow-up is required to analyze this outcome in more depth.

Chronic graft-versus-host disease

The cumulative incidence of any grade of chronic GVHD (defined by NIH criteria) was 53% (95% CI: 29-72%) in the SIR/TAC arm and 70% (95% CI: 42-86%) in the MTX/TAC arm (*P*=0.68). The incidence of moderate to severe chronic GVHD was 24% (95% CI: 7-47%) and 64% (95% CI 41-79%) in the SIR/TAC and MTX/TAC arms, respectively (*P*=0.008 (Figure 2). Cumulative incidence estimates are provided at 30 months post-HCT. Adjusting for age/donor type, moderate to severe chronic GVHD was significantly reduced among SIR/TAC-treated patients (HR 0.27, 95% CI 0.1-0.72, *P*=0.009). The predominantly involved organs were skin, mouth, eye, and liver, recapitulating previously published estimates.[28] Lung involvement was judged according to the proposed NIH criteria based on pulmonary spirometric and radiographic findings; diagnostic biopsies were not performed. The maximum grade of chronic GVHD differed significantly between the two treatment arms SIR/TAC *versus* MTX/TAC (Table 3). Chronic GVHD

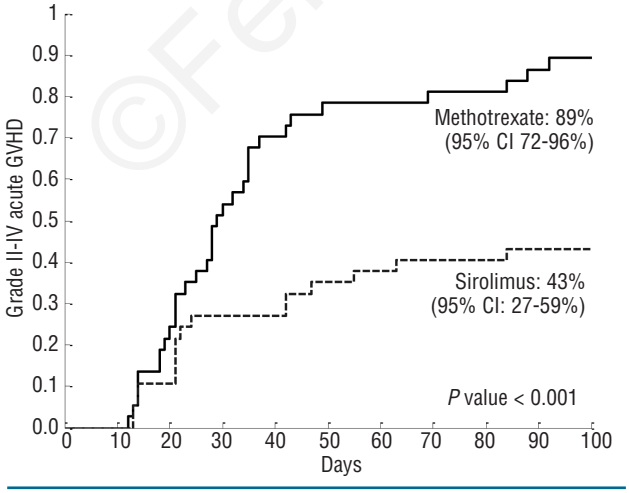

Figure 1. Cumulative incidence of grade II-IV acute GVHD over 100 days following HCT.

Table 3. Acute and chronic GVHD characteristics.

(A) Individual acute GVHD organ staging and overall acute GVHD grade

	MTX/TAC	SIR/TAC	*P* value
Skin stage			
0	15 (41%)	16 (43%)	*P*=0.48
1	17 (46%)	13 (35%)	
2	3 (8%)	7 (19%)	
3	2 (5%)	1 (3%)	
4	0 (0%)	0 (0%)	
GI stage			
0	8 (22%)	22 (59%)	*P*=0.003
1	27 (73%)	10 (27%)	
2	1 (3%)	3 (8%)	
3	1 (3%)	1 (3%)	
4	0 (0%)	1 (3%)	
Liver stage			
0	30 (81%)	35 (95%)	*P*=0.32
1	4 (11%)	1 (3%)	
2	2 (5%)	1 (3%)	
3	1 (3%)	0 (0%)	
4	0 (0%)	0 (0%)	
Overall grade			
0	2 (5%)	11 (30%)	*P*<0.001
I	2 (5%)	10 (27%)	
II	29 (78%)	11 (30%)	
III	4 (11%)	4 (11%)	
IV	0 (0%)	1 (3%)	

(B) Chronic GVHD scoring according to NIH Consensus Criteria: individual organ severity scores and global severity score

	MTX/TAC	SIR/TAC	*P* value
Skin			
0	20 (65%)	24 (73%)	*P*=0.62
1	7 (23%)	5 (15%)	
2	3 (10%)	4 (12%)	
3	1 (3%)	0 (0%)	
Mouth			
0	18 (58%)	22 (67%)	*P*=0.42
1	13 (42%)	10 (30%)	
2	0 (0%)	1 (3%)	
3	0 (0%)	0 (0%)	
Eyes			
0	21 (68%)	20 (61%)	*P*=0.27
1	5 (16%)	11 (33%)	
2	4 (13%)	2 (6%)	
3	1 (3%)	0 (0%)	
GI			
0	24 (77%)	32 (97%)	*P*=0.06
1	6 (19%)	1 (3%)	
2	0 (0%)	0 (0%)	
3	1 (3%)	0 (0%)	
Liver			
0	17 (55%)	29 (88%)	*P*=0.03
1	5 (16%)	2 (6%)	
2	8 (26%)	2 (6%)	
3	1 (3%)	0 (0%)	
Lung			
0	27 (87%)	32 (97%)	*P*=0.34
1	1 (3%)	0 (0%)	
2	1 (3%)	1 (3%)	
3	2 (7%)	0 (0%)	
Joints/fascia			
0	28 (90%)	31 (94%)	*P*=0.81
1	1 (3%)	1 (3%)	
2	2 (7%)	1 (3%)	
3	0 (0%)	0 (0%)	

