Lactose drives Enterococcus expansion to promote graft-versus-host disease

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Disruption of intestinal microbial communities appears to underlie many human illnesses, but the mechanisms that promote this dysbiosis and its adverse consequences are poorly understood. In patients who received allogeneic hematopoietic cell transplantation (allo-HCT), we describe a high incidence of enterococcal expansion, which was associated with graft-versus-host disease (GVHD) and mortality. We found that Enterococcus also expands in the mouse gastrointestinal tract after allo-HCT and exacerbates disease severity in gnotobiotic models. Enterococcus growth is dependent on the disaccharide lactose, and dietary lactose depletion attenuates Enterococcus outgrowth and reduces the severity of GVHD in mice. Allo-HCT patients carrying lactose-nonabsorber genotypes showed compromised clearance of posttransplant Enterococcus domination. We report lactose as a common nutrient that drives expansion of a commensal bacterium that exacerbates an intestinal and systemic inflammatory disease.

The healthy gut is inhabited by a diverse community of mostly anaerobic bacteria, and a hallmark of microbial imbalance (dysbiosis) observed in many disease states involves the expansion of facultative anaerobic bacteria (5). Enterococci are facultative anaerobes that colonize the intestines of almost every species, from insects to mammals (2), and make up a very small proportion (<0.1%) of the gut microbiota in healthy humans (3). However, enterococci are also pathogens; the species Enterococcus faecalis and Enterococcus faecium are important causes of multidrug-resistant infections in patients (4). In single-center studies, E. faecium has been observed to dominate the fecal microbiota of immunocompromised patients after allogeneic hematopoietic cell transplantation (allo-HCT), a curative-intent therapy for hematological malignancies (5–7). Moreover, fecal domination with vancomycin-resistant enterococci increases the risk of bloodstream infection in allo-HCT patients (5, 8). Patients with severe graft-versus-host disease (GVHD) after allo-HCT have poor outcomes with only ~30% long-term survival (9). Gut microbiota perturbations caused by broad-spectrum antibiotics and a reduction in microbial diversity are associated with increased transplant-related mortality and lethal GVHD in humans and mice (10–12). Besides causing infections, experimental studies in gnotobiotic mice have revealed that enterococci play an important role in colitis (14) by stimulating antigen-presenting cells and CD4+ T cells, which is associated with intestinal inflammation (15). In this study, we investigated the role of enterococci in the development of acute GVHD, both in allo-HCT patients and preclinical allo-HCT mouse models.

