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Novel role for surfactant protein A in gastrointestinal graft-versus-host disease

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Abstract

Background—Graft-versus-host-disease (GVHD) is a severe and frequent complication of allogeneic bone marrow transplantation (BMT) that involves the gastrointestinal tract and lungs. The pathobiology of GVHD is complex and involves immune cell recognition of host antigens as foreign. We hypothesize a central role for the collectin surfactant protein A (SP-A) in regulating the development of GVHD after allogeneic BMT.

Methods—C57BL/6 (*H2b*; WT) and SP-A deficient mice on C57BL/6 background (*H2b*; SP-$A^{-/-}$) mice underwent allogeneic (Allo) or syngeneic (Syn) BMT with cells from either C3HeB/FeJ (*H2k*; SP-$A^{-/-}$alloBMT or WTalloBMT) or C57Bl/6 (*H2b*; SP-$A^{-/-}$synBMT or WTsynBMT) mice. 5 weeks post BMT, mice were necropsied and lung and gastrointestinal (GI) tissue were analyzed.

Results—SP-$A^{-/-}$alloBMT or WTalloBMT had no significant differences in lung pathology however, SP-$A^{-/-}$alloBMT mice developed marked features of GI GVHD including decreased body weight, increased tissue inflammation 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 (Treg) cells.

Conclusions—Our results demonstrate the first evidence 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 alloBMT have a greater incidence of GI GVHD that is associated with increased Th17 cells and decreased Tregs. 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.

Keywords

Graft-versus-host disease; Surfactant Protein A; Th17; Regulatory T cells

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INTRODUCTION

Approximately 50,000 patients worldwide undergo bone marrow transplantation (BMT) each year for treatment of both malignant and non-malignant 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 naïve 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 cells (Treg), a specialized subpopulation of T cells which suppresses activation and/or extent of the proinflammatory T cell response, at mucosal surfaces, such as the GI tract, (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), time to parturition (13), while other collectins such as 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.

Herein 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 suggests that SP-A has an important immunological role in the prevention of gastrointestinal GVHD after BMT.

MATERIALS AND METHODS

Mice

Experiments were approved by the Duke Institutional Animal Care and Use Committees. Male 8–10 week-old C57BL/6 (*H2b*; WT) and SP-A deficient mice were maintained on C57BL/6 background (*H2b*;SP-A$^{-/-}$). C3HeB/FeJ (*H2k*;allogeneic) or C57BL/6 (*H2b*;syngeneic) mice were used as donors and were obtained from the Jackson Laboratories (Bar Harbor, ME). All animals were housed in a pathogen-free facility on LPS-free bedding (Shepherd Specialty Papers Inc., Kalamazoo, MI), with irradiated food (PicoLab Mouse Diet

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20-5058, Purina Mills, Richmond, IN) and antibiotic water (Sulfamethoxazole/Trimethoprim 1.2/0.24mg/mL) after BMT.

Murine bone marrow transplantation

Donor mice were euthanized using CO_2. Tibia and femur bone marrow and splenocytes were isolated, filtered twice through 70μm 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 lethally irradiated using a Cesium irradiator (9.25 Gy) and injected via retro-orbital route with 4×10^6 bone marrow cells and 1×10^6 splenocytes. Engraftment was evaluated 4 weeks post-transplantation using peripheral blood flow cytometry with anti-H2Db-FITC (clone KH95) and anti-H2Kk-PE (clone 36-7-5) (BD Biosciences). Animals were >95% engrafted with donor-derived cells.

Histologic Assessment of Acute Graft Versus Host Disease

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 weeks 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 hematoxylin and eosin (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 blinded pathologist (D.M.C for GI samples, S.M.P for lung samples). For samples of the GI tract (liver, distal small intestine, proximal large intestine and rectum) a semiquantitative scoring system was used to document the presence and severity of various GVHD associated morphologic features (23, 24). The specific parameters scored are listed in Table I. A grade was given for each parameter was as follows: 0 as normal, 0.5 as focal and rare, 1 as focal and mild, 2 as diffuse and mild, 3 as diffuse and moderate, and 4 as diffuse and severe. The grades were subsequently added to provide a total score for each specimen. For lung samples pathological severity of lymphocytic lung inflammation was graded on a 9-point scale as described previously (25).

