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Novel Role for Surfactant Protein A in Gastrointestinal Graft-versus-Host Disease

Kymberly M. Gowdy,* Diana M. Cardona,† Julia L. Nugent,‡ Charles Giambardino,§ Joseph M. Thomas,¶ Sambudho Mukherjee,∗ Tereza Martinu,∗ W. Michael Foster,* Scott E. Plevy,§ Amy M. Pastva,Jo Rae Wright,¶ and Scott M. Palmer∗

Graft-versus-host disease (GVHD) is a severe and frequent complication of allogeneic bone marrow transplantation (BMT) that involves the gastrointestinal (GI) tract and lungs. The pathobiology of GVHD is complex and involves immune cell recognition of host Ags as foreign. We hypothesize a central role for the collectin surfactant protein A (SP-A) in regulating the development of GVHD after allogeneic BMT. C57BL/6/H2b; WT) and SP-A–deficient mice on a C57BL/6 background (H2b; SP-A−/−−) mice underwent allogeneic or syngeneic BMT with cells from either C3HeB/FeJ (H2k; SP-A−/−−) mice that have undergone an allogeneic BMT [SP-A−/−−/alloBMT] or SP-A–sufficient recipient mice that have undergone an allogeneic BMT) or C57BL/6/H2b; SP-A−/−−− alloBMT mice that have undergone a syngeneic BMT or SP-A−/−−− recipient mice that have undergone a syngeneic BMT). Five weeks post-BMT, mice were necropsied, and lung and GI tissue were analyzed. SP-A−/−− alloBMT or SP-A−/−− recipient mice that have undergone an allogeneic BMT had no significant differences in lung histology, inflammatory cell infiltration, and lymphocytic infiltration. SP-A−/−− alloBMT mice also had increased colon expression of IL-1β, IL-6, TNF-α, and IFN-γ and as well as increased Th17 cells and diminished regulatory T cells. Our results demonstrate the first evidence, to our knowledge, of a critical role for SP-A in modulating GI GVHD. In these studies, we demonstrate that mice deficient in SP-A that have undergone an allogeneic BMT have a greater incidence of GI GVHD that is associated with increased Th17 cells and decreased regulatory T cells. The results of these studies demonstrate that SP-A protects against the development of GI GVHD and establishes a role for SP-A in regulating the immune response in the GI tract. The Journal of Immunology, 2012; 188: 4897–4905.

A pproximately 50,000 patients worldwide undergo bone marrow transplantation (BMT) each year for treatment of both malignant and nonmalignant disorders (1). Despite the use of potent immunosuppression, graft-versus-host disease (GVHD) is a frequent complication of allogeneic BMT, resulting in significant morbidity and mortality. GVHD typically involves the gastrointestinal (GI) tract, skin, and lungs (2). The specific mechanisms of how GVHD is initiated remain poorly characterized. However, a major component of GVHD is the presence of a milieu of proinflammatory cytokines such as IL-1, TNF-α, IFN-γ, and IL-6 that can lead to tissue damage (3). These cytokines have been shown to influence naive donor T cell differentiation into proinflammatory T cell populations such as Th1 and Th17 (4). In addition, GVHD is often associated with failure to develop regulatory T (Treg) cells, a specialized subset of T cells that suppresses activation and/or extent of the proinflammatory T cell response (5–7). The strong association between proinflammatory cytokine production and the absence of Treg cells during GVHD suggests that one mechanism by which to inhibit the detrimental effects associated with GVHD is to control the inflammation associated with tissue damage to thereby alter the T cell response.

The collectin surfactant protein A (SP-A) was first recognized as a molecule important in pulmonary host defense and homeostasis, but has recently been identified in other mucosal organs such as the uterus, eye, small intestine, and colon (8–11). Genetic polymorphisms that change the expression levels of surfactant proteins have been implicated in extrapulmonary disease pathology. Recent studies identified specific SP-A haplotypes associated with increased susceptibility of children to recurrent otitis media (12) and time to parturition (13), whereas other collectins such as surfactant protein D (SP-D) appear to be important for clearing GI pathogens (14) and in susceptibility to Crohn’s disease (15). These studies suggest that the expression of surfactant proteins in extrapulmonary mucosal surfaces is important to host defense. SP-A is also important in regulating the production of proinflammatory cytokines by immune cells. Multiple studies have reported that in the
absence of SP-A, there is a significant increase in pulmonary production of inflammatory cytokines such as TNF, IFN-γ, and IL-6 after pathogen challenge (16, 17). SP-A has also been shown to influence adaptive immune responses in pulmonary diseases by decreasing dendritic cell maturation and T cell proliferation (18–22). Taken together, SP-A is an important mediator of mucosal inflammation and immunity and may have a functional role in the development of GVHD after BMT.