We used 16S ribosomal RNA (rRNA) gene sequencing to study the fecal microbiota of 1325 adult allo-HCT recipients at four HCT centers: Memorial Sloan Kettering Cancer Center (MSKCC) (United States), Duke University (United States), Hokkaido University (Japan), and University Hospital Regensburg (Germany). Patient characteristics are shown in table S1. We observed high abundance of enterococci soon after transplantation in samples from all four transplant centers (Fig. 1B and fig. S1B). We defined Enterococcus domination as relative genus abundance ≥0.3 (≥30%) in any fecal sample, following a threshold we have used previously (5) (materials and methods and fig. S2C). The incidence of domination rose comparably across centers, with up to 65% of patients exhibiting a domination event after allo-HCT (Fig. 1A). E. faecium was the dominant species in both the MSKCC and the multicenter-validation cohort (Duke, Hokkaido, and Regensburg) (Fig. 1B, fig. S1, and table S2), where 40.1% of MSKCC patients (441 of 1101 patients) and 46.0% of multicenter-validation patients (103 of 224 patients) met criteria for domination at any time point between day −20 and day +80 relative to the date of allo-HCT, in which cells are infused on day 0. Fecal domination by Enterococcus in the early posttransplant period (day 0 to +21) was associated with significantly reduced overall survival and increased GVHD-related mortality in both the MSKCC and multicenter-validation cohort, as well as an increased risk of moderate-to-severe acute GVHD in the MSKCC cohort (Fig. 1, C and D, fig. S2, A and B, and table S3). The risk of relapse or disease progression was not associated with enterococcal domination in either cohort. The association of domination by genus Enterococcus with clinical outcomes in the MSKCC cohort remained significant in a multivariate analysis adjusted for graft source, disease, conditioning intensity, gender, and age (table S4). In a subset of MSKCC patients, the vanA operon was found in 152 (37.4%) of 406 patients that had samples available for analysis, indicating the presence of vancomycin-resistant enterococci (VRE) (fig. S2E). Notably, expansions of several different taxa were detected in fecal samples in this study, but the Enterococcus genus was the one most commonly observed dominating the microbiota in all four transplant centers (fig. S3 and tables S5 and S6).
To further investigate these clinical observations, we examined the fecal microbiota of mice early after transplantation using well-established mouse models of allo-HCT. In a major histocompatibility complex (MHC)-matched, minor-antigen-mismatched allo-HCT model [C57BL/6-to-129S1/Sv transplant (C57BL/6→129S1/Sv)], we performed 16S rRNA gene sequencing of fecal samples and found that *E. faecalis* dominated the fecal microbiota at posttransplant day +8 in mice who received T cell-replete grafts and developed lethal, acute GVHD (Fig. 2A and fig. S4A). In contrast to the patients who had protracted antibiotic exposures, this expansion of *E. faecalis* was independent of antibiotic administration and dependent upon GVHD, as it was not observed in control recipients of T cell-depleted allografts in which GVHD did not develop. This posttransplant expansion of enterococci was consistently found in two additional lethal GVHD models: C57BL/6→BALB/c mice (MHC-disparate model after irradiation conditioning) (Fig. 2B) and LP/J→C57BL/6 mice [MHC-matched, minor-antigen–mismatched after busulfan and cyclophosphamide conditioning (16)] (Fig. 2C). The expansion of enterococci in murine allo-HCT recipients with GVHD was accompanied by an increase in *Enterococcus* colony–forming units recovered from mesenteric lymph nodes, consistent with increased bacterial translocation (Fig. 2B).

Although we observed *E. faecium* domination in patients and a transient expansion of *E. faecalis* in GVHD mice, we hypothesized that both members of this genus might be associated with GVHD. Of note, *E. faecium* only recently became recognized as a major human pathogen; before the 1990s it was *E. faecalis* that caused >90% of clinical infections (17). Because *E. faecalis* expands in mice with GVHD and is the major *Enterococcus* species in laboratory mice, we next investigated whether *E. faecalis* contributes to GVHD. We colonized germ-free C57BL/6 mice with a community of six bacterial strains (*Akikermansia muciniphila*, *Lactobacillus johnsonii*, *Blaustia producta*, *Bacteroides sartori*, *Clostridium boltei*, and *Parabacteroides distasonis*; see materials and methods) (10, 18, 19) 21 days prior to allo-HCT (LP/J→germfree C57BL/6). One group of mice was colonized on day −21 with *E. faecalis* OG1RF, which remained detectable in mouse feces on days 0 and +7 (Fig. 2D, right panel, and fig. S4E). GVHD was exacerbated in *E. faecalis*–harboring mice.

