Mitigation of gastrointestinal graft-*versus*-host disease with tocilizumab prophylaxis is accompanied by preservation of microbial diversity and attenuation of enterococcal domination

Graft-*versus*-host disease (GvHD) of the gastrointestinal (GI) tract is a major driver of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT).[1] Profound alterations in intestinal microbiota composition are reproducibly observed in HSCT with an attendant loss of diversity and a shift from anaerobes to facultative aerobes.[2,3] Enrichment of *Enterococcus* is a characteristic feature of transplant-associated dysbiosis that predisposes patients to subsequent bacteremia and GvHD.[4-6] Patterns of microbial disruption have been shown to be consistent across different transplant centers and geographic locations, indicative of a stereotypical microbial community response during transplantation even across different patterns of antibiotic use.[7] Therefore, strategies to maintain diversity and remodel the intestinal microbiome offer the potential to attenuate the severity of this disease;[8] however, to date, there are no proven strategies to prevent dysbiosis in allogeneic HSCT recipients. In this report, we administered tocilizumab as adjunctive GvHD prophylaxis to patients at high risk for the development of GI-tract disease to assess the effect on transplant outcomes and on microbial diversity and composition.

This was a phase II study (clinicaltrials gov. Identifier: NCT03699631) in which the primary endpoint was the probability of GvHD-free/relapse-free survival (GRFS)[9] after matched related/unrelated donor peripheral blood allogeneic HSCT in patients that received busulfan-based myeloablative conditioning. GvHD prophylaxis consisted of tacrolimus and methotrexate plus tocilizumab administered on days -1 and day +100. The 12-months GRFS was compared to a prespecified historical control value of 20% at a one-sided 5% significance level.[9] Patients were eligible if they were >18 years of age and had a diagnosis of acute leukemia, chronic myelogenous leukemia, myeloproliferative disease, or myelodysplasia with <5% marrow blasts; and had a 10/10 matched sibling or 8/8 matched unrelated donor. Between November 21, 2018, and February 12, 2020, 29 patients were enrolled. The demographic data for this population is detailed in the *Online Supplementary Table S1*. All patients received a first dose of tocilizumab on day -1. Twenty-six patients were treated with a second dose at a median of 99 days post transplantation (range, 86-114 days). Three patients did not receive a second dose; one due to relapse prior to day 100, one developed grade 3-4 acute GvHD which triggered the GRFS endpoint, and the last patient refused due to concerns about acquiring SARS-CoV2 infection during outpatient tocilizumab administration. Median follow-up for surviving patients was 20.6 months (range, 14.4-29.2 months).

There were no cases of primary graft failure. The median time to engraftment was 16 days (range, 13-26 days) for neutrophils and 19 days (range, 10-35 days) for platelets. The cumulative incidence of grades 2-4 acute GvHD at day 180 (using Harris criteria)[10] was 10.5% (95% confidence interval [CI]: 2.6-24.9) (Figure 1A), whereas the incidence of grades 3-4 acute GvHD was 7.0% (95% CI: 1.2-20.4) (Figure 1B). One patient had grade 2 acute GvHD due to stage 3 skin involvement. Two patients developed grade 3 acute GvHD; one that involved the lower GI tract and another with stage 2 liver and biopsy-proven upper GI involvement. There were no other cases of lower GI-tract involvement within the first 12 months. Four of 29 patients developed bacteremia within the first 100 days (i.e., *Escherichia coli*, *Capnocytophaga sputigena*, *Streptococcus oralis*, and *Enterococcus faecalis*). The cumulative incidence of overall chronic GvHD (using NIH consensus criteria)[11] was 64.8% (95% CI: 42.4-80.3) (Figure 1C), whereas the incidence of severe chronic GvHD was 7.7% (95% CI: 1.3-22.1) at 12 months (Figure 1D). Organ involvement in the 17 patients who developed chronic GvHD consisted of liver (n=10), mouth (n=7), eyes (n=5), skin (n=4), lung (n=1), genital (n=1), and joints (n=1). The cumulative incidence of relapse was 10.3% (95% CI: 2.5-24.6) at 1 year (Figure 1E). Non-relapse mortality was 3.4% (95% CI: 0.2-15.3) at 12 months and occurred in one patient due to bronchiolitis obliterans (Figure 1F). Overall survival and relapse-free survival were identical at 12 months (86.2%; 95% CI: 67.3-94.6) (Figure 1G and H). The probability of GRFS at 1 year was 37.9% (95% CI: 20.9-54.9) which was significantly higher than the prespecified historical rate of 20% (P=0.023), indicating that the primary end point of the trial was met (Figure 1I).

