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Murine Syngeneic Graft-Versus-Host Disease Is Responsive to Broad-Spectrum Antibiotic Therapy

J. Anthony Brandon,* C. Darrell Jennings,†‡ Alan M. Kaplan,*‡ and J. Scott Bryson*‡§

Murine syngeneic graft-versus-host disease (SGVHD) initiates colon and liver inflammation following lethal irradiation, reconstitution with syngeneic bone marrow transplantation, and therapy with the immunosuppressive agent cyclosporine A. Previous studies have demonstrated that the inducible disease is mediated by CD4+ T cells with increased reactivity of peripheral and liver-associated lymphocytes against intestinal microbial Ags. In the current report, studies were performed to analyze the specificity of the CD4+ T cell response of T cells isolated from diseased animals and to determine the in vivo role of the microbiota to the development of SGVHD. Increased major histocompatibility Ag (MHC) class II-restricted responsiveness of SGVHD CD4+ T cells against microbial Ags isolated from the ceca of normal animals was observed. The enhanced proliferative response was observed in the CD62L− memory population of CD4+ T cells. To determine the role of the bacterial microbiota in the development of murine SGVHD, control and CsA-treated bone marrow transplantation animals were treated with broad-spectrum antibiotics (metronidazole, ciprofloxacin) after transplantation. Cyclosporine A-treated animals that were given antibiotic therapy failed to develop clinical symptoms and pathological lesions in the target tissues characteristic of SGVHD. Furthermore, the reduction in intestinal bacteria resulted in the elimination of the enhanced antimicrobial CD4+ T cell response and significantly reduced levels of the inflammatory cytokines, IFN-γ, IL-17, and TNF-α. The elimination of the disease-associated inflammatory immune responses and pathology by treatment with broad-spectrum antibiotics definitively links the role of the microbiota and microbial-specific immunity to the development of murine SGVHD. The Journal of Immunology, 2011, 186: 3726–3734.

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reatment of lethally irradiated rodents with CsA following syngeneic or autologous bone marrow (BM) transplantation (BMT) resulted in the development of a disease with target tissue range that was similar to graft-versus-host disease (GVHD) with inflammation occurring in the intestinal tract, liver, and skin (1–6). This syndrome was termed syngeneic GVHD (SGVHD) (1). Three conditions were shown to be required for the induction and development of SGVHD, as follows: the recipient animals had to have a thymus, they had to be irradiated prior to transplantation, and the recipients had to be given a posttransplant regimen of cyclosporine A (CsA) therapy (1, 2). It was shown in the rat and mouse that T cells are present in BM and periphery that could alter the development of CsA-induced SGVHD following syngeneic BMT. Initial studies done in the rat model of SGVHD demonstrated that CD4+ and CD8+ T cells present in the periphery and BM could inhibit the induction and adoptive transfer of SGVHD (7, 8), and more recently, CD4+CD25+ regulatory T cells were shown to modulate the resolution of rat SGVHD (9). In the mouse, Thy1+ BM cells were shown to regulate the induction of SGVHD in noninducible strains of animals (6, 10). The inducible disease develops within 2–3 wk of cessation of CsA therapy, and in the mouse clinical symptoms (weight loss, diarrhea) are evident in 70–100% of treated BMT animals (4, 11, 12).

Previous studies have demonstrated that a dichotomy exists between the rat and mouse models of CsA-induced SGVHD in relation to the phenotype and potential specificity of the effector cells that mediate disease. The rat model of SGVHD has been used to study the development of self-reactive immune responses. CD8+ effector T cells have been shown to mediate the induction and development of rat SGVHD (2, 13, 14), with CTLs specific for the CLIP being shown to be the predominant effector cell (15, 16). Where autoreactive CD8+ T cells are responsible for disease induction in the rat (1, 14, 15), CD4+ T cells were shown to be the major effector cell in murine SGVHD (17–19). Histologic analysis of tissue samples from SGVHD mice demonstrated lymphocytic infiltration of the target organs, with increases in CD4+ T cells being observed in the intestinal lesions (17). Similarly, increased numbers of intraepithelial and lamina propria CD4+ T lymphocytes were present in the colons of SGVHD versus control BMT mice (17–19). Based on these studies, it was shown by in vivo depletion and adoptive transfer studies that CD4+, but not CD8+ T cells played a dominant role in the development of murine SGVHD-associated colon and liver inflammation (17, 19).

During the development of murine SGVHD, animals develop a colitis-like disease (11, 20, 21) that is indistinguishable from other mouse models of inflammatory bowel disease (IBD) in relation to colon pathology, CD4+ effector T cell phenotype, and Th cell cytokine phenotype (reviewed in Refs. 22, 23). It has been shown that CD4+ T cells are the predominant effector cell in most
murine IBD models (22). IBD is thought to occur as a result of unregulated immune responsiveness against intestinal bacteria. In fact, the development of colitis in many of the murine models of IBD results from the unregulated response to intestinal microbiota. It was shown that in vitro responsiveness against microbial Ags isolated from fecal extracts of normal animals was increased in CD4⁺ T cells isolated from colitic animals (24–27). Naive CD4⁺ T cells reactive against gut microflora were also found in the periphery of normal mice (28, 29). These cells were found to be regulated in normal animals by cells with a regulatory T cell phenotype (CD4⁺CD25⁺) (29). Furthermore, long-term antimicrobial Ag-reactive T cell lines and clones were shown to transfer colitis into secondary recipient mice (30, 31).

