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Alteration of the Intestinal Microbiota by Broad-Spectrum Antibiotic Use Correlates with the Occurrence of Intestinal Graft-versus-Host Disease

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A B S T R A C T

Patients undergoing hematopoietic stem cell transplantation (HSCT) frequently receive empiric antibiotics during the neutropenic period before engraftment. Several recent studies have shown that anaerobes in the intestine are important mediators of intestinal homeostasis, and that commensal bacteria can be potent modulators of the severity of acute graft-versus-host disease (aGVHD). However, the relationships among the type of antibiotic used during the neutropenic period, changes in the intestinal microbiota, and subsequent occurrence of aGVHD are not clear. In this study, a total of 211 patients undergoing HSCT were stratified into 3 groups: patients not treated with any antibiotics during the neutropenic period (group 1; n = 43), patients treated with cefepime only (group 2; n = 87), and patients treated with carbapenem antibiotics, defined as meropenem or prepenem with or without previous cefepime therapy (group 3; n = 81). Intestinal microbiota analyses were performed on pre- and post-HSCT stool samples, and immunophenotypic analyses were performed on pre- and post-HSCT peripheral blood samples. Among the 211 patients, 95 (45%) developed aGVHD (grade ≥II), including 54 with intestinal GVHD. The incidence of intestinal GVHD was higher in group 3 compared with group 1 and group 2 (32.1%, 11.6%, and 26.4%, respectively; $P = .044$). After adjusting for potentially significant variables identified by univariate analysis, multivariate analyses identified broad-spectrum antibiotic use during the neutropenic period as associated with the occurrence of intestinal GVHD (hazard ratio, 3.25; 95% confidence interval, 1.13 to 9.34; $P = .029$). Accordingly, loss of bacterial diversity in terms of alterations in intestinal microbiota after HSCT was observed in patients who received broad-spectrum antibiotics. Moreover, alterations in the frequencies of several intestinal bacteria phyla were associated with the occurrence of intestinal GVHD. Evaluation of circulating immune cell subsets according to type of antibiotic used during the neutropenic period revealed delayed recovery of myeloid-derived suppressor cells in the broad-spectrum antibiotic use group. Our data indicate that the use of broad-spectrum antibiotics during the neutropenic period is associated with a higher incidence of intestinal GVHD via loss of microbiome diversity. Further studies are needed to determine whether maintaining bacterial diversity can help prevent the development of aGVHD.

INTRODUCTION

For many patients with malignant hematologic disorders and bone marrow failure, hematopoietic stem cell transplantation (HSCT) offers a curative treatment option [1]. Unfortunately, however, its success is still limited by the development of life-threatening complications, including acute graft-versus host disease (aGVHD) and infection. Patients with grade III and IV aGVHD have poor outcomes, with a ~30% and <5% probability of long-term survival, respectively [2]. Because neutropenia and mucosal injury after conditioning regimens often lead to neutropenic infections in HSCT recipients [3], most patients require treatment with broad-spectrum antibiotics. Early preclinical studies suggested that intestinal microflora contribute to the pathogenesis of aGVHD, and that growth suppression or eradication of intestinal bacteria can prevent the development of aGVHD, even in major histocompatibility complex antigen-mismatched transplants. These observations led to the practice of gut decontamination with oral nonabsorbable

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antibiotics in patients undergoing allogeneic HSCT as a method of aGVHD prophylaxis [4]. However, there is increasing evidence that use of broad-spectrum antibiotics may have a detrimental impact on intestinal microbiota composition and, consequently, the outcome of HSCT [5-8]. Previous studies have demonstrated that loss of bacterial diversity, in particular commensal Clostridiales, appears to contribute not only to the pathogenesis of gastrointestinal GVHD, but also to increased transplantation-related mortality (TRM) [9-11].

A healthy intestinal microbiota aids digestion and metabolism and protects against pathogen invasion and overgrowth of pathobionts, commensal bacteria that can intermittently reside as minor members of the microbiota but can also act as pathogens when the microbiota becomes disrupted [12]. Microbiome disruption is characterized by loss of bacterial diversity or protective species and overgrowth or dominance by a single organism. Although the widespread use of antibiotics has saved lives, incalculable numbers of bacteria, both pathogenic and commensal, are killed by these antibiotics. We are now beginning to understand the ways in which antibiotics reshape the ecology of the microbiome and the functional consequences of these changes [13], and strategies to prevent antibiotic-induced dysbiosis have been explored [7].

In the present study, we investigated the association between the spectrum of antibiotics administered during the neutropenic period and intestinal microbiota composition and aGVHD in a cohort of 221 Korean patients who underwent HSCT. We also explored the effects of broad-spectrum antibiotic use on immune reconstitution after HSCT. We found that administration of broad-spectrum antibiotics during the neutropenic period was associated with a higher incidence of intestinal GVHD via loss of bacterial diversity.

METHODS

Study Patients

The study cohort comprised 211 consecutive adult patients with hematologic malignancies who underwent HSCT at Seoul St Mary's Hospital between January 2013 and October 2014. Written informed consent was obtained from each patient before participation. The study was approved by the Institutional Review Board of Seoul St Mary's Hospital and The Catholic University of Korea (KC12SISE0585) and was conducted in accordance with the Declaration of Helsinki.

Transplantation Procedures

Donor selection was based on molecular typing for HLA-A, -B, -C, and -DRB1. Patients received either a myeloablative conditioning (MAC) regimen (n = 112) or a reduced-intensity conditioning regimen (n = 99). Overall, 33 patients received bone marrow and 178 received granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells. GVHD prophylaxis included a calcineurin inhibitor (cyclosporine for matched related transplants and tacrolimus for all unrelated transplants and haploidentical transplants) along with short-term administration of methotrexate. All patients received selective antibacterial and antifungal prophylaxis with ciprofloxacin (500 mg twice daily) and itraconazole oral solution (2.5 mg/kg twice daily) or i.v. micafungin (50 mg daily) from the starting day of conditioning until engraftment. Acyclovir (400 mg orally twice daily for transplants from sibling donors and 5 mg/kg i.v. twice daily for unrelated or HLA-mismatched familial donors) was administered from the starting day of conditioning to engraftment. Granulocyte colony-stimulating factor (lenograstim, 5 μg/kg/day) was administered s.c. to all patients from day +7 after transplantation until the absolute neutrophil count reached $>3.0 \times 10^9$/L. At our hospital, cefepime is the standard first-line antibiotic for empiric treatment of neutropenic fever, and carbapenem is used as a second-line treatment for neutropenic fever in patients with microbiological evidence of resistance to first-line treatment, as well as those with persistent fevers or clinical worsening of symptoms. Glycopeptides were also administered according to international guidelines for treatment [14]. Other general transplantation procedures were performed as described previously [15,16].

