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Intestinal Microbiota Can Predict Acute Graft-versus-Host Disease Following Allogeneic Hematopoietic Stem Cell Transplantation

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A B S T R A C T

The intestinal microbiome plays an important role in the development of acute graft-versus-host disease (aGVHD). However, whether intestinal microbiota can predict the development of aGVHD has been reported only rarely. Here we conducted a prospective study of microbiota in 141 patients after allogeneic hematopoietic stem cell transplantation. We found lower microbiota diversity in the aGVHD group compared with the non-aGVHD group at day 0 and day 15 ± 1 (P = .018 and .009, respectively). Diversity was negatively associated with conditioning intensity (P = .017, day 0; P = .045, day 15) and β-lactam antibiotic administration (P = .004, day 15). Intensified conditioning and β-lactam antibiotics were associated with a lower regulatory T (Treg)/T helper 17 (Th17) cell ratio at day 15 (P = .030 and .047, respectively). At day 15, the levels of the inflammatory factors (tumor necrosis factor α, interleukin [IL]-6, IL-17A, IL-1β, and lipopolysaccharide) were higher in the intensified conditioning group compared with the standard group (P < .05). The accumulated intestinal microbiota (AIM) score was defined as microbiota diversity and gradient of the 4 bacterials (Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, and Enterobacteriaceae) at day 15 post-transplantation. The AIM score was positively correlated with aGVHD grade (r = .481, P < .001), and the AIM score could be predictive of the development of aGVHD (grade II-IV aGVHD: area under the curve [AUC], .75, P < .001; grade III-IV aGVHD: AUC, .84, P < .001). These findings suggest that intestinal microbiota and conditioning might induce aGVHD by inflammatory factors and the Treg/Th17 balance. The constitution of the intestinal microbiota at neutrophil engraftment may predict the development of aGVHD.

INTRODUCTION

Acute graft-versus-host-disease (aGVHD) remains one of the major causes of mortality for patients who undergo allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1,2]. The frequency of aGVHD ranges from 50% to 70%, depending on HLA compatibility, donor and recipient characteristics, conditioning regimen, and strategy for aGVHD prophylaxis [2–6]. Once aGVHD appears, serious complications, such as infections and organ dysfunction, may follow, increasing the risk of mortality in allo-HSCT recipients [1,7,8].

An increasing number of studies have demonstrated that the intestinal microbiome plays an important role in the development of aGVHD, and that the loss of microbiota diversity is associated with aGVHD [9–12]. However, the relevant microbiota at the family or species level found to be related to aGVHD is not consistent across transplantation centers [12–14]. This may be because the intestinal microbiota can be affected by numerous factors, including antibiotic use, conditioning, diet, geographical environment, and race [12–16]. Recently, the use of the intestinal microbiota as a

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biomarker for predicting aGVHD has been discussed [17]. A new report from Seattle indicated that the intestinal microbiota at neutrophil engraftment was predictive of severe aGVHD after allo-HSCT [17].

In this study, we prospectively collected stool and blood samples from patients undergoing allo-HSCT at preconditioning, day 0, and day 15 ± 1 (ie, neutrophil engraftment) post-transplantation. The microbiota in the stool and inflammatory factor levels in the blood were detected by 16S rRNA gene sequencing and ProcartaPlex multiplex immunoassays, respectively. aGVHD was determined by a retrospective review of the clinical charts. Our results demonstrate that the intestinal microbiota is affected by conditioning and β-lactam antibiotics, and that the combination of diversity and the relative abundance of several specific organisms at neutrophil engraftment could serve as a predictive marker for the occurrence and grade of aGVHD.

METHODS

This study was approved by Nanfang Hospital's Medical Ethics Committee. All patients provided written informed consent for biospecimen collection and analysis. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki.

Samples

Stool and blood samples were collected from patients who underwent allo-HSCT at preconditioning, day 0, and post-transplantation day 15. The stool and plasma samples were tagged and stored at -80°C until retrieval for DNA extraction and cytokine detection, respectively [18]. T lymphocyte subsets in blood were directly examined by flow cytometry [18]. Any samples stored for >6 hours between collection and disposition were discarded.

Conditioning and GVHD Prophylaxis

Three different conditioning regimens were used for these patients, including 2 standard myeloablative regimens (BuCy [busulfan + cyclophosphamide] and TBI + Cy [total body irradiation] + Cy) and a sequential intensified regimen (fludarabine + Ara-C plus TBI + Cy + etoposide) [18–20]. The selection of conditioning regimens was based on disease type and status at transplantation. Generally, patients with acute myelogenous leukemia in complete remission (CR) received BuCY, and those with acute lymphoblastic leukemia in CR received TBI + CY, whereas those in non-CR received the intensified regimen. In addition, some high-risk patients also received the intensified regimen [18–20].

Cyclosporin A (CsA) and methotrexate (MTX) (on days +1, +3, and +6) were administered to patients who received a matched sibling donor transplant for GVHD prophylaxis. CsA + MTX + ATG (Thymoglobulin; Genzyme, Cambridge, MA) (total ATG dose, 7.5 mg/kg on days -3 to -1) were administered to patients who received a matched unrelated donor transplant. CsA + MTX + ATG + mycophenolate (MMF) (total ATG dose, 7.5-10 mg/kg on days -4 or -3 to -1) were administered to patients who received a haploidentical donor transplant for GVHD [18,19].

Table 1
Predictive Microbiota Scores for aGVHD

Scoring System (Relative Abundance, %)	Score
Diversity (A)	
≥2	0
<2	1
Lachnospiraceae (B)	
≥1.275	0
<1.275	1
Peptostreptococcaceae (C)	
≥.049	0
<.049	1
Erysipelotrichaceae (D)	
≥.092	0
<.092	1
Enterobacteriaceae (E)	
≥26.616	1
<26.616	0
AIM score*	
High	4-5
Low	0-3

* AIM score: A + B + C + D + E.

Infection Prophylaxis and Treatment

At our institution, oral sulfamethoxazole and norfloxacin were administered to all patients for infection prophylaxis before transplantation [19,20]. Sulfamethoxazole was administered for 1 week and discontinued before conditioning. Ganciclovir was given for 2 weeks before transplantation for the prophylaxis and treatment of cytomegalovirus (CMV) infection, and acyclovir was administered for other viruses. Antifungal agents were used for fungal infection prophylaxis. Fluconazole (.3 g/day) or itraconazole (.4 g/kg/day) was administered for up to 60 days post-transplantation to patients with no history of invasive fungal infection (IFI), identified according to revised definitions presented by the European Organization for Research and Treatment of Cancer/Mycoses Study Group [21], and those with a history of IFI received voriconazole (.4 g/day), itraconazole (.4 g/day), caspofungin (50 mg/day), or ambisome (2 mg/kg/day) intravenously. Oral voriconazole or itraconazole was substituted for the intravenous treatment when the peripheral white blood cell count was $>2.0 \times 10^9$/L and was discontinued after 90 days post-transplantation.

At our institution, β-lactam antibiotics include carbapenems (imipenem and meropenem), cephalosporin (cefoperazone/sulbactam), and piperacillin/tazobactam (Table 2). The median time of β-lactam antibiotic exposure was 7 days (range, 3 to 13 days) from conditioning to day 15 post-transplantation. Generally, patients were given imipenem alone or combined with amikacin as a first-line antibiotic for fever during neutropenia. Vancomycin or piperacillin/tazobactam were used as second-line antibiotics.

