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## Decreased Secretion of Paneth cell $\alpha$ -defensin in Graft-versus-host disease

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**Short title:** Fecal  $\alpha$ -defensins in GVHD

**Author contributions:** Y.E., K.N., and T.T. developed the conceptual framework of the study, designed the experiments, conducted studies, analyzed data and wrote the paper.

D.H. analyzed data and wrote the paper. S.S., N.S., K.A., and T.A. supervised experiments.

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## Abstract

*Background:* Intestinal microbial ecology is actively regulated by Paneth cell-derived antimicrobial peptides,  $\alpha$ -defensins. Graft-versus-host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation (SCT). We previously demonstrated that Paneth cells are targeted by GVHD and their expression of antimicrobial peptide  $\alpha$ -defensins is impaired, leading to a loss of physiological diversity among the microflora and development of bloodstream infection. Herein we evaluated whether fecal levels of  $\alpha$ -defensins could be surrogate marker of intestinal dysbiosis.

*Methods:* We directly measured  $\alpha$ -defensin cryptdin-1 (Crp1) in fecal pellets of mice with GVHD by using a novel enzyme-linked immunosorbent assay.

*Results:* Fecal levels of Crp1 were significantly decreased in mice with GVHD but unchanged in mice without GVHD after SCT. These were correlated with intestinal flora diversity.

*Conclusion:* We demonstrate a link between reduced secretion of Paneth cell  $\alpha$ -defensins and dysbiosis of intestinal flora in GVHD. Fecal levels of  $\alpha$ -defensins could be surrogate markers for intestinal microbial homeostasis.

**Key Word:** Graft-versus-host disease, Hematopoietic stem cell transplantation,  $\alpha$ -defensins, cryptidin

## Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is a curative therapy for various hematologic malignancies and other disorders, however; graft-versus-host disease (GVHD) and infections are the major complications. The intestine is the major interface between the environment and colonized by at least 1,000 distinct bacterial species(4). The pioneering studies by van Bekkum and others in 1960s-70s suggested a link between intestinal microflora and GVHD in mice (1-3). Intestinal microbial homeostasis is maintained by antimicrobial peptides produced by Paneth cells.  $\alpha$ -Defensins are the most potent antimicrobial peptides, accounting for 70% of the bactericidal peptide activity released from Paneth cells (5, 6).

We have recently demonstrated that Paneth cells are targeted by GVHD, resulted in a reduced expression of  $\alpha$ -defensins and a dysbiosis of the intestinal microflora(7). These findings have been confirmed in humans; Paneth cell loss and dysbiosis are hallmarks of intestinal GVHD(8-12). However, it is not easy to evaluate Paneth cell numbers or composition of intestinal flora in fecal samples. We have recently developed a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the mouse Paneth cell  $\alpha$ -defensin cryptdin (Crp) in fecal pellets(13). It is thus important to determine whether fecal levels of defensins could be surrogate markers of Paneth cell loss and dysbiosis. We herein addressed this issue in a mouse model. To our knowledge, this is the first report that measures levels of  $\alpha$ -defensins after

allogeneic SCT.

## Material and Methods

**Mice.** Female C57BL/6 (B6; H-2<sup>b</sup>), B6.SJL-*Ptprc*<sup>a</sup> (B6/SJL, H-2<sup>b</sup>, CD45.1<sup>+</sup>), and BALB.B (H-2<sup>b</sup>, CD45.2<sup>+</sup>) mice were purchased from Charles River Japan (Tokyo, Japan) or Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee.

**BMT.** Mice were transplanted as previously described(14). In brief, after 11Gy X-ray total body irradiation (TBI) delivered in 2 doses at 4 h intervals, BALB.B mice were intravenously injected with  $5 \times 10^6$  splenic T cells and  $5 \times 10^6$  T cell-depleted bone marrow (TCD-BM) cells from B6 or B6/SJL donors on day 0. Isolation of T cells and T-cell depletion were performed using the T cell isolation kit and anti-CD90-MicroBeads, respectively, and the AutoMACS (Miltenyi Biotec Japan, Tokyo, Japan). Mice were maintained in specific pathogen-free condition and received normal chow and autoclaved hyperchlorinated water (PH 4) for the first 3 weeks post-BMT and filtered water thereafter. Survival after BMT was monitored daily and the degree of clinical GVHD was assessed weekly by a scoring system as described previously(15).