In order to examine fecal microbial composition, we compared evaluable stool samples from trial participants (toci) with those from an observational cohort at Memorial Sloan Kettering Cancer Center (MSKCC) (control). The control population was assembled from demographically matched patients who contributed fecal samples while receiving an unmodified peripheral blood stem cell transplant following myeloablative conditioning with Flu/BU4 and received ta-

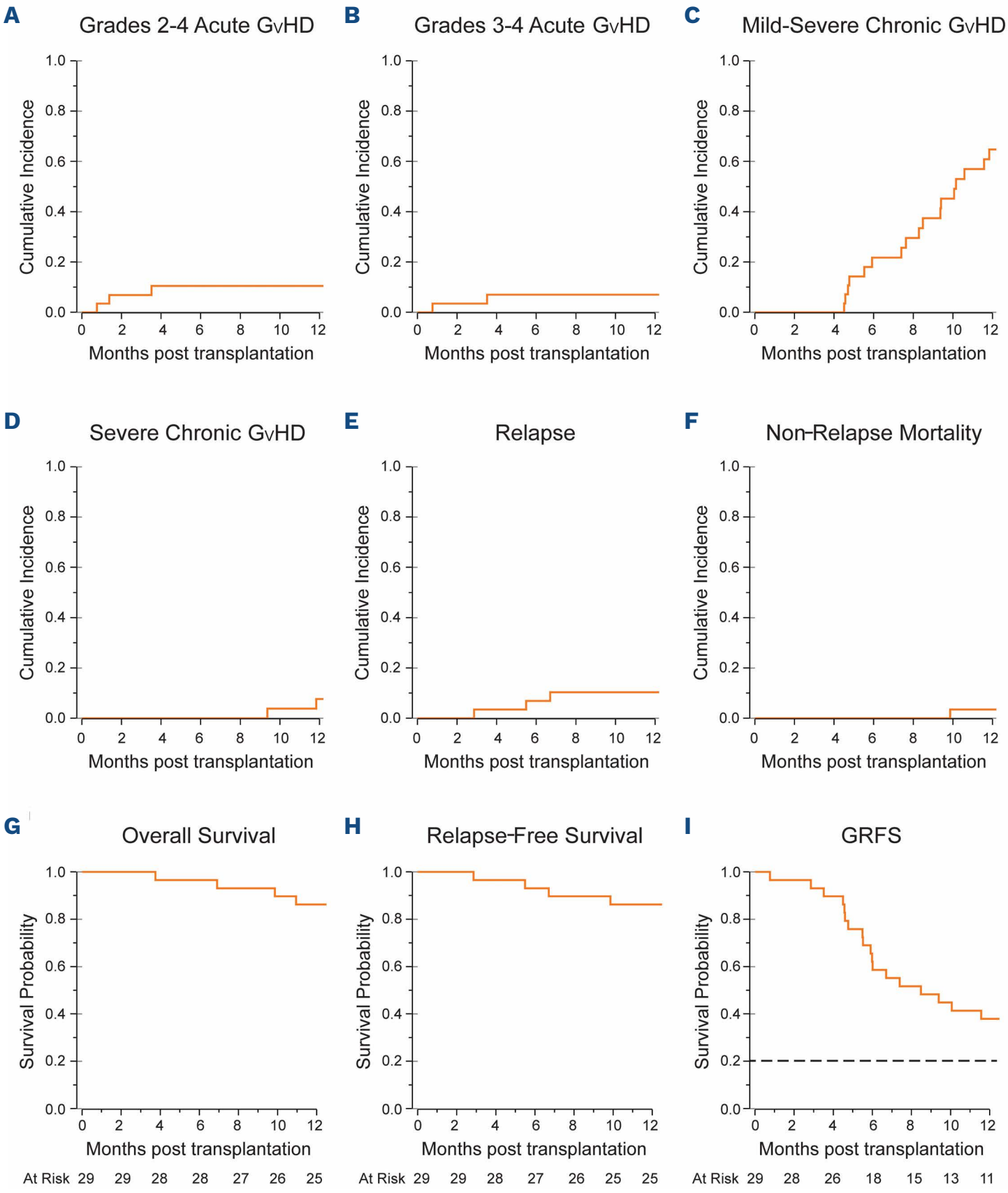
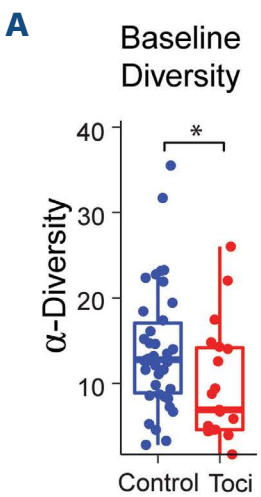
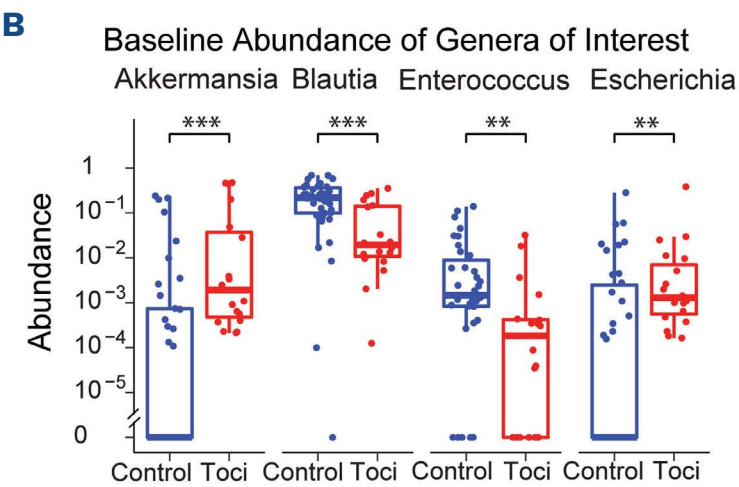
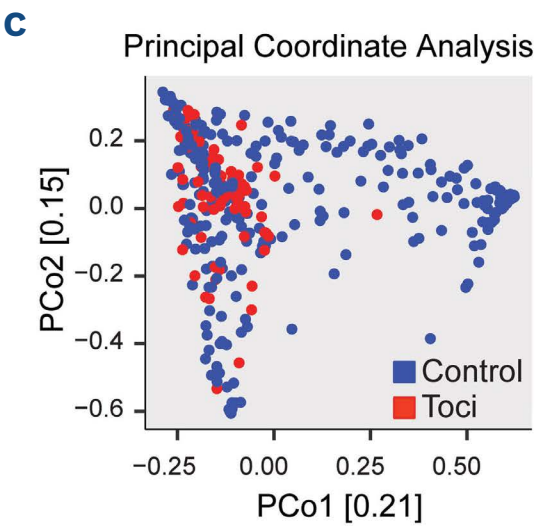