16S rRNA Gene Sequencing for Fecal Specimens

For each fecal specimen, DNA was extracted and purified, and the V3-V4 region of the 16S rRNA gene was PCR-amplified using modified universal bacterial primers [18]. Microbiome DNA concentrations were measured with a qPCR assay targeting the V3-V4 region of the 16S rRNA gene. Purified PCR products were sequenced with the Illumina HiSeq2500 PE250 platform [18]. Sequence data were compiled and processed using mothur version 1.31.2. Sequence data were screened and filtered for quality and then aligned to the full-length 16S rRNA gene, using the SILVA reference alignment as taxonomic units of 97% similarity [18].

Inflammatory Factors

TNF-α, IL-6, IL-17A, and IL-1β in plasma were detected with the ProcartaPlex Multiplex Immunoassay Kit (eBioscience, San Diego, CA) according to the manufacturer's protocol. Plates were read with the Luminex 200 system (Luminex, Austin, TX) and analyzed using ProcartaPlex software (eBioscience). The levels of lipopolysaccharide (LPS) in plasma were detected by the dynamic turbidimetric method [22]. Samples were measured with the EKT-5M LPS Assay Kit (JinshanChuan, China) with the MB-80 dynamic detection system, and the LPS content was automatically calculated.

T Lymphocyte Subsets

T lymphocyte subsets were detected as described previously [18]. Th17 cells were examined using the BD Intracellular Staining Kit (BD Pharmingen, San Diego, CA). Cells were incubated for 5 hours with phorbol-12-myristate-13-acetate (50 ng/mL) plus ionomycin (2.5 μg/mL; all reagents from Sigma-Aldrich, St Louis, MO) to stimulate IL-17A production, and the samples were supplemented with Golgistop (.7 μL/mL) during the last 4 hours to trap

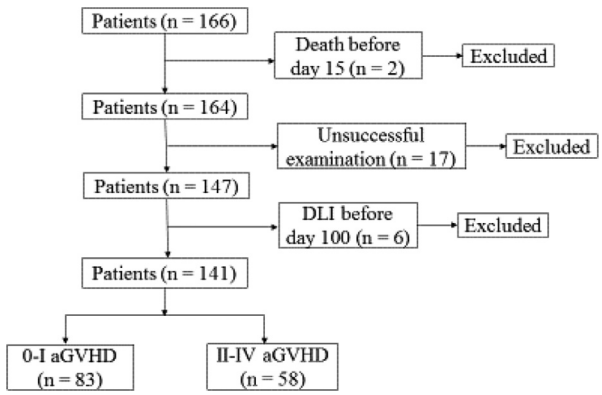

Figure 1. Diagram of patient groups enrolled in this study.

Table 2
Patient and Transplantation Characteristics

Variable	Grade 0-I aGVHD (N = 83)	Grade II-IV aGVHD (N = 58)	*P* Value
Age, yr, median (range)	33 (16-54)	33 (17-56)	.970
Age ≥33 yr, n (%)	45 (54.2)	32 (55.2)	.911
Female sex, n (%)	33 (39.8)	21 (36.2)	.669
Female donor, n (%)	24 (28.9)	12 (20.7)	.270
Underlying disease, n (%)			
AML	50 (60.2)	35 (60.3)	.995
ALL	29 (34.9)	20 (34.5)	
MDS	4 (5.1)	3 (5.2)	
Conditioning, n (%)			
Standard	53 (63.9)	26 (44.8)	.025
Intensified	30 (36.1)	32 (55.2)	
Disease status at transplantation, n (%)			
CR	61 (74.4)	42 (72.4)	.794
Non-CR	21 (25.6)	16 (27.6)	
Donor type, n (%)			
MSD	40 (48.2)	23 (39.7)	.547
HID	38 (45.8)	32 (55.2)	
MUD	5 (6.0)	3 (5.1)	
HLA-mismatch, n (%)			
0-1	45 (54.5)	27 (46.6)	.440
2-3	15 (18.1)	9 (15.5)	
4-5	23 (27.7)	22 (37.9)	
Graft source, n (%)			
PBSCs	30 (36.1)	21 (36.2)	1.000
BM	53 (63.9)	37 (63.8)	
Antibiotics, n (%)			
β-lactam	42 (50.6)	44 (75.9)	.002
Carbapenem	27 (32.5)	31 (53.4)	.013
Piperacillin/tazobactam	10 (12.0)	8 (13.8)	.760
Cephalosporin	5 (6.0)	5 (8.6)	.554
Vancomycin	28 (33.7)	24 (41.4)	.355
β-lactam + vancomycin	17 (20.5)	21 (36.2)	.038
Amikacin	26 (31.3)	22 (37.9)	.415

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BM, bone marrow; CR, complete remission; HID, haploidentical donor; MDS, myelodysplastic syndrome, MSD, matched sibling donor; MUD, matched unrelated donor; PBSCs, peripheral blood stem cells.

proteins in the cytoplasm. The proportions of T lymphocyte subsets (Tregs: $CD45^+CD3^+CD4^+CD25^+Foxp3^+$; Th17 cells: $CD45^+CD3^+CD4^+CD8\text{-}IL\text{-}17A^+$) in peripheral blood were analyzed by flow cytometry [18].

Clinical Metadata

All clinical data, including aGVHD, neutrophil engraftment, and prophylactic or therapeutic antibiotics were collected by a retrospective review of clinical charts by individuals blinded to the microbiota results of the participants. aGVHD was defined according to the 1994 Consensus Conference on aGVHD Grading and graded from I to IV [23]. Neutrophil engraftment was defined as the third day od an absolute neutrophil count $>.5 \times 10^9$/L post-transplantation. Based on aGVHD, the study groups were categorized into grade 0-I aGVHD (non-aGVHD) and grade II-IV aGVHD (aGVHD).

Microbial Diversity and Different Microbiota Taxa Analysis

Microbial diversity was estimated by the inverse Simpson Index, an ecological estimate of diversity calculated to represent the reciprocal of the expected probability of randomly selected bacterial sequences belonging to the same operational taxonomic unit [12]. The Mann-Whitney nonparametric test was used to compare the statistical significance of groups. Phylogenetic classification at the family level was analyzed based on a naive Bayesian classification scheme and the Greengenes reference database [24]. The nonparametric factorial Kruskal-Wallis rank-sum test was used to identify different microbiota taxa to detect features that differed significantly between the aGVHD and non-aGVHD groups. Linear discriminant analysis effect size (LEfSe) analysis was performed using LEfSe software. The nonparametric factorial Kruskal-Wallis rank-sum test was used in LEfSe analysis to detect characteristics between the 2 groups. The effect sizes of the identified characteristics were then analyzed using a linear discriminant analysis model [25].

Predicting aGVHD

The area under the receiver operating curve (ROC) from logistic regression analysis was used to present the correlations between aGVHD and microbiota biomarkers. Based on cutoff values for aGVHD, a score of 0 or 1 was given for the inverse Simpson Index and each bacterial abundance group (including Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, and Enterobacteriaceae), with 0 representing a negative association with aGVHD and 1 representing a positive association. The accumulated intestinal microbiota (AIM) score was then generated by summing the values [24,26]. A high score was defined as 4-5; a low score, as 0-3 (Table 1).