Stool Specimen Collection and Analysis

Stool specimens for the analysis of intestinal microbiota were collected 1 day before conditioning chemotherapy (pre-HSCT; n = 65) and after neutrophil engraftment, defined as the first of 3 consecutive days with an absolute neutrophil count $>.5 \times 10^9$/L (post-HSCT; n = 50). The median time to stool specimen collection after HSCT was 13 days (range, 10 to 27 days). The specimens from 9 patients were collected during antibiotic administration.

Isolation of Mononuclear Cells and Flow Cytometry Analysis

Blood samples for immune cell subsets analysis were collected 1 day before conditioning chemotherapy (n = 145) and after neutrophil engraftment (n = 50). Peripheral blood mononuclear cells were isolated from whole blood samples (10 mL) collected in EDTA-coated tubes by centrifugation in Ficoll-Paque and then processed immediately for analysis.

$CD3^+$, $CD4^+$, and $CD8^+$ T cells, natural killer (NK) cells ($CD3^-CD56^+$), natural killer T (NKT)-like cells ($CD3^+CD56^+$), regulatory T cells ($CD25^+CD127^{low}$ on $CD3^+CD4^+$ cells), mucosal-associated invariant T (MAIT; $CD161^+V\alpha\ 7.2^+$ on $CD8^+$) cells, and invariant NK cells (NKT-like cells marking $V\beta11^+CD3^+$) were analyzed by flow cytometry. Natural-killer receptor group 2, member D (NKG2D) expression on NK or NKT-like cells was also analyzed. Myeloid-derived suppressor cells (MDSCs) were classified as early-stage MDSCs (E-MDSCs) or monocytic MDSCs (M-MDSCs), as reported previously [17]. E-MDSCs were immunophenotyped as $HLA\text{-}DR^-Lin^-CD11b^+CD33^+$ populations, whereas M-MDSCs were defined as the $HLA\text{-}DR^-CD14^+$ population (Supplementary Figure 1). Each MDSC subtype was quantitated as a percentage of mononuclear cells.

Definitions and Statistical Analyses

aGVHD was diagnosed and graded according to clinical consensus criteria [18,19]. In this study, intestinal GVHD was clinically diagnosed based on classical clinical symptoms and the exclusion of alternative diagnoses, such as effects of drugs and infection [20-23]. Patients presenting with diarrhea and/or lower abdominal pain were clinically diagnosed with lower intestinal GVHD when routine stool tests for *Clostridium difficile*, including toxigenic culture and toxic polymerase chain reaction results, enteropathogenic bacterial culture, and parasite examination (including cryptosporidiosis and giardiasis) were negative, and confirmed pathologically by biopsy whenever feasible. Histological findings were assessed based on the proposed recommendations for biopsy reporting from the National Institutes of Health consensus project [24]. When immunohistochemical staining for cytomegalovirus (CMV) was positive, apoptotic bodies found only near CMV inclusions were considered to be only CMV disease. The primary treatment for aGVHD consisted mainly of methylprednisolone (2 mg/kg) or an equivalent dose of prednisone with a gradual taper [16,25].

The chi-square test and Fisher exact test were used to test the associations between categorical variables. The 2-tailed Student *t* test was used to analyze continuous variables, and one-way analysis of variance was used to compare continuous variables among the 3 groups. Risk factors for the occurrence of aGVHD or lower intestinal GVHD were assessed using logistic regression, and covariates with a *P* value $<.10$ in univariate analyses were added to the multivariate analysis model. Cumulative incidence was used to estimate the probability of TRM, and relapse was compared using the Gray test. Probabilities of overall survival (OS) and event-free survival were calculated using the Kaplan-Meier method and compared with the log-rank test.

RESULTS

Patient and Transplantation Characteristics

A total of 211 patients who underwent HSCT were stratified into 3 groups: patients not treated with any antibiotics during the neutropenic period (group 1; n = 43), patients treated with cefepime only (group 2; n = 87), and patients treated with a carbapenem antibiotic (meropenem or prepenem) with or without previous cefepime therapy (group 3; n = 81) (Figure 1). The median time to antibiotic treatment was 8 days after HSCT (range, -6 to 22 days), and the median duration of antibiotic treatment was 8 days (range, 2 to 37 days). The study group comprised 127 males and 84 females, with a median age of 45 years (range, 16 to 68 years). Patients with various hematologic malignancies received transplants from sibling donors (n = 98), unrelated donors (n = 71), or HLA-mismatched familial donors (n = 42). At the time of HSCT, 67 patients (31.8%) had advanced disease features, defined as acute leukemia beyond the first remission and high-risk myelodysplastic syndrome (International Prognostic Scoring System $\geq$intermediate-2). The proportion of patients receiving antibiotics during the neutropenic period differed according to the intensity of the pretransplantation conditioning regimen ($P = .046$). Patient and transplantation characteristics were similar across the 3 groups with the exception of conditioning intensity, as summarized in Table 1.

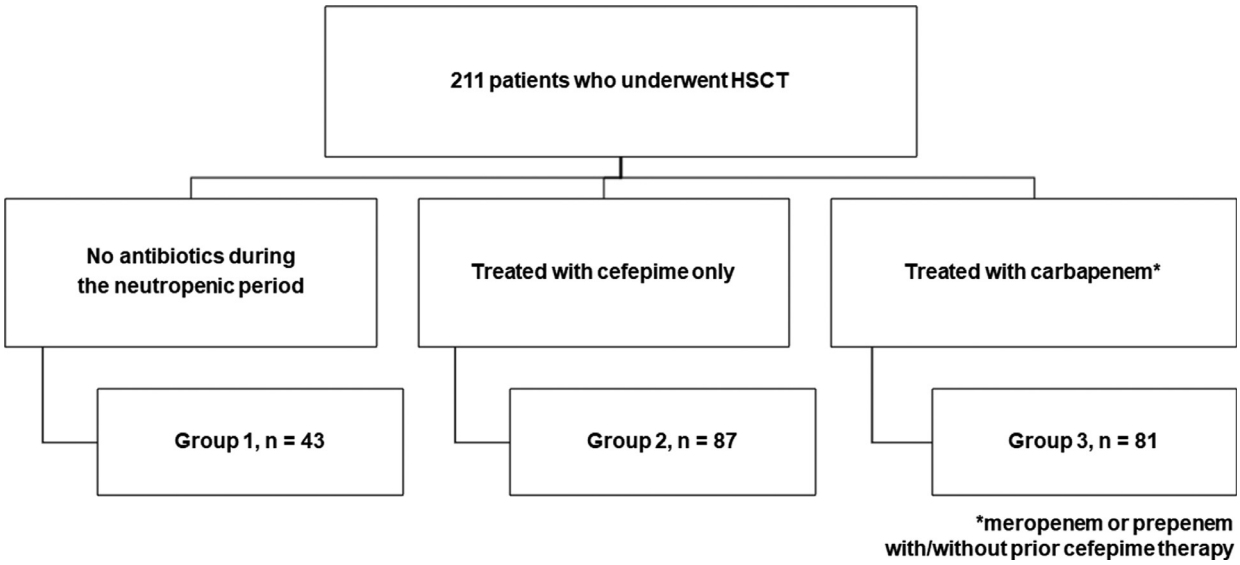

Figure 1. Patients included in this study.