In this study, we show that mice deficient in SP-A have a greater incidence and severity of colonic GVHD due to the potentiation of allogeneic T cell activation, increased Th17 cells in the GI tract, and decreased production of Treg cells. These data expand on the evolving literature suggesting extensive functional roles for surfactant proteins in extrapulmonary disease pathology. The data suggest that SP-A has an important immunological role in the prevention of GI GVHD after BMT.

Materials and Methods

Mice

Experiments were approved by the Duke Institutional Animal Care and Use Committees. Male 8–10 wk-old C57BL/6 (H2b; WT) and SP-A-deficient mice were maintained on a C57BL/6 background (H2b; SP-A−/−), C3H/HeJ (H2k; allogeneic) or C57BL/6 (H2k; syngeneic) mice were used as donors and obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in a pathogen-free facility on LPS-free bedding (Shepherd Specialty Papers, Kalamazoo, MI), with irradiated food (PicoLab Mouse Diet 20-5058; Purina Mills, Richmond, IN) and antibacterial water (sulfamethoxazole/trimethoprim 1:20.24 mg/ml) after BMT.

Murine BMT

Donor mice were euthanized using CO2. Tibia and femur bone marrow and spleenocytes were isolated, filtered twice through 70-μm filters (BD Biosciences, Franklin Lakes, NJ), counted, and resuspended in RPMI 1640 media containing 10% FBS (Hyclone, Logan, UT), 1% l-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% penicillin/streptomycin (Sigma-Aldrich). Recipient (WT or SP-A−/−) mice were maintained on a C57BL/6 background (H2b: SP-A−/−). C3H/HeJ (H2k: allogeneic) or C57BL/6 (H2k: syngeneic) mice were used as donors and obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in a pathogen-free facility on LPS-free bedding (Shepherd Specialty Papers, Kalamazoo, MI), with irradiated food (PicoLab Mouse Diet 20-5058; Purina Mills, Richmond, IN) and antibacterial water (sulfamethoxazole/trimethoprim 1:20.24 mg/ml) after BMT.

Histologic assessment of acute GVHD

The presence of acute GVHD was assessed by detailed morphologic analysis of lung, liver, distal small intestine, proximal large intestine, and rectum necropsy specimens. The specimens were harvested from mice 5 wk post-BMT and fixed in fresh neutral buffered formalin. Following fixation, the specimens were embedded in paraffin, cut into 5-μm-thick sections and stained with H&E. The slides were coded with a unique number that had no reference to mouse type or treatment status and graded by a single observer. The slides were stained with H&E. The slides were coded with a unique number that had no reference to mouse type or treatment status and graded by a single. The slides were stained with H&E. The slides were coded with a unique number that had no reference to mouse type or treatment status and graded by a single observer. The slides were stained with H&E. The slides were coded with a unique number that had no reference to mouse type or treatment status and graded by a single observer.

CD3+ immunohistochemistry

CD3 immunohistochemistry was performed with rabbit anti-mouse CD3 Ab (Thermo Scientific, Fremont, CA) used at a dilution of 1:150 and developed with HRP-conjugated streptavidin (Vector Laboratory, Burlingame, CA) and then dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA). Dianimobenzidine (DakoCytomation, Carpinteria, CA).