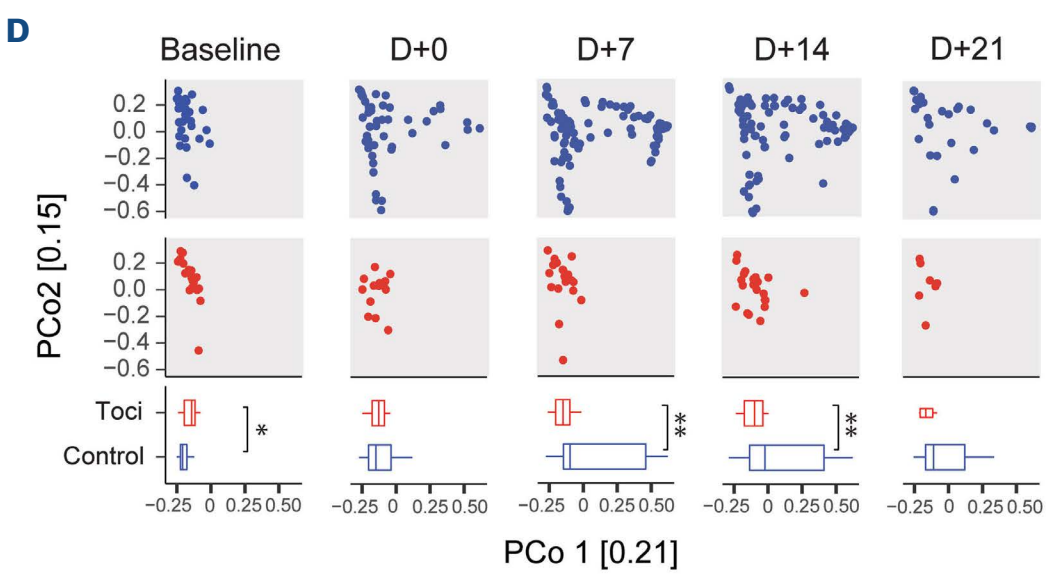
Figure 1. Transplant outcomes in patients that received graft-*versus*-host disease prophylaxis with tocilizumab. (A and B) Cumulative incidences of grades 2-4 (A) and 3-4 (B) acute graft-*versus*-host disease (GvHD). (C and D) Cumulative incidences of mild-severe and severe chronic GvHD. (E) Cumulative incidence of relapse. (F) Cumulative incidence of non-relapse mortality. (G and H) Probability of overall survival (G) and relapse-free survival (H). (I) Probability of GvHD-free/relapse-free survival (GRFS). Dotted line denotes the prespecified historical control rate of 20%.

