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Tocilizumab, tacrolimus and methotrexate for the prevention of acute graft-*versus*-host disease: low incidence of lower gastrointestinal tract disease

William R. Drobyski,[1] Aniko Szabo,[2] Fenlu Zhu,[1] Carolyn Keever-Taylor,[1] Kyle M. Hebert,[3] Renee Dunn,[3] Sharon Yim,[1] Bryon Johnson,[1] Anita D'Souza,[1] Mary Eapen,[1] Timothy S. Fenske,[1] Parameswaran Hari,[1] Mehdi Hamadani,[1] Mary M. Horowitz,[1] J. Douglas Rizzo,[1] Wael Saber,[1] Nirav Shah,[1] Bronwen Shaw[1] and Marcelo Pasquini[1]

[1]The Department of Medicine; [2]The Division of Biostatistics, Institute for Health and Society and [3]The Center for International Blood and Marrow Transplant Research, Medical College of Wisconsin, Milwaukee, WI, USA

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ABSTRACT

We conducted a phase 2 study in which patients undergoing allogeneic hematopoietic stem cell transplantation received tocilizumab in addition to standard immune suppression with tacrolimus and methotrexate for graft-*versus*-host disease prophylaxis. Thirty-five patients were enrolled between January 2015 and June 2016. The median age of the cohort was 66 (range: 22-76). All patients received busulfan-based conditioning, and were transplanted with human leukocyte antigen-matched related or matched unrelated bone marrow or peripheral stem cell grafts. The cumulative incidences of grades II-IV and III-IV acute graft-*versus*-host disease were 14% (95% CI 5-30) and 3% (95% CI 0-11) at day 100, and 17% (95% CI 7-31) and 6% (95% CI 1-16) at day 180, respectively. Notably, there were no cases of graft-*versus*-host disease of the lower gastrointestinal tract within the first 100 days. A comparison to 130 matched controls who only received tacrolimus and methotrexate demonstrated a lower cumulative incidence of grades II-IV acute graft-*versus*-host disease (17% *versus* 45%, *P*=0.003) and a significant increase in grades II-IV acute graft-*versus*-host disease-free survival at six months (69% *versus* 42%, *P*=0.001) with tocilizumab, tacrolimus and methotrexate, which was the primary endpoint of the study. Immune reconstitution was preserved in patients treated with tocilizumab, tacrolimus and methotrexate, as T-cell and B-cell subsets recovered to near normal levels by 6-12 months post-transplantation. We conclude that tocilizumab has promising activity in preventing acute graft-*versus*-host disease, particularly in the lower gastrointestinal tract, and warrants examination in a randomized setting. *clinicaltrials.gov Identifier:02206035*

Introduction

Graft-*versus*-host disease (GvHD) is the major complication arising from allogeneic hematopoietic stem cell transplantation (HSCT). GvHD is characterized by the overproduction of proinflammatory cytokines that induce target organ damage directly, or indirectly by activating other effector cell populations.[1-3] Interleukin 6 (IL-6) has emerged as an inflammatory cytokine that plays a pivotal role in the pathophysiology of GvHD and has become a potential therapeutic target.[4-6] Preclinical studies have demonstrated that IL-6 levels are increased early during GvHD and are present in all target tissues.[7] Moreover, blockade of the IL-6 signaling pathway using an antibody that binds to the IL-6 receptor has been shown to reduce the severity of GvHD and prolong survival in pre-clinical murine models.[7,8] In particular, IL-6 appears to have an important pathophysiological role in promoting inflammation in the gastrointestinal (GI) tract,[7] which is a major cause of morbidity and mortality during GvHD.

Correspondence:
wdrobysk@mcw.edu

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The importance of IL-6 in human GvHD is supported by studies which have shown that patients with elevated plasma levels of IL-6,[9,10] as well as those with a recipient or donor IL-6 genotype which results in increased IL-6 production,[11-14] have an increased incidence and severity of this disease. The Food and Drug Administration (FDA) approval of tocilizumab (Toc; Actemra™), which is a humanized anti-IL-6 receptor antibody that blocks both the membrane and soluble forms of the receptor for the treatment of severe active rheumatoid arthritis,[15,16] has allowed off-label use of this agent to determine whether blockade of IL-6 signaling attenuates GvHD. To that end, it was demonstrated that Toc induced clinical responses in patients with steroid refractory acute (a)GvHD that primarily involved the lower GI tract.[17] Furthermore, the addition of Toc to standard immune suppression resulted in a low incidence of aGvHD in a patient population which was comprised primarily of myeloid maliganancies,[18] providing evidence that inhibition of IL-6 might also be an effective approach for the prevention of GvHD. Notably, however, in the latter study myeloablative (MA) conditioning was carried out exclusively with total body irradiation (TBI) and cyclophosphamide (Cy), which is not a widely employed regimen for the treatment of myeloid malignancies. Since the intensity of the conditioning regimen is known to affect the magnitude of inflammatory cytokine production,[19] the relevance of these results to patients treated with alternative, more commonly utilized conditioning regimens is not clear. Furthermore, there was no corresponding demographically-matched control population against which to assess these results.

In the study herein, we sought to determine whether the addition of Toc to standard immune suppression was effective for the prevention of aGvHD in patients who received busulfan (Bu)-based conditioning regimens, with particular emphasis given to the lower GI tract given prior pre-clinical studies and the primacy of this organ in GvHD pathophysiology. To place our results in context, we interrogated the Center for International Blood and Marrow Transplant Research (CIBMTR) database to obtain a control population that was matched for relevant demographic and transplant characteristics. We also examined longitudinal immune reconstitution and inflammatory cytokine production as additional parameters by which to assess the effect of IL-6 inhibition in transplant recipients.

Methods

Patient population

Patients were eligible for this trial if they met the following criteria: age >18 years; a diagnosis of acute leukemia, chronic myelogenous leukemia, myeloproliferative disease, myelodysplasia with less than 5% of blasts in the bone marrow, or a diagnosis of chronic lymphocytic leukemia, non-Hodgkin lymphoma or Hodgkin lymphoma with chemosensitive disease; availability of a 10/10 matched sibling or 8/8 matched unrelated donor; ejection fraction at rest >45% for MA conditioning or >40% for reduced intensity conditioning (RIC); estimated creatinine clearance greater than 50 mL/minute; adjusted diffusing capacity for carbon monoxide (DLCO) ≥40% and forced expiratory volume in 1 second (FEV1) ≥50%; and total bilirubin < 1.5 x and alanine transaminase (ALT)/ aspartate transaminase (AST) < 2.5x the upper normal limit. Patients were excluded if they had had a prior allogeneic HSCT, Karnofsky Performance Score <70%, uncontrolled bacterial, viral or fungal infections at time of enrollment, prior intolerance or allergy to Toc, use of rituximab, alemtuzumab, antithymocyte globulin (ATG) or other monoclonal antibody at time of conditioning regimen, or history of diverticulitis, Crohn's disease, ulcerative colitis or a demyelinating disorder.