Statistical Analysis

The statistical analyses were performed in December 2017. The data are summarized as mean ± SD or median for continuous data. Comparisons of categorical variables were performed using Pearson's χ^2 test or Fisher's exact test when appropriate. Calculations of area under the ROC curve were used to evaluate the predictive performance for aGVHD. The Cox proportional hazards model was used to evaluate the risk for grade II-IV aGVHD in multivariate analysis. Factors associated with grade II-IV aGVHD with $P < .10$ in univariate analysis (grade of AIM score) or factors known to influence aGVHD (ie, patient age, donor sex, number of HLA mismatches, and vancomycin administration) were included in the final model. The median level of each cytokine and LPS at each time point were compared between the aGVHD and non-aGVHD groups using the Mann-Whitney nonparametric test. Treg and Th17 cell counts, and Treg/Th17 cell ratio, were analyzed using the Mann-Whitney nonparametric test. The Benjamini-Hochberg method [27] was applied for multiple comparison correction for different time points. Statistical correlations between the AIM score and aGVHD grade and the Treg/Th17 cell ratio and LPS level

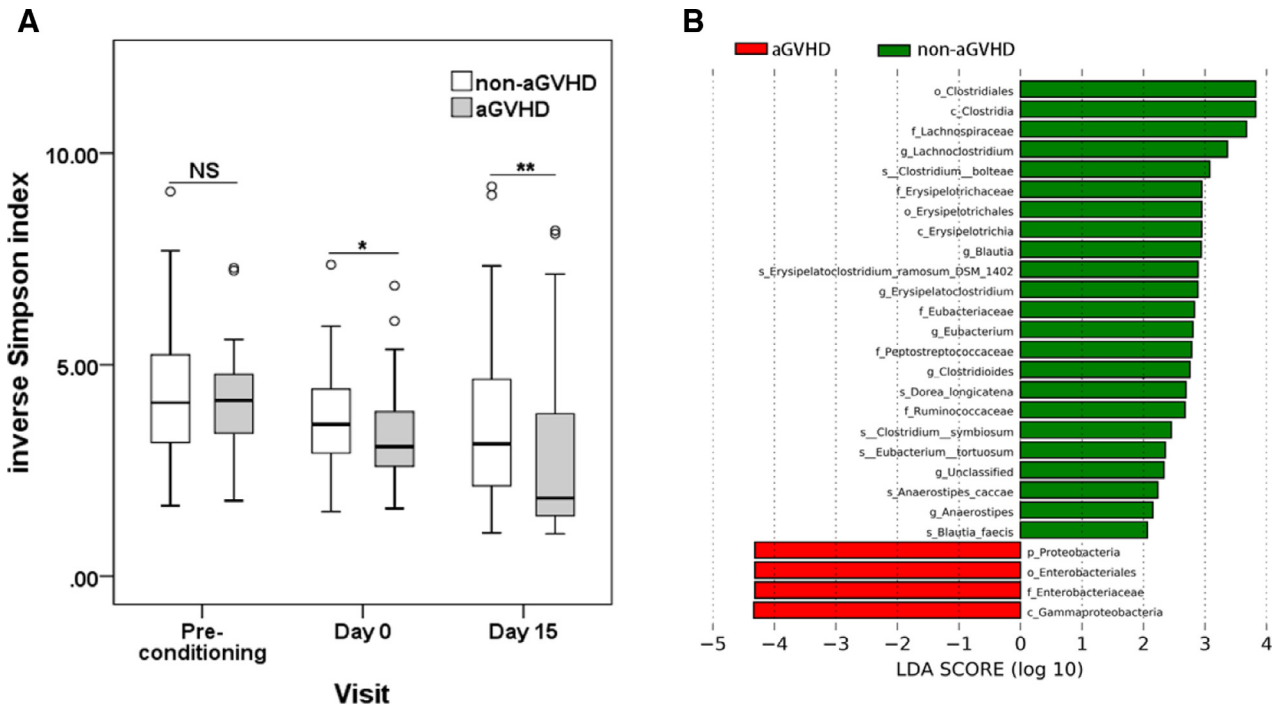

Figure 2. Microbiota diversity at 3 time points and constitution differences at day 15 between the groups. (A) Three time points were detected at preconditioning, day 0, and day 15 post-transplantation. The inverse Simpson index was compared between the groups. $^{*}P < .050$; $^{**}P < .010$. (B) A taxonomic histogram was generated using LEfSe analysis of the groups. All of the listed microbiota groups are significantly different ($P < .05$, Kruskal-Wallis test). p, phylum; c, class; o, order; f, family; g, genus; s, species.

were investigated using Spearman's bivariate correlation analysis. All *P* values were 2-sided, with the significance level fixed at .05. SPSS 19.0 (IBM, Armonk, NY) and R version 3.1.1 (R Institute for Statistical Computing, Vienna, Austria) were used for all data analysis. $P < .05$ was considered to indicate statistical significance.

RESULTS

Patient and Transplantation Characteristics

Between January 2016 and June 2017, the original cohort of 166 patients who underwent allo-HSCT were enrolled in this prospective study, of whom 141 were retained for analysis.

Table 3
Phylogenetic Changes in the aGVHD and Non-aGVHD Groups

	Median Relative Abundance (%)‡				
		Day 0		Day 15	
Bacterial taxon†	Control§ (N = 141)	Non-aGVHD (N = 83)	aGVHD (N = 58)	Non-aGVHD (N = 83)	aGVHD (N = 58)
Firmicutes	26.20	22.15	20.78	17.69	12.54***
Proteobacteria	19.14	20.45	26.13	22.53	63.55***
Proteobacteria. Gammaproteobacteria	17.23	18.76	23.85	21.29	59.56***
Firmicutes. Clostridia	15.96	14.68	12.29	11.61*	.86***
Firmicutes. Clostridia. Clostridiales. Lachnospiraceae	6.14	4.89	3.24**	2.66**	.02***
Proteobacteria. Gammaproteobacteria. Enterobacteriales. Enterobacteriaceae	15.38	16.78	20.56	19.33	57.20***
Firmicutes. Erysipelotrichia. Erysipelotrichales. Erysipelotrichaceae	.15	.17	.11	.22	.01***
Firmicutes. Clostridia. Clostridiales. Peptostreptococcaceae	.16	.15	.10	.13	<.01***
Firmicutes. Clostridia. Clostridiales. Lachnospiraceae. Blautia	2.16	1.78	1.35	.07***	<.01***
Firmicutes. Clostridia. Clostridiales. Lachnospiraceae. Lachnoclostridium	1.54	1.33	1.19	.55*	<.01***
Firmicutes. Erysipelotrichi. Erysipelotrichales. Erysipelotrichaceae. Erysipelatoclostridium	.03	.04	.01	.06	<.01***

$^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$, compared with control.

† For each bacterial taxon, classification at each preceding level is separated by periods.

‡ Median proportion of bacteria belonging to the specified taxon.

§ Patients at preconditioning.