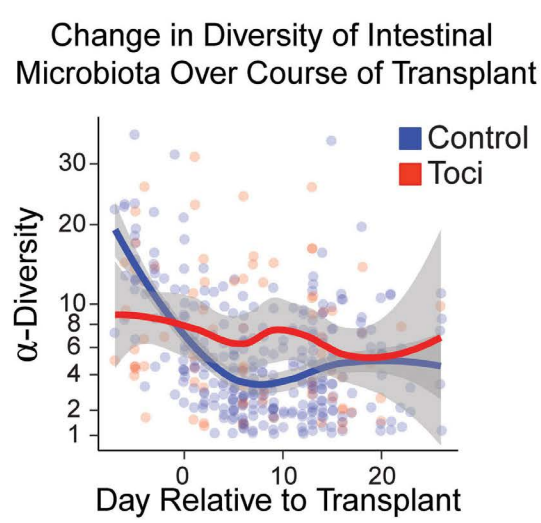
crolimus and methotrexate as sole GvHD prophylaxis (*Online Supplementary Table S2*). A comparison of baseline microbiomes demonstrated that α-diversity was significantly lower in toci patients prior to transplantation (Figure 2A). We then examined the relative baseline abundances of bacteria from four genera of interest (*Blautia, Enterococcus, Eschericia, and Akkermansia)* that were selected based on prior studies which have described associations for *Blautia* with lower rates of GvHD-related mortality,[12] *Enterococcus* as a driver of GvHD in humans and murine recipients,[4,6] *Escherichia* as a cause of bloodstream infections in patients,[5,13] and *Akkermansia* as an emergent bacterium in mouse models of GvHD.[14] We observed that the toci cohort had a lower abundance of genus *Blautia* and *Enterococcus*, whereas there was a higher abundance of *Akkermansia* and *Eschericia* (Figure 2B). Principal-coordinate analysis (PCoA) of global composition revealed that variation along the first coordinate was attributable primarily to the control cohort