Control population

The control population was derived from cases reported to the CIBMTR.[20] Eligibility criteria for the control cohort consisted of having received a first allogeneic transplant at an American center, excluding The Medical College of Wisconsin (MCW), and meeting the same eligibility as the study population, with the exception of receiving only tacrolimus (Tac)/ methotrexate (MTX) as GvHD prophylaxis. Controls were selected from the years 2010-2015. This selection process resulted in the screening of 1,442 patients to define an optimally-matched control cohort.

Conditioning regimens and GvHD prophylaxis

All patients received Bu as part of the preparative regimen. Patients receiving MA conditioning were treated with either Bu 3.2 mg/kg/day (days -7 to -4) and Cy 60 mg/kg/day (days -3 and -2) or Bu 3.2 mg/kg/day (days 5- to -2) and fludarabine (Flu) 30 mg/m^2/day (days -5 to -2). Bu dosing was modified after the fifth dose, if necessary, to achieve a targeted level of 900±100 ng/mL. RIC was with Flu 30 mg/m^2/day (days -6 to -2) and Bu 3.2 mg/kg/day (days -5 and -4). Patients received either T-cell replete bone marrow or granulocyte colony factor-stimulated peripheral blood stem cell grafts. For GvHD prevention, Tac was administered intravenously at a dose of 0.03 mg/kg/day starting on day –3 to maintain a level of 5-15 ng/mL. MTX was given at the doses of 15 mg/m^2 IV on day +1, and 10 mg/m^2 IV on days +3, +6 and +11 after hematopoietic stem cell infusion. Toc was infused intravenously at a dose of 8 mg/kg (maximum dose of 800 mg) once on day-1 approximately 24 hours prior to the hematopoietic stem cell infusion, as per Kennedy and colleagues.[18]

Study design

The primary objective of this study was to compare the probability of grades II-IV aGvHD-free survival at day 180 post-transplant between recipients of Toc, Tac and MTX and a contemporary control population who received Tac/MTX-based GvHD prophylaxis. Pre-specified secondary objectives of the study were to compare chronic (c)GvHD, transplant related mortality (TRM), disease relapse or progression, disease-free survival (DFS) and overall survival (OS) between Toc/Tac/MTX and Tac/MTX CIBMTR controls. *Online Supplementary Table S1* contains the definition of the events, censorings, and competing risks for all time-to-event outcomes. Additionally, secondary objectives included description of the incidence of grades ≥3 toxicities according to Common Terminology Criteria for Adverse Event (CTACAE) v4, neutrophil and platelet engraftment, characterization of infections, proportion of donor chimerism, extent of immune reconstitution, and production of proinflammatory cytokines among patients who received Toc/Tac/MTX. A population that consisted of patients who were otherwise eligible for the trial but did not receive Toc in addition to Tac/MTX for GvHD prophylaxis was employed as a control for the cytokine analysis. These patients were consented and enrolled on a separate study. Both protocols were approved by the Institutional Review Board at the MCW.

Outcome assessments

Neutrophil recovery was defined as the first of three consecutive days with an absolute neutrophil count (ANC)>500. Platelet engraftment was defined as the first day of a sustained platelet count above 20,000 without any platelet transfusions for the preceding seven days. Five patients who received reduced intensity

transplants never dropped their platelet count below 20,000. In these patients, the date of platelet recovery was defined as the first day that the platelet count increased after its nadir. The grade of aGvHD was determined using the Glucksberg scale.[21] cGvHD was graded using the National Institutes of Health (NIH) Chronic GvHD Consensus criteria.[22]

Statistical analysis

The probabilities of DFS, aGvHD-free survival, and OS were calculated using the Kaplan-Meier estimator. The probabilities of neutrophil and platelet engraftment, TRM, disease progression/relapse, and aGvHD and cGvHD were calculated using the cumulative incidence estimator. GvHD was calculated using disease progression/relapse or death as competing risks. Matching criteria for controls consisted of age within 5 years; performance score (≥90 *vs.* <90); Bu-based regimen (Flu/Bu reduced intensity, Bu/Cy MA, or Flu/Bu MA); disease, and donor type (human leukocyte antigen [HLA]-matched sibling vs. HLA-matched unrelated donor). Up to four matches per case were selected whenever possible; when more controls were available preference was given to identical stem cell source (bone marrow, peripheral blood) and closest age. With respect to stem cell source, 124 of the 135 CIBMTR control patients were also matched for this variable. The follow up of the control patients was administratively censored at 22 months, which corresponded to the longest follow up of the trial patients. With 35 patients enrolled in the trial and 140 controls, there was an 80% power to detect an improvement of 20% on day 180 aGvHD-free survival. The outcomes were compared between groups using a stratified log-rank test or Gray's test for survival and competing risk outcomes, respectively, with matched sets defining strata. Stratified Cox or Fine-Gray regression was used to obtain hazard ratios with 95% confidence intervals. Each cytokine was analyzed separately in the combined sample, and by conditioning regimen (ablative *vs.* RIC). Cytokine values were shifted by half of the smallest non-zero value and then log-transformed to improve normality of the residuals. First, a separate analysis was conducted for each treatment group (Toc and Control) followed by a joint analysis. A repeated measures analysis was performed using a mixed effects model with a random subject-specific intercept to incorporate within-subject dependence. All time-points were compared to the baseline value with Dunnett-Hsu adjustment for multiple testing. The estimates were back-transformed to the original scale for reporting.

Table 1. Patient characteristics.

Variable	Value
N	35
Age, median (range)	66 (22-76)
Sex (M/F)	22/13
Disease (n, %)	
AML	14 (40)
CR1	11
CR2	3
ALL	4 (11)
CR1	3
CR2	1
Secondary AML, CR1	3 (9)
Therapy-Related AML, CR1	2 (6)
MDS	3 (9)
CMML	5 (14)
Myelofibrosis	1 (3)
CML, CP2	1 (3)
T-cell Lymphoma, CR2	1 (3)
NK/T-cell Lymphoma, CR2	1 (3)
Donor Type (n, %)	
MRD	14 (40)
MUD	21 (60)
Preparative Regimen (n, %)	
Bu/Cy	5 (14)
Flu/Bu4	13 (34)
Flu/Bu2	7 (51)
Graft Source (n, %)	
Bone Marrow	6 (17)
Peripheral Blood	29 (83)
Disease Risk Index (n, %)	
Low	4 (11)
Intermediate	22 (63)
High	9 (26)
CMV Serostatus (n, %)	
Donor–/Recipient–	12 (34)
Donor+/Recipient–	11 (31)
Donor+/Recipient+	4 (11)
Donor–/Recipient+	8 (23)

AML: acute myelogenous leukemia; ALL: acute lymphoblastic leukemia; MDS: myelodysplasia; CMML: chronic myelomonocytic leukemia; CML: chronic myelogenous leukemia; MRD: matched related donor; MUD: matched unrelated donor; CMV: cytomegalovirus; Bu: busulfan; Cy: cyclophosphamide; Flu: fludarabine; CR: complete remission; CP2: second chronic phase.