**Fig. 1.** *Enterococcus* domination occurs globally and increases risk of GVHD and mortality after allo-HCT. Fecal microbiota were profiled using 16S rRNA gene sequencing of 1325 adult allo-HCT recipients. The patients attended one of four HCT centers in different countries: MSKCC (United States), Duke University (United States), Hokkaido University (Japan), and University Hospital Regensburg (Germany). (A) (Left) Cumulative incidence of patients who experienced at least one instance of genus *Enterococcus* domination of the gut microbiota [domination defined as a genus relative abundance of ≥0.3 (on a unitless scale from 0 to 1) over the course of allo-HCT (day −20 to +24 relative to HCT; using 7-day sliding windows) at different transplant centers]. (Right) Fraction of fecal specimens with enterococcal domination of the gut microbiota. (B) Relative abundance of different *Enterococcus* spp. in the microbiota of allo-HCT patients from the MSKCC and multicenter-validation cohort over the course of HCT, determined by 16S rRNA gene sequencing of fecal samples. Each point represents a fecal sample, and color indicates the different *Enterococcus* spp.; the red dotted line indicates the threshold for domination set at a relative abundance ≥0.3. (C) Overall survival (left) and cumulative incidence of GVHD-related mortality (right) in the T cell replete graft recipients in the MSKCC patient cohort (see table S3), stratified into nondominated and *Enterococcus*-dominated groups (domination is defined as the relative genus abundance ≥0.3 in at least one sample between day 0 and +21). n, number of individuals. (D) Overall survival (left) and cumulative incidence of *Enterococcus*-related mortality (right) in *Enterococcus*-dominated (at genus level) versus nondominated allo-HCT patients in the combined multicenter-validation cohort (table S3). Clinical outcomes in (C) and (D) were analyzed using the R packages `survival` and `cmprsk`. Wald values of *P* <0.05 signify higher risks (HR, hazard ratios) of mortality among patients with *Enterococcus* domination as compared with those without domination.
Fig. 2. Enterococcus dominates mouse gut microbiota after HCT and can exacerbate GVHD. (A) (Left) High-density sampling and 16S rRNA gene sequencing of fecal microbiota from 129S1/Sv mice (1 box = 1 mouse) receiving bone marrow (BM; upper row) or T cell–replete bone marrow (BM+T (2 × 10^6 T cells); lower row). (Right) BM+T transplanted mice develop lethal GVHD as shown by survival analysis. rel., relative. (B) (Left) Relative abundance of the genus Enterococcus in BALB/c host mice transplanted with C57BL/6 BM or BM+T (1 × 10^6 T cells) at different time points relative to HCT. (Middle) Colony-forming units (CFUs) of enterococci in fecal samples and in mesenteric lymph nodes (mLN). Scatter plot data show means ± SEM. (Right) Survival of BALB/c recipient mice after HCT [BM versus BM+T (1 × 10^6 T cells)]. (C) (Left) Schematic showing HCT of LP/J BM versus BM+T (4 × 10^6 T cells) into C57BL/6 mice after chemotherapy conditioning. (Middle) Relative abundance of the genus Enterococcus in the feces of transplanted mice at different time points relative to HCT. (Right) Comparison of overall survival between BM and BM+T mice. (D) (Left) Schematic showing colonization of germ-free C57BL/6 mice with a 6-strain minimal microbiota with (+EF) or without (-EF) E. faecalis OG1RF (2 × 10^7 CFU per mouse); after 14 days, colonized mice received chemotherapy conditioning with busulfan and cyclophosphamide and, subsequently, an HCT of LP/J BM versus BM+T (4 × 10^6 T cells). (Middle) Comparison of overall survival. (Right) Relative abundances of E. faecalis spiked to the minimal microbiota in the EF+ group with samples collected at the day of HCT (day 0) and 7 days later (n = 4 to 11 mice per group; P = 0.09, paired testing of relative abundances of enterococci of day 0 versus BM+T day +7). Scatter plot data are presented as means ± SEM **P < 0.01, ***P < 0.001 (independent t test for BM versus BM+T); survival data were statistically analyzed using Mantel-Cox log-rank test.
Fig. 3. Metagenomic and metabolomic analyses of Enterococcus-dominated fecal specimens in human HCT patients and mice. (A) (Left) Differential abundances of shotgun-sequenced and HUMAnN2-annotated bacterial metabolic pathways between paired pre- and post-HCT fecal samples from MSKCC patients who received allo-HCT for acute myeloid leukemia (AML) analyzed by linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe). pre HCT, day −8 to −1 before allo-HCT; post HCT, day +3 to +25 after allo-HCT. (Right) Pie chart showing mean relative abundances of bacterial genera (analyzed by MetaPhlAn2) found in patient fecal samples pre- and post-HCT; data are aggregated across all patients. non-dom., nondominated; domin., dominated.