$CD3^+$ Immunohistochemistry

CD3 immunohistochemistry was performed with rabbit anti-mouse CD3 antibody (Thermo Scientific, Fremont, CA) used at a dilution of 1:150 and developed with horseradish peroxidase-conjugated streptavidin (Vector Laboratory, Burlingame, CA) and then diaminobenzidine (Dako, Carpinteria, CA). To quantify the amount of intraepithelial lymphocytes, the most densely inflamed areas (away from prominent lymphoid follicles) were identified for each colon sample. The number of CD3 positive intraepithelial lymphocytes were counted in 10 consecutive fields at 400× magnification and averaged per sample.

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 Laboratories). For additional transcript analysis, cDNA was transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). 50ng of cDNA was used for qPCR (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 (4352933) (Applied Biosystems). Ct values were determined using ABI 7500 Real Time PCR System with SDS software 1.3.1. Change in expression was calculated

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using the $2^{-\Delta\Delta Ct}$ method normalized to β-actin expression and expressed as fold-change compared to the control group.

Serum ELISA

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

Peyer's patches and mesenteric lymph node flow cytometry

Peyer's patches and mesenteric lymph node were isolated, filtered through a 70μm filter (BD Biosciences), washed, and resuspended in PBS with 3% FBS, 0.05% sodium azide (VWR International, West Chester, PA), and 10mM EDTA. Live cells were counted using 0.4% Trypan-Blue (Sigma-Aldrich) dead-cell exclusion. Cells were blocked using 5% normal-mouse-serum, 5% normal-rat-serum (Jackson ImmunoResearch Laboratories Inc., 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 phorbol myristate acetate and 500 ng/ml ionomycin (Sigma) for 4 hours before staining and incubated with GolgiStop (BD Biosciences) during the last 3 hours of stimulation. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and labeled with antibodies against IL-17A-PE (clone eBio17B7, eBioscience) and IFN-γ-APC (clone XMG1.2, eBioscience). For staining of surface antigens, cells were labeled with anti-mouse anti-CD3-FITC, anti-CD8-PE-Cy7, anti-CD11b-APC-Cy7, and anti-CD4-PE-Cy5 (eBioscience). For intracellular 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 BD FACSCantoII flow cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star Inc., 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.

Mixed Leukocyte Reaction

T cells from C57BL/6 were isolated from spleens by negative magnetic bead selection (Pan T cell beads; Miltenyi Biotec Inc., Auburn, CA). Isolated T cells were washed in media, filtered through 70 μm filters (BD, Franklin Lakes, NJ), counted on a hemocytometer, and resuspended in RPMI 1640 (Gibco, Grand Island, NY) containing Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO), L-Glutamine (Sigma-Aldrich, St. Louis, MO) 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 co-cultured with 5×10^5 C57Bl/6 responder T cells in the presence of 20μg/ml of exogenous SP-A (purified as previously described (26)) or 20μg/ml of purified mouse control IgG (Jackson Immunoresearch, West Grove, PA) for 4 days. 1 uCi of ^{3}H-thymidine was added for the last 18 hours of culture. 4 days following initial culture, splenocytes were harvested (TomTec harvester 96 Mach III M, Hamden, CT) and radioactivity was measured using a β-counter (Wallac Trilux 1450 Microbeta counter, Waltham, MA). Results are reported as counts per minute (cpm) and proliferation was determined by subtracting cpm of responders alone from cpm in wells containing both responders and stimulators.

Colonic tissue explant cultures

Colonic tissue fragments (0.5 g dry weight) were harvested 5 weeks 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 enzyme-linked immunosorbent assay 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 Graph Pad Prism Software (Version 5.03, La Jolla, CA). P-values of <0.05 were considered significant. The study was repeated 5 independent times with an n of 5–8 mice per group per experiment. The data shown here is 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) donor cells were assessed for perivascular and peribronchiolar lymphocytic inflammation (Figure 1 and Supplemental Figure 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 (SP-A$^{-/-}$alloBMT or WTalloBMT). This trend was also reflected in the BAL cell counts and T cell infiltration into the airspace (data not shown). Minimal pathology was seen with mice that had undergone syngeneic BMT (SP-A$^{-/-}$synBMT or WTsynBMT) (Figure 1 and Supplemental Figure 1). This data suggested that SP-A does not regulate alloimmune lung injury after allogeneic bone marrow transplantation.