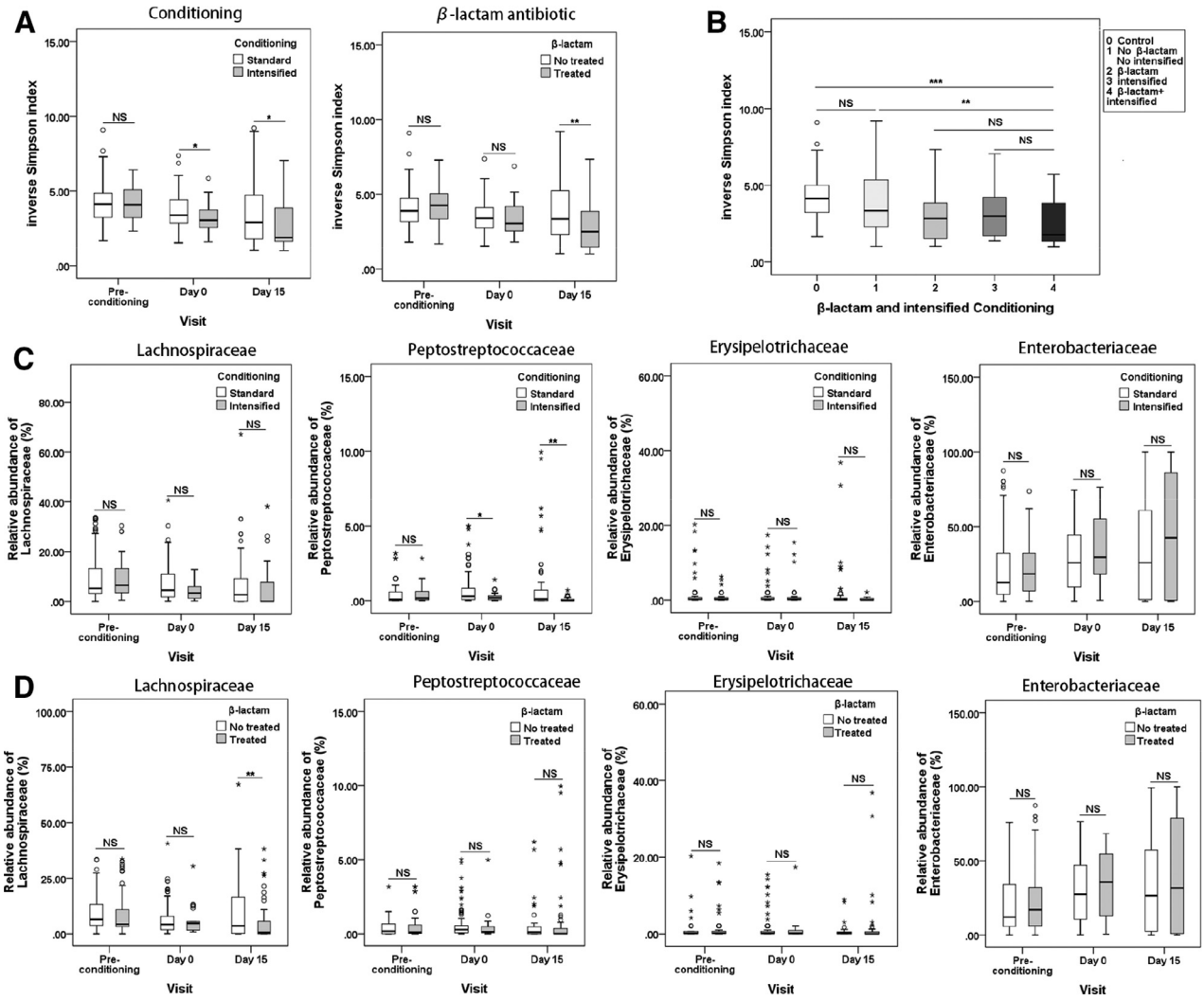

Figure 3. Changes in the microbiota are associated with both conditioning intensity and β-lactam during allo-HSCT. (A) Changes in microbiota diversity are associated with intensified conditioning and β-lactam antibiotic administration. (B) The microbiota diversity at day 15 was influenced by both intensified conditioning and β-lactam antibiotics. Control group, the total group of patients at preconditioning. (C and D) The effects of conditioning intensity (C) and β-lactam antibiotics (D) on Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, and Enterobacteriaceae. $^{*}P < .050$; $^{**}P < .010$; $^{***}P < .001$.

Twenty-five patients were excluded, including 2 patients who died before engraftment, 17 patients without data on fecal specimens, and 6 patients who received donor lymphocyte infusion (DLI) within 100 days post-transplantation (Figure 1). Patient and transplantation characteristics are summarized in Table 2. The cohort included 54 females and 87 males, with a median age of 33 years (range, 16 to 56 years). The primary diseases included acute lymphoblastic leukemia, acute myelogenous leukemia, and myelodysplastic syndrome. A total of 65 patients received an HLA-matched sibling donor transplant, 67 received a haploidentical donor transplant, and 9 received an HLA-matched unrelated donor transplant. Ninety-eight patients were treated with a standard myeloablative regimen, and 43 received an intensified myeloablative regimen. Within 100 days post-transplantation, 58 patients had grade II-IV aGVHD, including 19 patients with grade III-IV aGVHD. The characteristics of the patients with and without aGVHD are summarized in Table 2. There were differences in the conditioning intensity and antibiotics between the 2 groups (55.2% versus 36.1%, 75.9% versus 50.6%, respectively; $P = .025$ and .002, respectively).

aGVHD-Associated Intestinal Microbiota Characteristics at Engraftment

To investigate differences in the intestinal microbiota between the aGVHD and non-aGVHD groups, we analyzed the diversity and richness of the ecosystem in the 2 groups. At preconditioning, the results demonstrated no significant between-group difference in α diversity of the microbiota ($P = .674$; Figure 2A) or in the constitution of the microbiota. At day 0, the diversity was lower in the aGVHD group compared with the non-aGVHD group ($P = .018$; Figure 2A), and the Lachnospiraceae family of Clostridiales was decreasing in the aGVHD group ($P < .01$; Table 2). At day 15, the diversity was lower in the aGVHD group (median, 1.83; range, 1.00 to 8.17) compared with the non-aGVHD group (median, 3.21; range, 1.02 to 9.21; $P = .005$) (Figure 2A).

To further determine whether the absence or presence of a specific bacterial taxa was associated with aGVHD, we compared the microbiota constitution of the patients at engraftment in the 2 groups. The constitution of the intestinal microbiota differed between the aGVHD and non-aGVHD groups (Table 3 and

Table 4
Multivariate Analysis of aGVHD

Factor	Grade II-IV aGVHD	
	Univariate, *P* Value (HR)	Multivariate, *P* Value (HR; 95% CI)
Patient age	.736 (1.094)	.637 (1.157; .632-2.120)
<33 yr		
≥33 yr		
Donor sex		
Female	.308 (1.392)	.177 (.619, .309-1.241)
Male		
HLA mismatch (%)		
0-1	.153 (1.232)	.703 (1.077, .735-1.579)
2-3		
4-5		
Graft source		
BM + PBSC	.557 (1.181)	.452 (1.323, .638-2.742)
PBSC		
Conditioning		
Intensified	.002 (2.311)	.037 (1.818, 1.038-3.186)
Standard		
β-lactam	.003 (2.523)	.011 (2.280, 1.210-4.297)
Vancomycin (i.v.)	.325 (1.300)	.742 (1.101, .622-1.949)
AIM score*		
High (4-5)	< .001 (3.373)	< .001 (2.709, 1.559-4.708)
Low (0-3)		

HR, hazard ratio.
* AIM score according to Table 1.