(B) LEfSe analysis of bacterial metabolic pathway abundances in HCT day +7 fecal samples of 129S1/SV mice transplanted with C57BL/6 BM versus BM+T (2 × 10^6 T cells) (see Fig. 2A). (C) Pie charts with metabolic pathway abundances determined by whole-genome sequencing of E. faecalis (isolated from feces of a BALB/c GVHD mouse, day +7 after HCT; upper panel) and E. faecium (human isolate; ATCC #700221; lower panel); only pathways with an abundance ≥2% are shown in both panels. (D) In vitro growth of E. faecalis (mouse GVHD isolate; upper panel) and E. faecium (ATCC #700221; lower panel) in nontreated BHI broth or in BHI broth pretreated with lactase. (E) Butyrate concentrations (means ± SEM) in cecal contents of BALB/c mice transplanted with C57BL/6 BM or BM+T (1 × 10^6 T cells) at day +7 after HCT. Statistical analysis: *P < 0.05 [paired t test (E) or independent t test (F)].
(Fig. 2D and fig. S4B). Serum interferon-γ concentrations were significantly elevated in *E. faecalis*-colonized mice (fig. S4C), and we observed a significantly increased number of donor T cells, an increase of activated and proliferating CD4+ T cells (fig. S4D; CD4+CD25+; CD4+Ki67+), and an increased number and percentage of CD4+ RORγ+ TH17 cells in colon lamina propria (fig. S4D). Posttransplant administration of *E. faecalis* OG1RF to conventionally housed, T cell–replete bone marrow (BM+T)–transplanted BALB/c mice also aggravated GVHD (fig. S5A). These findings indicate that *E. faecalis* can aggravate GVHD severity.

**Fig. 4. Lactose-free diet reduces experimental GVHD and lactase genotypes associated with microbiota dynamics after allo-HCT in humans.** (A) (Left) Schematic showing that BALB/c recipient mice received C57BL/6 BM or BM+T (5 × 10^6 T cells) and were fed control chow (ctr) versus lactose-free chow (LF) from day –7 to +14 relative to transplant. Comparison of survival between BM and BM+T mice (middle) and relative abundance of the genus *Enterococcus* in mouse feces (right) are shown. Scatter plot data presented as means ± SEM; *P < 0.05 (independent t test). (B) (Left) Schematic showing HCT of LP/J BM versus BM+T (4 × 10^6 T cells) into C57BL/6 mice after chemotherapy conditioning. Comparison of survival between BM and BM+T mice (middle) and relative abundance of the genus *Enterococcus* at different time points relative to HCT (right) are shown. Scatter plot data presented as means ± SEM; *P < 0.05 (independent t test). (C) (Left) Relative abundance (log10) of *Enterococcus* (genus) by days relative to the day of antibiotic cessation (broad-spectrum antibiotics for neutropenic fever: intravenous piperacillin-tazobactam, intravenous imipenem-cilastatin, or intravenous meropenem). Box plot inserts display the median relative abundances of the genus *Enterococcus* of time binned in the indicated day ranges relative to antibiotic cessation; whiskers represent maximum and minimum. Statistical analysis of box plot data: *P < 0.05 (Wilcoxon rank test). (D) Cumulative incidence of acute GVHD grade 2 to 4 in rs4988235 SNP-genotyped MSKCC patients (T cell–depleted grafts excluded; graft source: BM/PBSC unmodified = 213 patients; cord blood = 102 patients; C/C = 175, T/C+T/T = 140). The cumulative incidence of grade 2 to 4 acute GVHD was compared between genotype groups using the R package cmprsk.
We next considered whether posttransplant defects of mucosal defense mechanisms facilitate enterococcal expansion. Immunoglobulin A (IgA) coating of intestinal bacteria can be protective in colitis and is important for maintaining mucosal integrity (20). However, we did not observe members of the genus Enterococcus to be enriched in either IgA-negative or IgA-positive fecal fractions, even though total fecal IgA was significantly reduced in allo-HCT recipients with GVHD (Fig. S5, B to D). Reduction of IgA by transplanting IgA-deficient bone marrow (BM) from activation-induced cytidine deaminase knockout mice did not further increase enterococcal expansion (Fig. S5E). Intestinal antimicrobial peptides of the Reg3 family can suppress the growth of VRE (21) and are reported to play a major role in GVHD (22). Accordingly, we found that both Reg3B/G transcripts and interleukin-22 protein, which regulates Reg3 expression (23), were reduced in the ileum of GVHD mice (Fig. S5F).