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 approximately 85% of starting body weight after transplantation. This observation was not seen in WTalloBMT, syngeneic, or NT controls. After BMT, all treatment groups lost approximately 20% of body weight at 2 weeks post transplantation. WT mice that had undergone an alloBMT and syngeneic controls were able to regain back to 100% of starting body weight by 4 weeks post BMT (Fig. 2).

Given the severe weight loss in SP-A$^{-/-}$alloBMT mice, we were interested to examine the pathology of the gastrointestinal tract to describe a cause for this weight loss. The liver, small and large intestines were examined for features of gastrointestinal GVHD (Fig. 3A, B, and D). 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 Figure 2A and B). These gross pathologic changes were reflected in increased gross colon weight of the SP-A$^{-/-}$alloBMT as compared to WTalloBMT controls (Fig. 3D). Histologic examination revealed that SP-A$^{-/-}$alloBMT mice had a significant increase in inflammation and edema of the musclaris propria and lamina propria in both the small and large intestine when compared to the WTalloBMT mice (Fig. 3 E–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 and E).

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To determine when GI pathology develops after BMT, large intestinal pathology was assessed at 2, 3, and 4 weeks post-transplant. 2 weeks post alloBMT, both WT and SP-A$^{-/-}$ recipients has similar colon pathology (Fig. 4). However, at 3 weeks post BMT, SP-A$^{-/-}$alloBMT mice had a significant increase in pathology score and this difference was accentuated at 4 weeks post BMT (Fig. 4). The exacerbated GVHD pathology seen at 4 weeks 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 qPCR. 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 to WTalloBMT controls (Fig. 5A–D). This increase in cytokine expression was unique to allogeneic BMT since there was no increase in any of the mediators after syngeneic BMT in SP-A sufficient or deficient recipients (Fig. 5). The upregulation of proinflammatory cytokines occurs only in the GI tissue since serum cytokine levels such as TNF-α were no different in SP-A deficient recipients when compared to sufficient controls (Fig. 5E).

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

Since 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 (29, 30), 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, a mixed lymphocyte reaction (MLR) in the presence of exogenous SP-A was performed. Allogeneic T cell proliferation was decreased in the presence of SP-A when compared to 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 (Figure 6B and Supplemental Figure 3). Minimal CD3^{+} T cell infiltration was seen in WTalloBMT, SP-A$^{-/-}$synBMT, and WTsynBMT (Figure 6B and Supplemental Figure 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 or not SP-A deficiency had an effect on CD4 or CD8 T cell numbers in the secondary lymph node structures of the GI tract, mesenteric lymph nodes (MLNs) and Peyer's patches (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 to WTalloBMT mice (Fig. 7A and B; Supplemental Figure 4).

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SP-A deficiency decreases Tregs and increases Th17 cells in gut-associated lymphoid tissue after allogeneic BMT

Since $CD4^+$ 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 approximately 18% (18 ± 3.2) of $CD4^+$ T cells that were $CD25^+FoxP3^+$ (Tregs) in the MLNs and 12% (12 ± 1.3) of $CD4^+$ T cells in PPs (Fig. 8C and D). However, when recipients were deficient in SP-A, Tregs were decreased to approximately half (MLN 10% ± 1.8; PP 7% ± 1.6) of what was seen in WTalloBMT mice (Fig. 8A and B). This decrease was not seen in the MLNs or PPs of $SP\text{-}A^{-/-}$synBMT mice when compared to WTsynBMT (data not shown). These trends were also reflected in the absolute numbers of Tregs in the MLNs and PPs (Fig. 8E).

Th17 cells have been associated with the severity of GVHD (31, 32), therefore, we examined whether or not SP-A deficiency is associated with the development of Th17 cells in the secondary lymphoid structures of the GI tract. After WTalloBMT, $CD3^+CD4^+IL\text{-}17^+$ (Th17) cells were not readily identified within the MLNs or PPs (Fig. 9C and D). However, in MLNs and PPs of $SP\text{-}A^{-/-}$alloBMT, approximately 5% of $CD4^+$ T cells produced IL-17 (Fig. 9A and B). 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 $SP\text{-}A^{-/-}$alloBMT when compared to WT and syngeneic controls (Fig. 9F). Together, these data suggest that there is an alteration in T cell polarization towards Th17 and away from regulatory T cells in SP-A deficient mice after allotransplant, which may predispose them to the development of GI GVHD.