Transplantation Outcomes

Engraftment was achieved in all patients. The median time to neutrophil engraftment after HSCT was 12 days (interquartile range [IQR], 11 to 13 days), and that for platelet engraftment was 14 days (IQR, 12 to 15 days). Table 2 summarizes transplantation outcomes. At a median of 33.0 days (IQR, 33 to 54 days) after transplantation, 130 patients (45.0%) experienced aGVHD grade ≥II (32.6% in group 1 versus 43.7% in group 2 versus 53.1% in group 3; $P = .087$). Forty-five patients (21.3%) had grade III-IV aGVHD (9.3% in group 1 versus 23.0% in group 2 versus 25.9% in group 3; $P = .088$). Intestinal GVHD developed in 54 patients (25.6%), and its occurrence differed according to the use and spectrum of antibiotics (11.6% in group 1 versus 26.4% in group 2 versus 32.1% in group 3; $P = .044$). Among the 54 patients with intestinal GVHD, 5 (9.3%) did not receive antibiotics before engraftment, 23 (42.6%) were treated with cefepime alone, and 26 (48.1%) were treated with broad-spectrum antibiotics (Figure 2). However, the incidence of steroid-refractory intestinal GVHD was not significantly different across the groups (1 of 5 [20%] in group 1, 3 of 23 [13%] in group 2, and 5 of 26 [19%] in group 3; $P = .827$).

Among the patients who received antibiotics during the neutropenic period, longer duration of antibiotic treatment was associated with higher TRM ($P < .001$) and lower rate of relapse ($P = .022$) and showed a tendency toward greater intestinal GVHD ($P = .080$) and lower OS ($P = .080$).

Risk Factors for the Occurrence of Intestinal GVHD

The results of univariate analysis for potential risk factors affecting the occurrence of intestinal GVHD are listed in Table 3. Group 2 showed a trend toward an increased risk for intestinal GVHD compared with group 1 (relative risk [RR], 2.73; 95% CI, .96 to 7.78; $P = .060$). Patients in group 3 had a significantly higher rate of intestinal GVHD than those in group 1 (RR, 3.59; 95% CI, 1.27 to 10.19; $P = .016$). After adjusting for potential contributing variables identified by univariate analysis, multivariate analyses revealed that use of carbapenem antibiotics (group 3) during the neutropenic period was associated with the occurrence of intestinal GVHD (RR, 3.25; 95% CI, 1.13 to 9.34; $P = .029$).

Changes in Microbiota Composition Following HSCT

Because the intestinal microbiota is known to play a key role in the pathogenesis of aGVHD [11,26,27], and based on our finding that carbapenem antibiotic use during the neutropenic period was associated with the occurrence of aGVHD, especially intestinal GVHD, we were interested in changes in the intestinal tract microbiota during the process of HSCT. Comparisons of bacterial abundance at the phylogenetic level of genus by 16S rRNA gene sequencing showed distinct patterns in pre-HSCT (n = 65) and post-HSCT stool samples (n = 50) (Figure 3A).

Bacterial composition at the phylum level was significantly different between pre-HSCT and post-HSCT samples, in particular the phyla Firmicutes and Proteobacteria (Figure 3A). At the genus level (Figure 3B to F), *Lachnospira* was significantly down-regulated post-HSCT. In contrast, *Parabacteroides, Streptococcus, Lactonifactor*, and *Macellibacteroides* were up-regulated post-HSCT.

Effect of Antibiotics on Intestinal Microbiota Composition

We next investigated whether the use of antibiotics during the neutropenic period was associated with changes in the composition of the intestinal microbiota after HSCT. A significant reduction in diversity of commensal bacterial flora as measured by Pielou's evenness index was observed in patients treated with carbapenem, but not in patients who did not receive antibiotics and those who received cefepime only (Figure 4A). Bacterial composition patterns at the phylum level differed across the 3 groups. In group 3, Bacteroidetes, Cyanobacteria, and Verrucomicrobia were significantly reduced after transplantation (Figure 4B). The use of carbapenem was associated with expansion of *Melissococcus*and *Anaerotruncus* at the genus level (Figure 4C and D).

Relationships between Intestinal Microbiota and Intestinal GVHD

We next investigated the effect of loss of bacterial diversity on the occurrence of aGVHD. Twenty-one patients with available paired stool samples were divided according to the occurrence of aGVHD into an intestinal GVHD group (n = 10) and a no intestinal GVHD group (n = 11). In each group, the change of microbial diversity, as reflected by the Chao1 richness index, was analyzed (Figure 5A). There was no significant difference in pre-HSCT and

Table 1
Patient and Transplantation Characteristics

Characteristic	Total Cohort (N = 211)	Groups According to Antibiotic Use During the Neutropenic Period			
		No Antibiotics (N = 43)	Cefepime Only (N = 87)	Carbapenem (N = 81)	*P* Value
Age, yr, median (range)	45 (16-68)	49 (17-68)	42 (16-68)	45 (18-65)	.097
Patient sex, n (%)					.154
Female	84 (39.8)	21 (48.8)	37 (42.5)	26 (32.1)	
Male	127 (60.2)	22 (51.2)	50 (57.5)	55 (67.9)	
Donor sex, n (%)					.092
Female	87 (41.2)	24 (55.8)	32 (36.8)	31 (38.3)	
Male	124 (58.8)	19 (44.2)	55 (63.2)	50 (61.7)	
Sex pair*, n (%)					.161
Female to male	53 (25.1)	15 (34.9)	17 (19.5)	21 (25.9)	
Others	158 (74.9)	28 (65.1)	70 (80.5)	60 (74.1)	
Diagnosis, n (%)					.136
ALL	52 (24.6)	7 (16.3)	26 (29.9)	19 (23.5)	
AML	104 (49.3)	25 (58.1)	43 (49.4)	36 (44.4)	
MDS	37 (17.5)	6 (14.0)	10 (11.5)	21 (25.9)	
Others	18 (8.5)	5 (11.6)	8 (9.2)	5 (6.2)	
ABO match, n (%)					.395
Match	109 (51.7)	19 (44.2)	44 (50.6)	46 (56.8)	
Mismatch	102 (48.3)	24 (55.8)	43 (49.4)	35 (43.2)	
Pre-HSCT disease status, n (%)					.095
Advanced†	67 (31.8)	9 (20.9)	26 (29.9)	32 (39.5)	
Standard	144 (68.2)	34 (79.1)	61 (70.1)	49 (60.5)	
Conditioning intensity, n (%)					.046
MAC	112 (53.1)	16 (37.2)	47 (54.0)	49 (60.5)	
RIC	99 (46.9)	27 (62.8)	40 (46.0)	32 (39.5)	
TBI-containing conditioning regimen, n (%)					.890
Yes	142 (67.3)	28 (65.1)	58 (66.7)	56 (69.1)	
No	69 (32.7)	15 (34.9)	29 (33.3)	25 (30.9)	
ATG-containing conditioning regimen, n (%)					.611
Yes	122 (57.8)	22 (51.2)	52 (59.8)	48 (59.3)	
No	89 (42.2)	21 (48.8)	35 (40.2)	33 (40.7)	
GVHD prophylaxis, n (%)					.099
CS-based	98 (46.4)	24 (55.8)	33 (37.9)	41 (50.6)	
FK506-based	113 (53.6)	19 (44.2)	54 (62.1)	40 (49.4)	
Donor type, n (%)					.188
FMT	42 (19.9)	7 (16.3)	19 (21.8)	16 (19.8)	
Sibling	98 (46.4)	24 (55.8)	32 (36.8)	42 (51.9)	
Unrelated	71 (33.6)	12 (27.9)	36 (41.4)	23 (28.4)	
Graft source, n (%)					.662
BM	33 (15.6)	6 (14.0)	12 (13.8)	15 (18.5)	
PBSCs	178 (84.4)	37 (86.0)	75 (86.2)	66 (81.5)	