Next, we analyzed microbiota-intrinsic factors and used shotgun metagenomic sequencing to characterize the metabolic potential of the Enterococcus-dominated fecal microbiota. Pre- and posttransplant fecal samples from MSKCC patients who received allo-HCT for acute myeloid leukemia were selected for sequencing on the basis of having a highly diverse pre-HCT microbiota and posttransplant Enterococcus faecium domination (by 16S rRNA gene sequencing). We focused on microbial metabolic pathways that specifically characterize domination by comparing them with the highly diverse pretransplant microbiota from the same patients. Pathways involved in DNA synthesis and, notably, in lactose and galactose degradation were enriched in the E. faecium–dominated, posttransplant microbiota. In contrast, amino acid synthesis and starch-degradation pathways were more prevalent in pretransplant specimens (Fig. 3A). The lactose-and-galactose degradation pathway was also significantly enriched in the posttransplant E. faecalis–dominated samples of mice with GVHD (Fig. 3B). Comparison of whole-genome sequencing from isolates of E. faecium (from a human allo-HCT patient) and of E. faecalis (from a mouse with GVHD) revealed that genes encoding lactose and galactose metabolism accounted for ~3% of their genomes (Fig. 3C). In silico analysis of these Enterococcus genomes and publicly available genomes of other members of the gnotobiotic six-strain consortium revealed that enterococci are specifically enriched in enzymes of the tagatose-type galactose pathway for galactose-to-glucose degradation (24) (materials and methods and Fig. S6, A and B). Enterococcal growth depends on lactose in vitro, as both E. faecalis and E. faecium strains cultured in brain-heart infusion (BHI) broth depleted of lactose (by lactase; fig. S7A) did not grow (Fig. 3D). Growth was reinstated upon transfer to regular BHI, excluding antibacterial effects of lactate treatment (Fig. 3D). Enterococcal expansion after allo-HCT was accompanied by a loss of Clostridium spp. in the microbiota of allo-HCT patients (Fig. 3, A and F) and of mice with GVHD (Fig. S8, A to C, and table S7). This may be important for allo-HCT patients, as high abundances of clostridia are associated with better survival and lower incidence of GVHD (12, 25). Commensal clostridia are known to produce large amounts of butyrate (26), which mitigates lethal GVHD in mice through protecting energy homeostasis of enterocytes (27). We observed that posttransplant enterococcal domination and a loss of clostridia were accompanied by a significant reduction in fecal butyrate in both allo-HCT patients and mice with GVHD (Fig. 3, E and G) (28). A loss of this key metabolite may contribute to the poor outcomes in Enterococcus-dominated patients and mice.