RNA analysis

After necropsy, tissue samples were preserved in RNAlater (Ambion/Applied Biosystems, Austin, TX). RNA was extracted (Ambion/Applied Biosystems); quantity was measured spectrophotometrically, and quality was analyzed using Bio-Rad Experion chips (Bio-Rad). For additional transcript analysis, cDNA was transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). A total of 50 ng cDNA was used for quantitative PCR (in triplicate) using TaqMan probe-and-primer combinations for IL-6 (Mm00475162_m1), IL-1β (Mm01336189_m1), IFN-γ (Mm01168134_m1), and the endogenous β-actin (4552933) (Applied Biosystems). Threshold cycle (Ct) values were determined using the ABI 7500 Real Time PCR System (Applied Biosystems) with SDS software 1.3.1. Change in expression was calculated using the 2−ΔΔCt method normalized to β-actin expression and expressed as fold change compared with the control group.

Serum ELISA

Blood was harvested 5 wk post-BMT, and murine TNF-α levels were measured in serum with a cytokine-specific ELISA kit (R&D Systems, Minneapolis, MN).

Peyer’s patches and mesenteric lymph node flow cytometry

Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs) were isolated, filtered through a 70-μm filter (BD Biosciences), washed, and resuspended in PBS with 3% FBS, 0.05% sodium azide (WVR International, West Chester, PA), and 10 mM EDTA. Live cells were counted using 0.4% trypsin blue (Sigma-Aldrich) dead-cell exclusion. Cells were blocked using 5% normal mouse serum, 5% normal rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA), and 1% Fc-receptor-block (anti-mouse CD16/32; eBioscience, San Diego, CA). For intracellular cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h before staining and incubated with Golgistop (BD Biosciences) during the last 3 h of stimulation. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and labeled with Abs against IL-17A–PE (clone eBio17B7; eBioscience) and IFN-γ–allophycocyanin (clone XM21.2; eBioscience). For staining of surface Ags, cells were labeled with anti-mouse anti-CD3–FITC, anti-CD8–PE–Cy7, anti-CD11b–APC–Cy7, and anti-CD4–PE–Cy5 (eBioscience). For intra-cellular staining of transcription factor Foxp3, after surface staining, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) and then labeled with anti-Foxp3–PE (clone FJK-16s; eBioscience). Fluorescence was measured using a BD FACSCantoII flow cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Ashland, OR); a singlet gate was used to exclude cell aggregates, followed by an all-cell gate to exclude small debris and dead cells. Cell percentages are expressed as percentage of all cells and converted to absolute numbers by multiplying by live-cell counts. MLC

T cells from C57BL/6 were isolated from spleens by negative magnetic bead selection (Pan T cell beads; Miltenyi Biotec, Auburn, CA). Isolated T cells were reconstituted with cells from alloBMT or synBMT mice and sacrificed 5 wk after transplant. Lung pathology was evaluated, and pathology grades were determined in a blinded fashion using a semiquantitative scoring system based on the thickness of perivascular and peribronchioral inflammation as well as the overall percentage of lung involved. Graph is representative of one experiment, n = 5–8/group; data were replicated in five independent experiments.

FIGURE 1. SP-A deficiency does not affect the development of pulmonary GVHD after allogeneic BMT. SP-A−/− or -sufficient (WT) mice were reconstituted with cells from alloBMT or synBMT mice and sacrificed 5 wk after transplant. Lung pathology was evaluated, and pathology grades were determined in a blinded fashion using a semiquantitative scoring system based on the thickness of perivascular and peribronchioral inflammation as well as the overall percentage of lung involved. Graph is representative of one experiment, n = 5–8/group; data were replicated in five independent experiments.
were washed in media, filtered through 70-μm filters (BD Biosciences), counted on a hemocytometer, and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) containing penicillin/streptomycin (Sigma-Aldrich), L-glutamine (Sigma-Aldrich), and 10% FBS (Hyclone, Logan, UT). C3HeB/FeJ (allogeneic) or C57BL/6 (syngeneic) 5 × 10^5 stimulator splenocytes (irradiated in a Cesium irradiator at 20 Gy) were cocultured with 5 × 10^5 C57BL/6 responder T cells in the presence of 20 μg/ml exogenous SP-A [purified as previously described (26)] or 20 μg/ml purified mouse control IgG (Jackson ImmunoResearch Laboratories) for 4 d. A total of 1 μCi [3H]thymidine was added for the last 18 h of culture. Four days following initial culture, splenocytes were harvested (TomTec harvester 96 Mach III M; TomTec, Hamden, CT), and radioactivity was measured using a β-counter (Wallac Trilux 1450 Microbeta counter; Wallac, Waltham, MA). Results are reported as counts per minute, and proliferation was determined by subtracting counts per minute of responders alone from counts per minute in wells containing both responders and stimulators.