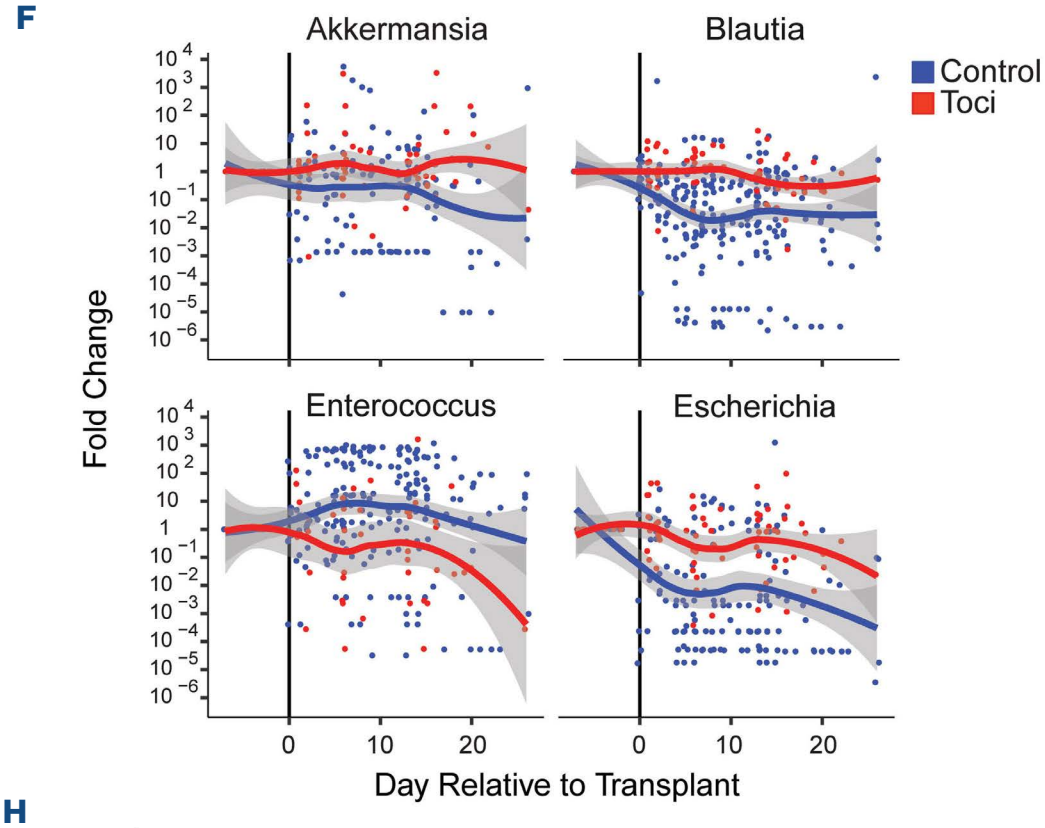


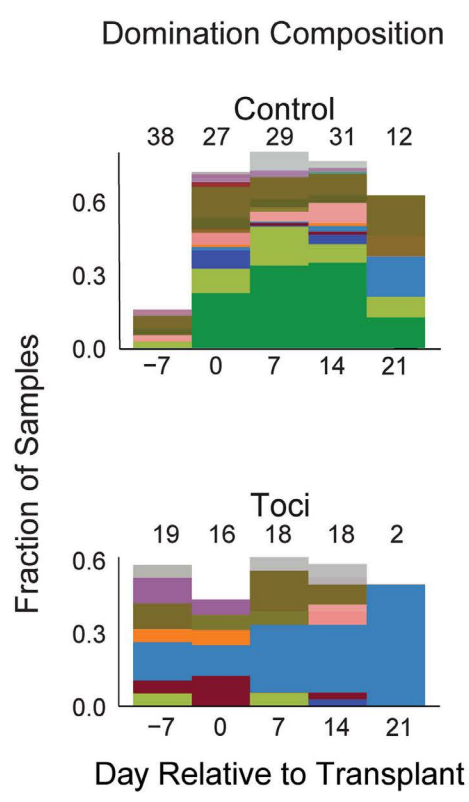












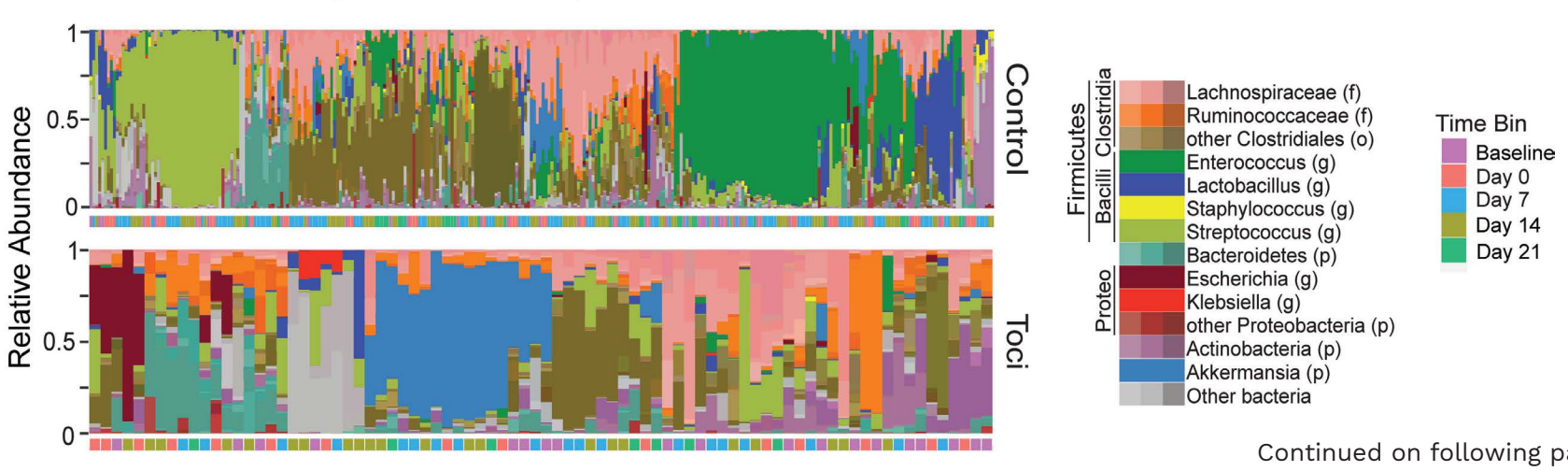


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Figure 2. Microbial diversity and composition in tocilizumab and control cohorts. (A) Baseline α-diversity, as measured by the inverse Simpson index, in study patients that received toci *vs*. microbiome controls. Baseline samples were collected between day -7 and day -1. (B) Baseline abundance plots of *Akkermansia*, *Blautia*, *Enterococcus*, and *Escherichia coli* in these 2 cohorts. (C). Principal-coordinate analysis of Bray-Curtis dissimilarities between samples in which each point is a fecal sample. Samples from toci recipients are largely on the left-hand side of the graph, while samples from the microbiome control are spread throughout the principal-coordinate space. (D) Ordination data from (C) split by specified time windows illustrating the dynamics of fecal compositional shifts along the PC1 axis. In box plots depicted in (B and D), the horizontal line in each box represents the median, the lower and upper boundaries of the boxes the interquartile range, and the end of the whisker lines the minimum and maximum values within 1.5 times the interquartile range. (E) α-diversity plot over time in tocilizumab recipients compared with microbiome controls. A generalized estimating equation (GEE) was used to compare the α-diversity trends, revealing significant differences between groups both in terms of the trajectory over time (P=0.0005) and the more linear response shape in the toci cohort (P=0.001). Each point is a fecal sample; the curves are smoothed averages, and the gray shading are 95% confidence intervals. See the *Online Supplementary Table S3* for a summary of the GEE model. (F) Fold change in relative abundances, compared to baseline, of selected genera are plotted and depicted. Each point represents a sample; curves are smoothed averages and shaded areas show 95% confidence intervals. For each fold-change analysis, we considered only patients with detectable abundances of the indicated feature at baseline. (G) Taxa contributing to domination events in samples from the 2 cohorts. Total stacked-bar height shows