**Preparation of fecal samples.** Fecal samples were prepared as previously described(13). In brief, samples were air dried, powdered using a bead beater-type homogenizer (Beads Crusher  $\mu$ T-12, TAITEC, Saitama, Japan), and vortex mixed with

PBS for 1 h at 4 °C. After centrifugation at 20,000g for 20 min, supernatant was collected to measure Crp.

**ELISA.** In the sandwich ELISA, we used 2 anti-Crp1 monoclonal antibodies, designated 77-R5 and 77-R20, generated following immunization of rats with synthetic oxidized-Crp1. The sandwich ELISA was carried out, as previously described(13). In brief, 100 µl of samples were added to the wells coated with Crp1 mAb (77R-5, 1µg/mL) and incubated at 25 °C for 2 h. After washing, 100 µl of 0.5 µg/ml biotinylated detection antibody (77R-20, 0.5 µg/mL) was added at 25 °C for 1 h. Subsequently, the wells were incubated with 100 µl of Streptavidin-HRP conjugate (GE Healthcare Biosciences, Piscataway, NJ) at 25 °C for 1 h. After final washes, 100 µl of TMB chromogen substrate buffer was added and incubated at 25 °C for 30 min. The reaction was stopped and absorbance values were determined at 450 nm using a microplate reader.

**DNA extraction and amplification of 16S ribosomal RNA (rRNA) gene.** Total DNA was isolated from fecal pellets by using a QIAamp DNA stool mini kit (QIAGEN Tokyo, Japan) as described(7). Briefly, baked 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) were added to each aliquot, incubated at 95 °C, and processed for 1 min at speed 5.5 on Fastprep system (Qbiogene, Carlsbad, CA). Bacterial 16S rRNA genes were amplified with bacterial-universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') labeled at the 5' end with

6-carboxyfluorescein (6-FAM) and 1492R (5'-GGTTACCTTGT TACGACTT-3')(16). PCR amplification was performed using *EX Taq* (Takara Bio, Tokyo, Japan) as described(7). Amplicons were purified using a QIAquick PCR Purification kit (QIAGEN).

**Restriction fragment length polymorphism (RFLP) analysis.** RFLP was performed as previously described(7). DNA products were digested with 10 U of either *HhaI* or *MspI* (Takara Bio) at 37°C for 3 h. The fluorescently labeled fragments (T-RFs) were separated by size on an ABI 3130 genetic analyzer (Applied Biosystems Japan, Tokyo) and analyzed with GeneMapper version 4.0 software (Applied Biosystems). Each unique RELP pattern was designated as an operational taxonomic unit (OTU). OTUs with a peak area of less than 0.5% of the total area were excluded from the analysis. Proportion of *E. coli* was defined as the ratio of area of OTU for *E. coli* to total areas of OTUs. Diversity of the microbial community corresponding to the RFLP banding pattern was calculated using the Simpson's index of diversity  $1 - D$  ( $D = \sum p_i^2$ ) (17) and Shannon diversity index  $H'$  ( $H' = -\sum p_i \ln(p_i)$ ) (18) and where  $p_i$  is the proportion of total number of species made up of its species.

**Statistical analysis.** Mann-Whitney U tests were used to compare data, the Kaplan-Meier product limit method was used to obtain survival probability, and the log-rank test was applied to compare survival curves. To determine the statistically significant correlation, the Spearman rank correlation coefficient (R) was adopted. All

tests were performed with the SigmaPlot Version 10.0 software.  $P < .05$  was considered statistically significant.

## Results

### Reduced fecal levels of Crp1 in GVHD

BALB.B mice were intravenously injected with  $5 \times 10^6$  TCD-BM cells alone (control group) or with  $5 \times 10^6$  spleen T cells (GVHD group) from B6 donors on day 0 following 11 Gy TBI. In this major histocompatibility complex (MHC)-matched, minor histocompatibility antigen-mismatched model of GVHD, recipient mice exhibit typical clinical signs of acute GVHD, including ruffled hair, diarrhea, and weight loss in a T-cell dose dependent manner (19). The allogeneic animals developed severe GVHD and all of these mice died within 80 days after BMT, whereas all TCD-BM controls survived through this period (Fig. 1A). The surviving allogeneic animals showed significantly more severe signs of clinical GVHD than controls (Fig. 1B). An analysis of the donor cell engraftment in the spleens of BALB.B (CD45.2<sup>+</sup>) recipients transplanted from B6/SJL (CD45.1<sup>+</sup>) donors showed complete donor engraftment on day 50 showed complete donor engraftment in allogeneic recipients (data not shown).