AML indicates acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; ATG, antithymocyte globulin; BM, bone marrow; CS, cyclosporine; FMT, HLA-mismatched familial donor; MDS, myelodysplastic syndrome; PBSCs, peripheral blood stem cells; RIC, reduced-intensity conditioning; TBI, total body irradiation

* Sex pair is expressed as donor to recipient.

† Advanced disease status at transplantation includes acute leukemia beyond the first remission, high-risk myelodysplastic syndrome (international prognostic scoring system≥intermediate-2).

post-HSCT microbial diversity in the no intestinal GVHD group, but a marked reduction in diversity pre-HSCT and post-HSCT in the intestinal GVHD group. In addition, principal component analysis showed a dissimilarity of gut flora post-HSCT between the no intestinal GVHD and intestinal GVHD groups (Figure 5B). Decreased abundance of *Ruminococcus* and increased abundance of *Eubacterium* and *Escherichia* were associated with the occurrence of intestinal GVHD (Figure 5C).

Effect of Antibiotics on Immune Cell Recovery

The role of microbiota in immune homeostasis has been explored recently [7]. To further explore the impact of broad-spectrum antibiotic dysbiosis on intestinal GvHD, we analyzed the frequency of circulating immune subsets according to the use of antibiotics. The frequency of $CD3^+$, $CD4^+$, and $CD8^+$ T cells; regulatory T cells; MAIT cells; NK, NKT, and type 1 NKT cells; and MDSCs (E- and M-MDSCs) were analyzed in both pre-HSCT (n =145) and post-HSCT (n = 50) blood samples (Figure 6). Patients who received carbapenem treatment had a lower frequency of $CD4^+$ T cells in pre-HSCT samples compared with patients who did not receive antibiotics. In post-HSCT samples, the use of carbapenem was associated with a higher frequency of $CD8^+$ T cells and lower ratios of E-MDSCs and M-MDSCs to T cells.

Table 2
Transplantation Outcomes

Outcome	Total (N =211)	Groups According to Antibiotic Use During the Neutropenic Period			
		No Antibiotic (N = 43)	Cefepime Only (N = 87)	Carbapenem (N = 81)	*P* Value
Engraftment kinetics					
Days to ANC >.5 × 10^9/L, mean ± SD	12.8 ± 3.3	12.3 ± 3.2	12.5 ± 3.4	13.3 ± 3.1	.153
Days to platelets >20 × 10^9/L, mean ± SD	15.6 ± 13.9	13.5 ± 3.8	14.7 ± 9.0	17.6 ± 20.1	.215
aGVHD grade II-IV, n (%)	95 (45.0)	14 (32.6)	38 (43.7)	43 (53.1)	.087
aGVHD grade III-IV, n (%)	45 (21.3)	4 (9.3)	20 (23.0)	21 (25.9)	.088
Intestinal GVHD, (%)	54 (25.6)	5 (11.6)	23 (26.4)	26 (32.1)	.044
CMV reactivation, (%)	122 (57.8)	21 (48.8)	47 (54.0)	54 (66.7)	.104
Cumulative incidence of relapse, %, mean ± SD					
3 mo	3.8 ± 1.3	4.7 ± 3.3	1.1 ± 1.1	6.2± 2.7	.368
1 yr	15.4 ± 2.6	17.7 ± 6.3	15.8 ± 4.1	13.6 ± 4.1	.944
Overall	24.8 ± 4.6	17.7 ± 6.3	30.0 ± 10.5	24.7 ± 5.9	.983
Cumulative incidence of TRM, %, mean ± SD					
3 mo	3.8 ± 1.3	7.0 ± 3.9	3.4 ± 2.0	2.5 ± 1.7	.282
1 yr	11.5 ± 2.3	9.3 ± 4.5	10.7 ± 3.4	13.5 ± 4.0	.672
Overall	14.4 ± 2.6	9.3 ± 4.5	12.3 ± 3.7	19.3 ± 5.0	.527
Event-free survival, %, mean ± SD					
3 mo	90.5 ± 2.0	83.7 ± 5.6	94.3 ± 2.5	90.1 ± 3.3	.159
1 yr	68.4 ± 3.5	73.0 ± 7.1	69.0 ± 5.2	65.5 ± 5.9	.940
Overall	60.8 ± 4.8	73.0 ± 7.1	57.7 ± 10.0	56.6 ± 6.7	.698
OS, %, mean ± SD					
3 mo	93.4 ± 1.7	88.4 ± 4.9	95.4 ± 2.2	93.8 ± 2.7	.307
1 yr	77.6 ± 3.1	78.4 ± 7.0	77.5 ± 4.7	77.3 ± 5.1	.999
Overall	69.2 ± 4.1	78.4 ± 7.0	66.8 ± 6.5	67.5 ± 7.1	.893

ANC indicates absolute neutrophil count.

DISCUSSION

Here we investigated the effects of different spectrum antibiotics on the intestinal flora of Korean patients who underwent HSCT. We found that the use of antibiotics with broad-spectrum activity, such as carbapenem, during the neutropenic period increased the occurrence of intestinal GVHD in Korean patients undergoing HSCT compared with the use of a narrow-spectrum antibiotic, such as cefepime, or no use of antibiotics during this period. Broad-spectrum antibiotic treatment was not correlated with a higher TRM and worse OS compared with narrow-spectrum antibiotic treatment (cefepime alone) or no antibiotic treatment. Early exposure to narrower- or broader-spectrum antibiotics had no impact on infectious TRM, suggesting that early exposure to antibiotics is not simply a surrogate parameter observed in more vulnerable patients who are at risk of early death from infectious complications. Rather, it is likely that early antibiotic treatment during the neutropenic period induces microbiota disruptions and is itself a risk factor for intestinal GVHD, the diagnosis and treatment of which can be particularly difficult in a clinical setting.