DISCUSSION

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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 regimens. The failure of standard immunosuppressive regimens necessitates an understanding of specific mechanisms by which the host develops GVHD after bone marrow transplant in order to effectively treat GVHD. We present, in this current study, novel data that suggest that SP-A protects against the development of GI GVHD presumably by a reduction in the production of proinflammatory cytokines. In addition, we highlight a potential mechanistic role for SP-A in maintaining specific T cell polarization in mucosal tissues after alloBMT.

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The enhanced allogeneic T cell proliferation and exacerbated GI pathology in the SP-A deficient mice was unexpected in light of what is known about SP-A expression and production. Classically, SP-A is defined as a principle component of the lung alveolar 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 (IPS) 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 IPS model used in the previous studies not only

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used a much higher dose of T cells at the time of BMT than the model presented here. 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 intestine and cytokine production consistent with GI GVHD. Despite the predominant expression within the lung, SP-A expression is present at 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 (39–45). Murray et al. (46) demonstrated that a lack of SP-D in the gastric mucosa significantly increased incidence of, and inflammation associated with, Helicobacter pylori infection (14). Human SNPs in SP-D have also been associated with susceptibility to Crohn's disease (47). 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. identified that neonatal mice deficient in SP-A had significant intestinal inflammation and pathology after endotoxin exposure (43). This 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 molecular weight as the SP-A found in the lungs. As a result of similarities among collectins as well as containing major blood group antigenic epitopes (48), 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 which demonstrate that deficiencies of this protein leads to extrapulmonary diseases (42, 45, 49, 50) supporting the protective role of SP-A in multiple organs. The mechanism by how 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 since 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 since 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 (51–53), as well as enhanced antigen presenting cell (APC) recruitment and maturation (54). SP-A has also been shown to influence adaptive immune responses by preventing T cell proliferation (55) and influencing pulmonary phenotypes (19), through interaction with not only T lymphocytes but also APCs (20). We extend these observations by demonstrating that SP-A is important for limiting the expansion of lymphocyte populations after allogeneic bone marrow transplantation. We also identify that there is a

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preferential polarization from Tregs to Th17 cells in the SP-A deficient recipients. Recent studies have shown that *in vitro* differentiated Th17 cells can mediate acute GVHD with severe pathology in the mucosal organs (32). The mechanism of graft tolerance has also been shown to involve Treg cells and their capability to confer immune tolerance of allo- as well as auto-antigens (56). Therefore, the balance between Th17 and Tregs appear to be critical for immune response responsible for GVHD. Our data suggests 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 regulatory T 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Allo	allogeneic
Syn	syngeneic
SP-A	surfactant protein A
SP-D	surfactant protein D
BMT	bone marrow transplant
GI	gastrointestinal
GVHD	graft-versus-host disease
APC	antigen presenting cell
H&E	hematoxylin and eosin
WT	wild-type
MHC	major histocompatibility complex

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SP-A$^{-/-}$ alloBMT	SP-A deficient recipient mice that have undergone an allogeneic BMT
SP-A$^{-/-}$ synBMT	SP-A deficient recipient mice that have undergone a syngeneic BMT
WTalloBMT	SP-A sufficient recipient mice that have undergone an allogeneic BMT
WTsynBMT	SP-A sufficient recipient mice that have undergone a syngeneic BMT
NT	nontransplanted
Treg	regulatory T cells
Th17	T cells producing IL-17