Whereas the pathology and effector cell phenotype are similar between murine SGVHD and IBD models, the specificity of the effector cells responsible for murine SGVHD induction has not been determined. We have previously demonstrated that T cells from the periphery (19) and liver-associated mononuclear cells from SGVHD mice (21) did not respond against normal syngeneic APC in an autoreactive response, but displayed enhanced proliferative responses against microbial Ags. As murine SGVHD presents with similar immune pathology to murine IBD models, studies were undertaken to further characterize the reactivity of CD4⁺ T cells from diseased animals and to determine the role that the microbiota might play in the SGVHD model. CD4⁺ T cells from SGVHD animals exhibited enhanced, MHC class II-restricted proliferation against syngeneic APC from normal mice that were pulsed with a cecal Ag preparation when compared with T cells isolated from BMT control mice. These cells did not respond against unpulsed syngeneic APC or APC pulsed with food, colonial epithelial, or nominal protein Ags. Importantly, SGVHD was not inducible in BMT, CsA-treated animals in which the colonial microbiota had been reduced with broad-spectrum antibiotics. The development of this inducible disease.

Materials and Methods

Mice

Female C3H/HeN mice for use as BM donors or recipient mice were purchased from Harlan (Indianapolis, IN) at 19–21 d of age and were used within 1 wk of arrival. Animals were housed in sterile microisolator cages (Lab Products, Maywood, NJ) and were fed autoclaved food and acidified water ad libitum. All animal protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Induction of SGVHD

BM was isolated from the femurs and tibias of syngeneic age-matched mice. The donor BM suspensions were prepared in RPMI 1640 (Cellgro, Herndon, VA) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μM glutamine (Life Technologies, Grand Island, NY). The BM cell suspension was depleted of Thy-1⁺ cells (ATBM) using anti-Thy1.2 (HO-13-4) and Low Tox M rabbit complement (Cedarlane Laboratories, Westbury, NY), as previously described (6). Recipient mice were lethally irradiated (900 rad) in a Mark I 137Cs irradiator (J.L. Shepard and Associates, Glendale, CA). Following irradiation, the animals were reconstituted i.v. with 5 × 10⁶ ATBM 4–6 h after conditioning. Beginning on the day of BMT, the mice were treated daily i.p. with 15 mg/kg/day of CsA or the diluent olive oil (Sigma-Aldrich, St. Louis, MO) for 21 d. Upon cessation of CsA therapy, the BMT control and CsA-treated animals were weighed three times per week and monitored for the development of clinical symptoms of SGVHD (weight loss, diarrhea). Animals that developed clinical symptoms for three consecutive weighings were considered positive for the induction of SGVHD. Clinical symptoms were typically observed by 2–3 wk after the end of CsA therapy.

Isolation of peripheral lymphoid cells

At 2–4 wk after cessation of CsA therapy, BMT control and CsA-treated SGVHD mice exhibiting clinical symptoms of disease (typically 80–100% of CsA-treated animals) were euthanized, and the spleen and mesenteric lymph nodes (MLN) were removed and placed into RPMI 1640 containing penicillin/streptomycin/glutamine and 5% FBS (Atlanta Biologicals, Norcross, GA). Single-cell suspensions were prepared from pooled spleen and MLN within each group, and the RBC were lysed by treatment with 0.83% Tris-buffered NH₄Cl. The donor cell suspensions were placed into aliquots for flow cytometric analysis (see below). The spleen and MLN cells were then pooled together within each treatment group. CD4⁺ T cell subsets were positively selected utilizing MACS magnetic beads with the cells being selected on an AutoMACS system (Miltenyi Biotec, Auburn, CA). The purity of the MACS-selected donor cells was monitored by flow cytometry and was found to be >95% CD4⁺ for T cells.

Ag preparations

A cecal Ag (CeAg) preparation was prepared according to the procedure described by Cong et al. (24). Briefly, the ceca were removed from animals and placed into a petri dish containing PBS. The cecal contents were removed and collected by low-speed centrifugation. The contents were resuspended in PBS containing DNase 1 (10 μg/ml; Sigma-Aldrich) and glass beads, equal to one-fifth the volume of the material in the tube, were added to the cecal content preparation. The preparation was sonicated for 5 min (15 s on, 15 s off) in a Fisher 550 Dismembrator (Thermo Fisher, Waltham, MA). After sonication, the Ag preparation was centrifuged for 15 min at 10,000 rpm in a Fisher accuSpin microcentrifuge (Thermo Fisher) to remove debris. A food Ag preparation was prepared by dissolving normal mouse chow into PBS, sonication, as described above, and the particulate material was removed by centrifugation. To prepare a colonial epithelial cell Ag preparation, a lysate from the C3H colonial epithelial cell line MODE-K (32) (provided by D. Cohen, University of Kentucky), the cells were washed in PBS and suspended in 5 mM MgCl₂ with 2 mM PMSF and 10 mM Tris. The cells were then lysed by freeze thawing in dry ice/ethanol bath. The lysate was spun at 14,000 rpm for 30 min, and the supernatant was collected. Finally, purified OVA was used as a nominal Ag (Sigma-Aldrich, St. Louis, MO). All Ag preparations were filter sterilized, and the protein concentration was determined by Bio-Rad assay.

Generation of BM-derived dendritic cells

BM cells were isolated from C3H/HeN mice. RBCs were removed by treatment with Tris-buffered NH₄Cl. Following removal of RBC, the BM cells were cultured in 5% complete RPMI 1640 media (5% FCS, 1% penicillin/streptomycin/glutamine, 25 mM 2-ME) and 20 ng/ml murine rGM-CSF (PeproTech, Rocky Hill, NJ). The nonadherent cells were removed on days 3 and 5, and GM-CSF–containing media was replaced. The cells were harvested between days 8 and 10 and were used as APC. Greater than 90% of these cells were CD11c⁺.