Figure 2B). At the phylum level, the microbiota community in the aGVHD group had a greater abundance of Proteobacteria and less abundance of Firmicutes compared with the non-aGVHD group ($P < .001$ and $P = .019$, respectively). At the class level, the aGVHD group had a greater abundance of Gammaproteobacteria and less abundance of Clostridia compared with the non-aGVHD group ($P < .001$ for both). At the family level, there was a greater abundance of Enterobacteriaceae and less abundance of Lachnospiraceae, Peptostreptococcaceae, and Erysipelotrichaceae in the aGVHD group compared with the non-aGVHD group ($P < .001$ for all). At the genus level, the abundances of *Blautia, Lachnoclostridium, Erysipelatoclostridium*, and *Eubacterium* were lower in the aGVHD group compared with the non-aGVHD group ($P < .001$ for all).

Patient and Transplantation Characteristics with Intestinal Microbiota

To explore latent factors influencing the intestinal microbiota, we investigated variables including conditioning and antibiotics. The results indicated that the conditioning regimen had an effect on the intestinal microbiota diversity at days 0 and 15 (intensified versus standard: median, 3.39 versus 3.04 [$P = .017$] at day 0 and 3.17 versus 1.89 [$P = .045$] at day 15; Figure 3A). A total of 96 patients received antibiotics for treatment of infections before neutrophil engraftment, including 24 patients before day 0. The diversity at day 0 was not associated with the use of antibiotics, including β-lactam antibiotics and vancomycin ($P = .407$ and .284, respectively; Figure 3A). However, an effect of antibiotic use on diversity was seen at day 15 (β-lactam versus no β-lactam: median, 3.33 versus 2.48 [$P = .004$]; vancomycin versus no vancomycin [$P = .004$]) (Supplementary Figure S1). Furthermore, intensified conditioning plus β-lactam antibiotics had an additive effect on diversity at day 15 compared with preconditioning ($P < .001$; Figure 3B). In addition, other variables, including patient age and sex, disease status at transplantation, donor type, and use of ATG for GVHD prophylaxis, were not significantly associated with the intestinal microbiota ($P = .093$, .555, .549, .548, and .273, respectively).

To determine which specific bacterial taxa was influenced by conditioning (intensified conditioning versus standard conditioning) and antibiotics (treated versus not treated), we compared the microbiota composition of the patients in the 2 groups at the 3 points (Figure 3C and D and Supplementary Figure S2). At the family level for preconditioning, the microbiota composition, including Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, and Enterobacteriaceae, was not different between intensified and standard conditioning ($P > .05$; Figure 3C) and antibiotic (β-lactam, vancomycin) treatment and no treatment ($P > .05$, Figure 3D and Supplementary Figure S2). Whereas the abundance of Peptostreptococcaceae was influenced by intensified conditioning according to abundance at days 0 and 15 ($P = .042$ and .003, respectively; Figure 3C), Lachnospiraceae, Erysipelotrichaceae, and Enterobacteriaceae were not associated with conditioning intensity ($P > .05$; Figure 3C). In addition, Lachnospiraceae were also injured by β-lactam antibiotics and vancomycin at day 15 ($P = .006$ and .030, respectively; Figure 3D and Supplementary Figure S2). The abundances of Peptostreptococcaceae and Erysipelotrichaceae were not associated with the use of β-lactam antibiotics and vancomycin on day 15 ($P > .05$; Figure 3D and Supplementary Figure S2). Enterobacteriaceae expansion was associated with vancomycin administration ($P = .009$; Supplementary Figure S2).

Risk Factors for aGVHD

According to the Cox regression model for multivariate analysis of grade II-IV aGVHD in Table 4, high AIM score, intensified conditioning, and β-lactam antibiotics were identified as independent risk factors for grade II-IV aGVHD ($P < .001$, $P = .037$, and $P = .011$, respectively; hazard ratio, 2.709 [95% confidence interval (CI), 1.559 to 4.708], 1.818 [95% CI, 1.038 to 3.186], and 2.280 [95% CI, 1.210 to 4.297], respectively). Patient age, donor sex, number of HLA mismatches, and i.v. vancomycin administration were not identified as independent risk factors for grade II-IV aGVHD ($P > .05$).

Conditioning and Antibiotics Associated with Treg/Th17 Cell Balance

To investigate why conditioning and β-lactam antibiotics influenced aGVHD, we studied the frequencies and absolute numbers of Tregs and Th17 in peripheral blood at preconditioning and day 15 after transplantation. Tregs and Th17 were quantified with the following gating strategy (Figure 4A and B). At day 15, the aGVHD group harbored a fewer amount of Treg cells (proportion: 2.49% versus 3.27%, $P < .001$; absolute numbers: 5.19 versus 7.02×10^6/L, $P = .006$) and a greater proportion of Th17 cells compared with the non-aGVHD group (proportion: 3.50% versus 2.65%, $P < .001$; absolute numbers: 5.16 versus 5.65×10^6/L, $P = .056$), although there was no between-group difference at preconditioning (Figure 4C). The results also showed that intensified conditioning and β-lactam antibiotics were associated with a lower Treg/Th17 cell ratio at day 15 ($P = .030$ and .047, respectively; Figure 4D).

To further explore the associations among conditioning, β-lactam antibiotics, and Treg/Th17 cell ratio, the levels of TNF-α, IL-6, IL-17A, IL-1β, and LPS in blood were measured