the fraction of samples in 7-day bins exhibiting >30% domination by a single amplicon sequence variant (ASV), and the color shows the dominating ASV's taxonomy according to the color scheme shown below in (H). (H) Taxonomic composition of all samples is presented as stacked bar graphs and color coded according to the scheme described in Peled *et al.*[11] Samples are ordered by hierarchical clustering of the β diversity matrix for each cohort. (A and B) Control cohort includes 38 samples from 38 unique patients, toci cohort includes 19 samples from 19 unique patients. (C to H) Control cohort considers 308 samples from 38 unique patients; toci cohort considers 82 samples from 19 unique patients. Statistics: *P<0.05; **P<0.01; ***P<0.001.

(Figure 2C). Subsetting this PCoA plot by time intervals demonstrated that at baseline, samples from both the toci and control cohorts clustered at lower values of PCo1. A shift to the right of the plot, (i.e., higher values of PCo1) were observed over time more prominently among control (blue) than toci (red) samples, particularly at days 7 and 14 (Figure 2D).

Loss of α-diversity in the first 2 weeks was significantly more rapid in the control cohort compared to toci patients (Figure 2E). This was statistically significant as early as day 0 and thereafter at weekly intervals. Examination of temporal alterations in specific taxonomic groups of interest revealed a significant reduction in *Blautia* in both cohorts; however, this relative decline occurred at earlier time points in control patients (Figure 2F). In addition, *Akkermansia* which had higher baseline abundance in toci patients (Figure 2A) further increased by week 3, whereas fold change was unaltered in the control cohort. Most notably, enterococcal domination was observed in approximately a third of control patients but was absent in the toci population (Figure 2G, dark green). The differences in domination patterns are illustrated in stacked barplots of taxonomic composition, where frequent dominations by *Enterococci* (dark green) and *Streptococci* (light green) were observed in the control patients; in contrast *Akkermansia* dominations were commonly observed in the toci cohort (Figures 2G and H, light blue).