Proliferation assay

To determine the proliferative capacity of CD4⁺ T cells from control or SGVHD mice or long-term CD4⁺ T cell lines isolated 8–10 d after in vitro stimulation, 1–2 × 10⁶ T cells were placed in 10% complete RPMI 1640 media and were cultured in 96-well flat-bottom microtiter plates with 2 × 10⁵ irradiated splenic APC or 1 × 10⁶ dendritic cells (DC). Ag-pulsed C3H/HeN splenic APC (2 × 10⁵/ml) or DC (1–2 × 10⁵/ml) were incubated with the various Ags overnight, washed, placed into 10% complete RPMI 1640 media, and irradiated with 2000 cGy γ irradiation. As a control, syngeneic APC were treated with LPS or polysorbolic-polyacytidyl acid (pLc) overnight, as described above. In some experiments, control or Abs against MHC class II were added to the cultures. Proliferation was measured by the addition of [³H]thymidine during the last 18 h of a 96-h culture.

Intracellular cytokine analysis

Lymphoid cells were isolated from the MLN of donor mice and activated for 8 h with anti-CD3 (1:5000 dilution of 2C11 ascites). During the last 4 h of culture, 1 μg/ml monensin was added to the cultures to block secretion of cytokines. The cells were harvested and placed into staining buffer (PBS containing 1% FBS, 0.1% NaN₃). To minimize nonspecific staining, the cells were incubated with Ab against CD11b/CD32 (2.4G2, Fc block; BD Pharmingen, San Diego, CA). The cells were then surface stained with CD4 Ab (RM-4-5; Caltag, Burlingame, CA). After washing, the cells were permeabilized and incubated with anti-IL-17 (eBioTC11), IFN-γ (XMG1.2), and TNF-α (MP6-XT22) Ab, according to manufacturer’s directions (eBioscience, San Diego, CA), and then analyzed by flow
cytometry. In some experiments, after isolation of CD4+ T cells, the cells were stained with labeled anti-CD62L mAb (BD Pharmingen) and then sorted using a MoFlo cell sorter into CD62L+ and CD62L- populations. The purity of these populations was >98% pure.

Antibiotic treatment after BMT

SGVHD was induced, as described above. Beginning 7 d after transplantation, BMT control or CsA-treated recipient mice were given 0.660 mg/ml ciprofloxacin (Amresco, Solon, OH) and 2.5 mg/ml metronidazole (Baxter Healthcare, Deerfield, IL) in drinking containing 20 mg/ml sugar-sweetened grape Kool Aid mix (Kraft Foods) (27). The sweetened antibiotic drinking water was sterilized through a 0.22-μm filter and was replaced two to three times/week, and the amount of water consumed was monitored. The animals remained on the treated drinking water throughout the course of the experiment. Beginning on the last day of CsA therapy, BMT control and CsA-treated mice were monitored for clinical symptoms of SGVHD, as described.

Histological analysis of SGVHD

Colon and liver samples were obtained from control and antibiotic-treated animals at 4–5 wk after cessation of CsA. The tissues were fixed in 10% buffered formalin and embedded into paraffin, and 4- to 6-μm sections were cut and mounted onto a glass slide. All slides were stained with a standard H&E protocol and were graded blindly without the knowledge of treatment group, according to a previously published grading scale (33), as follows: colon, 1/2, rare crypt cell necrosis; 1, definite scattered single-cell necrosis in crypts; 2, several necrotic cells in gland, crypt abscesses present; 3, confluent destruction of glands; 4, loss of mucosa with formation of granulation tissue and pseudomembrane; liver, 1/2, minimal lymphocyte infiltrate in portal area, rare bile duct epithelial cell degeneration; 1, sparse, but definite portal lymphocyte infiltrate; occasional necrotic bile duct epithelial cell; 2, diffuse infiltrate in portal area and invasion of bile ducts by inflammatory cells; 3, heavy infiltrate partially obscuring bile ducts and focal bile duct destruction; 4, all of the above plus secondary changes in hepatocytes, bile stasis, hepatocyte necrosis, and disordered architecture.

Analysis of plasma from SGVHD animals

Plasma levels of alanine aminotransferase (ALT) were performed, according to manufacturer’s recommendations. Briefly, 100 μl serum was mixed with 1 ml 37°C prewarmed ALT reagent (Pointe Scientific, Canton, MI) and then incubated at 37°C for 1 min before the absorbance at 340 nm was read. An additional two absorbance readings (340 nm) were taken 1 min apart, with the sample being incubated at 37°C between readings. The ALT concentration (IU/l) was calculated by multiplying the average absorbance difference per minute by the factor 1708. Cytokine analysis of plasma was performed using IL-17, IFN-γ, and TNF-α via ELISA kits, as per manufacturer’s instructions (Ready-SET-Go; eBioscience).

Statistical analysis

Statistical differences between groups were determined using one-way ANOVA. Induction of SGVHD was monitored by Kaplan-Meier method and log rank test or Fisher’s exact test. Differences ≤0.05 were considered statistically different.

Results

Characterization of the proliferative response of CD4+ T cells isolated from SGVHD mice

It had been shown that CD4+ T cells reactive against bacterial Ags were responsible for the development of colitis in several murine models of IBD (24–26), and preliminary studies demonstrated that increased numbers of enteric bacterial Ag-reactive T cells were present in SGVHD versus BMT control animals (19, 21). Given the role of CD4+ T cells in the SGVHD model (17, 19), as well as in various models of IBD, studies were performed to further characterize the in vitro responsiveness of CD4+ T cells isolated from SGVHD mice.

CD4+ T cells isolated from diseased animals had an increased proliferative response against CeAg-pulsed APC when compared with T cells isolated from transplant control animals (Fig 1). The antimicrobial Ag T cell proliferative response was observed when the spleen cells from normal syngeneic animals were pulsed over a range of 50–400 μg/ml CeAg (Fig. 1A) obtained from syngeneic C3H/HeN animals. An Ag dose response was observed consistently over the range of 50–200 μg/ml. Because pulsing the APC with 200 μg/ml gave consistent results with less Ag relative to the upper levels tested, it was used for the remainder of the experiments presented in this manuscript. In addition to the use of primary CD4+ T cells from BMT control and SGVHD animals, multiple long-term CD4+ T cell lines were generated from diseased animals by repeated stimulation with CeAg-pulsed APC. These cell lines exhibited similar proliferative responses to those observed with primary T cells (Supplemental Fig. 1).