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Genital			
0	0 (0%)	0 (0%)	
1	0 (0%)	0 (0%)	
2	0 (0%)	0 (0%)	
3	0 (0%)	0 (0%)	
Other			
0	30 (97%)	33 (100%)	*P*=0.48
1	0 (0%)	0 (0%)	
2	0 (0%)	0 (0%)	
3	1 (3%)*	0 (0%)	
Overall global score			
0	11 (36%)	17 (52%)	*P*=0.001
1	1 (3%)	10 (30%)	
2	11 (36%)	5 (15%)	
3	8 (26%)	1 (3%)	

**pericardial effusion.*

therapy was not mandated by the protocol, but was given according to usual clinical practice. None of the patients with chronic GVHD in the study had completely discontinued taking the original prophylactic immune suppressive agents by the time of onset of chronic GVHD. These prophylactic agents were, therefore, continued upon development of chronic GVHD. Overall moderate-severe chronic GVHD was an indication for escalating systemic therapy, while overall mild chronic GVHD was treated as possible with local/topical therapies.

Overall survival, non-relapse mortality, disease relapse, and patient-reported outcomes

The median follow-up for surviving patients at the time of analysis was 20 months (range, 4-32) for SIR/TAC-treated patients and 17 months (range, 4-32) for MTX/TAC-treated ones. Overall survival did not differ significantly between the two groups: the 2-year overall survival rate was 61% (95% CI: 41-77%) in the SIR/TAC group and 69% (95% CI: 48-83%) in the MTX/TAC group (*P*=0.66). We also did not observe significant differences in primary disease relapse: the 2-year cumulative incidence of relapse was 18% in the SIR/TAC group and 31% in the MTX/TAC group (*P*=0.09). Adjusting for age/donor type, the hazard for relapse was not different between the two arms (HR 0.41; 95% CI: 0.15-1.14; *P*=0.09). Relapse of malignancy was the primary cause of death for two patients in the SIR/TAC arm and seven patients in the MTX/TAC arm. The 2-year incidence of non-relapse mortality was 28% and 8% in the SIR/TAC and MTX/TAC arms, respectively (*P*=0.025). Adjusting for age/donor type, the hazard for non-relapse mortality among SIR/TAC patients (reference MTX/TAC) was increased (HR 4.95; 95% CI: 1.1-22.3; *P*=0.04). Eight patients in the SIR/TAC arm died of causes other than relapse (septicemia in two, hepatic VOD, multi-organ failure, acute GVHD, chronic GVHD and hepatic failure, influenza and respiratory failure, and respiratory syncytial virus pneumonia in one each), as did two patients in the MTX/TAC arm (alveolar hemorrhage, and unknown).

The FACT-BMT quality of life questionnaire was completed by patients at baseline, and then at serial time points following HCT. While the scores among SIR/TAC- *versus* MTX/TAC-treated patients were significantly lower for functional well-being (mean 15.66, SE 0.95 *versus* mean 19.7, SE 0.96, respectively; *P*<0.01) and FACT-G (mean 77.58, SE 2.48 *versus* mean 86, SE 2.18; *P*<0.05) at baseline prior to