Other detailed methods

Serum cytokine and immune reconstitution analyses are described in *Online Supplementary Methods.*

Results

Patient characteristics

From January 29, 2015 to June 30, 2016, 35 patients were enrolled in the study. The demographic data for this population is detailed in Table 1. The median age of the cohort was 66 (range: 22-76). Diseases consisted of *de novo* acute myeloid leukemia (AML; n=14), acute lymphoblastic leukemia (ALL; n=4), secondary or therapy-related AML (n=5), myelodysplastic syndrome (MDS; n=3), chronic myelomonocytic leukemia (CMML; n=5), myelofibrosis (n=1), T-cell lymphoma (n=1), chronic myeloid leukemia (CML; n=1), and natural killer (NK)/T-cell lymphoma (n=1). The disease status of patients with acute and chronic leukemia are further specified in Table 1. Four of the 14 patients with AML in first remission had FLT3/ITD mutations, two additional recipients had evidence of minimal residual disease at the time of transplant, and another patient had a monosomal karyotype. All three patients with ALL in complete remission 1 (CR1) were Philadelphia chromosome positive. Using the adjusted disease risk index,[23] patients were classified as low (n=4), intermediate (n=22), or high (n=9) risk.

Engraftment and chimerism

There were no cases of graft rejection. The median time to an ANC>500 was 18 days (range: 14-26) (Figure 1A).

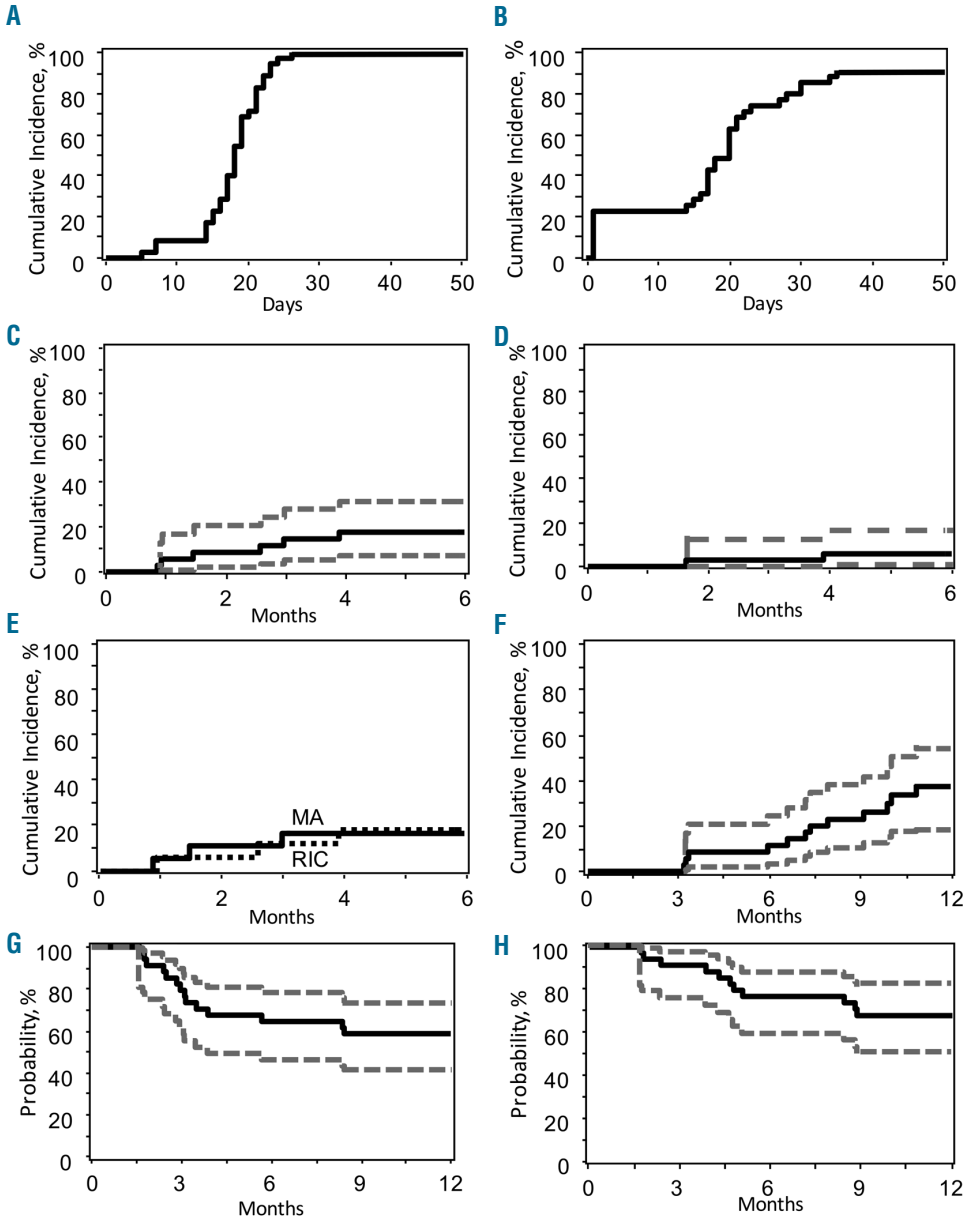

Figure 1. Engraftment, GvHD, disease-free survival, and overall survival. (A). Cumulative incidence of achieving an absolute neutrophil count >500/mm³ for three consecutive days. (B). Cumulative incidence of patients that achieved an unsupported platelet count > 20,000/mm³. Five patients never dropped their platelet count below 20,000. (C,D). Cumulative incidence of grades II-IV and grades III-IV aGvHD. (E). Cumulative incidence of grades II-IV aGvHD in patients that received myeloablative (MA) *versus* reduced intensity (RIC) preparative regimens. (F). Cumulative incidence of NIH-defined cGvHD. (G) Probability of disease-free survival and (H) overall survival in patients that received Toc/Tac/MTX as GvHD prophylaxis. Dashed gray lines indicate 95% confidence interval bands.