After pulsing with CeAg overnight, DC expressed increased levels of MHC class II and costimulatory molecules (data not shown). The maturation/activation of the DC was most likely due to ligation of TLR on the APC with pathogen-associated molecular patterns that are present in the CeAg (microbial Ag) preparation. To ensure that the increased proliferative response was not due to the activation of the Ag-pulsed DC by TLR, the proliferation of control and SGVHD CD4+ T cells against DC that were treated with two TLR ligands, LPS (TLR4) and pI:C (TLR2), was measured. Whereas increased proliferation was observed against CeAg-pulsed APC, overnight treatment/activation of DC with the TLR ligands LPS and pI:C did not induce proliferation (Fig. 1B).

To further analyze the Ag specificity of the responding CD4+ T cells from SGVHD mice, the cells were cultured with DC pulsed with

![FIGURE 1.](http://www.jimmunol.org/)

Enhanced antimicrobial Ag responsiveness of CD4+ T cells isolated from SGVHD animals. C3H/HeN mice were irradiated, reconstituted with syngeneic BM, and injected daily for 21 d with olive oil or CsA (15 mg/kg/day). Animals were monitored for clinical symptoms of SGVHD, weight loss, and/or diarrhea. A, CD4+ T cells were isolated from BMT control and SGVHD mice 2–4 wk after cessation of CsA therapy. APC were pulsed with varying concentrations of CeAg (data representative of four experiments) or 200 μg Ag preparations derived from normal mouse chow or the syngeneic colon epithelial line MODE-K (representative of two experiments). The APC were irradiated and placed into culture with CD4+ T cells in 96-well flat-bottom microtiter plates, and proliferation was measured by the addition of [3]Hthymidine during the last 18 h of a 96-h culture. *Statistically different from control CD4 T cells cultured with same DC source, p < 0.001. B, CD4+ T cells were isolated from control BMT or SGVHD animals, and were stimulated with APC that were pulsed overnight with 200 μg CeAg, 10 μg LPS, or 100 μg pI:C. Proliferation was determined in a 96-h assay. Data presented are representative of two experiments for each Ag source. Statistical significance was determined by one-way ANOVA.
Ag preparations prepared from normal laboratory chow, a murine colon epithelial cell tumor line (MODE-K), or the nominal protein Ag, OVA. As shown in Fig. 1A, a minimal response was observed against DC that were pulsed with food or epithelial cell Ag, which was significantly less than that observed when SGVHD T cells were stimulated with CeAg-pulsed APC. SGVHD T cells did not respond to the nominal protein Ag OVA (data not shown). Finally, the possibility existed that the CeAg preparation could act as a superAg resulting in nonspecific activation of the CD4+ effector cells. Two pieces of information suggest that this is not the case. First, if the Ag preparation was acting as a superantigen, similar responses should be observed between CD4+ T cells isolated from control and diseased animals. Such is the case when BMT control T cells and SGVHD T cells are stimulated with a polyclonal activator such as anti-CD3 (data not shown). Secondly, when the Ag preparation was added at the time of initiation of culture of long-term CeAg T cell lines from SGVHD mice and unpulsed DC, no increase in proliferation of SGVHD T cells above those cultured with DC alone was observed (Supplemental Fig. 1A). These results demonstrated that the enhanced proliferative response of primary SGVHD T cells and long-term SGVHD T cell lines was specific for an undefined microbial Ag that was present in the CeAg preparation. Finally, the reduced autoreactive response against self Ags presented by normal syngeneic spleen or BM-derived DC suggested that few, if any, autoreactive T cells were present in the cells from SGVHD animals.

It was expected that the enhanced SGVHD CD4+ T cell proliferative response would be restricted via MHC class II presentation of microbial Ags due to the restriction pattern between CD4+ T cells and MHC class II Ag. However, as the autoreactive T cell response in rat SGVHD is mediated by CD8+ T cells that respond to self Ag presented by MHC class II Ag (14, 15, 34), we analyzed the MHC restriction of this T cell proliferative response. To determine whether this response exhibited MHC class II restriction, CD4+ T cells from control and SGVHD mice were placed into culture with CeAg-pulsed DC in the presence of anti-class II mAbs (class II), control rat IgG, or a mAb specific for an irrelevant mouse class II haplotype (I-A<sup>β</sup>). The addition of a pan-specific anti-class II mAb (Ms/114) or the addition of anti-I–A<sup>β</sup> (10-2-16) and anti-I–E<sup>α</sup> (14-4-4S) to the cultures inhibited the proliferation of SGVHD T cells (Fig. 2). Conversely, the addition of an anti-class II mAb with a specificity for a different MHC class II haplotype did not affect the proliferative response. Similar results were also observed with the long-term CD4+ T cell lines isolated from SGVHD mice (Supplemental Fig. 1B). These results demonstrated that, as expected, the anti-CeAg proliferative response was restricted to the MHC class II Ia<sup>β</sup> molecules.

In vitro SGVHD CeAg-specific response was restricted to the CD4+CD62L<sup>−</sup> subset

We had previously demonstrated that increased percentages (>75%) of the CD4+ T cells isolated from the periphery of SGVHD were activated when compared with control animals and had lost expression of the CD62L marker (17, 19). We therefore wanted to determine which subset of the CD4+ T cells from SGVHD mice, based on CD62L expression, responded to CeAg-pulsed DC in vitro. Peripheral lymphoid tissues were isolated from BMT control and SGVHD mice, and the CD4+ T cells were isolated by cell sorting into CD4+CD62L<sup>+</sup> or CD4+CD62L<sup>−</sup> populations. These cells were then cultured with syngeneic DC or DC pulsed with CeAg, and the proliferative response was determined. As shown in Fig. 3, only the CD4+CD62L<sup>−</sup> cells from SGVHD animals responded with a significant in vitro response. No response was observed from the CD4+CD62L<sup>+</sup> isolated from SGVHD mice or from the CD4+CD62L<sup>−</sup> or CD4+CD62L<sup>+</sup> cells from BMT control animals. These results suggest that the increased presence of activated CD4+ T cells in the periphery of SGVHD animals (>75%) (19) most likely resulted from the in vivo activation of these cells via an Ag-specific response against microbial Ags.