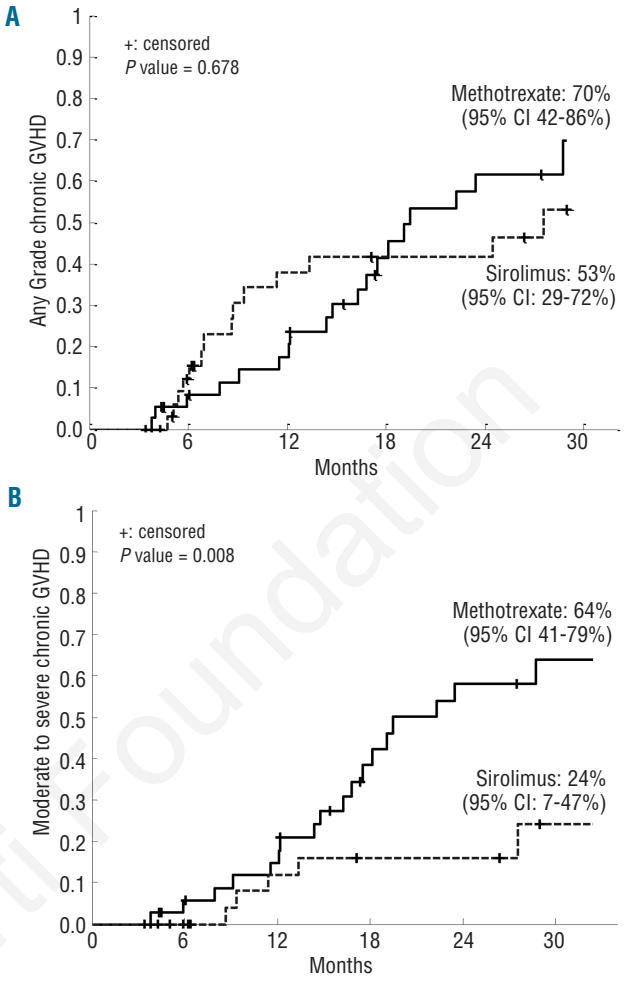

Figure 2. Cumulative incidence of (A) any grade chronic GVHD and (B) moderate to severe chronic GVHD according to NIH criteria.

HCT, no significant differences were detected at day 30 or 90 post-HCT in any individual domain or summary score. Further follow-up is ongoing for later time points.

Regulatory T cell reconstitution and suppressive function

Samples were obtained from all patients at the pre-specified time points to determine the number of Treg in peripheral blood. There were significantly greater proportions of Treg/total CD4$^+$ cells at days 30 and 90 in SIR/TAC-treated patients (Figure 3). There were increased absolute numbers of Treg and decreased absolute numbers of non-Treg CD4$^+$ cells at these time points (*Online Supplementary Figures S2* and *S3*). In subsets of patients from the SIR/TAC (n=4) and MTX/TAC (n=5) groups, functional assays were performed on samples taken on day 90 (SIR n=2, MTX n=1), day 180 (SIR n=2, MTX n=3) and day 360 (MTX n=1). All patients were on systemic immune suppression at the time these samples were obtained. The systemic immune suppression among SIR/TAC-treated patients included SIR (n=4), TAC (n=3), and prednisone (n=2), at doses from 0.17 – 1 mg/kg/day, whereas that for the MTX/TAC-treated patients included TAC (n=5), SIR (n=1), and prednisone (n=2), at doses from 0.1 – 0.83 mg/kg/day. For an increasing ratio of

sorted Treg to T responder cells, we observed increasing percentage suppression. While these Treg were functional, we did not observe significant differences in suppressive function between the SIR/TAC- and MTX/TAC-treated patients.

Discussion

We observed that SIR/TAC led to significantly less grade II-IV acute GVHD compared to MTX/TAC, which has been considered a standard of care in GVHD prevention.[1,2] The major benefit observed was a reduction in overall grade II acute GVHD, driven by GI manifestations. SIR/TAC did not appear to offer significant advantages in reducing grade III-IV acute GVHD or specifically skin or hepatic acute GVHD. The prevention of grade III-IV acute GVHD is a particularly relevant therapeutic goal, as it is associated with a greater risk of non-relapse mortality. These data add evidence to results from prior single-arm phase II trials that SIR/TAC is active in GVHD prevention.[5-7] Given that acute GVHD remains a significant source of early transplant-associated morbidity, mortality, and impaired quality of life, more effective acute GVHD prevention is an important clinical goal. The burden of acute GVHD still experienced by SIR/TAC-treated patients in this trial indicates that further investigation and novel approaches for GVHD prevention are still needed.

Late morbidity, symptom burden, disability and mortality from chronic GVHD also pose major threats to the long-term success of HCT. We found that SIR/TAC significantly reduced the incidence of moderate-severe chronic GVHD, suggesting that SIR-based immune suppression, and particularly the intentionally prolonged administration of SIR for 1 year post-HCT, may favorably modify the biology and resulting presentation of chronic GVHD without increasing the incidence of malignancy relapse. This finding is particularly noteworthy as previous trials examining SIR/TAC resulted in a greater burden of chronic GVHD: specifically, Cutler *et al.* and Rodriguez *et al.* suggested that the incidence of chronic GVHD is comparable to that previously reported following MTX/TAC.[5,7] It should be noted that our study design mandated at least 1 year of SIR therapy for SIR/TAC-treated patients, with the intention of limiting the risk of chronic GVHD development and promotion of immune tolerance. Longer follow-up is needed to analyze time to immune suppression discontinuation and freedom from chronic GVHD and to assess whether prolonged administration of SIR facilitates immune tolerance.

