Influence of Donor Microbiota on the Severity of Experimental Graft-versus-Host-Disease

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The link between microbial flora and the shaping of immune responses is being increasingly appreciated, and recent data have uncovered a role for recipient microbiota in the severity of graft-versus-host disease (GVHD). The impact of donor microbiota on T cell–mediated alloresponses and GVHD is not known, however. Using multiple clinically relevant murine models, we analyzed the effect of donor microbiota on the severity of GVHD induced by T cells from specific pathogen-free and germ-free donors, and found that donor microbiota does not alter the expansion or differentiation of alloreactive T cells or the severity of GVHD.

INTRODUCTION

Development of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation is the major obstacle to the effective use of this potentially curative therapy against many hematologic diseases, including leukemia. The composition of the alloantigens and antigen-presenting cells, inflammatory milieu, age, and several other host-specific factors play important roles in the biology of GVHD. These factors notwithstanding, the frequency of the alloreactive donor T cells and their responses to alloantigens are fundamental factors affecting the induction and severity of GVHD [1].

Countless microbes colonize the intestine and skin and form part of the host microbial ecology. Cross-talk of the microbiota with various immune cell subsets of the host immune system is known to modulate the immune responses and affect autoimmunity, metabolic diseases, and response to pathogens [2,3]. In line with this, previous and recent data have uncovered a role for the host (recipient) microbiome in the severity of GVHD. Studies in mice have shown a reduction of GVHD with gut-decontaminating antibiotics [4] and transplantation under germ-free (GF) conditions [5]. Data have demonstrated shifts in the gut flora in GVHD recipients [6,7]. Furthermore, breaching of the epithelial barriers and translocation of pathogen-associated molecular patterns have been shown to enhance proinflammatory cytokines released from damaged host tissue, enhance donor T cell alloreactivity, and aggravate GVHD [8]. Resident microbiota plays key roles in the development and maturation of T cells. $CD4^+$ T cells from GF donors have been found to cause colitis of similar or greater severity as T cells from conventionally housed donors [9,10]. However, the influence of the cross-talk in microbiota in shaping donor T cell alloresponses and GVHD has not been reported previously. Using well-characterized murine models, we compared the alloreactivity of T cells from GF donors and from conventionally housed specific pathogen-free (SPF) donors to induce GVHD, and found that donor microbiota does not affect the severity of GVHD in hosts.

MATERIALS AND METHODS

Mice

SPF C57BL/6J (SPF-B6; H-2^b) mice were purchased from Jackson Laboratory (Bar Harbor, ME), and BALB/c (H-2^d) and B6D2F1 (H-$2^{b/d}$) mice were purchased from the National Cancer Institute (Frederick, MD). GF C57BL6/J (GF-B6) mice were maintained in a GF mouse facility at the University of Michigan, where they were housed in soft-sided plastic isolators in which they remained free of all bacteria, exogenous virus, fungi, and parasites until just before euthanasia. All animal studies were performed in accordance with protocols approved by the University of Michigan's Committee on the Use and Care of Animals.

Antibodies and Flow Cytometry

Our analyses used FITC-, PE-, PerCP-Cy5.5–, and APC-conjugated mAbs to mouse CD4, CD8a, CD25, H-$2K^b$, H-$2K^d$, FoxP3 (all from eBioscience, San Diego, CA), and anti-mouse TCR-β (BD Pharmingen, San Diego, CA). Flow cytometry was performed as described previously [11], and the cells were analyzed on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA).