**Antibiotic treatment inhibits the development of murine SGVHD**

Several models of colitis and colon inflammation have been shown to be sensitive to treatment with broad-spectrum antibiotics (27, 35, 36). The increased proliferative responsiveness of SGVHD CD4+ T cells against microbial Ags suggested that the microbiota participated in the pathology associated with murine SGVHD. Studies were therefore undertaken to determine whether a reduction in the microflora of CsA-treated animals by treatment with broad-spectrum antibiotics would alter the development of SGVHD. C3H/HeN mice were lethally irradiated, reconstituted with T cell-depleted BM, and treated daily with diluent or CsA, as described. Beginning 1 wk after BMT, control and CsA-treated mice were given broad-spectrum antibiotics (ciprofloxacin, metronidazole) (27) in the drinking water for the remainder of the experiment. The animals were monitored for clinical symptoms (weight loss, diarrhea) of SGVHD, as described. As shown in Fig. 4, antibiotic treatment significantly reduced the CD4+CD62L<sup>−</sup> cells from SGVHD animals with activated/memory phenotype are responsive in vitro against microbial Ags. Peripheral lymphoid tissues were isolated pooled within groups from control BMT and SGVHD animals 2–4 wk after cessation of CsA therapy. The single-cell suspensions were stained with fluorochrome-labeled mAb against CD4 and CD62L (L-selectin), sorted into CD4<sup>+</sup>CD62L<sup>−</sup> and CD4<sup>+</sup>CD62L<sup>+</sup> populations, and stimulated as described with unpulsed DC or CeAg-pulsed DC, and proliferation was determined during a 96-h assay. SGVHD CD4+CD62L<sup>−</sup> significant against control CD4<sup>+</sup>CD62L<sup>+</sup>, control CD4<sup>+</sup>CD62L<sup>−</sup>, or SGVHD CD4<sup>+</sup>CD62L<sup>−</sup> responses, as determined by one-way ANOVA, p < 0.001.
4A, >80% (11 of 12) of the CsA-treated animals developed clinical symptoms of SGVHD. Conversely, a significant reduction in the development of SGVHD in CsA-treated animals given broad-spectrum antibiotics was observed (1 of 12; \( p < 0.0001, \) log rank test). The induction was reduced to the level of BMT control and BMT control antibiotic-treated animals (\( p > 0.05 \)). Upon physical examination of target tissues, it was shown that development of SGVHD was associated with a significant reduction of colon length compared with BMT control animals (Fig. 4B). Importantly, the length of the colons from antibiotic-treated, CsA-treated animals was similar to those of control mice, demonstrating the lack of an inflammatory response resulting in a lack of colon thickening/length in these animals. Furthermore, we have recently shown that the liver inflammation that develops during SGVHD is associated with significant increases in ALT in the plasma/serum of diseased animals (21). Similar results were observed in the current study, and the ALT levels were significantly reduced in the antibiotic-treated, CsA-treated animals (Fig. 4C).

Antibiotic treatment altered colon length and reduced the level of liver enzymes following antibiotic therapy of CsA-treated animals, suggesting that SGVHD-induced tissue pathology was reduced in the treated animals. To monitor the effects of antibiotic treatment on SGVHD-mediated colon and liver inflammation, tissues from BMT control, BMT control, antibiotic-treated, SGVHD, and CsA-antibiotic–treated animals were examined. Target tissues were isolated 53 d after BMT, stained with H&E, analyzed, and scored for SGVHD pathology (33) (Fig. 5). Minimal inflammation and normal tissue architecture were observed in the colons (Fig. 5A) and livers (Fig. 5E) isolated from BMT control animals. Tissues from control animals treated with antibiotics demonstrated histology similar to the control BMT animals (Fig. 5B, 5F). However, significant colonic mucosal infiltrate with crypt abscess formation (arrow) and glandular destruction was observed in tissues isolated from SGVHD animals (Fig. 5C) relative to control BMT animals. In addition, periporal inflammation with bile duct infiltration (arrow) was seen in the livers isolated from SGVHD animals (Fig. 5G). Interestingly, and in line with the colon length and plasma ALT data, broad-spectrum antibiotic therapy completely eliminated colon inflammation (Fig. 5D) and significantly reduced the periporal response in the livers (Fig. 5H) of antibiotic, CsA-treated animals compared with SGVHD animals. When the tissues from these animals were quantitatively graded (33), an increase in the pathology grade was observed in the colons and livers (Fig. 5I, 5J, respectively) of SGVHD versus BMT control animals. Conversely, a significant reduction of SGVHD grade was observed in the colon and livers of antibiotic, CsA-treated animals compared with SGVHD animals. Finally, the pathology grade between the antibiotic-treated BMT control and the antibiotic, CsA-treated BMT animals was not significantly different (>0.05), further demonstrating the antibiotic responsiveness of the induction of murine SGVHD.