Fecal pellets were collected from each mouse and Crp1 levels were measured once a week.  $\alpha$ -Defensin gene family coding for approximately 20 different Crp isoforms in mice(20). Crp1 levels after TCD BMT were constantly equivalent to those in naïve mice. In contrast, these were gradually decreased in mice with GVHD and reached markedly low levels at 3 weeks posttransplant (Figure 2).

### **Alteration in the intestinal microbiota in GVHD**

Paneth cell-derived  $\alpha$ -defensins are essential regulators of the microbiota composition in the intestine(5). We evaluated changes in the composition of the intestinal microflora during the course of GVHD once a week by RFLP analysis of bacteria-specific 16S rRNA genes that were constructed from each sample of fecal pellets, as previously described(7). Although there was little changes in the RFLP patterns in controls, the numbers and heights of the RFLP patterns were markedly reduced in the mice with GVHD. Diversity of the microbial community, which corresponds to the RFLP banding patterns, was significantly reduced in mice with GVHD at all-time points, as assessed by Simpson's index of diversity(17) and Shannon's diversity index (18) (Fig. 3A, B). Of note, an aberrant single high peak at 368 bp was emerged in GVHD mice and was identified as *E. coli* derived by DNA sequencing, as previously described(7). The proportion of *E. coli* in the microbiota, which was defined as the ratio of the area of OTU for *E. coli* to the total areas of all OTUs, was significantly higher 14 days after BMT in mice with GVHD and remained higher throughout the entire observation period (Fig. 3C).

### **Discussion**

Intestinal GVHD is a risk for septicemia and is critical for determining the outcome of allogeneic BMT. We recently demonstrated that Paneth cells are targeted by GVHD, resulting in marked reduction in the expression of the major enteric antimicrobial peptides,  $\alpha$ -defensins, a loss of physiological diversity among the microflora with the overwhelming expansion of otherwise rare bacteria, and development of bloodstream infection in mice(7). Subsequently, these findings have been confirmed in humans; Paneth cell loss and dysbiosis are hallmarks of intestinal GVHD(8-12). However, problem is that it is not easy to evaluate Paneth cell numbers in small biopsied specimens or whole composition of intestinal flora in fecal samples. In this study, we demonstrated that fecal levels of  $\alpha$ -defensins were significantly reduced during GVHD and correlated with the intestinal microflora diversity.  $\alpha$ -Defensins shape the composition of the microbiota in the entire intestine. Importantly,  $\alpha$ -defensins have selective bactericidal activity against noncommensals such as *Salmonella enterica*, *E. coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, while exhibiting minimal bactericidal activity against commensals (20, 21). Such bacteria-dependent bactericidal activities of  $\alpha$ -defensins are in tune with intestinal environment and are likely associated with the alterations in the intestinal microbiota in GVHD in the absence of  $\alpha$ -defensins.

Our results suggest that fecal levels of  $\alpha$ -defensins are easy and useful surrogate markers for Paneth cell numbers and intestinal microbial homeostasis. Fecal samples can be noninvasively collected and their levels of  $\alpha$ -defensins can be easily and

quickly determined using our methods. Thus, monitoring of fecal  $\alpha$ -defensins will greatly enhance our understanding of the mutual association between GVHD and intestinal microbiota in human.