The risks of hepatic VOD and TMA were similar in both treatment arms. In contrast to previously published evidence in the setting of busulfan/cyclophosphamide conditioning,[27] or that of largely intravenous busulfan/fludarabine but with the combination of TAC/everolimus,[29] we did not observe a significantly increased risk of hepatic VOD among patients treated with SIR/TAC and busulfan-based conditioning. However, the overall incidence of this complication was low and this risk was likely mitigated by avoiding cyclophosphamide, pharmacokinetic targeting of intravenous busulfan, initiation of SIR on day -1 after conditioning therapy was completed, and VOD prophylaxis with ursodeoxycholic acid in all patients. Observing these precautions may allow safe co-administration of SIR and busulfan.

We have demonstrated that, compared to MTX/TAC, SIR/TAC supports better reconstitution of Treg following

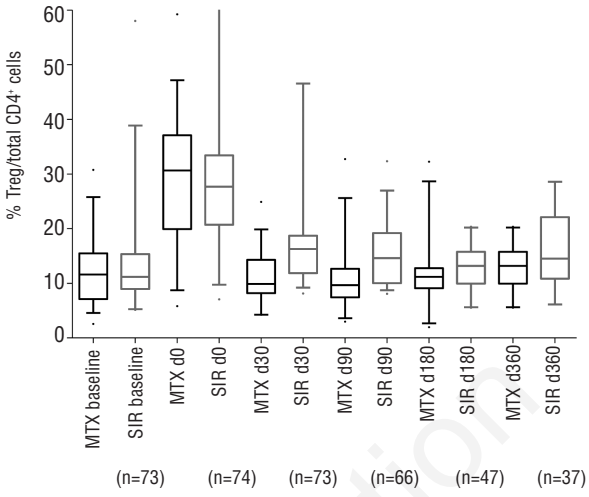

Figure 3. Proportion of Treg (% Treg/total CD4⁺ cells) compared between patients in the SIR/TAC and MTX/TAC groups at baseline, and on days 0, 30, 90, and 360 following HCT. Day 30 (*P*<0.0001), day 90 (*P*=0.0009), day 180 (*P*=0.07), otherwise, *P*=NS. (box and whisker plot: box margins = interquartile range, line = median value, whiskers = 95% confidence interval, dots = outliers).

HCT. While our primary end-point of interest was the proportion of Treg/total CD4⁺ cells, we also observed increased absolute numbers of Treg and decreased absolute numbers of non-Treg CD4⁺ lymphocytes (*Online Supplementary Figures S2 and S3*) among SIR/TAC- *versus* MTX/TAC-treated patients. The most striking differences occurred at earlier time points (i.e. days 30 and 90 post-HCT), during a time frame in which MTX/TAC-treated patients would have been exposed to higher systemic levels of TAC. The numbers of both Treg and non-Treg CD4⁺ lymphocyte tended to increase at later time points with ongoing immunological reconstitution and TAC tapering. The net benefit observed in Treg reconstitution among SIR/TAC-treated patients is likely due to both the suppression of non-Treg CD4 T cells achieved by SIR, as well as lower TAC exposure in these patients than in MTX/TAC-treated patients. These prospective data advance knowledge about Treg reconstitution following clinical HCT beyond that provided by previously reported correlative studies,[30,31] support the concept that SIR suppresses non-Treg CD4⁺ cells,[10] and indicate that the SIR/TAC combination may serve as a platform for Treg adoptive therapy.

While these data offer significant insights, we acknowledge the following limitations. First, as Treg are dependent on interleukin-2 signaling, we recognize that concurrent administration of TAC may counter the beneficial effects of SIR on Treg. Although a calcineurin inhibitor-free regimen would be most attractive, current evidence does not support the feasibility of this approach for GVHD prophylaxis after adult stem cell grafts.[32] Second, we acknowledge the risk of a biased classification. Although this was a randomized clinical trial with acute and chronic GVHD graded prospectively by treating physicians, blinding was not possible. Next, our adherence to the proposed NIH criteria classification and severity grading of chronic GVHD limits comparisons with prior literature. The concurrent control of MTX/TAC-treated patients does, however, place the observed incidence of chronic GVHD following SIR/TAC therapy in context. Finally, these findings were generated in patients con-

ditioned with chemotherapy-only regimens. Results from the national phase III CTN trial (CTN 0402) will provide comparative evidence of SIR *versus* MTX in patients conditioned with cyclophosphamide and total body irradiation, and transplanted with grafts from sibling donors.

In summary, these data demonstrate that SIR/TAC mitigates the risk of grade II-IV acute GVHD and moderate-severe chronic GVHD, and supports the reconstitution of Treg after HCT.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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