**FIGURE 4.** Broad-spectrum antibiotic therapy inhibits the development of SGVHD. C3H/HeN mice were lethally irradiated, reconstituted with syngeneic BM, and then treated with diluent olive oil or CsA daily for 21 d. Beginning 7 d after BMT, groups of control BMT and CsA-treated animals were given metronidazole and ciprofloxacin in the drinking water for the duration of the experiment. After cessation of CsA therapy, animals were weighed three times per week and monitored for the induction of SGVHD (three consecutive weight loss, diarrhea, mortality) (A). CsA versus control, control antibiotic, or CsA antibiotic, \( p < 0.0001 \), as determined by log rank test. Pooled data from two experiments with \( n = 12 \) per treatment group. B. The colons were isolated on day 53 post-BMT; the length was measured, and statistical differences were determined by one-way ANOVA (\( n = 11 \)). Data represent pooled data from two experiments; CsA versus control BMT, \( p < 0.001 \); CsA versus CsA antibiotic, \( p < 0.001 \); CsA antibiotic versus control antibiotic, \( p > 0.05 \). C. As a measure of liver inflammation, plasma ALT concentration was determined. Plasma was isolated 53 d after BMT and analyzed for ALT levels, as described in Materials and Methods. Significance was determined using one-way ANOVA, using pooled samples from two experiments. \( p < 0.001 \), CsA versus control; \( p < 0.01 \), CsA versus control antibiotic; \( p < 0.05 \), CsA versus CsA antibiotic; \( p > 0.05 \), CsA antibiotic versus control antibiotic.

**Antibiotic treatment alters the immune responses associated with induction of SGVHD**

Previous studies (19) and studies outlined in the current manuscript have demonstrated increased proliferation of CD4+ T cells or liver-associated mononuclear cells (LAM) (21) from SGVHD mice against APC pulsed with microbial Ags isolated from the ceca of normal syngeneic animals. As the induction of SGVHD was shown to be responsive to therapy with broad-spectrum antibiotics, studies were undertaken to monitor the immune responses that were present in antibiotic, CsA-treated versus SGVHD mice. Initial studies analyzed the ability of CD4+ T cells or LAM from the various treatment groups to respond to DC pulsed with CeAg. As shown in Fig. 6A, CD4+ T cells isolated from the periphery of SGVHD animals demonstrated a significant increase in proliferation against CeAg-pulsed DC compared with T cells isolated from BMT control animals. Similarly, LAM from diseased animals also exhibited enhanced proliferation against CeAg-pulsed DC (Fig. 6B). Minimal T cell proliferation was observed.
against unpulsed, BM-derived DC regardless of the source of the responder lymphoid population. Interestingly, and in support for a role of antimicrobial CD4\(^+\) T cell reactivity in the development of murine SGVHD, T cells and LAM isolated from antibiotic, CsA-treated animals displayed in vitro reactivity that was at or below that observed when lymphoid cells from BMT control animals were used in the cultures as responding cells. Thus, antibiotic treatment most likely removed the antigenic stimulus, reducing the antimicrobial reactivity of T cells isolated from treated animals.

In addition to the increase of in vitro reactivity of SGVHD CD4\(^+\) T cells against microbial Ags, we have demonstrated that an increase in Th1 and Th17 immunity was observed in SGVHD animals (11, 12, 17–21). Th1 and Th17 effector CD4\(^+\) T cells have been shown to participate in a variety of models of colon and liver inflammation. Similar to published studies, increased numbers of Th1 (IFN-\(\gamma\))-producing and Th17 (IL-17 and TNF-\(\alpha\))-producing CD4\(^+\) T cells were observed in cells isolated from the MLN from SGVHD versus control animals (Supplemental Fig. 2). The increase in these putative effector populations was significantly decreased in CsA-treated BMT animals that were treated with broad-spectrum antibiotics. In addition, CsA, antibiotic-treated animals had reduced levels of circulating (plasma) TNF-\(\alpha\), IFN-\(\gamma\), and IL-17 compared with SGVHD mice (Fig. 7). Because

**FIGURE 5.** Antibiotic therapy decreased tissue pathology associated with development of SGVHD. SGVHD was induced, and groups of control BMT and CsA-treated animals were given antibiotics in the drinking water, as described. The colons (A–D) and liver (E, F) were isolated 53 d after BMT, placed in buffered formalin, and embedded in paraffin, and 4- to 5-\(\mu\)m sections were stained with a standard H&E procedure. A, Control BMT colon; B, colon from control BMT antibiotic-treated animal; C, CsA-treated SGVHD colon demonstrating glandular loss and crypt abscess formation (arrow); D, colon from CsA-treated animal given antibiotics throughout course of experiment; E, liver isolated from control BMT mouse; F, liver tissue from antibiotic-treated control BMT animal; G, liver from CsA-treated SGVHD mouse demonstrating bile duct infiltration (arrow); H, liver obtained from CsA-treated animal given antibiotics throughout the course of the experiment. All tissues were photographed at \(\times\)200 magnification and are representative of animals within each treatment group. The pathology grading of colon (I) and liver (J) tissues was performed according to previously published grading scale (33). Data represent the mean \(\pm\) SEM of samples from a single experiment (\(n = 6\)), and the significance was determined using the one-way ANOVA.
CD4+ T cells mediate the development of SGVHD (17, 19), the reduction in in vitro CD4+ antimicrobial reactivity and the reduction of effector cytokines by treatment with broad-spectrum antibiotics demonstrated the dependence of the development of SGVHD on the antigenic stimulus by the microflora in the post-transplant BMT recipient animals treated with CsA.

**Discussion**

To our knowledge, the data presented in this work demonstrated for the first time that the development of murine SGVHD was dependent on the intestinal microbiota. An enhanced proliferative response of CD4+ T cells isolated from SGVHD animals, relative to T cells from control BMT mice, was observed against APC pulsed with microbial Ags. This response was MHC class II restricted and was predominantly mediated by the CD62L− memory subset of CD4+ T cells. In line with the antimicrobial proliferative data was the demonstration of a functional role for the intestinal microbiota in the development of murine SGVHD. Treatment with broad-spectrum antibiotics eliminated the induction of clinical symptoms, immunopathology, and in vitro immune responsiveness that is associated with this inducible disease.