Given the known association between antibiotic exposure and microbiome injury in HSCT,[4,5,7,14] we examined the possibility that differences in the incidence of febrile neutropenia might explain this observation since the onset of febrile neutropenia triggers additional antibiotic administration. We observed that the cumulative incidence of febrile neutropenia between the two cohorts was comparable (Figure 3A), and that differences in α-diversity trends (Figure 2E) remained statistically significant even when samples collected after onset of febrile neutropenia were excluded (P=0.001) (Figure 3B). Moreover, the rightward shift observed in the PCoA plot (Figure 2D) was not attributable solely to samples collected after the onset of febrile neutropenia (Figure 3C). We also explored individual taxa that exhibited relative expansions or contractions in multivariable models that highlighted an expansion of *Enterococcus* in the control cohort relative to the toci cohort (*Online Supplementary Figure S1A*). In a separate model that considered exposures to antibiotic classes common between the two cohorts, many associations were observed, as expected, between antibiotics and specific taxa (*Online Supplementary Figure S1B*). The number and percent of patients exposed to each antibiotic class are tabulated in the *Online Supplementary Figure S1C*.

This study demonstrated that tocilizumab prophylaxis was associated with a low incidence of GI-tract GvHD as only one of 29 patients developed lower tract disease within the first year, providing evidence that IL-6 inhibition suppresses inflammation in the GI tract. We hypothesized that attenuation of intestinal inflammation by IL-6 blockade would mitigate the microbiome injury that accompanies allogeneic HSCT. Although the control cohort was assembled *post hoc* from patients at another center, transplant characteristics were well-matched with trial participants, and samples from both centers were centrally sequenced and computationally analyzed. Even though baseline diversity was higher in the control cohort, the loss of diversity was significantly attenuated in tocilizumab-treated patients. The dramatic shifts in global microbiome composition that has been well described in allogeneic HSCT recipients[2,7] was observed in the control