Given that the optimal growth of enterococci depends on lactose availability in vitro, we investigated whether enterococcal expansion can be mitigated by feeding mice lactose-free chow (fig. S7B and table S8). In the C57BL/6→BALB/c model, the absence of dietary lactose significantly reduced posttransplant Enterococcus bloom and mitigated GVHD (Fig. 4A and fig. S9B). Flow cytometric analysis of donor T cells on day +14 revealed a reduction in the percentage of activated and proliferating CD4+ T cells (CD4+CD69+; CD4+Ki67+) as well as a reduction in the percentage of CD4+Tbet+ (T1/1) T cells (fig. S9). The effect of a lactose-free diet on enterococcal outgrowth and GVHD was replicated in the LP/J→C57BL/6 mouse model (Fig. 4B and table S9 for changes in non-enterococcal taxa). Intestinal mucosal damage by irradiation or allo-reactive T cells may affect the expression of lactase, the enzyme found on small-intestine enterocytes that facilitates lactose absorption through disaccharide cleavage. Duodenal lactase transcript abundance progressively declined in BM+T recipients over the course of transplantation (fig. S9C), which may induce a lactose-intolerant–like state in mice, allowing nondigested lactose to reach the lower intestinal tract and serve as a carbon source for bacteria.

Next, we explored whether enterococcal expansion is associated with lactose tolerance in human allo-HCT patients by genotyping 602 patients from the MSKCC cohort with available pretransplant germline DNA samples for the gene polymorphism rs4988235 (13910*T). This single-nucleotide polymorphism (SNP) regulates lactase expression and predicts lactose absorption and/or tolerance (C/T or T/T alleles) and malabsorption (C/C alleles) in the upper gut (29). Although abundance of the genus Enterococcus increased comparably during exposure to broad-spectrum antibiotics in both lactose absorbers and malabsorbers, enterococcal domination was significantly prolonged in malabsorbers after cessation of antibiotics (Fig. 4D and fig. S10). This finding suggests that the maintenance of enterococcal domination and microbiota recovery after broad-spectrum antibiotic exposure is significantly modulated by the luminal availability of lactose as a growth substrate.

Fecal domination by Enterococcus spp. is a significant risk factor for the development of acute GVHD and for increased overall and GVHD-related mortality after allo-HCT. Our findings extend previous reports from smaller single-center analyses that posttransplant VRE bacteriaemia and fecal domination are associated with worse outcomes after allo-HCT (7, 8, 30). In gnotobiotic mouse models, enterococci exacerbate GVHD, consistent with previous reports of aggravated colitis in models of inflammatory bowel disease (14) or systemic autoimmune responses (31). We previously identified Blautia abundance (a genus within class Clostridia) as a predictor of protection from lethal GVHD (12), whereas here we describe Enterococcus domination as a risk factor for GVHD. These two findings are noteworthy in light of our recent observation that a B. producta strain can inhibit VRE growth via the production of a lantibiotic protein (32). We identified a microbiota-intrinsic mechanism that is dependent on lactose utilization and favors the expansion of enterococci. This process may be triggered through a loss of lactate produced by enterococci damaged by conditioning or allo-reactive T cells. We validated this concept experimentally, by showing that depletion of lactate in vitro and in vivo inhibited enterococcal expansion and mitigated GVHD, and clinically, by showing that patients harboring a lactose-malabsorption allele experienced prolonged Enterococcus domination after antibiotic exposure. These observations in mice and allo-HCT patients provide proof-of-concept for a novel, non–antibiotic-based therapeutic strategy, such as a lactose-free diet, to attenuate the outgrowth of pathobionts like enterococci and possibly improve clinical outcomes by modulating dietary sources of nutrients for pathogenic bacteria.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS

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Lactose drives *Enterococcus* expansion to promote graft-versus-host disease


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Lactose can fuel GVHD

Allogeneic hematopoietic cell transplantation (allo-HCT) is used to treat certain hematopoietic malignancies, but patients have a risk of developing graft-versus-host disease (GVHD). Stein-Thoeringer *et al.* performed a large-scale analysis of more than 1300 patients treated with allo-HCT across four clinical centers (see the Perspective by Zitvogel and Kroemer). High levels of bacteria from the *Enterococcus* genus were associated with greater incidence of GVHD and mortality. Lactose appears to provide a substrate for *Enterococcus* growth, and patients with a lactose-malabsorption genotype had a greater abundance of *Enterococcus*. A lactose-free diet limited *Enterococcus* growth, reduced the severity of GVHD, and improved survival in gnotobiotic mouse models.

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