SGVHD was first described in the rat and was shown to be mediated by CD8+ T cells (2, 13, 14). The autoreactive effector cells were shown to be specific for the MHC-associated CLIP protein (15, 16). In contrast, the Ag specificity of the effector cells responsible for the development of the murine form of this inducible disease remained unknown. Murine SGVHD has many similarities to other models of murine colitis. Similar pathologies in the colon, effector cytokines, and the predominant role for CD4+ T cells in the development of the two types of colonic inflammation suggested that spontaneous and inducible colitis and SGVHD could have similar effector cell specificities. It has been shown that many of the inducible models of mouse colitis develop as a result of unregulated CD4+ T cell responses against microbial Ags (24–27). Likewise, we have demonstrated that CD4+ T cells mediate the induction of SGVHD (17, 19). Furthermore, preliminary studies had demonstrated that T cells from SGVHD demonstrated enhanced proliferative responses against microbial Ags derived from the ceca of normal mice (19, 21). Given the similarity of the two colonic inflammatory diseases, studies were

**FIGURE 6.** Broad-spectrum antibiotic therapy eliminates the antimicrobial proliferative response in CsA-treated animals. A, CD4+ T cells were isolated 52 d after BMT from control BMT and SGVHD and control and CsA-treated animals that were treated with broad-spectrum antibiotics. The cells were placed into culture with unpulsed DC or CeAg-pulsed DC, and proliferation was analyzed, as described. B, Similarly, LAM were isolated from control, diseased, and antibiotic-treated control and CsA-treated animals and tested for responsiveness against unpulsed or CeAg-pulsed DC in a 96-h proliferation assay. Data presented are representative of two experiments. Statistical differences were determined by one-way ANOVA. *p < 0.001, SGVHD DC Ag versus control BMT DC-Ag, control DC-Ag versus control DC, SGVHD DC-Ag versus CsA-antibiotic DC-Ag.

**FIGURE 7.** Antibiotic therapy inhibited proinflammatory cytokine production during the development of SGVHD. C3H/HeN were induced for the development of SGVHD, as described. Beginning 7 d after BMT, control and CsA-treated mice were given metronidazole and ciprofloxacin in the drinking water through the end of the experiment, day 53 post-BMT. The animals were bled and plasma was prepared. The levels of (A) IL-17A, (B) IFN-γ, and (C) TNF-α were determined by ELISA. n = 7–12. Statistical analysis was performed by one-way ANOVA.
initiated to further characterize the specificity of the antimicrobial immune responses was present in SGVHD animals. SGVHD CD4\(^+\) T cells proliferated at a significantly elevated level to APC that were pulsed with colonic microbial Ags (CeAg) relative to unpulsed APC or APC pulsed with nominal Ags or food or self Ags from colonic epithelial tumors relative to T cells isolated from BMT control animals. As expected, based on classical MHC restriction patterns, the addition of mAb against the appropriate MHC class II haplotype significantly eliminated the CD4\(^+\)-mediated in vitro proliferative response against microbial Ag. Finally, when CD4\(^+\) T cells were isolated from control and SGVHD animals and sorted into naive (CD62L\(^+\)) and activated/memory (CD62L\(^-\)) subsets, the proliferative response against microbial Ags was solely present in the CD62L\(^-\) subset of the SGVHD CD4\(^+\) T cells. Previous studies had demonstrated a significant skewing of the CD4\(^+\) T cell population to the CD62L\(^-\) subset (17–19). This response suggested that the responsive cells were activated in vivo and that few naive precursor cells were present in either the control or SGVHD animals, as evidenced by lack of reactivity of the CD62L\(^+\) subset.

To determine whether the enhanced in vitro reactivity of the SGVHD T cells against bacterial Ags was functional in vivo, control BMT and CsA-treated animals were treated with broad-spectrum antibiotics. It has been shown that murine colitis models are sensitive to treatment with broad-spectrum antibiotics (27, 35, 36). Although it was unlikely that antibiotic therapy completely eliminated the microbiota in these animals, a reduction in aerobic and anaerobic bacteria in the gut most likely reduced the appropriate antigenic stimuli required for Ag-specific stimulation of naive microbial-specific CD4\(^+\) T cells and ultimately reduced immunopathology. In combination with the enhanced proliferative responsiveness against microbial Ags in colitis models (24–27), elimination of clinical disease following antibiotic therapy (27, 35, 36), as well as a reduction in colitis in germ-free IL-10\(^-/-\) mice (37), demonstrated a role for the microbiota in the development of murine colitis (38). Similarly, the bacterial microflora was shown to be involved in the development of intestinal lesions during the development of allogeneic GVHD (39, 40). With these findings in mind, we chose to treat control BMT and CsA-treated BMT animals with broad-spectrum antibiotics beginning 1 wk after BMT (during the induction period of SGVHD [days 0–21]) through development of disease. The broad-spectrum antibiotic regimen consisted of ciprofloxacin and metronidazole, antibiotics that target aerobic and anaerobic bacteria, respectively (27). Antibiotic therapy of CsA-treated BMT completely inhibited the development of murine SGVHD relative to BMT animals given CsA alone. This was demonstrated by the elimination of clinical symptoms (weight loss, diarrhea), a significant reduction in colon pathology, and the elimination of the CD4\(^+\) T cell proliferative response against CeAg compared with SGVHD animals. Furthermore, it has been shown that significant increases occur in Th17/Th1 immune responses during the development of SGVHD (11, 12, 17–21). Following treatment with ciprofloxacin/metronidazole, a significant reduction in these inflammatory cytokine responses was observed. These findings demonstrated an association between the development of the effector inflammatory cytokine response and the microbiota of the intestine.