Bone Marrow Transplantation

Bone marrow (BM) transplantations (BMTs) were performed as described previously [12]. In brief, recipient mice were irradiated (^{137}Cs source) with 8 Gy total body irradiation (BALB/c recipients) or 11 Gy total body irradiation (B6D2F1 recipients) 1 day before BMT. BM cells were harvested from femurs and tibias of 7- to 8-week-old SPF-B6 mice and were depleted of T cells using MACS CD90.2 microbeads and an LS column (Miltenyi Biotec, Auburn, CA). T cells were harvested from the spleens of 7- to 8-week-old SPF-B6 or GF-B6 mice. GF-B6 donor mice were euthanized immediately after being transferred into the SPF environment, after which T cells were isolated. BM cells and T cells were transplanted into allogeneic experimental mice housed in sterilized microisolator cages under SPF conditions and maintained on acidified water (pH < 3) for 3 weeks [13]. Survival was monitored daily, and clinical GVHD was assessed weekly [14].

Antibiotic Treatment

SPF C57BL/6J mice were provided with a cocktail of broad-spectrum oral antibiotics, including metronidazole 0.5 mg/mL, neomycin 0.5 mg/mL, ampicillin 0.5 mg/mL (Sigma-Aldrich, St. Louis, MO), and vancomycin 0.5 mg/mL, along with Splenda (McNeil Nutritional, Fort Washington, PA), in autoclaved drinking water for 10-14 days before use as a source of donor T cells.

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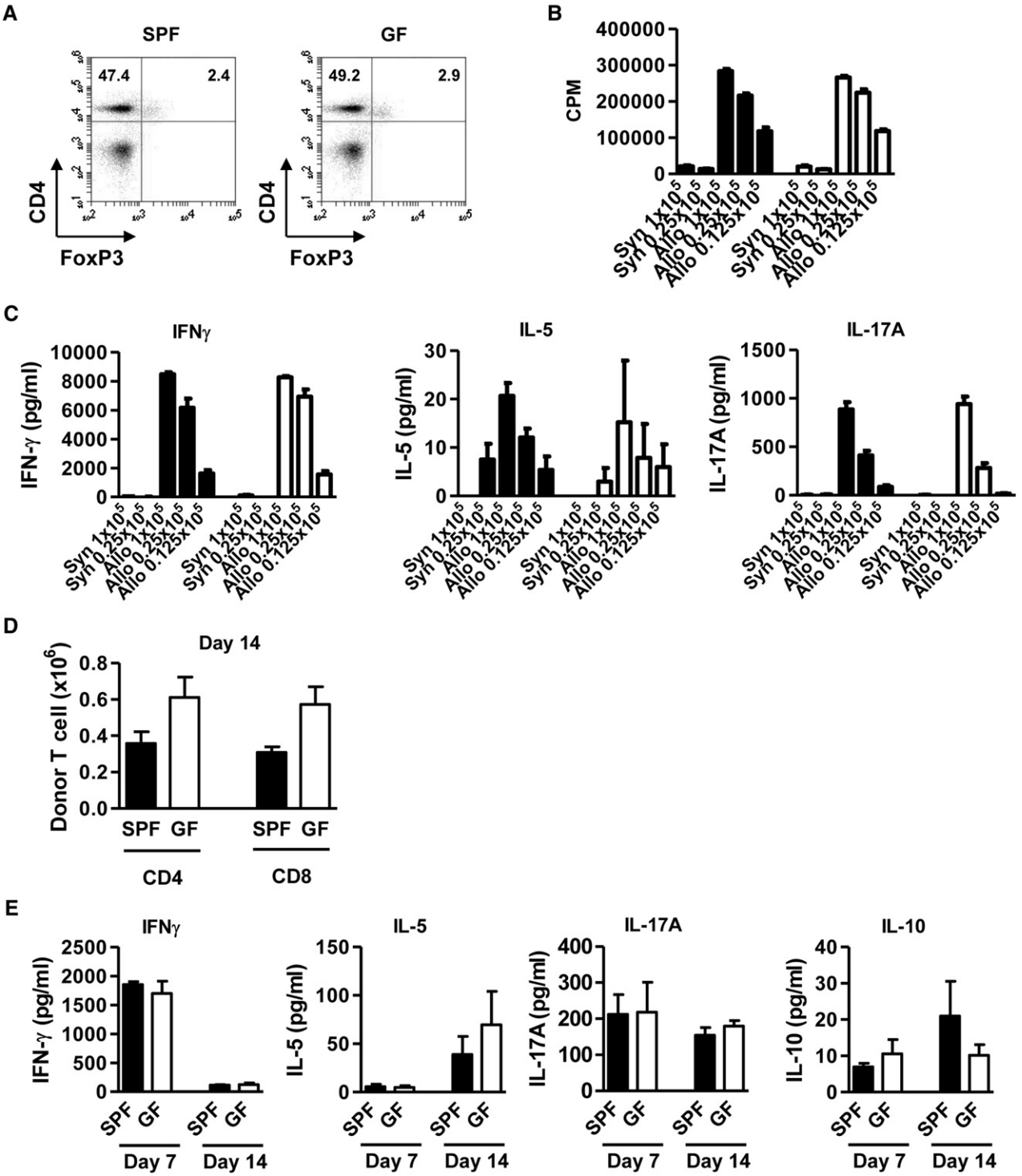