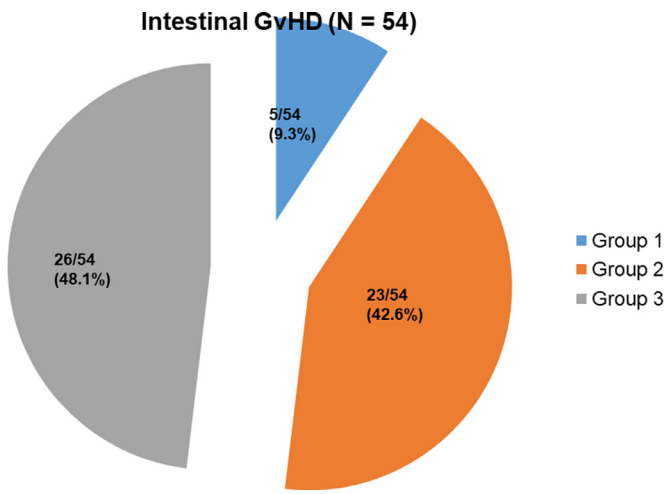

Figure 2. Rate of intestinal GVHD by group. Group 1, patients not treated with any antibiotics during the neutropenic period except for prophylaxis; group 2, patients treated with cefepime only; group 3, patients treated with carbapenem antibiotics, defined as meropenem or prepenem with/without previous cefepime therapy.

Table 3
Risk Factors for the Occurrence of Intestinal GVHD

Factor	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Age (years), continuous	.97 (.95-1.00)	.034	.98 (.95-1.00)	.070
Sex of patient (male versus female)	1.05 (.56-2.00)	.873	-	-
Sex of donor (male versus female)	1.41 (.75-2.71)	.296	-	-
Sex pair (others versus female to male)	1.24 (.61-2.66)	.570	-	-
Diagnosis (AML versus ALL)	.71 (.34-1.51)	.369	-	-
(MDS versus ALL)	.72 (.27-1.85)	.506	-	-
(others versus ALL)	.64 (.16-2.13)	.491	-	-
ABO match (mismatch versus match)	1.09 (.59-2.03)	.777	-	-
Pre-HSCT disease status (standard versus advanced)	.91 (.47-1.78)	.773	-	-
Conditioning intensity (RIC versus MAC)	.71 (.38-1.33)	.293	-	-
TBI-containing (yes versus no)	1.54 (.79-3.16)	.221	-	-
ATG-containing (no versus yes)	.61 (.31-1.14)	.129	-	-
GVHD prophylaxis (FK506-based versus CS-based)	1.87 (.99-3.59)	.056	1.83 (.95-3.55)	.072
Donor type (sibling versus FMT)	.64 (.28-1.50)	.294	-	-
(unrelated versus FMT)	1.12 (.49-2.65)	.787	-	-
Graft source (PBSCs versus BM)	1.33 (.57-3.51)	.531	-	-
Antibiotic use groups (cefepime only versus no antibiotics)	2.73 (.96-7.78)	.060	2.21 (.76-6.43)	.146
(carbapenem versus no antibiotics)	3.59 (1.27-10.19)	.016	3.25 (1.13-9.34)	.029

Infections in neutropenic patients can occur with minimal symptoms and progress rapidly, leading to hypotension and/or other life-threatening complications. Early recognition of neutropenic fever and prompt initiation of empiric systemic antibacterial therapy are critical to avoid progression to sepsis syndrome and possibly death [14]. Here we demonstrate that

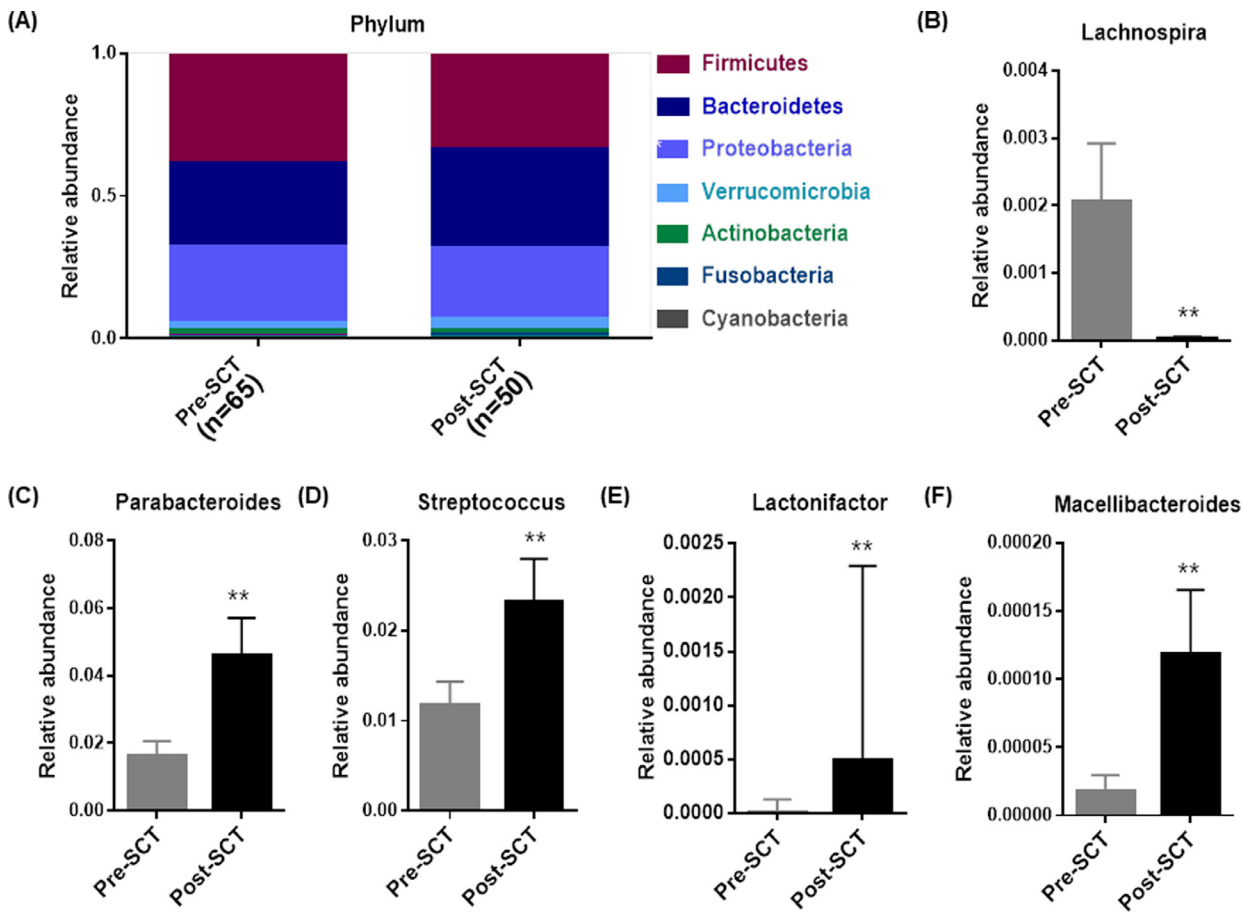

Figure 3. Alterations in gut microbiota after HSCT. (A) Different bacterial composition at the phylum level in the pre-HSCT (n = 65) and post-HSCT (n = 50) groups. (B-F) Shifts in bacterial composition after HSCT at the genus level. Statistical significance is based on a *t* test comparison of pre-HSCT and post-HSCT group values. $^{*}P < .1$; $^{**}P < .05$.