Twenty-one patients received granulocyte-colony stimulating factor (G-CSF) for 1-3 days on days 14-19 post-transplantation to accelerate white blood cell (WBC) recovery. The platelet count in five patients never dropped below 20,000. Two additional patients died before achieving engraftment of platelets. In the remaining patients, the median time to platelet engraftment was 17 days (range: 10-103) (Figure 1B). Chimerism studies conducted on day 28 post-transplantation were available for 33 patients. Median donor CD3 chimerism was 88% (range: 34-100), while median CD33 chimerism was 100% (range: 97-100). Day 100 studies were performed in 27 patients and revealed a median donor CD3 chimerism of 91% (range: 43-100) and CD33 chimerism of 100% (range: 91-100).

GvHD, transplant-related mortality, relapse, and survival

The cumulative incidence of grades II-IV aGvHD at days 100 and 180 was 14% (95% CI 5-30%) and 17% (95% CI 7-35%), respectively, (Figure 1C). The incidence of grades III-IV aGvHD at these same time points was 3% (95% CI 0-11%) and 6% (95% CI 1-16), respectively, (Figure 1D). All patients who developed ≥ grade II aGvHD within the first 100 days had isolated involvement of the skin or upper GI tract. Three patients had GvHD of the skin, one of whom developed grade IV disease, which proved to be fatal. Three patients had upper GI tract involvement, which was resolved in each case with modest doses of steroids. There were no cases of aGvHD involving the liver or lower GI tract within this time interval. One patient did develop grade II-IV aGvHD involving the lower GI tract between days 100-180 post-transplantation. A second patient with a prior history of overall grade III skin involvement developed upper GI tract disease in the duodenum on day 177. The cumulative incidence of grades II-IV aGvHD for each individual tissue site is shown in *Online Supplementary Figure S1*. There was no difference in the incidence of

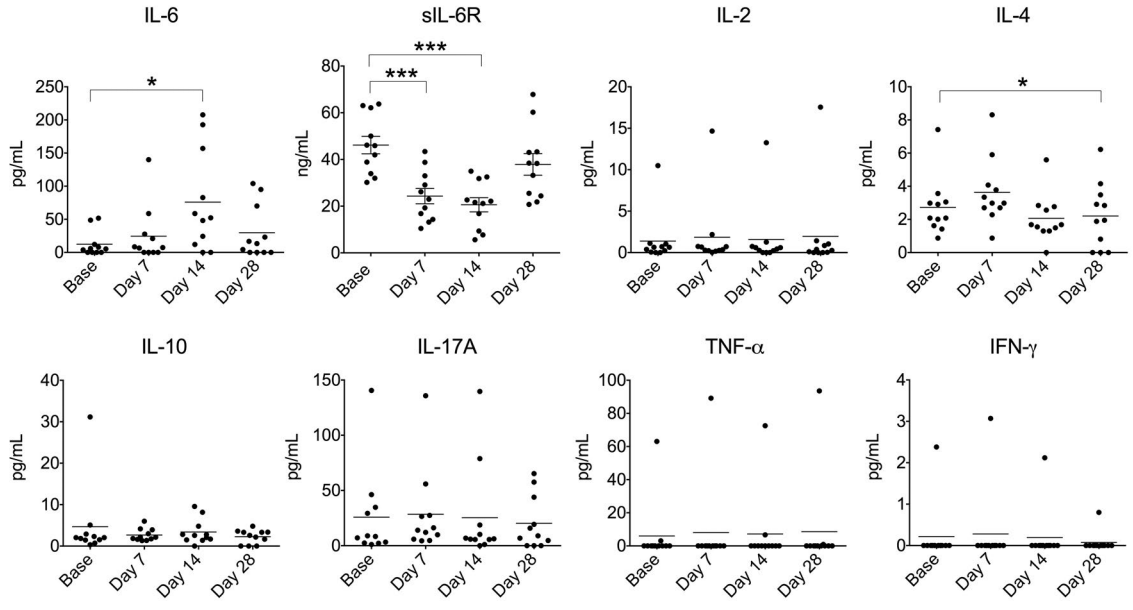

Figure 2. Cytokine levels in control population that received tacrolimus and methotrexate for GvHD prophylaxis. Concentration of IL-6, sIL-6R, IL-2, IL-4, IL-10, IL-17, TNF-α, and IFN-γ in the serum of patients (n=11) who received Tac/MTX for the prevention of aGvHD prior to the start of conditioning, and at days 7, 14 and 28. **P*<0.05, ****P*<0.001. IL: interleukin; sIL: soluble interleukin; TNF-α: tumor necrosis factor α; IFN-γ: interferon γ.

grades II-IV aGvHD at day 180 between patients who received MA (17%, 95% CI 4-37) *versus* reduced intensity (18%, 95% CI 4-39) conditioning regimens (Figure 1E). The median time to onset for aGvHD in recipients of MA *versus* reduced intensity transplants was 44 and 78 days, respectively. The incidence of cGvHD at 12 months was 38% (95% CI 21-55) in this patient population (Figure 1F). The median follow up for surviving patients was 15 months. TRM was 14% (95% CI 5-28) at 12 months and was attributable to GvHD (n=3), sepsis (n=1), aspergillus pneumonia (n=1), idiopathic pneumonia syndrome (n=1), and respiratory failure (n=1). The one-year cumulative incidence of relapse was 29% (95% CI 15-44). DFS and OS at 12 months was 57% (95% CI 42-70) and 68% (95% CI 53-79), respectively, (Figures 1G,H).

Side effects and infectious complications

There were no infusion-related reactions associated with the administration of Toc. The major immediate Toc-associated side effect within the first 28 days post-transplantation was the development of grade III liver toxicity. Nine patients (26%) had ≥ grade III ALT elevations, two patients (6%) had ≥ grade III AST levels and one patient had a grade IV bilirubin elevation. Transaminase elevations typically peaked 7-10 days after infusion, and were transient in all patients, eventually returning to baseline. The marked bilirubin level in one patient was ascribed to total parenteral nutrition administration after a biopsy revealed no evidence of GvHD or any other underlying pathology. No patient developed veno-occlusive disease of the liver. A total of 23 grade III or higher infectious complications were observed in 13 patients during the first 100 days. Fifteen of these were due to bacterial infections, of which 11 were bloodstream (staphylococcus epidermidis [n=6], bacillus cereus [n=1], strep oralis [n=1], strep mitis [n=1], polymicrobial sepsis [n=1], vancomycin-resistant enterococcus [n=1]), two urinary tract (*staphylococcus epidermidis* [n=1], vancomycin-resistant enterococcus [n=1]), one respiratory (*staphylococcus epidermidis*), and one attributable to clostridium difficile. Cytomegalovirus (CMV) reactivation occurred in two of 12 (17%) seropositive recipients. Other viral infections consisted of human herpes virus 6 (HHV-6) encephalitis (n=1), enterovirus (n=1), and BK virus (n=3). One patient developed invasive aspergillus pneumonia.