Colonic tissue explant cultures

Colonic tissue fragments (0.5 g dry weight) were harvested 5 wk post-BMT and processed as previously described (27). Tissue-fragment supernatants were collected after 24 h for cytokine analysis. Murine IL-17a levels were measured in supernatants with a cytokine-specific ELISA kit (R&D Systems, Minneapolis, MN).

Statistical analysis

Data are expressed as means ± SEM. Between-group comparisons were performed to specifically determine if SP-A−/− BMT mice were significantly different from WT BMT (allogeneic or syngeneic) mice. Comparisons were performed using a one-way ANOVA in GraphPad Prism software (version 5.03; GraphPad, La Jolla, CA). The p values < 0.05 were considered significant. The study was repeated five independent times, with n = 5–8 mice per group per experiment. The data shown in this study are representative of one study.

Results

SP-A deficiency does not affect development of pulmonary GVHD after BMT

SP-A-deficient (SP-A−/−) or sufficient (WT) recipient mice that had undergone BMT with either allogeneic (alloBMT) or syngeneic (synBMT) donors were reconstituted with cells from alloBMT or synBMT mice and sacrificed 5 wk after transplant. Liver, small intestine, and colon pathology was evaluated, and pathology grades were determined in a blinded fashion using a semiquantitative scoring system described in Table I. Quantitative scoring of liver pathology (A), small intestine (B), and colon (E) 5 wk post-BMT. (C) Gross pathology of SP-A−/− alloBMT (left panel) and WT alloBMT (right panel). (D) Weights of colons 5 wk post-BMT. Representative colon histology sections are shown for alloBMT groups; H&E stain, original magnification ×40: SP-A−/− alloBMT (F) and WT alloBMT (G); H&E stain, original magnification ×200: SP-A−/− alloBMT (H) and WT alloBMT (I). Graph is representative of one experiment, n = 5–8/group; data were replicated in five independent experiments. *p < 0.05, **p < 0.0001. LP, Lamina propria; MM, muscularis mucosa; MP, muscularis propria.
neic (synBMT) donor cells were assessed for perivascular and peribronchiolar lymphocytic inflammation (Fig. 1, Supplemental Fig. 1). Mice that had not undergone a BMT (nontransplanted [NT]), whether deficient (SP-A^{-/-}) or sufficient (WT) in SP-A, did not have any significant pulmonary pathological changes. After alloBMT, comparable levels of perivascular and peribronchiolar inflammation were seen in both SP-A–deficient and –sufficient (WT) recipient mice that have undergone an allogeneic BMT (SP-A^{-/-} alloBMT or WTalloBMT). This trend was also reflected in the bronchoalveolar lavage cell counts and T cell infiltration into the airspace (data not shown). Minimal pathology was seen with SP-A^{-/-} or –sufficient (WT) recipient mice that have undergone a syngeneic BMT (SP-A^{-/-} synBMT or WTsynBMT) (Fig. 1, Supplemental Fig. 1). These data suggested that SP-A does not regulate alloimmune lung injury after allogeneic BMT.

SP-A deficiency increases the severity of small and large intestine GVHD after allogeneic BMT

Despite no difference in pulmonary pathology, we did observe that SP-A^{-/-} alloBMT were only able to regain ~85% of starting body weight after transplantation. This observation was not seen in WTalloBMT, syngeneic, or NT controls. After BMT, all treatment groups lost ~20% of body weight at 2 wk posttransplantation. WT mice that had undergone an alloBMT and syngeneic controls were able to regain back to 100% of starting body weight by 4 wk post-BMT (Fig. 2).