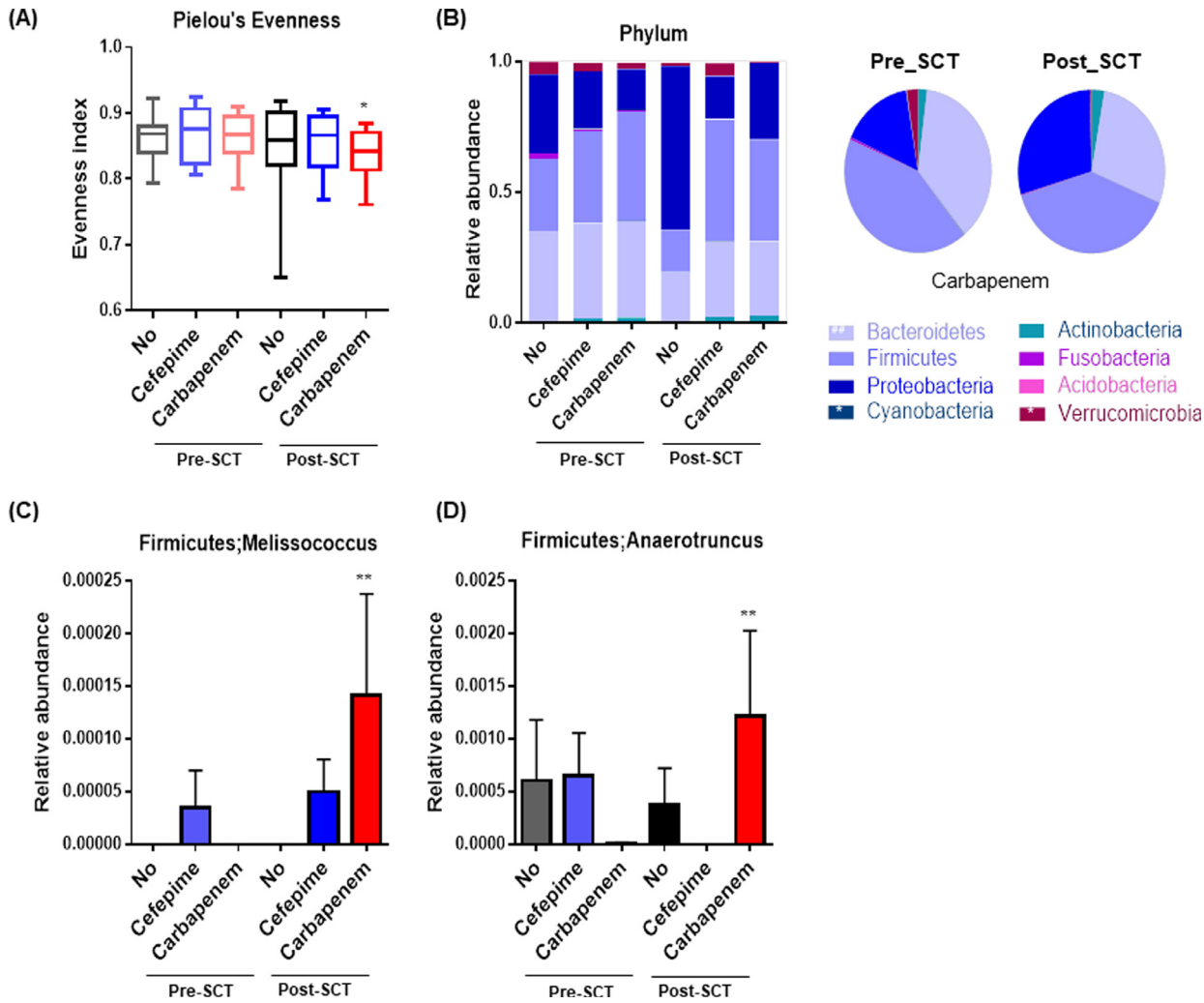

Figure 4. Impact of antibiotic administration on gut microbiota after HSCT. (A) Diversity of commensal bacterial flora was assessed using Pielou's evenness index. (B) Differences in bacterial composition were observed at the phylum level in patients treated with carbapenem, patients treated with cefepime only, and patients not treated with antibiotics. (C and D) Altered intestinal bacteria were classified at the genus level in patients treated with carbapenem. Statistical significance is based on a *t* test of the pre-HSCT and post-HSCT samples in each antibiotic group and on analysis of variance among the antibiotic groups within pre-HSCT and post-HSCT samples. The number of tested samples was 15 for the no-antibiotic group, 22 for the cefepime-only group, and 28 for the carbapenem group pre-HSCT and 14, 25, and 11, respectively, post-SCT. $^{*}P < .1$; $^{**}P < .05$.

different antibiotics used to treat neutropenic fever have different effects on intestinal microbiota composition. Broad-spectrum antibiotic use resulted in a loss of microbiome diversity, which was significantly associated with the occurrence of intestinal GVHD, suggesting that selecting antibiotics with a more limited spectrum of activity (especially against anaerobes) could attenuate intestinal GVHD, presumably due to less severe microbiota changes.

Other important reasons for maintaining anaerobes in HSCT are immune reconstitution and antitumor activity. Given that the gut microbiota are considered major regulators of immune homeostasis [4], microbiota injury may result in impaired immune reconstitution after HSCT. In this study, we explored the effects of antibiotic treatment on immune recovery in HSCT recipients. Reduced recovery of E- and M-MDSCs were observed after the administration of broad-spectrum antibiotics. Considering that indoleamine 2,3-dioxygenase expressed by MDSCs has been implicated in suppression after HSCT [28], we analyzed urine levels of L-kynurenine, the tryptophan metabolite of indoleamine 2,3-dioxygenase, and found a positive correlation between L-kynurenine and MDSCs (Supplementary Figure 2). This result supports the impact of broad-spectrum antibiotic use on increased intestinal GVHD. Similarly, defects in hematopoiesis have been reported in germ-free mice; specifically, the intestinal microbiota promotes steady-state myeloid cell development and increases the potential of myeloid stem cell differentiation to granulocyte and/or monocyte progenitors [29]. Recently, Sheih et al [30] investigated the effect of the intestinal microbiota on reconstitution of MAIT cells after HSCT and found that a deficiency in the Lachnospiraceae family was associated with poor MAIT cell recovery and a possibly increased risk of aGVHD. We also observed a trend toward a lower frequency of MAIT in the broad-spectrum antibiotic treatment group.