Inflammatory cytokine analyses

To examine how the administration of Toc altered inflammatory cytokine production, we assayed serum cytokine levels (IL-2, IL-4, IL-6, IL-10, IL-17A, tumor necrosis factor α [TNF-α] and interferon γ [IFN-γ]) and soluble (s)IL-6R levels in the peripheral blood of patients who received Toc (n=35) as well as a control population (n=11) which had the same trial eligibility criteria, but did not receive this agent (see patient demographics in *Online Supplementary Table S2*). In the control population, we observed that IL-6 was the only cytokine that was increased above baseline during the first 28 days (i.e., nine-fold increase on day 14 post-transplantation) (Figure 2). Conversely, sIL-6R levels were significantly decreased on days seven and 14 before rebounding back to baseline on day 28. In the Toc cohort, IL-6 levels were increased in patients who received Toc at days seven, 14, and 28 when compared to baseline (Figure 3A). Levels were augmented above baseline in both MA and RIC recipients (Figure 3A), although IL-6 concentrations were higher in patients who received ablative compared to reduced intensity regimens on days seven and 14, but not day 28 (*Online Supplementary Figure S2A*). sIL-6R levels were also significantly increased beginning on day seven post-transplantation, and were still elevated by day 28 (Figure 3B). Levels

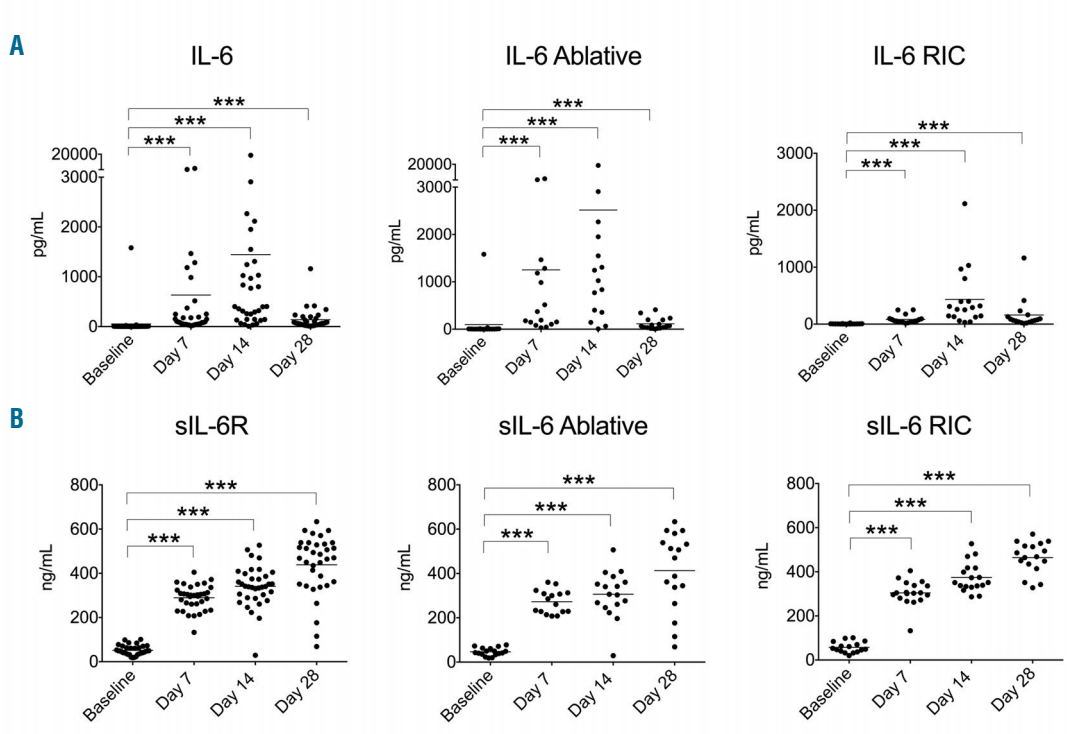

Figure 3. Effect of tocilizumab administration on interleukin 6 and soluble interleukin 6 receptor levels based on conditioning regimen. (A). Concentration of IL-6 in the serum from patients that were treated with tocilizumab and received myeloablative or reduced intensity conditioning. (B). Concentration of soluble IL-6 receptor in the serum from patients that were treated with tocilizumab and received myeloablative or reduced intensity conditioning. ***P<0.001. IL: interleukin; sIL: soluble interleukin; RIC: reduced intensity conditioning.

were augmented in recipients of reduced intensity as opposed to MA regimens on day 14, but otherwise there were no differences at other time points (*Online Supplementary Figure S2B*). Of the other cytokines measured in the blood, marginal increases were observed in IL-2 at day seven and IL-10 at day 28 (*Online Supplementary Figure S3*). A direct comparison of cytokine levels between control and Toc-treated patients demonstrated a marked increase in IL-6 and sIL-6R levels in the latter group at all time points (Figure 4). There were also significant but very modest decreases in IL-2, IL-4 and IL-10 in these patients.

Immune reconstitution

Patients were tested by multi-parameter flow cytometry for reconstitution of lymphocytes and major lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$ T cells, B cells, and NK cells) at four intervals over the first year (Figure 5A,B). Patients recovered lymphocyte subsets to, or near, healthy control levels between six months and one year post-transplant, except for NK cells, which recovered early. The percentage of both regulatory T cells (Tregs) and T helper 17 (TH17) cells were within the expected range of healthy donors, and while Treg levels gradually decreased over the post-transplant period, the absolute number of TH17 cells remained stable (*Online Supplementary Figure S4*). The percentages and absolute number of B cells were particularly low at the one-month and three-month assessments (*Online Supplementary Figure S5)*, and a subset analysis revealed other imbalances. Specifically, a functionally immature/transitional $CD21^-$ subset found to be elevated in association with autoimmunity,[24-26] infection,[27] and a subset of patients with cGvHD[27,28] was increased throughout the assessment period. However, this subset as well as most other abnormalities (including the percentage of antigen inexperienced naïve B cells) recovered through the first year. There were no significant differences seen when comparing patients experiencing aGvHD or cGvHD compared to those who did not experience GvHD for any assessment (*data not shown*).