Given the severe weight loss in SP-A^{-/-} alloBMT mice, we were interested to examine the pathology of the GI tract to describe a cause for this weight loss. The liver, small, and large intestines were examined for features of GI GVHD (Fig. 3A, 3B, 3D, Table I). Within the liver, no significant pathologic alterations were seen in any of the treatment groups (Fig. 3A). Upon necropsy, SP-A^{-/-} alloBMT mice had increased colonic wall thickness, decreased length and unformed fecal pellets (Fig. 3C), as well as common features of GI GVHD such as ulceration, neutrophil infiltration, and increase in apoptotic cells (Supplemental Fig. 2A, 2B). These gross
Table I. A semiquantitative scoring system was used to document the presence and severity of various GVHD-associated morphologic features.

<table>
<thead>
<tr>
<th>Liver</th>
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<td>Portal triads</td>
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<tr>
<td>Portal tract expansion</td>
<td>50</td>
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<tr>
<td>Bile ducts/ductules</td>
<td>50</td>
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<tr>
<td>Mononuclear infiltrate of epithelium</td>
<td>50</td>
</tr>
<tr>
<td>Pyknotic/apoptotic duct cells</td>
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<tr>
<td>Intraluminal epithelial cells</td>
<td>50</td>
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<tr>
<td>Vascular</td>
<td>50</td>
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<tr>
<td>Endothelialitis</td>
<td>50</td>
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<tr>
<td>Mononuclear cells around central vein</td>
<td>50</td>
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<tr>
<td>Hepatocellular damage</td>
<td>50</td>
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<tr>
<td>Confluent necrosis</td>
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<tr>
<td>Acidophilic bodies</td>
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<tr>
<td>Mitotic figures</td>
<td>50</td>
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<tr>
<td>Neutrophil accumulations</td>
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<tr>
<td>Macrophage aggregates</td>
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<tr>
<td>Cholestasis</td>
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<tr>
<td>Steatosis</td>
<td>50</td>
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<tr>
<td>Small and large bowel</td>
<td>50</td>
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<tr>
<td>Architecture</td>
<td>50</td>
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<tr>
<td>Villus blunting</td>
<td>50</td>
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<td>Crypt regeneration</td>
<td>50</td>
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<td>Ulceration</td>
<td>50</td>
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<td>Lamina propria inflammation</td>
<td>50</td>
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<td>Crypt loss</td>
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<td>Epithelial cytology</td>
<td>50</td>
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<tr>
<td>Apoptosis</td>
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<tr>
<td>Sloughing into lumen</td>
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<tr>
<td>Lymphocytic infiltrate</td>
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<td>Neutrophilic infiltrate</td>
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<td>Vascular</td>
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<td>Edema</td>
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Pathologic changes were reflected in increased gross colon weight of the SP-A−/− alloBMT as compared with WTalloBMT controls (Fig. 3D). Histologic examination revealed that SP-A−/− alloBMT mice had a significant increase in inflammation and edema of the muscularis propria and lamina propria in both the small and large intestine when compared with the WTalloBMT mice (Fig. 3E–I). However, injury was more severe in colon than in the small intestine, possibly due to the slower rate of repair in the large intestine after radiation injury (28). Minimal GI pathology was seen in SP-A−/− synBMT or WTsynBMT or NT mice (Fig. 3B, 3E).

To determine when GI pathology develops after BMT, large intestinal pathology was assessed at 2, 3, and 4 wk posttransplant. Two weeks post-alloBMT, both WT and SP-A−/− recipients had similar colon pathology (Fig. 4). However, at 3 wk post-BMT, SP-A−/− alloBMT mice had a significant increase in pathology score, and this difference was accentuated at 4 wk post-BMT (Fig. 4). The exacerbated GVHD pathology seen at 4 wk post-BMT was associated with an increase in hallmarks of GVHD such as neutrophil and lymphocyte recruitment in the colons of SP-A−/− alloBMT that was not seen in WTalloBMT (data not shown).

**SP-A deficiency increases proinflammatory cytokine expression in large intestine after allogeneic BMT.**

To identify mediators that may potentiate the GI GVHD seen in SP-A−/− alloBMT mice, RNA was isolated from the colon, and proinflammatory cytokine mRNA expression was determined by quantitative PCR. In correlation with more severe pathological findings, SP-A−/− alloBMT mice had a significant increase in TNF-α, IL-1β, IL-6, and IFN-γ mRNA when compared with WTalloBMT controls (Fig. 5A–D). This increase in cytokine expression was unique to allogeneic BMT because there was no increase in any of the mediators after syngeneic BMT in SP-A−/− deficient recipients (Fig. 5). The upregulation of proinflammatory cytokines occurs only in the GI tissue because serum cytokine levels such as TNF-α were no different in SP-A−/− deficient recipients when compared with sufficient controls (Fig. 5E).