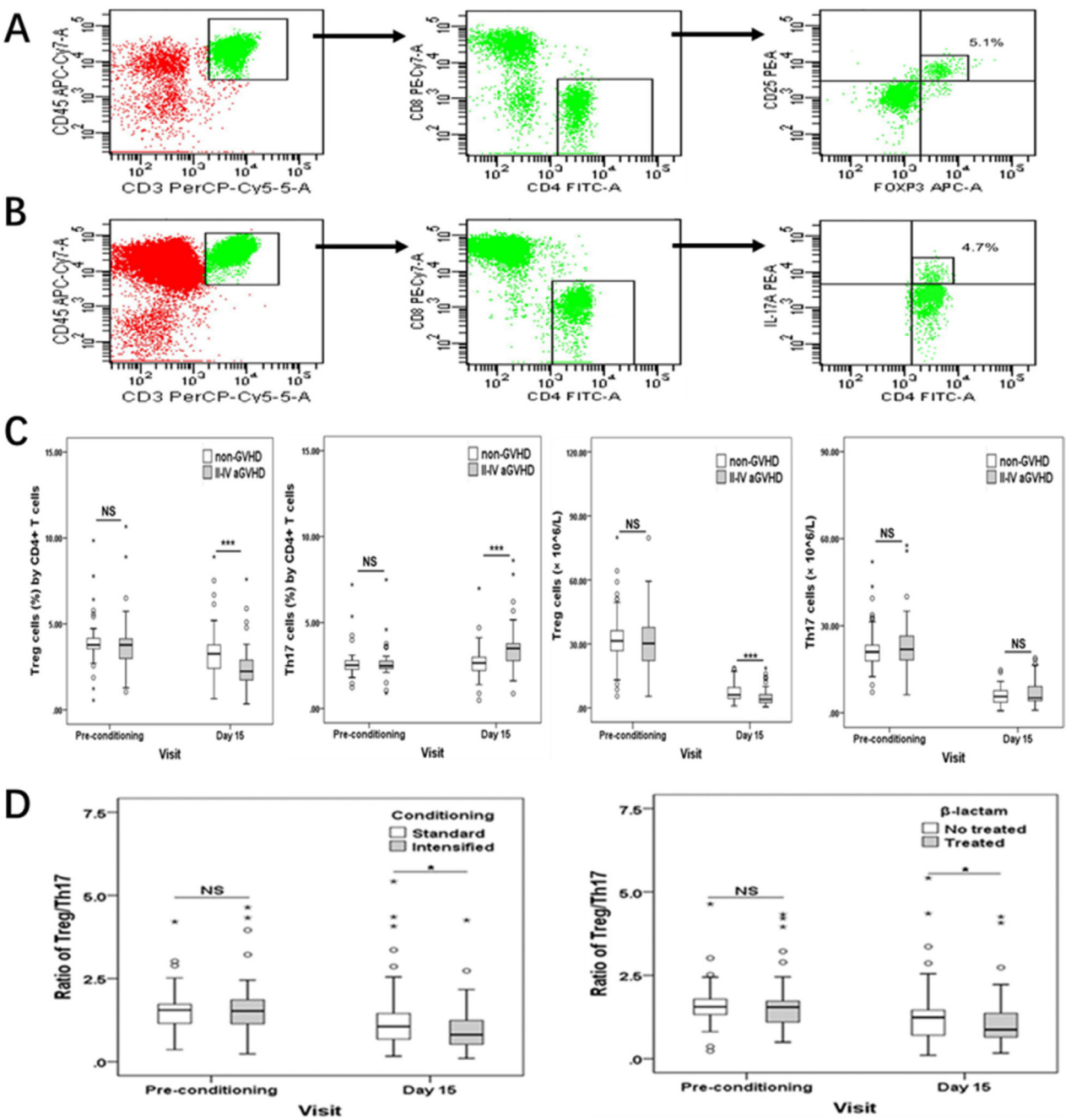

Figure 4. Conditioning intensity and β-lactam antibiotics were associated with the ratio of Treg/Th17 cells. (A and B) Sequential gating strategy for Treg (A) and Th17 (B) identification using flow cytometry. (C) The percentage and absolute numbers ($\times 10^6$/L) of Tregs and Th17 cells is shown for the 2 groups at preconditioning and day 15. (D) Intensified conditioning and β-lactam antibiotics were associated with a lower Treg/Th17 cell ratio at day 15 post-transplantation. NS, not significant. $^*P < .050$; $^{***}P < .001$.

(Figure 5A). At day 0, levels of TNF-α, IL-6, and LPS were higher in the intensified conditioning group compared with the standard conditioning group ($P = .015$, .004, and .007, respectively), but IL-17A and IL-1β levels were not different between the 2 groups ($P = .081$ and .168, respectively). At day 15, the total levels of the inflammatory factors were higher in the intensified conditioning group compared with the standard conditioning group ($P < .05$).

Regarding the effects of β-lactam antibiotics on inflammatory factors, the IL-17A, IL-1β, and LPS levels were associated with β-lactam antibiotic administration at day 15 ($P = .032$, .005, and .021, respectively; Figure 5B), although there was no difference between β-lactam antibiotic administration and no β-lactam antibiotic administration at day 0, possibly related to the fact that only 24 patients received β-lactam antibiotics before day 0.

Treg and Th17 Cell Balance Was Associated with aGVHD

The Treg/Th17 cell ratio was higher in the non-aGVHD group compared with the aGVHD group at day 15 ($P < .001$) but similar in the 2 groups at preconditioning ($P = .115$; Table 5). In addition, the levels of inflammatory factors, including TNF-α, IL-6, IL-17A, IL-1β, and LPS, at day 15 were associated with aGVHD ($P < .010$; Table 5).