Figure 1. GF mouse T cells respond against allogeneic stimulator, similar to SPF mouse T cells in vitro and in vivo. (A) $CD90^+$ T cells were isolated from SPF-B6 mice (left) or GF-B6 mice (right), and frequencies of $CD4^+FoxP3^+$ regulatory T cells were analyzed by flow cytometry. (B) Isolated $CD90^+$ T cells from SPF-B6 (filled bar) or GF-B6 (open bar) mice were plated at 4×10^5/well in a 96-well flat-bottomed plate and cocultured with different numbers of syngeneic B6 or allogeneic BALB/c irradiated (30 Gy) spleen cells for 72 hours. ^{3}H-thymidine (1 μCi/well) incorporation during last 6 hours of culture was measured. (C) Cytokine levels in the supernatant of the 66-hour culture was determined by ELISA. (D and E) B6D2F1 recipients were irradiated (11 Gy) on day -1 and injected with allogeneic 5×10^6 SPF-B6 TCDBM cells plus 2×10^6 SPF-B6 $CD90^+$ T cells (solid bar; n = 3 for each time point) or allogeneic 5×10^6 SPF-B6 TCDBM cells plus 2×10^6 GF-B6 $CD90^+$ T cells (open bar; n = 3 for each time point). Spleen cells and sera were collected from recipients on day 7 or day 14. (D) Spleen cells were counted; stained with anti–H-$2K^d$, -CD4, and -CD8 mAbs; and analyzed by flow cytometry. Donor CD4 and CD8 T cell expansion was determined based on spleen cell count and the percentage of CD4 and CD8 positivity in gated ($H\text{-}2Kd^-$) cells. (E) Cytokine levels in the sera were determined by ELISA.