**SP-A deficiency increases lymphocytic inflammation in large intestine after allogeneic BMT.**

Because GVHD is also associated with the development of excessive T cell inflammation, (3, 7) and SP-A has previously been shown to influence T cell proliferation (19, 29), it was of interest to identify if SP-A attenuates T cell alloproliferation and tissue infiltration. To determine if SP-A can influence allogeneic T cell proliferation, an MLR in the presence of exogenous SP-A was performed. Allogeneic T cell proliferation was decreased in the presence of SP-A when compared with media with IgG. Syngeneic T cell proliferation was minimal, and no difference was seen with the addition of exogenous SP-A (Fig. 6A). To examine if this effect was seen in vivo, large intestine sections were stained for CD3+ cells and quantified as previously described. After alloBMT, SP-A−/− deficient recipients had a significant increase in intraepithelial and lamina propria CD3+ T cells (Fig. 6B, Supplemental Fig. 3A–D). Minimal CD3+ T cell infiltration was seen in WTalloBMT, SP-A−/− synBMT, and WTsynBMT (Supplemental Fig. 3 and data not shown, respectively).

**SP-A deficiency increases CD4 and CD8 T cells in gut-associated secondary lymphoid tissue after allogeneic BMT.**

To establish whether SP-A deficiency had an effect on CD4 or CD8 T cell numbers in the secondary lymph node structures of the GI

![FIGURE 6. SP-A deficiency leads to excessive T cell proliferation in the colon after allogeneic BMT. (A) MLR with T cells isolated from spleens of C3H/FeJ mice (2 × 10^6 cells/ml, Allo T cells) or C57BL/6J mice (Syn T cells) and stimulated with irradiated splenocytes from C57BL/6J mice (5 × 10^6 cells/ml) and cultured in media or media plus 20 μg/ml of exogenous human SP-A. Proliferation was assessed by [3H] incorporation and expressed as counts per minute. SP-A−/− or −sufficient (WT) mice were reconstituted with cells from alloBMT or synBMT mice and sacrificed 5 wk after transplant. Colon tissue was stained for CD3+ cells. Lymphocyte infiltration was quantified by averaging 10 × 400 fields in a blinded fashion. (B) Average number of CD3+ cells in colon in a ×400 field of all groups. Graph is representative of one experiment, n = 5–8/group; data were replicated in five independent experiments. *p < 0.05.**
tissue, SP-A
ferences were noted in the size or number of secondary lymphoid populations were examined by flow cytometry. Although no dif- Because CD4+ T cells have been implicated in the development in GALT after allogeneic BMT
SP-A deficiency decreases Treg cells and increases Th17 cells
Supplemental Fig. 4A–D).

A
B
C
D
E

MLN
Peyer’s Patches

CD25+

SP-A–/– alloBMT
WT alloBMT

SP-A is defined as a principle component of the lung alveolar
tract, MLNs and PPs were isolated, and CD4+ and CD8+ T cell populations were examined by flow cytometry. Although no differences were noted in the size or number of secondary lymphoid tissue, SP-A–/– alloBMT mice had a significant increase in the percent and total number of CD4+ and CD8+ T cells in the MLNs and PPs when compared with WTalloBMT mice (Fig. 7A, 7B, Supplemental Fig. 4A–D).