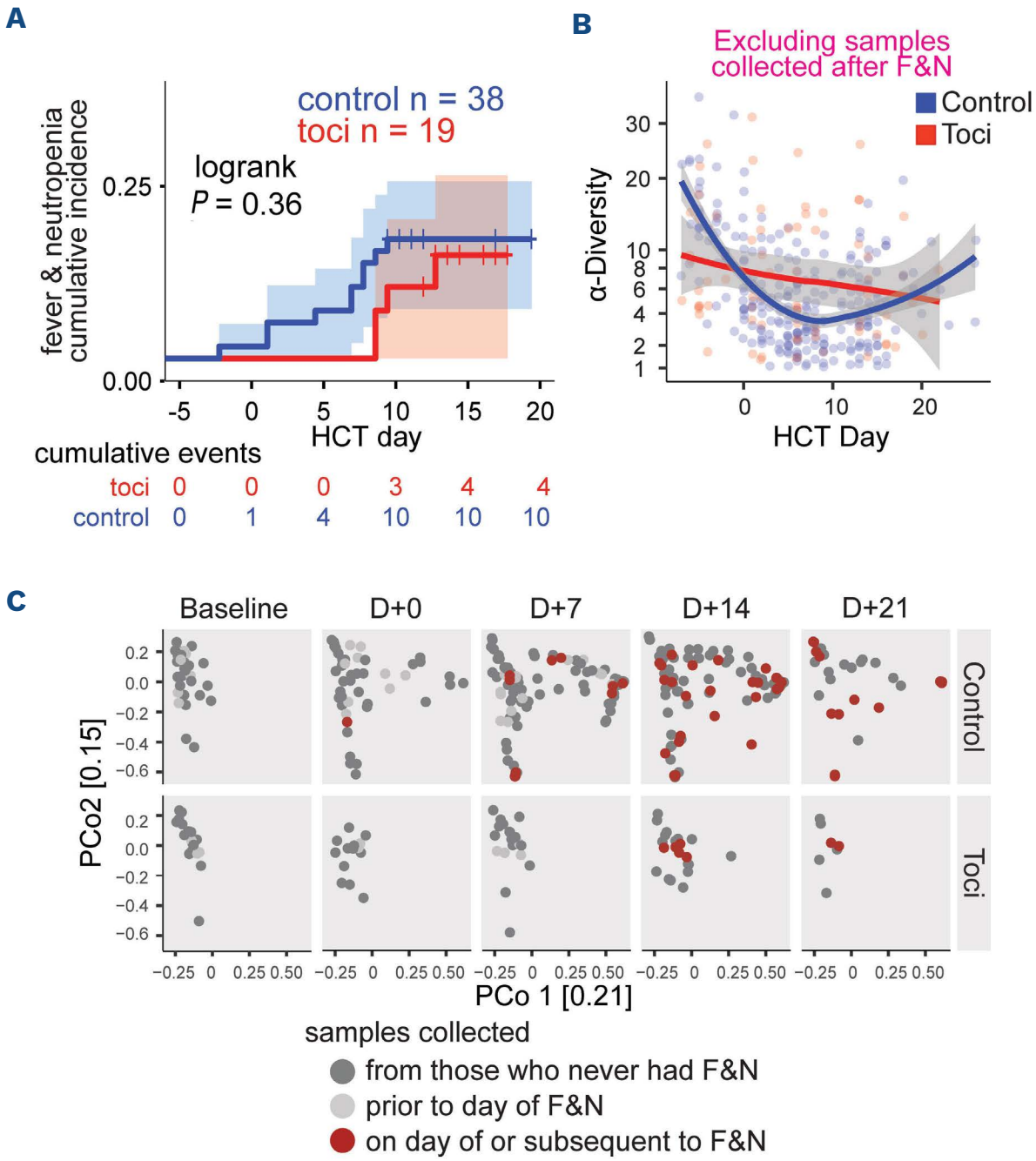

Figure 2. Mitigation of microbiome injury by tocilizumab is not attributable to differences in febrile neutropenia. (A) Cumulative incidence of febrile neutropenia (F&N) in the 2 cohorts. The time of origin was set to hematopoietic stem cell transplantation (HSCT) day -6 (approximate time of conditioning start) to accommodate an event that occurred prior to cell infusion. (B) The trajectories in α-diversity over time remained significantly different in the 2 cohorts even when excluding samples collected after onset of F&N (GEE model, P=0.001). (C) The PCoA plot in Figure 2D is repeated, now color-coded to indicate timing of sample collection relative to F&N. The positions on the horizontal axis of samples collected after F&N were indistinct from those collected prior to fever onset or from patients who never developed F&N; this was the case in the day -14 and day -21-time bins in both the cohorts (Wilcoxon P>0.2 for all 4 comparisons).

cohort, but to a markedly lesser extent in tocilizumab recipients. Importantly, we saw little evidence of enterococcal dominance and only one case of enterococcal bacteremia in tocilizumab-treated patients. This contrasted with the control cohort in which enterococcal domination occurred in approximately 30% of recipients, consistent with previous observations in a much larger patient population at this same institution.[7] Interestingly, phylogenetic analysis revealed a preponderance of *Akkermansia*, whose presence has been shown to increase the production of antimicrobial metabolites and proliferation of intestinal stem cells,[15] suggesting a potential salutary role in preserving intestinal barrier function.

In summary, we conclude that an extended course of tocilizumab administration is effective for the prevention of lower GI-tract GvHD, and that loss of microbial diversity and enterococcal domination is attenuated in tocilizumab-treated recipients. Thus, GvHD prophylaxis with adjunctive tocilizumab represents a possible therapeutic approach for remodeling the intestinal microbiome and preventing the emergence of potentially pathogenic organisms.

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Contributions

SC designed the study, enrolled patients on the trial, provided clinical care of patients in the study, analyzed data, and wrote the paper. AS and DE provided statistical analysis. KM and LS assisted in the acquisition of data. SA, WL, PNH, MH, NNS, LR and JHJ enrolled patients on the trial, provided clinical care of patients, and edited the paper. AC, NW, GA, TF, JUP and MvdB performed microbiome computational analyses and edited the paper. WRD designed the study, provided clinical care, analyzed data, and wrote the paper.

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Data-sharing statement

Data can be obtained by contacting the corresponding author WD.

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