Comparison to a matched control population

From the CIBMTR database, four controls were identified for 30 patients, three controls for three patients, and two and one control for one patient each. The baseline characteristics for the patients in the phase 2 trial and the control cohort are detailed in Table 2. Median follow up was 15 months (range: 9-20 months) for patients receiving Toc/Tac/MTX and 13 months (range; 3-72 months) for the control cohort. The incidence of grades II-IV aGvHD at day 180 was significantly lower in the Toc/Tac/MTX cohort when compared to the Tac/MTX control population (17% *versus* 45% at day 180, HR=0.34 [0.17-0.69], P=0.003) (Figure 6A). Furthermore, corresponding probabilities of grade II-IV aGvHD-free survival were significantly higher in patients who received Toc/Tac/MTX than the matched cohort (69% *versus* 42% at day 180, HR=0.37, [0.21-0.67], P=0.001) (Figure 6B). There was no difference in the incidence of cGvHD between recipients in the Toc/Tac/MTX *versus* the Tac/MTX groups (38% *versus* 45% at 12 months, HR=0.65, [0.37-1.13], P=0.13) (Figure 6C). There was also no difference in TRM,

Table 2. Demographics of tocilizumab trial patients and matched controls,

	Toc/Tac/MTX (n=35)	Tac/MTX (n=130)
Age, median (range)	66 (23-76)	64 (23-74)
KPS ≥ 90	12 (34)	48 (37)
HCT-CI		
0	9 (26)	20 (15)
1-2	11 (31)	40 (31)
3+	15 (43)	70 (54)
Disease		
AML (*de novo*)	14 (40)	56 (43)
AML (secondary)	5 (14)	20 (15)
ALL	4 (11)	14 (11)
CMML/CML	6 (17)	23 (18)
MDS/MPD	4 (11)	12 (9)
NHL	2 (6)	5 (4)
Donor Type		
MRD	13 (37)	48 (37)
MUD	22 (63)	82 (63)
Conditioning Regimen		
Flu/Bu2	17 (49)	63 (48)
Bu/Cy	5 (14)	17 (13)
Flu/Bu4	13 (37)	50 (38)
Graft Source		
BM	6 (17)	22 (17)
PBSC	29 (83)	108 (83)
Median Follow up Surviving Patients	15 months (9-20)	13 months (3-72)

AML: acute myelogenous leukemia; ALL: acute lymphoblastic leukemia; CMML: chronic myelomonocytic leukemia; CML: chronic myelogenous leukemia; MDS: myelodysplasia; MPD: myeloproliferative disorder; NHL: non-Hodgkin's lymphoma; MRD: matched related donor; MUD: matched unrelated donor; BM: bone marrow; PBSC: peripheral blood stem cells; KPS: Karnofsky Performance Score; HCT-CI: Hematopoietic cell transplantation - specific comorbidity index; Bu: busulfan; Cy: cyclophosphamide; Flu: fludarabine; Toc: tocilizumab; Tac: tacrolimus; MTX: methotrexate.

relapse, or DFS at 12 months between the two groups (Figures 6D-6F).

Discussion

Inflammatory cytokine production is a proximate event in the pathophysiology of aGvHD.[1-3] While a number of inflammatory molecules are produced as a consequence of the conditioning regimen and the activation and expansion of alloreactive donor T cells, IL-6 has emerged as an important cytokine mediator of tissue damage.[4,7,8] In the study herein, we demonstrate that inhibition of IL-6 signaling by the administration of Toc in addition to standard immune suppression resulted in a significant reduction in grades II-IV aGvHD and an increase in grades II-IV aGvHD-free survival, when compared to a matched control population. The administration of Toc was also observed to be safe when given in the setting of MA or reduced intensity Bu-based conditioning regimens. Moreover, adverse events were largely confined to transient elevations in transaminase values, and infectious complications were not dissimilar to what we have previously observed in this patient population treated with standard immune suppression only.

The results of the current study extend those reported by Kennedy and colleagues,[18] who also examined the efficacy of Toc for the prevention of aGvHD. These investigators observed an incidence of grades II-IV and III-IV aGvHD at day 100 of 12% and 3%, respectively, which was similar to what we observed (14% and 3% for these same endpoints). There were, however, several important differences between the two studies, which suggest that the results may be more broadly generalizable to allogeneic HSCT recipients. First of all, the median age of patients in our report was substantially higher (66 *versus* 48), indicating that Toc administration appears to have activity in older patients who comprise an increasing percentage of the transplant population.[20] Secondly, patients in the current report received Bu-based conditioning regimens, whereas those in the study by Kennedy *et al*. were treated with either total body irradiation and Cy (MA conditioning) or Flu and melphalan (RIC). Since the intensity of the conditioning regimen affects the degree of inflammatory cytokine production[19,29] and incidence of aGvHD,[30] the fact that promising results were observed in patients who received different MA and reduced intensity regimens is evidence that inhibition of IL-6 may have activity across a spectrum of preparative regimens. Finally, we were able to provide additional context to our data by demonstrating a reduced incidence of grades II-IV aGvHD as well as an increase in grades II-IV GvHD-free survival when com-

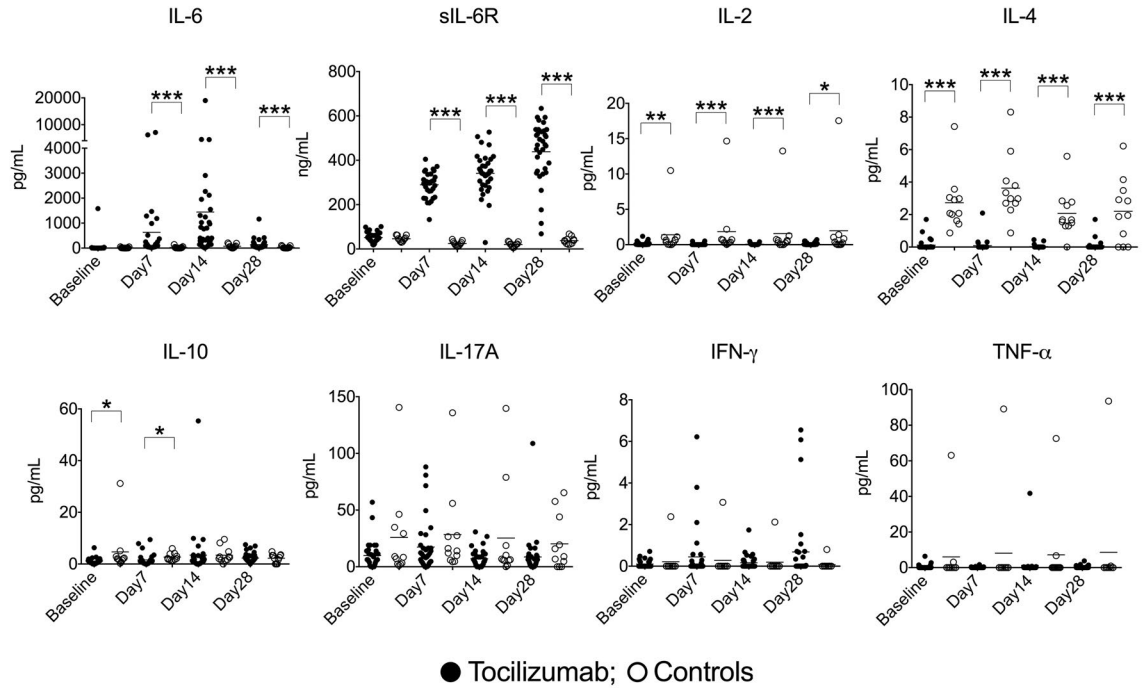

Figure 4. Comparative analysis of serum cytokine production in tocilizumab-treated *versus* patients that received Tac/MTX only. Concentration of IL-6, sIL-6R, IL-2, IL-4, IL-10, IL-17, IFN-γ, and TNF-α in the serum of patients that were treated with Toc/Tac/MTX (●, n=35) or Tac/MTX (control) (O, n=11) for the prevention of aGvHD prior to the start of conditioning and at days 7, 14 and 28. **P*<0.05, ***P*<0.01, ****P*<0.001. IL: interleukin; sIL: soluble interleukin; TNF-α: tumor necrosis factor α; IFN-γ: interferon γ.

pared to a matched control population that only received Tac/MTX.