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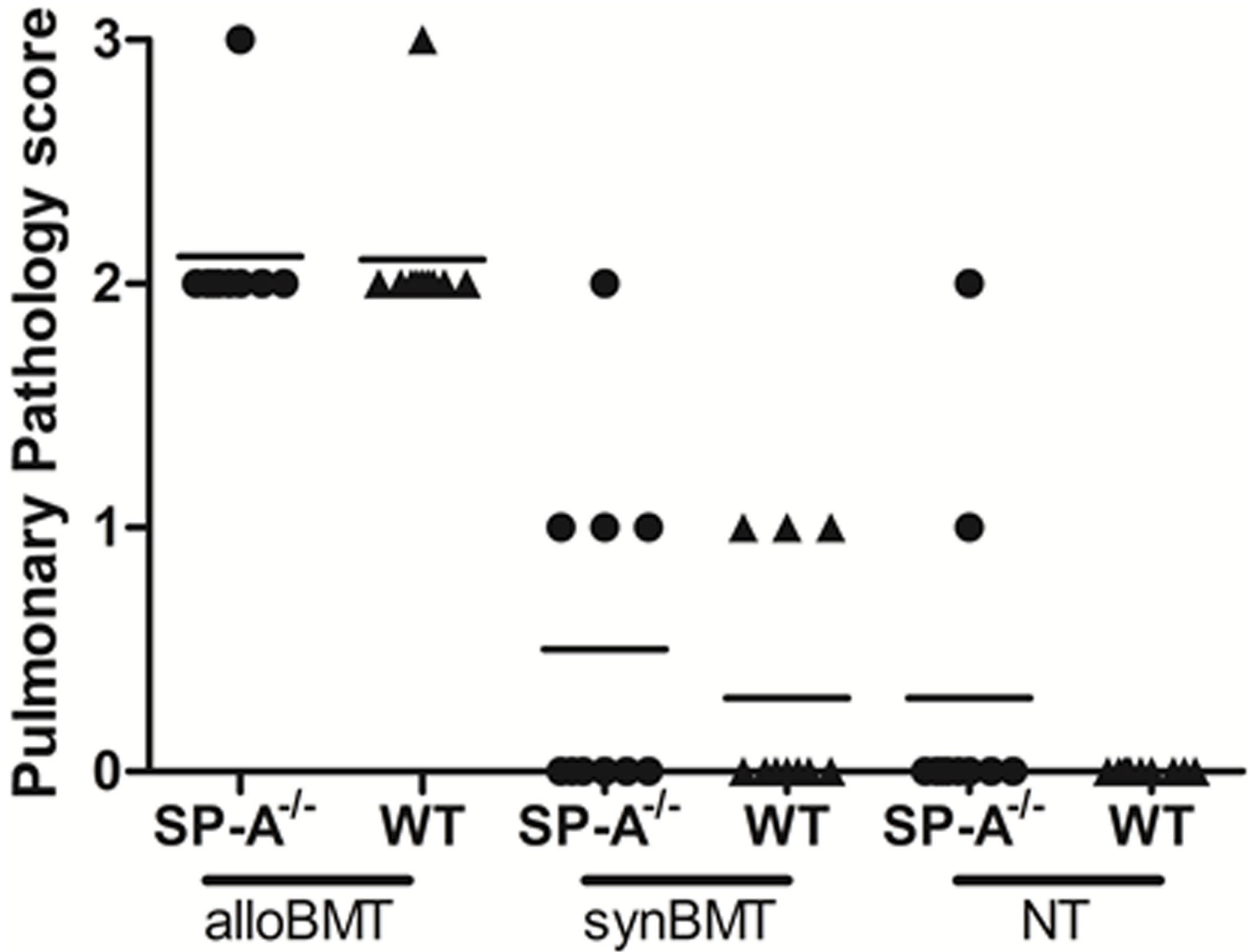

FIGURE 1. Surfactant Protein A (SP-A) deficiency does not affect the development of pulmonary GVHD after allogeneic BMT

SP-A deficient (SP-A$^{-/-}$) or sufficient (WT) mice were reconstituted with cells from allogeneic (alloBMT) or syngeneic (synBMT) mice and sacrificed 5 weeks after transplant. Lung pathology was evaluated and pathology grades were determined in a blinded fashion using a semi-quantitative scoring system based on the thickness of perivascular and peribronchiolar inflammation as well as the overall percentage of lung involved. (A) Representative histology of lymphocytic perivascular and peribronchiolar inflammation (B–E) (H&E stain, 100×) (B) SP-A$^{-/-}$alloBMT; (C) WTalloBMT (D) SP-A$^{-/-}$synBMT; (E) WTsynBMT; (graph is representative of 1 experiment n=5–8/group, data was replicated in 5 independent experiments).