It is possible that the inhibition of SGVHD by antibiotic treatment is due to anti-inflammatory properties of ciprofloxacin. A single high dose (167–214 mg/kg) injection of ciprofloxacin has been shown to inhibit LPS-induced mortality and proinflammatory cytokine production in vivo (41). Similarly, Ogino et al. (42) demonstrated that a single injection of ciprofloxacin prior to LPS challenge inhibited production of TNF-\(\alpha\), but not of other inflammatory cytokines. Moreover, between 10 and 100 \(\mu\)g/ml ciprofloxacin was required to inhibit the in vitro TNF-\(\alpha\) production by murine peritoneal macrophages. In the current study, based on the chronic administration of antibiotics in the drinking water (86 mg/kg/day) and the 60-min \(\text{t}_{1/2}\) for ciprofloxacin in mice (43, 44), an estimated plasma antibiotic concentration of 1.6 \(\mu\)g/ml, which is substantially lower than the reported levels for anti-inflammatory effects in vitro (42), would be achieved in the SGVHD animals. Thus, the observed effects of antibiotic therapy on the SGVHD model are most likely not to be due to the inhibitory effects of ciprofloxacin on the immune responses associated with the development of SGVHD.

Interestingly, we have recently demonstrated that a unique aspect of the SGVHD model was an absolute linkage between chronic colon and liver inflammation (21). We had proposed that the enterohepatic association may be the result of microbial-specific CD4\(^+\) T cells being activated in the colon, then migrating to the liver, as has been proposed in clinical liver inflammation that is associated with IBD (45). It was shown that antibiotic therapy of CsA-treated mice also eliminated the development of chronic liver inflammation and the increased microbial-specific proliferative responses that are generated by CD4\(^+\) liver-associated lymphocytes. These findings further strengthen our previous findings of the enterohepatic linkage that is present in the development of chronic colon and liver inflammation in this inducible inflammatory disease. Thus, these results demonstrate that SGVHD develops as a response to microbial Ags that is eliminated by broad-spectrum antibiotic therapy, and support the findings that the induction of murine SGVHD is significantly different from the autoimmune-like disease that is present in lethally irradiated rats treated with CsA therapy.

Murine SGVHD appears to develop as a CD4\(^+\) T cell response against microbial Ags. Recent findings by Feng et al. (46) have demonstrated that in addition to Ag-specific responses, microbiota-derived signals that activate innate immune responses via TLRs also drive the spontaneous proliferation of T cells. Thus, both Ag-specific and spontaneous proliferative responses were required for colitis to develop when naive CD4\(^+\) T cells were adoptively transferred into immunodeficient rag\(^{-/-}\) mice. It is not clear whether both Ag-specific and spontaneous T cell proliferation are required for the development of the chronic inflammation that develops during SGVHD. We have previously shown that innate immunity is activated to a heightened level during the induction of murine SGVHD (12). Treatment with sublethal doses of LPS resulted in increased production of proinflammatory cytokines and mortality. However, LPS appeared not to participate in disease induction as similar levels of SGVHD were induced in LPS-responsive and hyporesponsive animals (12). This, however, does not preclude the possibility that other TLR ligands participate in the development of SGVHD and that antibiotic therapy reduces the microbiota, thus reducing the proinflammatory responses induced via TLR stimulation of innate immunity. We have recently demonstrated that TLR expression is increased in SGVHD animals (21). It is also clear that following lethal irradiation and CsA therapy, the treated animals are lymphopenic, and it is likely that expansion of CD4\(^+\) T cells occurs via specific and nonspecific proliferative responses. Thus, the results presented in the current manuscript cannot rule out a role for spontaneous proliferation of T cells in the development of SGVHD.

Overall, the findings presented support the hypothesis that the intestinal microflora drives the development of murine SGVHD. In line with these findings, the elimination of colon and liver inflammation by treatment with broad-spectrum antibiotics that reduce aerobic and anaerobic bacteria significantly reduced the
induction, pathology, and enhanced Ag-specific immune responses that develop in SGVHD animals and demonstrated clearly that the intestinal microbiota contribute to the development of this inducible disease. We propose that murine SGVHD will be useful to determine the role of the microbiota in the development of chronic intestinal and liver inflammation in this inducible model.

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Disclosures

The authors have no financial conflicts of interest.

References


**Supplemental Figure 1.** Long-term SGVHD CD4\(^+\) T cell line microbial antigen-induced proliferation is MHC class II restricted. Long-term CD4\(^+\) T cell lines were generated from SGVHD animals. CD4\(^+\) T cells were isolated from the periphery of SGVHD animals 2-4 weeks after cessation of CsA therapy. The cells were stimulated with CeAg-pulsed syngeneic APC every 8-10 days. A) SGVHD CD4\(^+\) T cells were isolated 9 days after stimulation and cultured with unpulsed spleen cells or spleen cells that were pulsed with 200 µg CeAg or with unpulsed spleen cells to which CeAg was added to the well at the time of initiation of culture (representative of two experiments) and proliferation monitored by the addition of [\(^{3}\)H]-thymidine during the last 18 hours of a 96 hour culture. B) SGVHD CD4\(^+\) T cell lines were placed into culture with spleen or CeAg-pulsed spleen cells in the presence or absence of 1:20 dilution of anti-I-A\(^{k}\) and anti-I-E\(^{k}\) mAb. Proliferation was determined as described. Data is representative of four experiments. Statistical significance was determined by one-way ANOVA.
Supplemental Figure 2. Broad-spectrum antibiotic therapy reduced the number of IL-17- IFN-γ- and TNF-α-producing CD4+ T cells. Animals were induced for SGVHD as described. Antibiotic therapy was initiated on control BMT and CsA-treated mice 7 days after BMT and continued throughout the course of the experiment. At the time of active disease mice were euthanized, and the MLN were removed process individually and stimulated with anti-CD3 for 8 h. Monensin was added during the last 4 h of culture to block cytokine secretion. The cells were then processed for intracellular cytokine staining of IL-17 and IFN-γ. Graphs represent mean ± SEM and is pooled data from 2 experiments. Significance determined using one-way ANOVA.