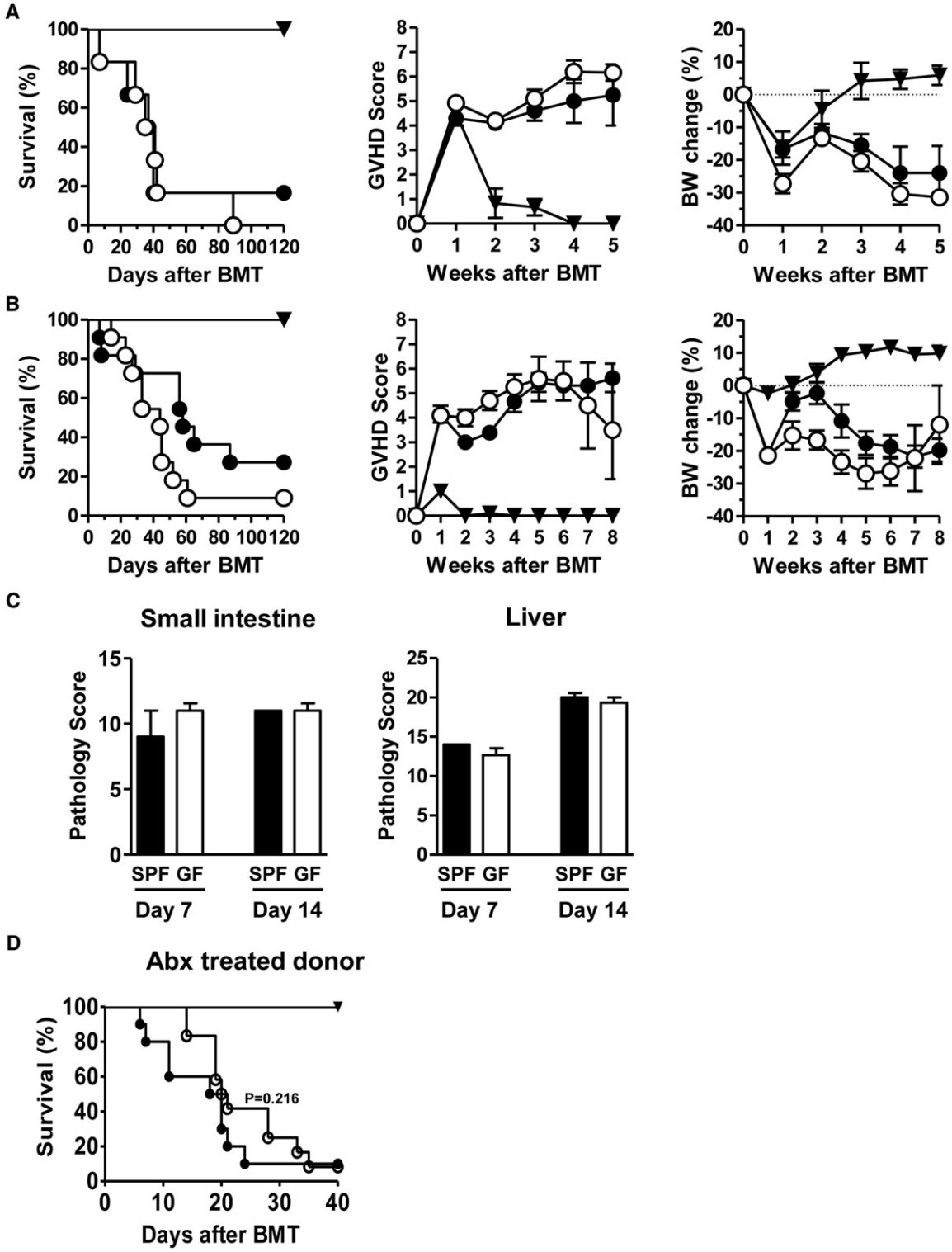

Figure 2. GF mouse T cells and SPF mouse T cells induce GVHD to a similar extent. (A) BALB/c recipients were irradiated (8 Gy) on day -1 and injected with syngeneic 5×10^6 BALB/c BM cells (▼; n = 3), allogeneic 5×10^6 SPF C57BL/6J (SPF-B6) TCDBM cells plus 5×10^5 SPF-B6 CD90$^+$ T cells (●; n = 6), or allogeneic 5×10^6 SPF-B6 TCDBM cells plus 5×10^5 GF C57BL/6J (GF-B6) CD90$^+$ T cells (○; n = 6). Survival was monitored daily, and GVHD clinical score and body weight change were monitored weekly. (B) B6D2F1 recipients were irradiated (11 Gy) on day -1 and injected with syngeneic 5×10^6 B6D2F1 BM cells (▼; n = 5), allogeneic 5×10^6 SPF-B6 TCDBM cells plus 2×10^6 SPF-B6 CD90$^+$ T cells (●; n = 11), or allogeneic 5×10^6 SPF-B6 TCDBM cells plus 2×10^6 GF-B6 CD90$^+$ T cells (○; n = 11). Survival was monitored daily, and GVHD clinical score and body weight change were monitored weekly. (C) B6D2F1 recipients were irradiated (11 Gy) on day -1 and injected with allogeneic 5×10^6 SPF-B6 TCDBM cells plus 2×10^6 SPF-B6 CD90$^+$ T cells (solid bar; n = 3 for each time point) or allogeneic 5×10^6 SPF-B6 TCDBM cells plus 2×10^6 GF-B6 CD90$^+$ T cells (open bar; n = 3 for each time point). GVHD target tissue (small intestine and liver) were collected from recipients on day 7 or day 14. Histopathological scores were determined for the small intestine (left) and liver (right). (D) Antibiotic treatment of donors does not mitigate GVHD in the recipients. B6 donor mice were treated with a cocktail of oral antibiotics (open circles; n = 10) or autoclaved water alone (solid circles; n = 11), as described in Materials and Methods. The donor T cells were harvested 10-14 days later and transplanted into allogeneic BALB/c or syngeneic B6 animals (inverted triangle) after conditioning with 10 Gy along with TCDBM from SPF B6 donors. *P* = not significant. ○ versus ●. Combined data from 2 similar experiments are shown.

In Vitro Proliferation Assay