SP-A deficiency decreases Treg cells and increases Th17 cells in GALT after allogeneic BMT
Because CD4+ T cells have been implicated in the development of GVHD (31), we wanted to determine if there was a difference in T cell polarization in secondary lymph node structures after alloBMT in SP-A–deficient recipients. WTalloBMT mice had ~18% (18 ± 3.2) of CD4+ T cells that were CD25+Foxp3+ (Treg cells) in the MLNs and 12% (12 ± 1.3) of CD4+ T cells in PPs (Fig. 8C, 8D). However, when recipients were deficient in SP-A, Treg cells were decreased to approximately half (MLN 10 ± 1.8%; PP 7 ± 1.6%) of what was seen in WTalloBMT mice (Fig. 8A, 8B). This decrease was not seen in the MLNs or PPs of SP-A–/–/– synBMT mice when compared with WTsynBMT (data not shown). These trends were also reflected in the absolute numbers of Treg cells in the MLNs and PPs (Fig. 8E).

Th17 cells have been associated with the severity of GVHD (31, 32); therefore, we examined whether SP-A deficiency is associated with the development of Th17 cells in the secondary lymphoid structures of the GI tract. After WTalloBMT, CD3+CD4+IL-17+ (Th17) cells were not readily identified within the MLNs or PPs (Fig. 9C, 9D). However, in MLNs and PPs of SP-A–/– alloBMT, ~5% of CD4+ T cells produced IL-17 (Fig. 9A, 9B). The presence of Th17 cells was not seen in either secondary lymphoid structure after synBMT in SP-A–sufficient or –deficient recipients (data not shown). The increase in Th17 cells in the MLNs and PPs after alloBMT in SP-A–sufficient and –deficient recipients was also confirmed in the absolute number of Th17 cells (Fig. 9E). Colon explant cultures for IL-17a also reflected a similar trend with a significant increase in tissue production as seen in the secondary lymphoid structures from SP-A–/– alloBMT when compared with WT and syngeneic controls (Fig. 9F). Together, these data suggest that there is an alteration in T cell polarization toward Th17 and away from Treg cells after allotransplant, which may predispose them to the development of GI GVHD.

Discussion
GVHD pathophysiology is complex and not well understood, but is critically dependent on the differentiation of donor-derived T cells into proinflammatory subsets (3, 33) leading to marked tissue injury, morbidity, and mortality. This morbidity and mortality persists despite aggressive immunosuppressive regimens, which suggest that inflammatory pathways exist in this disease process that are poorly responsive to standard immunosuppressive regi-
lining fluid. For this reason, we expected to observe enhanced lung injury after allogeneic BMT in SP-A–deficient recipients. Contrary to this expectation, SP-A–deficient recipients did not have observable lymphocytic inflammation consistent with enhanced pulmonary GVHD in our model. This lack of exacerbated pulmonary GVHD in SP-A–deficient recipients could be explained by compensation from the other collectins such as SP-D. Previous reports using an idiopathic pneumonia syndrome model have noted that recipient mice lacking SP-A or both SP-A and SP-D have a significant increase in lung disease after an allogeneic BMT with a higher dose of T cells (34–37). The idiopathic pneumonia syndrome model used in the previous studies not only used a much higher dose of T cells at the time of BMT than the model presented in this study, but also a chemotherapeutic agent to condition the mice and a different route of injection for transplantation was used. Any of these factors could account for the variations in pulmonary pathology in SP-A–deficient mice.

Despite the lack of pulmonary pathology, SP-A–deficient recipients in the current study clearly developed enhanced morbidity after BMT, as evidenced by profound weight loss. This suggested that GVHD was occurring in other organ systems. We made the novel observation that allogeneically transplanted SP-A–deficient mice developed pathology in both the small and large intestines and cytokine production consistent with GI GVHD. Despite the predominant expression within the lung, SP-A expression is present within extrapulmonary tissues, though at lower levels than seen in the lung (38). Prior work suggests that collectins are present in the GI system and appear to have a role in the prevention of intestinal inflammation and pathology (9, 11, 37–41). Murray et al. (14) demonstrated that a lack of SP-D in the gastric mucosa significantly increased incidence of, and inflammation associated with, Helicobacter pylori infection (14). Human single nucleotide polymorphisms in SP-D have also been associated with susceptibility to Crohn’s disease (15). These works also indicate that SP-D may not be present in the GI tract to compensate for SP-A deficiency, though this hypothesis was not tested in the reported experiments. Despite the data on SP-D in GI pathology, less is known about a functional role for SP-A in the intestines. A recent study examined the immunoregulation by SP-A in the intestines. George et al. (11) identified that neonatal mice deficient in SP-A had significant intestinal inflammation and pathology after endotoxin exposure. These data and ours cumulatively suggest that surfactant proteins regulate inflammation and adaptive immunity in the GI system.