Despite the reduction in grades II-IV aGvHD, however, there was no difference in TRM or OS between these two groups. There are several possible explanations for this observation. First, this was not a randomized trial, and the matching process could have resulted in unperceived differences between the two cohorts that could have impacted transplant outcome. Secondly, we observed two late GvHD deaths beyond six months, suggesting that the salutary effects conferred by Toc may be temporally limited. That there was no difference in the incidence of cGvHD is compatible with this interpretation. This does not, in our view, diminish the results, but rather highlights that effective prophylactic strategies for GvHD are likely to require a multi-tiered approach of which the mitigation of aGvHD within the first six months would be one important step.

A notable finding in this study was the very low incidence of aGvHD that occurred in the lower GI tract. Specifically, there were no cases within the first 100 days, and only one case which occurred by day 180. Pre-clinical studies have shown that IL-6 messenger ribonucleic acid (mRNA) levels are significantly increased in the colons of mice,[7] and the blockade of the IL-6 signaling pathway is able to significantly reduce the severity of GvHD in this tissue site.[7,8] IL-6 has also been identified as a plasma biomarker that predicts for severity and non-relapse mortality in patients with GI GvHD.[31] Our findings further support the premise that IL-6 plays an important role in mediating tissue damage in the lower GI tract. Given that the incidence of liver GvHD has been declining over time,[32] involvement of the GI tract has emerged as the primary driver of morbidity and mortality in patients with this disease. In fact, the development of lower GI tract GvHD carries significant prognostic implications for OS. Patients with lower tract GvHD are more likely to be steroid-resistant,[33] which itself is associated with increased mortality.[34] Furthermore, patients with higher clinical and histological grades of lower GI tract GvHD have an increase in non-relapse mortality that results in reduced OS.[35,36] Thus, given the poor prognosis associated with severe GI GvHD,[37,38] therapeutic strategies that are focused on preventing the development of this complication have the potential to impact the overall course of this disease and improve transplant outcome. While we observed patients with upper GI tract GvHD, recent studies have shown that disease in this tissue site is generally responsive to modest doses of steroids, and does not impact OS.[39,40]

We observed that IL-6 was the only measured serum cytokine that was significantly increased above baseline in a control population of patients that received Tac and MTX but not Toc. The administration of Toc resulted in much higher serum IL-6 levels, above that seen in the control population, in recipients of both MA and RIC regimens. This was likely due to decreased consumption of IL-6 when Toc binds to the IL-6R. IL-6 signaling occurs through two distinct mechanisms; IL-6 can bind to a membrane receptor that is expressed on hematopoietic cells and hepatocytes,[41] and also bind to a soluble form of the IL-6 receptor, which can in turn bind to glycoprotein (gp)130, which is ubiquitously expressed on most cells.[42,43] As further support for this premise, we observed that sIL-6R levels were also significantly augmented in patients who received both MA and RIC regimens. Of note, the