$CD90^+$ T cells were isolated from spleen cells of SPF-B6 and GF-B6 mice (4×10^5) and incubated with syngeneic (C57BL/6J) or allogeneic (BALB/c) irradiated (30 Gy) spleen cells for 72 h. Proliferation was measured by the incorporation of ^{3}H-thymidine (1 μCi/well).

In Vivo Donor T Cell Expansion

B6D2F1 recipient mice were irradiated and transplanted with SPF-B6 T cell–depleted BM (TCDBM) and 2×10^6 $CD90^+$ T cells from either SPF-B6 or GF-B6 mice. On day 14 post-BMT, spleen cells were harvested and analyzed for donor T cells ($H\text{-}2K^{d-}$) by flow cytometry.

Cytokine Analysis

Levels of IFN-γ, IL-5, IL-17A, and IL-10 in sera and culture supernatant were determined by ELISA (BD Pharmingen) in accordance with the manufacturer's instructions.

Histology

Formalin-preserved gut and liver were embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin for histological examination. Slides were coded and examined in a blinded fashion by a pathologist (Chen Liu). A semiquantitative scoring system was used to assess for the abnormalities known to be associated with GVHD [13].

Statistical Analysis

The Student *t* test was used for statistical analysis of in vitro data, and the log-rank test was used for analysis of survival data. A *P* value $<.05$ was considered statistically significant.

RESULTS AND DISCUSSION

We first evaluated the in vitro alloresponses and regulatory T cell (Treg) frequencies of T cells harvested from the spleens of GF mice. The $CD90^+$ T cells from SPF-B6 and GF-B6 mice were analyzed for the frequency of $CD4^+FoxP3^+$ Tregs. The frequency of $Foxp3^+$ Tregs in the $CD90^+$ T cell fraction was similar in SPF-B6 and GF-B6 mice (Figure 1A). We next analyzed the alloresponses of the T cells in vitro with mixed leukocyte reaction (MLR) against allogeneic BALB/c irradiated spleen cells at different responder/stimulator ratios. SPF-B6 and GF-B6 T cells demonstrated equivalent proliferation, as well as similar levels of IFN-γ, IL-5, and IL-17A (signature cytokines for Th1, Th2, and Th17) in the supernatants (Figure 1B and C).