One limitation of the present work is that we are unable to reliably identify SP-A in the intestines of mice. Epithelial cells in the intestines of rats and humans have been shown to produce both SP-A mRNA and protein (9, 10). This protein has the same charge and m.w. as the SP-A found in the lungs. As a result of similarities among collectins as well as containing major blood group antigenic epitopes (42), the reliability of detecting SP-A poses a challenge and is difficult based on current available reagents. In our hands, we were able to detect transcripts for SP-A in the GI tract, but were not able to discern SP-A versus other collectins in the GI tract (data not shown) using previously established protocols. However, there is a body of literature that identified SP-A in other tissues besides the lung and demonstrates that deficiencies of this protein lead to extrapulmonary diseases (37, 40, 43, 44),

**FIGURE 9.** SP-A deficiency promotes Th17 cell polarization in the GI tract after allogeneic BMT. After BMT, MLNs and PPs were isolated, and cells were analyzed by flow cytometry. Representative flow cytometric plots show CD3⁺CD4⁺IL-17⁺ (Th17) populations for each experimental group (MLNs): SP-A⁻/⁻alloBMT (A) and WTalloBMT (B); PPs: SP-A⁻/⁻alloBMT (C) and WTalloBMT (D). (E) Absolute numbers of Th17 cells in MLNs and PPs. (F) IL-17a production by colon explant cultures was measured by ELISA at 24 h postharvest. Graph is representative of one experiment, n = 5–8/group; data were replicated in three independent experiments. *p < 0.05.
supporting the protective role of SP-A in multiple organs. The mechanism by which SP-A regulates susceptibility to GI GVHD could be an indirect effect (spillover of inflammation from the lung) or a local effect (production by the GI tract). We favor the local effect because serum cytokines were unaltered, and the lungs of the SP-A^{-} alloBMT mice had no significant changes in pathology, cell counts, or T cell infiltrates. An indirect effect could be considered because SP-A does have an important role in controlling inflammation and T cell proliferation in other organs, although our findings do not support this thus far.

SP-A has been reported to influence both innate and adaptive immune responses. Mice lacking SP-A challenged with respiratory pathogens have not only delayed clearance but also significant increase in proinflammatory cytokines such as IL-6 and TNF-α by alveolar macrophages (45–47), as well as enhanced APC recruitment and maturation (48). SP-A has also been shown to influence adaptive immune responses by preventing T cell proliferation (49) and influencing pulmonary phenotypes (19) through interaction with not only T lymphocytes, but also APCs (50). We extend these observations by demonstrating that SP-A is important for limiting the expansion of lymphocyte populations after allogeneic BMT. We also identify that there is a preferential polarization of Treg cells to Th17 cells in the SP-A−/− recipients. Recent studies have shown that in vitro-differentiated Th17 cells can mediate acute GVHD with severe pathology in the mucosal organs (31). The mechanism of graft tolerance has also been shown to involve Treg cells and their capacity to confer immune tolerance of allo- as well as autoantigens (50). Therefore, the balance between Th17 and Treg cells appears to be critical for immune response responsible for GVHD. Our data suggest that decreased SP-A levels or function in the GI tract may allow excessive APC maturation and increased allogeneic T lymphocyte response, promoting the elaboration of proinflammatory cytokines and thereby causing GI GVHD.

In conclusion, our findings in this study describe an entirely novel role for SP-A in regulating the immune response in the development of GI GVHD. Although our current study did not determine the precise mechanisms of how SP-A influences the development of GI disease after allogeneic BMT, the presence of increased Th17 cells in the GI tract in association with a subsequent decrease in Treg cells suggests a potential role for SP-A in influencing these T cell subsets in extrapulmonary tissues. Restoring SP-A specifically in the GI tract may be a viable approach to pursue in future studies in an attempt to develop new avenues for treating patients and reducing the burden of GVHD after BMT.

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References


Corrections


The sixth author’s name was published incorrectly. The correct spelling is Sambuddho Mukherjee.

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