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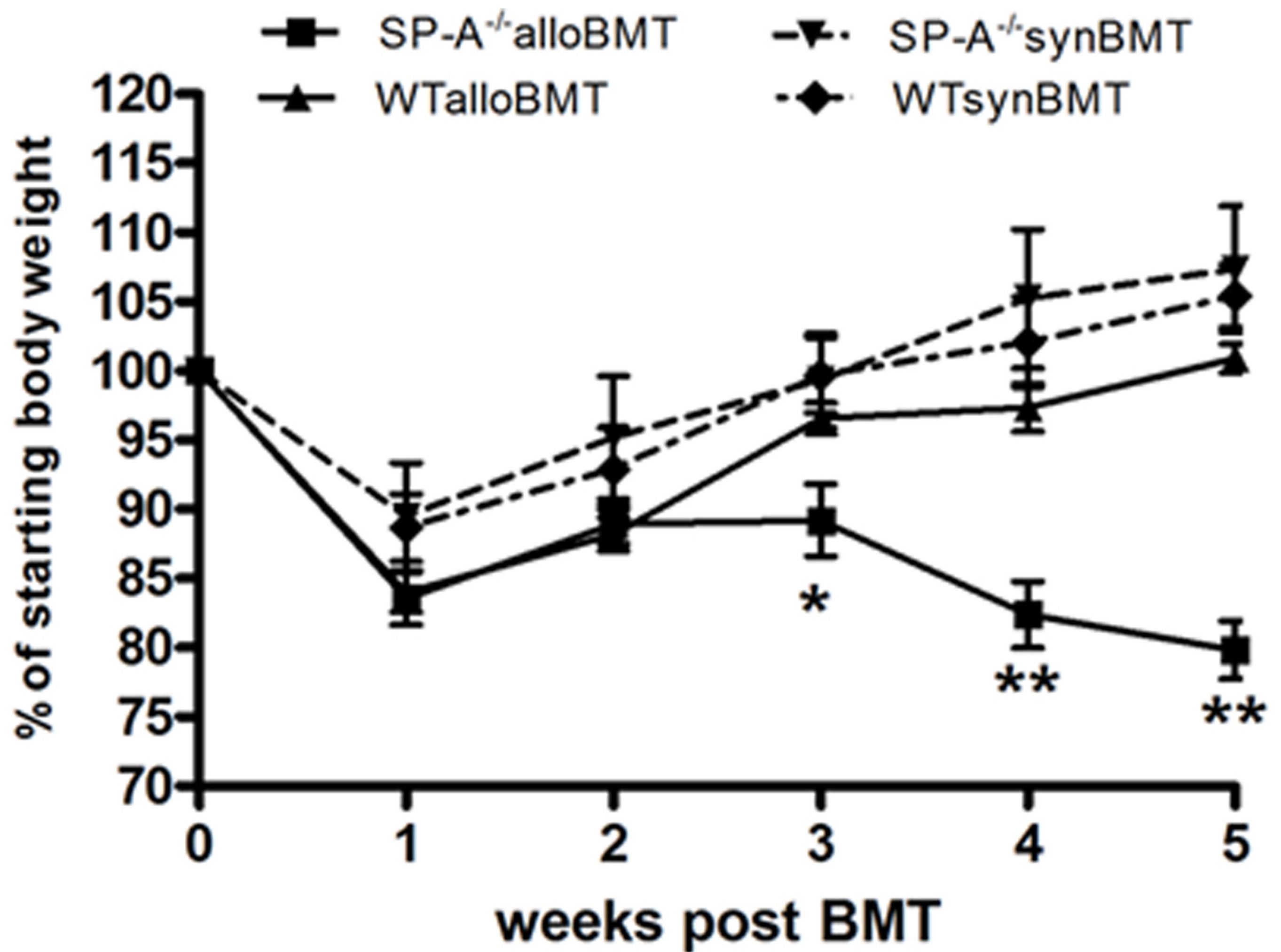

FIGURE 2. Surfactant Protein A (SP-A) deficiency decreases body weight regain after allogeneic BMT

Percent of starting body weights after BMT at weeks 1, 2, 3, 4, and 5 post transplant (* = p-value less than 0.05, ** = p-value less than 0.001) (graph is representative of 1 experiment n=5–8/group, data was replicated in 5 independent experiments).