During HSCT, the gastrointestinal mucosa is damaged, and colonizing bacteria are impacted, leading to an impaired intestinal microbiota with reduced diversity. The association between an MAC regimen and broad-spectrum antibiotics use in our

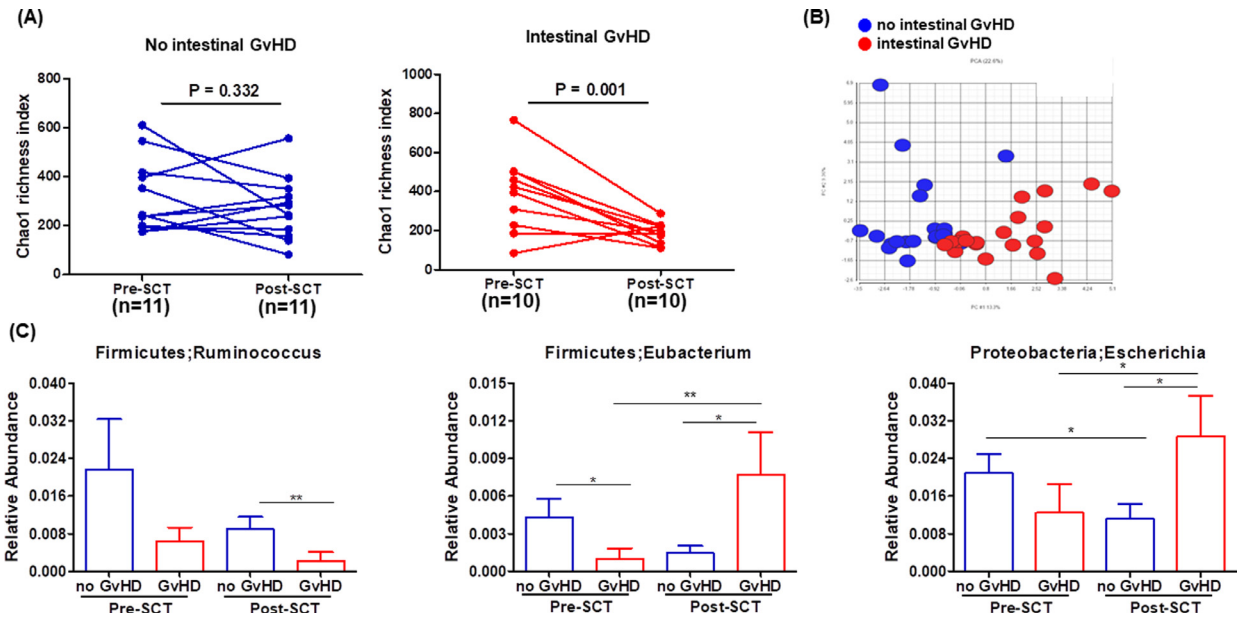

Figure 5. Differences in gut microbiota between patients without intestinal GVHD and those with intestinal GVHD. (A) The Chao1 index showed different patterns of change in patients with intestinal GVHD (n = 10) and patients without intestinal GVHD (n = 11) after HSCT. (B) Principal component analysis showing dissimilarity of the gut flora between patients with and without intestinal GVHD after HSCT. (C) Differentially enriched bacteria were classified at the genus level in patients who developed intestinal GVHD. Statistical significance is based on a paired *t* test between pre-HSCT and post-HSCT samples and on a *t* test between no GVHD and GVHD groups. $^{*}P < .1$; $^{**}P < .05$.

study, possibly due to increased mucosal injury from MAC, leading to secondary infections. As shown by the comparison of 16S rRNA gene sequences in patients before and after HSCT, loss of microbiome diversity was observed. Notably, patients not treated with antibiotics and those treated with narrow-spectrum antibiotics did not have a significant loss of microbiome diversity pre-HSCT and post-HSCT, whereas major changes in microbiome diversity occurred during the neutropenic period in patients treated with broad-spectrum antibiotics, such as carbapenems. In particular, clinical use of broad-spectrum antibiotics strongly suppressed commensal flora. Antibiotics have previously been reported to have a deleterious effect on GVHD occurrence [26,31] and GVHD-related mortality [32].

Although the question of whether dysbiosis is sufficient to cause GVHD remains to be addressed, previous studies have shown that loss of bacterial diversity affected GVHD. Holler et al [5] found a shift to enterococci and a corresponding drop in Firmicutes and other commensal phyla in patients who subsequently developed intestinal GVHD and at the time of active GVHD. Another study from Taur et al [10] showed increased GVHD-related mortality according to microbial diversity group. Regarding the role of specific bacteria in the development of GVHD, an increased abundance of Lactobacillales and a reduced abundance of Clostridiales during GVHD was observed in stool specimens of patients with GVHD [9], whereas an increased abundance of *Blautia* was significantly associated with less GVHD-related mortality and improved OS [33]. Gorshein et al [34] found higher abundances of the genera *Blautia* and *Ruminococcus* in the family Lachnospiraceae in patients with GVHD compared with patients without GVHD. Furthermore, Weber el al [6] found that early exposure to antibiotics was associated with decrease in the fecal abundance of Clostridiales and with increased TRM, compared with the group with no antibiotics or late exposure to antibiotics. Consistent with their findings, we observed a significant reduction in relative abundance of Clostridiales in patients treated with antibiotics during the neutropenic period after HSCT, but no significant changes in the control group (Supplementary Figure 3). In addition, the abundance of *Ruminococcus*, order Clostridiales, was reduced in patients who subsequently developed intestinal GVHD. Meanwhile, *Eubacterium* and *Escherichia* were associated with the occurrence of intestinal GVHD (Table 4).

Furthermore, we demonstrated associations among antibiotic treatment, altered gut microbiota, and clinical occurrence of intestinal GVHD. Our results indicate that exposure to broad-spectrum antibiotics should be avoided, and that antibiotic use should be restricted in low-risk patients who show a good therapeutic response. Strategies to overcome the impact of antibiotics based on probiotics applied in parallel to broader-spectrum antibiotics should be developed. Alternatively, rapid restoration of microbial diversity early after cessation of antibiotic treatment by either fecal microbiota transplantation or artificially composed microbiota may be a feasible treatment option [6]. However, we acknowledge that because changes in microbiota are really rapid, the variance in the timing of stool collection and measurement at only a single point are inherent flaws of our study. As such, validation in a large independent cohort is needed.