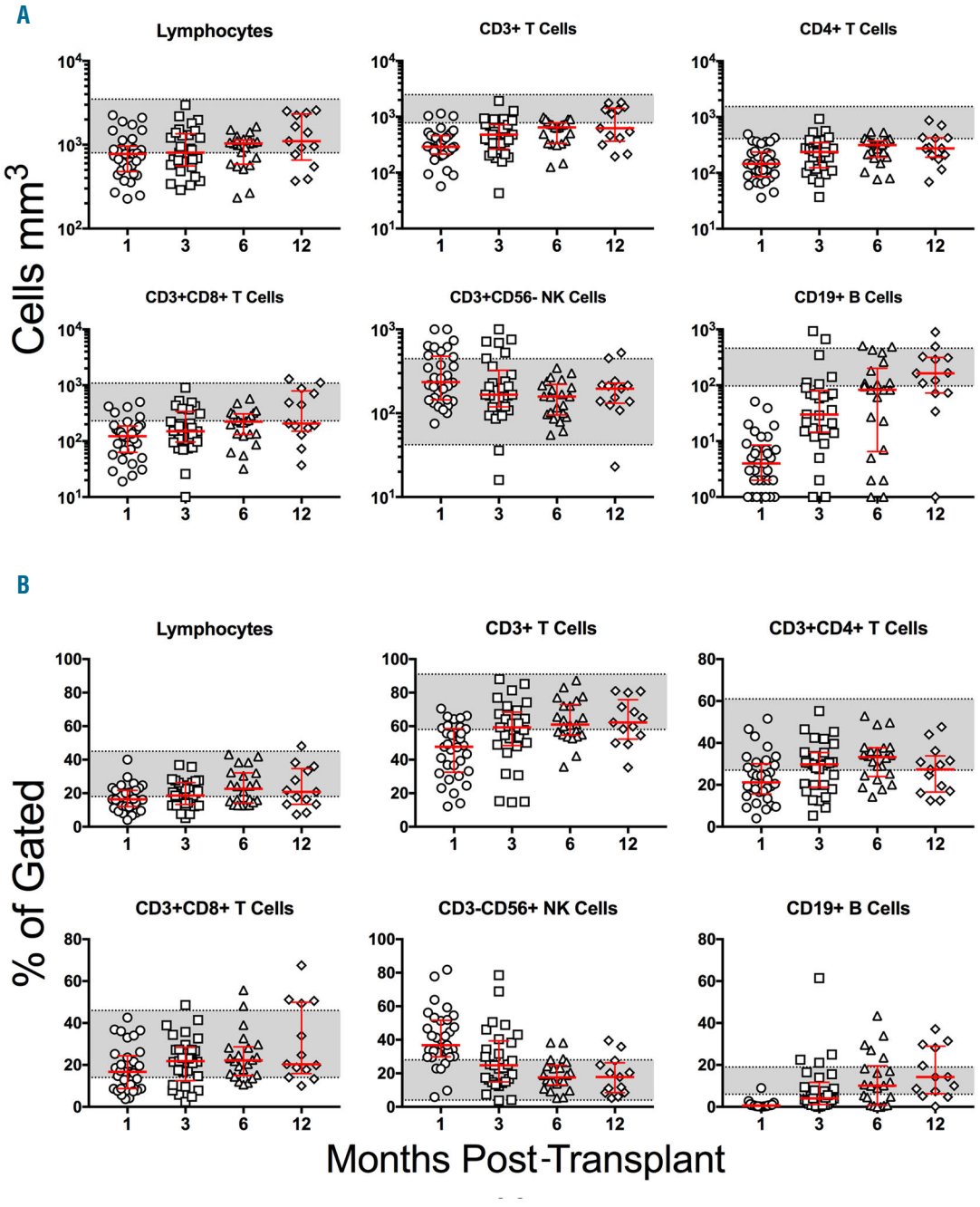

Figure 5. Reconstitution of major lymphocyte subsets in patients who received tocilizumab for GvHD prophylaxis. (A,B). The absolute number of cells per mm^3 (i.e., microliter) is shown in panel A, and the percentage of the gated cells is shown in panel B. Data are shown for individual patients together with the median and 25th and 75th quartiles (red bars). Gray shading represents the upper and lower range expected for healthy control subjects. Samples were obtained at one month (n=33), three months (n=29), six months (n=22), and 12 months (n=13). Lymphocytes were gated on total CD45$^+$ white blood cells, and all other subsets were gated on lymphocytes. NK: natural killer.

immunoassay used to detect sIL-6R levels captures free sIL-6R, IL-6R bound to IL-6, and IL-6R bound to Toc.[44] Therefore, we cannot distinguish the composition of the sIL-6R complex, but it is likely that a significant component is attributable to the binding of Toc to IL-6R, given that a prior study showed that Toc can be detected for up to one month in allogeneic stem cell transplant recipients.[18] This would therefore also explain the high IL-6 levels in these patients, as free IL-6 may have been precluded from binding to the Toc/sIL-6R complex. The fact that IL-6 levels were higher in patients treated with MA *versus* RIC, however, suggests that the conditioning regimen itself also contributed to the increase in IL-6 levels. Notably, we did not observe meaningful increases in any of the other cytokines that we examined (i.e., IL-2, IL-4, IL-10, TNF-α, IFN-γ and IL-17) in control or Toc-treated patients, providing evidence that dysregulation of IL-6 is an important early event post-transplantation. Toc had no

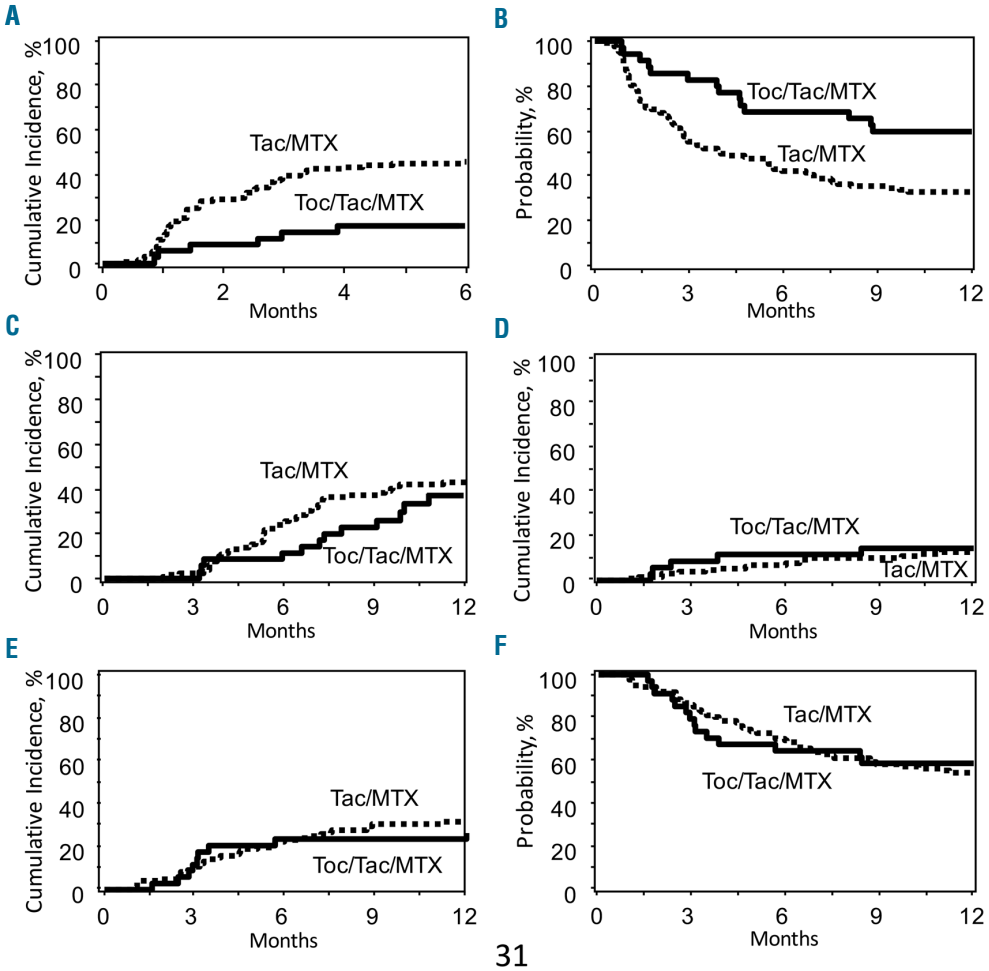

Figure 6. GvHD and transplant outcomes in patients treated with tocilizumab *versus* a matched CIBMTR control population. (A). Cumulative incidence of grades II-IV aGvHD in patients treated with tocilizumab *versus* the matched control cohort. (B). Probability of grades II-IV aGvHD-free survival. (C). Cumulative incidence of cGvHD, (D) transplant-related mortality, and (E) relapse. (F) Probability of disease-free survival in patients treated with tocilizumab *versus* the matched control cohort. Toc: tocilizumab: Tac: tacrolimus; MTX: methotrexate.

discernible adverse effect on immune reconstitution, as patients achieved near normal T-cell and B-cell subset numbers by 6-12 months post-transplantation.

In summary, this study demonstrates that Toc can be safely administered in conjunction with standard immune suppression to an older aged patient cohort treated with a Bu-based conditioning for the prevention of GvHD. The administration of Toc resulted in a low incidence of aGvHD, which was particularly evident within the lower GI tract, and was significantly less than that observed in a matched control population. There was, however, no difference in the incidence of cGvHD or a reduction in TRM. We conclude that Toc has activity for the prevention of aGvHD, and warrants further examination in a randomized setting.

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