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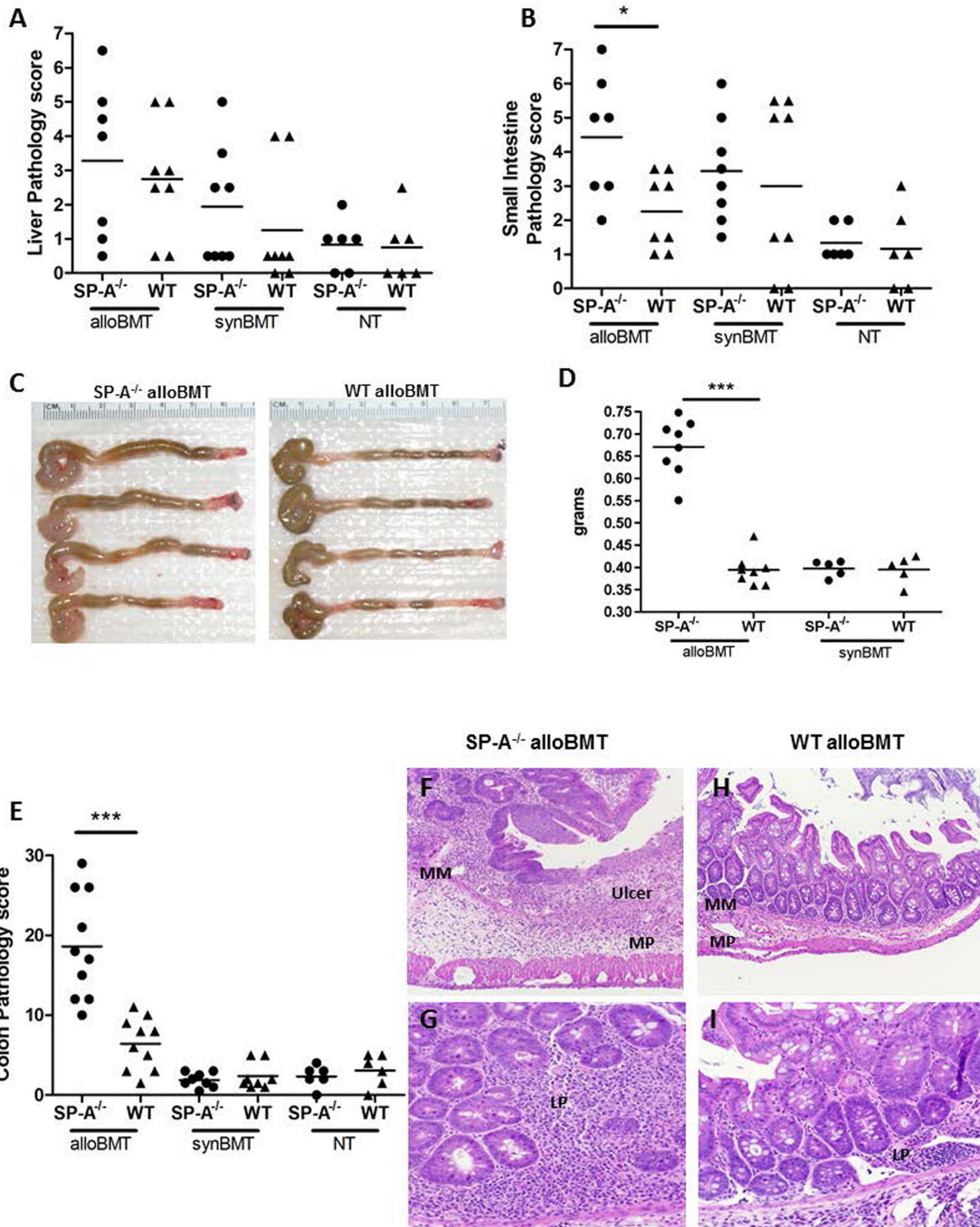

FIGURE 3. SP-A deficiency exacerbates features of gastrointestinal GVHD after allogeneic BMT

SP-A deficient (SP-A$^{-/-}$) or sufficient (WT) mice were reconstituted with cells from allogeneic (alloBMT) or syngeneic (synBMT) mice and sacrificed 5 weeks after transplant. Liver, small intestine and colon pathology was evaluated and pathology grades were determined in a blinded fashion using a semi-quantitative scoring system described in table I. Quantitative scoring of (A) liver pathology; (B) small intestine; and (E) colon 5 weeks post BMT. (C) Gross pathology of SP-A$^{-/-}$alloBMT (left panel) and WTalloBMT (right panel). (D) Weights of colons 5 weeks post BMT. (F–I) Representative colon histology sections are shown for alloBMT groups (H&E stain, 40×) (F) SP-A$^{-/-}$alloBMT; (G) WTalloBMT; (H&E stain, 200×) (H) SP-A$^{-/-}$alloBMT; (I) WTalloBMT. (* = p-value less

than 0.05, *** = p-value less than 0.0001) (graph is representative of 1 experiment n=5–8/ group, data was replicated in 5 independent experiments).