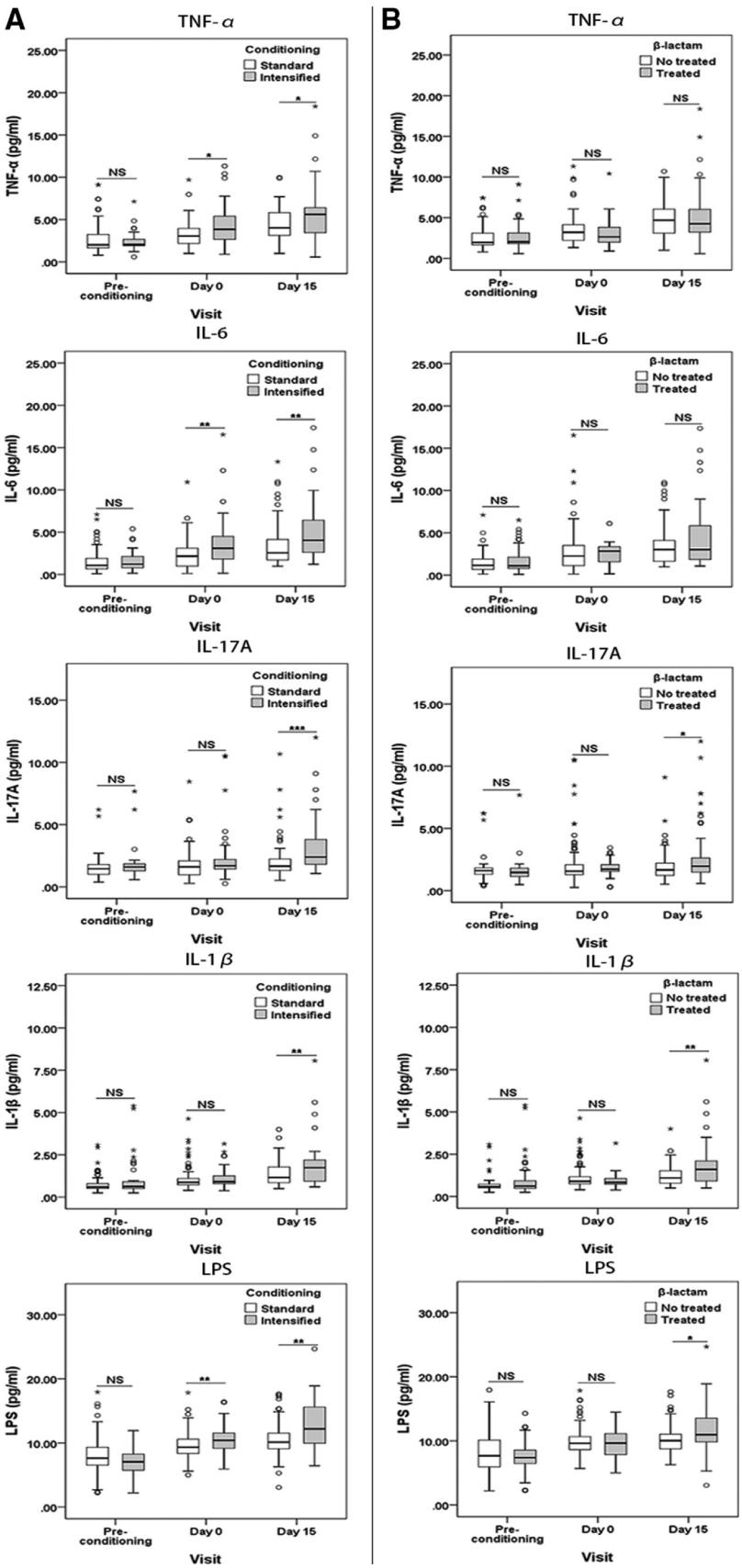

Figure 5. Conditioning intensity and β-lactam antibiotics were associated with inflammatory factors. Changes in TNF-α, IL-6, IL-17A, IL-1β, and LPS levels were associated with intensified conditioning (A) and β-lactam antibiotics (B). $^{*}P < .050$; $^{**}P < .010$; $^{***}P < .001$.

Table 5
Inflammatory Factors and the Treg/Th17 cell Ratio Associated with aGVHD

Factor	Preconditioning			Day 0			Day 15		
	Non-aGVHD (N = 83)	aGVHD (N = 58)	*P* Value	Non-aGVHD (N = 83)	aGVHD (N = 58)	*P* Value	Non-aGVHD (N = 83)	aGVHD (N = 58)	*P* Value
TNF-α	2.10	2.02	.836	3.02	3.82	.023	3.92	5.36	.001
IL-6	1.14	1.07	.599	2.16	2.70	.092	2.41	4.03	<.001
IL-17A	1.59	1.51	.927	1.56	1.67	.236	1.75	2.39	.003
IL-1β	.58	.62	.389	.85	.94	.057	1.01	1.85	<.001
LPS	7.60	7.55	.773	9.40	10.07	.017	9.96	13.01	<.001
Treg/Th17 cell ratio	1.56	1.38	.115	-*	-*	-	1.41	.65	<.001

* The Treg and Th17 cell counts could not be successfully detected at day 0 due to lower T cell counts.

Microbiota Predictive of aGVHD

To determine suitable predictors for aGVHD, the microbiota at day 15 post-transplantation was evaluated. We then selected organisms that were significantly positively or negatively correlated with subsequent aGVHD. As determined by the area under the curve (AUC) in ROC plots, the inverse Simpson Index of 4 families of microbiota, including Enterobacteriaceae, Lachnospiraceae, Erysipelotrichaceae, and Peptostreptococcaceae, were determined (AUC >.60 and $P < .05$), and others were excluded because of an AUC ≤.60 or *P*value ≥ .05. The results show that the AUC of the 4 bacterials could be predictive of the subsequent development of grade II-IV and grade III-IV aGVHD (Figure 6A). Based on the cutoff values for aGVHD, the inverse Simpson Index and the abundance of Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, and Enterobacteriaceae were scored as 1 or 0 (Table 1). To better define the potential clinical utility of the microbiota markers, an AIM score of the inverse Simpson Index and the 4 types of bacterials for aGVHD was used. The results of our AUC analysis of ROC curves for the predictive model according to the AIM score indicate that the AIM score can serve as a predictor for the eventual development of aGVHD grade II-IV (AUC = .75; $P < .001$) and grade III-IV (AUC = .84, $P < .001$; cutoff value, 3.5), with sensitivity and specificity of the latter for grade III-IV aGVHD of .84 and .75, respectively (Figure 6B).

Based on the AIM score, post-transplantation patients were divided into 5 groups. As shown in Figure 6C, the 0-1 scoring group consisted primarily of patients with grade 0-I aGVHD (77.8% to 80.0%); 17.8% to 22.2% of these patients had grade II GVHD, and only 1 patient had grade III-IV aGVHD. In the 2-3 scoring group, 33.3% to 35.0% of patients had grade II aGVHD, and 5.0% to 8.4% of the patients had grade III-IV aGVHD. However, in the 4-5 scoring group, 27.3% to 41.7% of the patients had grade III-IV aGVHD. Importantly, the AIM score was closely correlated with aGVHD grade on Spearman correlation analysis ($r = .481$, $P < .001$; Figure 6C).

DISCUSSION

The relationship between intestinal microbiota and aGVHD has garnered increasing attention in recent years. Taur et al [12] reported that lower intestinal microbiota diversity was positively correlated with aGVHD and worse mortality. Golob et al [17] reported that Actinobacteria and Firmicutes were positively correlated with aGVHD, whereas Lachnospiraceae was negatively correlated at neutrophil recovery. Simms et al [28] reported a significant decline in anti-inflammatory Clostridia in pediatric patients with aGVHD. In this study, we found a loss of Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae and a bloom of Enterobacteriaceae in patients with aGVHD, which is consistent with previous studies [12,28,29].

Several previous studies have indicated that the constitution of intestinal microbiota at neutrophil engraftment might be a predictor for the development of aGVHD. Weber et al [30] found that urinary 3-indoxyle sulfate, which is a metabolite of intestinal Clostridiales, could be a biomarker for predicting aGVHD. Golob et al [17] showed that a gradient of 20 types of bacterial species could predict (AUC = .83) severe aGVHD by calculating a gradient of the sum of the relative abundance of positively correlated bacteria minus the sum of the relative abundance of negative correlates. In this study, our results show that microbiota diversity combined with the gradients of the 4 bacterials (Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, and Enterobacteriaceae) can serve as a potent predictive marker not only for the development of aGVHD, but also for grade of aGVHD. Our findings are more economical and effective in clinical applications compared with previous studies [17,30]. A limitation of this study is that the AIM score that we propose is based on a retrospective analysis, and it remains to be validated in larger, prospective cohorts.

Some studies have reported numerous factors affecting the intestinal microbiota during allo-HSCT, including patient age, conditioning regimen, antibiotic use, and diet [12–14,18]. In a previous study, we reported the effects of antibiotics on the intestinal microbiota [18]. We demonstrated that administration of β-lactam antibiotics was associated with loss of Lachnospiraceae, and that vancomycin was associated with expansion of Enterobacteriaceae. Other studies found that conditioning had significant effects on the intestinal microbiota, with less diversity in recipients of a myeloablative regimen compared with recipients of a nonmyeloablative regimen [12,31]. In the present study, we also found that intensified conditioning decreased the diversity and abundance of Peptostreptococcaceae families compared with standard conditioning. These results may be interpreted as demonstrating an association between conditioning intensity and the destruction of the mucosal barrier and release of inflammatory factors [31–33]. Moreover, we observed that intensified conditioning combined with antibiotics may have additive effects on microbiota diversity.