In summary, our results provide insight into the risks and benefits of specific antibiotic regimens during the neutropenic period in HSCT recipients. Our study shows that microbiome diversity after HSCT is conserved in patients given no antibiotics and is adversely affected in patients given broad-spectrum antibiotics. Regulation of the intestinal microbiota may reduce intestinal inflammation and alleviate the occurrence of intestinal GVHD. To change current standard care routines for HSCT, randomized, prospective clinical studies involving multiple centers are indispensable.

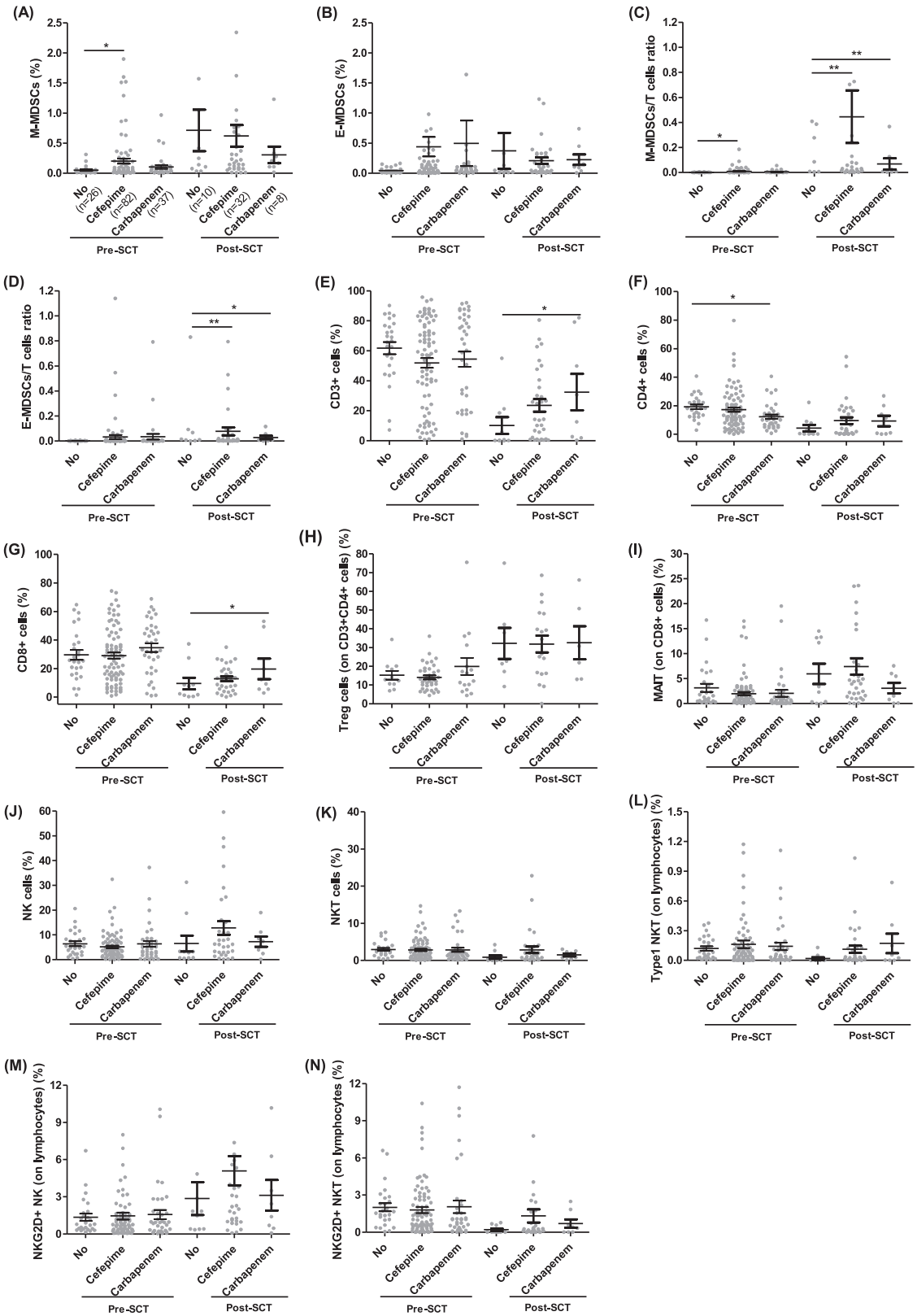

Figure 6. Immune cell populations according to antibiotic use. The frequency of immune cell populations in peripheral blood was determined for patients in each antibiotic group. The frequency of each cell population pre-HSCT (n = 145) and post-HSCT (n = 50) was analyzed. $CD3^+$, $CD4^+$, and $CD8^+$ T cells; regulatory T cells; natural killer (NK), NKT, and type 1 NKT cells; and myeloid-derived suppressor cells (MDSCs; including monocytic and early-stage MDSCs) were analyzed by flow cytometry in both pre-HSCT and post-HSCT blood samples. Data are presented as median values. Statistical significance is based on a *t* test comparing pre-HSCT and post-HSCT samples in each antibiotic use group, and on analysis of variance among the groups within pre-HSCT and post-HSCT samples. $^*P < .1$; $^{**}P < .05$.

Table 4
Reported Human Studies on Bacterial Taxa Related to GVHD and/or GVHD-Related Mortality

Study	Taxonomic Rank*	Decreased GVHD and/or GVHD-Related Mortality	Increased GVHD and/or GVHD-Related Mortality
Jenq et al, 2015 [33]	6	*Blautia*	
	5	Lachnospiraceae	
	4	Clostridiales	
	3	Clostridia	
Jenq et al, 2012 [9]	4	Clostridiales	Lactobacillales
Holler et al, 2014 [5]	2		Enterococcus (especially *E. faecium*)†
Golob et al, 2017 [11]	6	*Erysipelatoclostridium ramosum, Bacteroides thetaiotaomicron, Blautia luti, Candidatus soleaferrea, Butyricicoccus* spp, *Blautia* spp, *Dorea* spp, *Ruminococcus* spp, *Clostridium clostridioforme, Bacteroides ovatus, Bacteroides caccae*	*Roseburia hominis, Eisenbergiella* spp, *Veillonella parvula, Enterococcus faecium* or *hirae*-like, *Lactobacillus salivarius, Lactobacillus fermentum, Bacteroides dorei, Solobacterium moorei, Rothia mucilaginosa*
Weber et al, 2017 [6]	4	Clostridiales	
Gorshein et al, 2017 [34]	6	*Blautia, Ruminococcus*	
Present study	6	*Ruminococcus*	*Eubacterium, Escherichia*

* 1, kingdom; 2, phylum; 3, class; 4, order; 5, family; 6, genus.
† With a complementary decrease in other Firmicutes.

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SUPPLEMENTARY MATERIALS

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2019.06.001.

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