We next evaluated the in vivo alloresponses of SPF-B6 and GF-B6 T cells. Using an MHC-mismatched B6→B6D2F1 model, we performed allogeneic BMT with donor T cells from either GF-B6 or SPF-B6 animals. The recipient sera and spleens were harvested on day 7 or day 14 after BMT and analyzed for allogeneic T cell expansion and differentiation. Donor T cell expansion was similar in the allogeneic recipients for both CD4 ($P = .1220$) and CD8 ($P = .0594$) subsets (Figure 1D). Serum levels of IFN-γ, IL-5, IL-17A, and IL-10 (Th1, Th2, Th17, and Tr1) cytokines were also equivalent (Figure 1E). These data indicate that the absence of cross-talk with microbial flora in donors does not alter T cell alloreactivity in vitro and in vivo.

To further determine the functional and clinical relevance of the microbiome on donor T cell alloreactivity, we analyzed the severity of GVHD induced by T cells isolated from SPF-B6 mice and GF-B6 mice using well-established MHC-mismatched B6→BALB/c GVHD model. We transplanted 5×10^5 T cells from either SPF-B6 or GF-B6 donors along with 5×10^6 SPF-B6 TCDBM. The clinical severity and mortality from GVHD was similar in the 2 groups ($P = .95$; Figure 2A). To rule out potential strain- and model-dependent artifacts, we analyzed the induction of GVHD in a second well-established B6→B6D2F1 model. We found no statistically significant difference in GVHD-mediated mortality between the SPF-B6 and the GF-B6 allorecipient mice ($P = .1061$; Figure 2B). To further examine whether the absence of donor microbiota might affect the ability of donor T cells to cause differential GVHD target organ damage, we analyzed GVHD-specific histopathology scores of small intestine and liver from allogeneic B6D2F1 recipients on day 7 or day 14 after BMT (Figure 2C), and found no significant difference in GVHD severity. We next analyzed whether treatment of SPF donor mice with a cocktail of broad-spectrum oral antibiotics known to reduce and alter gut microbial flora would alter donor T cell–mediated GVHD mortality in the recipient animals [15,16]. Treatment of B6 donor mice with broad-spectrum antibiotics induced GVHD mortality at a rate similar to that seen in control SPF donor mice. These data suggest that reducing and altering the microbial flora in the donors had no affect on their T cell alloreactivity and induction of GVHD after allogeneic BMT.

Emerging data demonstrate an important role for recipient microbiota on the outcome of GVHD following allogeneic BMT from conventional donors. The role of donor microbiota on the induction of alloreactivity and GVHD in conventional recipients had not been studied previously, however. We found similar magnitudes of proliferation, differentiation, and GVHD in T cells from GF donors and T cells from conventional or SPF mice. These data demonstrate that in contrast to host microbiota, donor microbial flora do not affect the induction and severity of GVHD. Our data are also consistent with the idea that after adoptive transfer, the T cells from GF mice can induce pathogenic autoimmune responses, such as colitis, and collectively suggest either that donor microbial status does not affect T cell response or that once transferred into conventional settings, the previous lack of interaction with microbiota does not influence subsequent responses [10].

Our donor mice were housed under stringent GF conditions and fed normal chow; however, the potential lipopolysaccharide content of the diet was not controlled for. Hrncir et al. [17] reported no significant impact on systemic immune phenotype regardless of lipopolysaccharide contamination of chow in GF mice. It is possible that specific components of or type of microbiota dysbiosis in donors might have differential effects on the ability of donor T cells to respond to alloantigens. As such, it is important to note that our investigation did not address the impact of any unique microbial genus on T cells in causing GVHD, but instead analyzed the impact of a lack of all microbial flora in the donors.

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Authorship Statement: Isao Tawara designed and performed research, analyzed data, and wrote the manuscript; Chen Liu performed research and analyzed data; Hiroya Tamaki performed research; Tomomi Toubai performed research; Yaping Sun performed research; Rebecca Evers performed research; Evelyn Nieves performed research; Nathan Mathewson performed research; Gabriel Nunez provided intellectual input and contributed new reagents and mice; and Pavan Reddy conceived the study, designed experiments, analyzed data, and wrote the manuscript.

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