It has been reported that commensal microbiota can influence immune homeostasis. Atarashi et al [34,35] suggested that anti-inflammatory Clostridia could regulate the Treg/Th17 balance in healthy mice. Furusawa et al [36] found that butyrate derived from commensal microbiota induced the differentiation of colonic regulatory T cells in mice. Gaboriau et al [37] reported that the overrepresentation of segmented filamentous bacteria in mice led to increased levels of Th17, Th1, IFN-γ, and IL-17, thereby stimulating the immune response. Zeng et al [38] found that LPS from pathogenic Enterobacteriaceae could promote Th17-mediated inflammation. It is well known that Th17 participates in the pathogenesis of aGVHD. Previous studies have demonstrated that circulating Th17 cells increase at day 17 to 19 after allo-HSCT in patients who develop aGVHD [39–41]. Varelias et al [42] revealed that IL-17-sensitive gut microbiota could aggravate aGVHD in a mouse model.

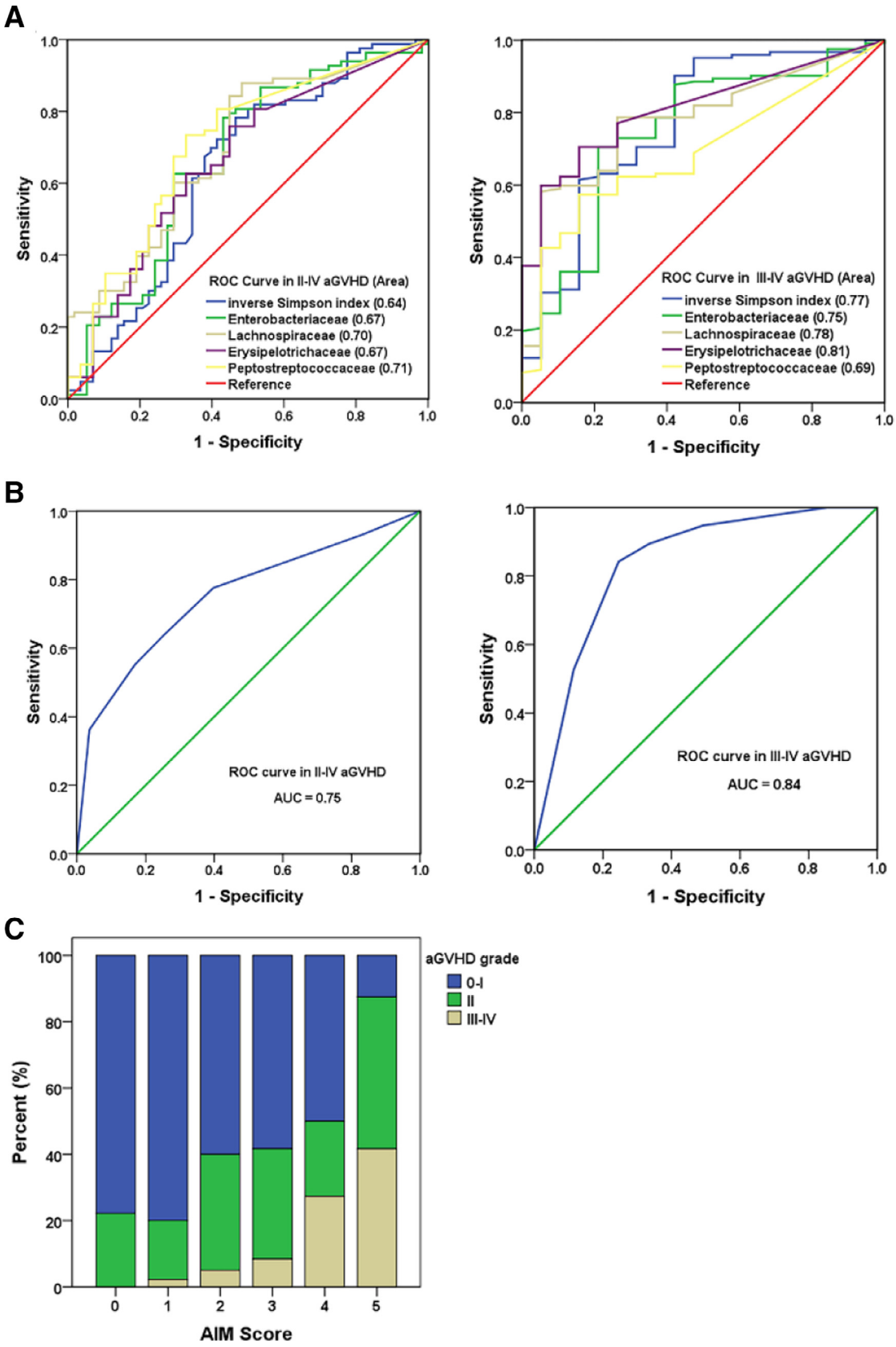

Figure 6. The microbiota and AIM score at day 15 could predict aGVHD by 100 days post-transplantation. (A and B) ROC curves of the diversity and different microbiota (A), and the AIM score (B) at day 15 for the prediction of aGVHD (grade II-IV and grade III-IV). (C) The AIM score correlated with the grade of aGVHD by Spearman correlation analysis ($r = .481$; $P < .001$). The different colors represent different grades of aGVHD.

Swimm et al [43] demonstrated that indole metabolites produced by the intestinal microbiota act via type I IFNs to limit intestinal inflammation and damage in an aGVHD mouse model. In our previous study, we demonstrated that the intestinal microbiota influences the Treg/Th17 balance in patients with aGVHD [18]. In this study, we revealed that conditioning intensity is associated with elevated levels of TNF-α, IL-6, and LPS at day 0 and with elevated levels of TNF-α, IL-6, LPS, IL-17A, and IL-1β at engraftment. Furthermore, use of β-lactam antibiotics is associated with elevated levels of LPS, TNF-α, IL-17A, and IL-1β, but not IL-6, at engraftment. Based on our findings, we suggest that these inflammatory factors might induce aGVHD by influencing the Treg/Th17 balance.

Future studies are essential to explore how the alterations of microbiota influence the metabolic pathway and inflammation environment of the host. A promising approach involves altering certain microbiota species or the microbiota metabolism to improve the inflammatory response and adaptive immunity in aGVHD. In addition, since microbiota changes occur rapidly and are readily affected by diet, antibiotics, and other factors, a study of microbiota ecology would elucidate the real impact of microbiota on GVHD.

CONCLUSIONS

This study demonstrates that conditioning intensity and antibiotics have significant effects on the microbiota in recipients of allo-HSCT. The intestinal microbiota and conditioning might induce aGVHD by inflammatory factors and influence the Treg/Th17 balance. The constitution of the intestinal microbiota at neutrophil engraftment may be a predictive marker for the development and grade of aGVHD. It will be helpful for clinicians to determine which allo-HSCT recipients are at greatest risk for severe aGVHD and to target these patients for more aggressive interventions.

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Authorship statement: L.H., Q.F.L., and H.J., designed the study and wrote the paper; F.H., Z.F., and M.D. supervised the collection of samples and recorded the clinical data; H.Z. and Q.Y.L. were responsible for DNA extraction from stool samples; S.C., K.Z., X.Z., and J.P. contributed to the examinations of the inflammatory factors and T lymphocyte subsets; Y.L. and Z.J. assisted in the preparation and writing of the manuscript; L.Z. and L.X. performed data analysis. All authors read and approved the final manuscript. L.H., H.Z., S.C., and L.Z. contributed equally to this work.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2019.07.006.

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