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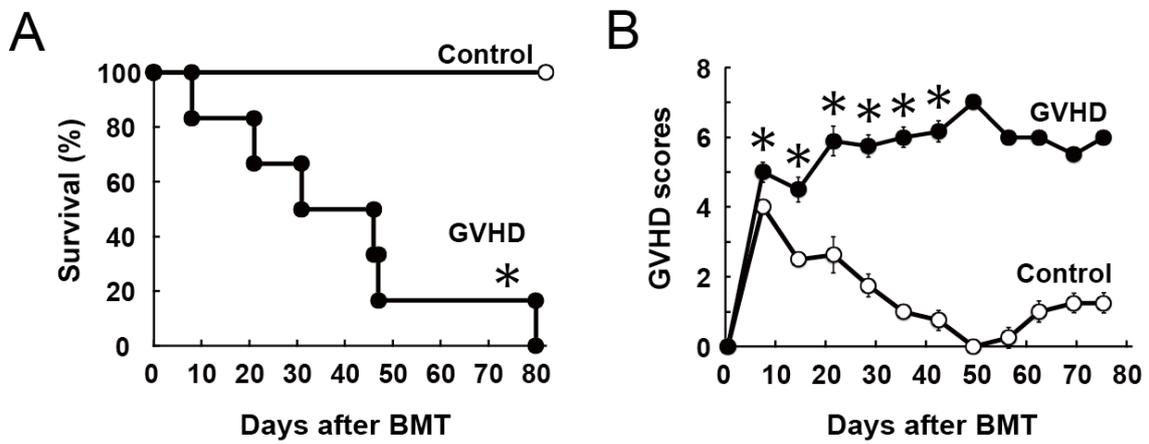
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## Figure Legends

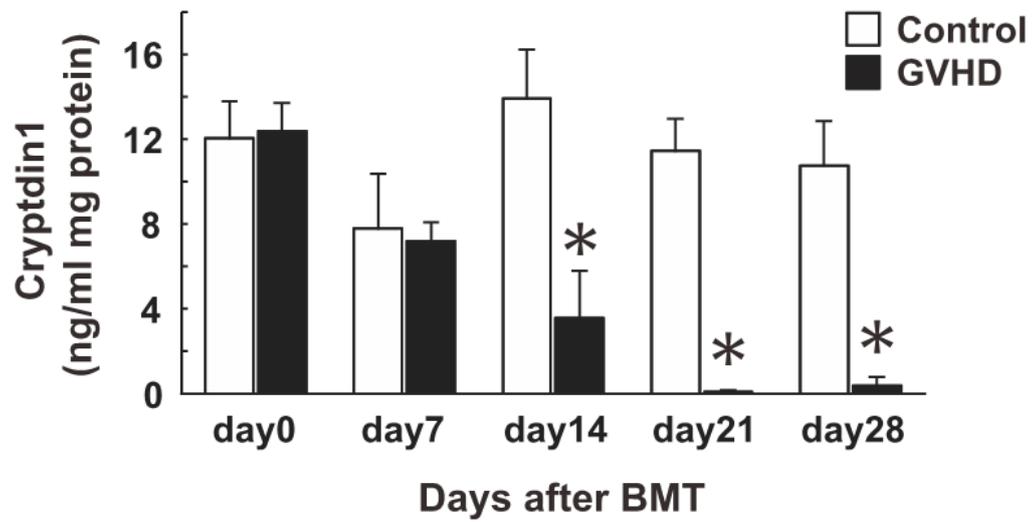
### Figure 1. Mortality and morbidity of GVHD

Lethally irradiated BALB.B mice were transplanted with  $5 \times 10^6$  TCD BM cells without (control group,  $n = 6$ ) or with  $5 \times 10^6$  T cells (GVHD group,  $n = 6$ ) from B6 donors on day 0. (A-B) Survival (A) and clinical GVHD scores (B, means  $\pm$  SE) are shown. \* $P < .05$ .



**Figure 2. Reduced fecal levels of Crp1 in GVHD**

Lethally irradiated BALB.B mice were transplanted with  $5 \times 10^6$  TCD BM cells without (control group, n = 6) or with  $5 \times 10^6$  T cells (GVHD group, n = 6) from B6 donors on day 0. Fecal samples were collected and Crp1 levels were measured. \* $P < .05$ .



### Figure 3. Dysbiosis of the intestinal microflora in GVHD

Fecal pellets were collected before and after BMT weekly and intestinal microbiota was characterized by RFLP analysis of 16S rRNA gene libraries constructed from each sample of fecal pellets and digested with *HhaI* (n=6 / group). (A, B) Time course changes in flora diversity after BMT determined by using Simpson index (A) and Shannon index (B), and (C) those in the proportion of *E. coli*. Data are representative of 3 similar experiments. \* $P < .05$ .