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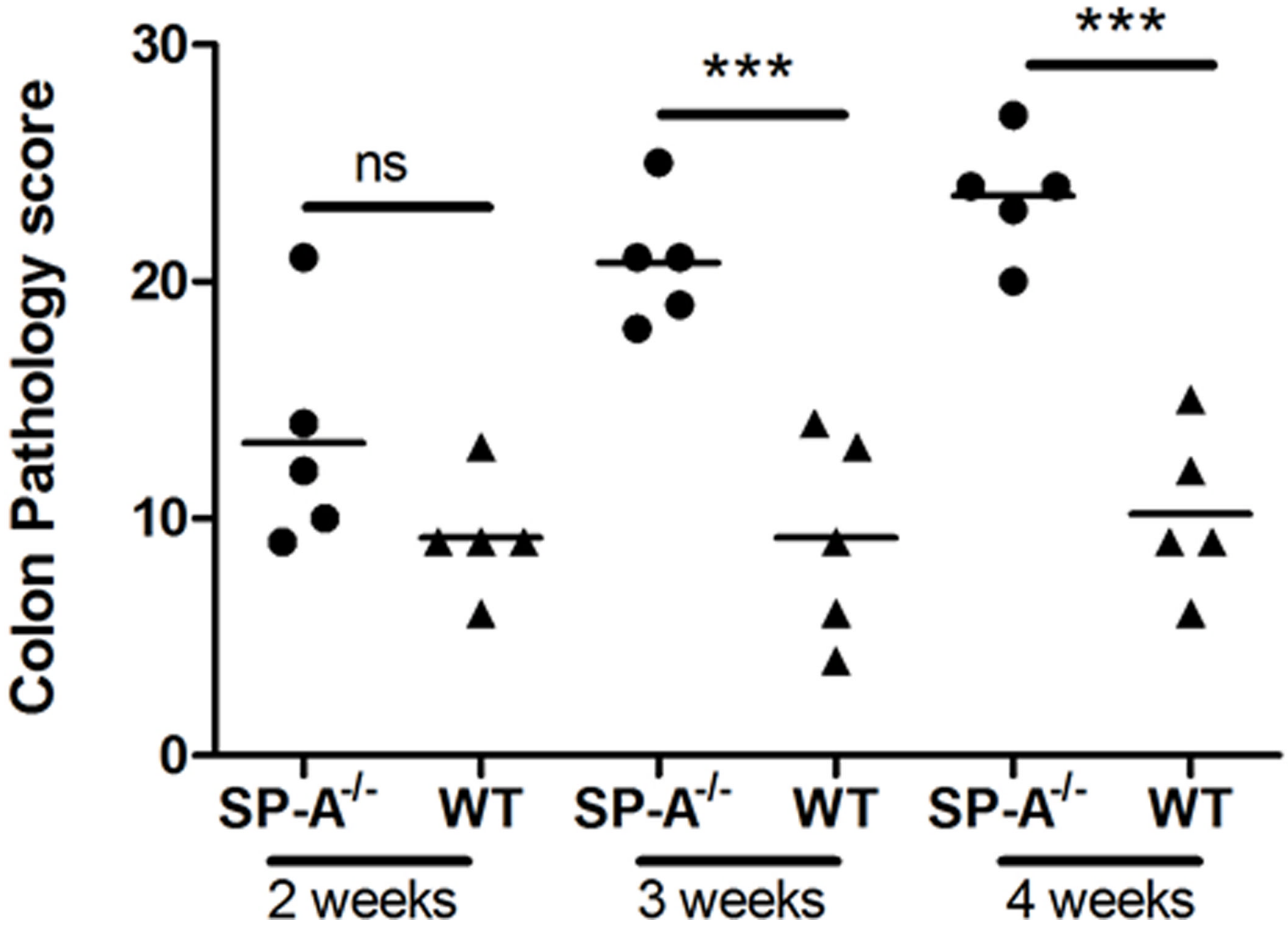

FIGURE 4. SP-A deficiency leads to features of gastrointestinal GVHD after allogeneic BMT by week 3 post BMT

SP-A deficient (SP-A$^{-/-}$) or sufficient (WT) mice were reconstituted with cells from allogeneic (alloBMT) or syngeneic (synBMT) mice and sacrificed at 2, 3, and 4 weeks after transplant. Colon pathology was evaluated at all time points and pathology grades were determined in a blinded fashion using a semi-quantitative scoring system described in table I. (*** = p-value less than 0.0001, ns= not significant) (graph is representative of 1 experiment n=5–8/group, data was replicated in 5 independent experiments).

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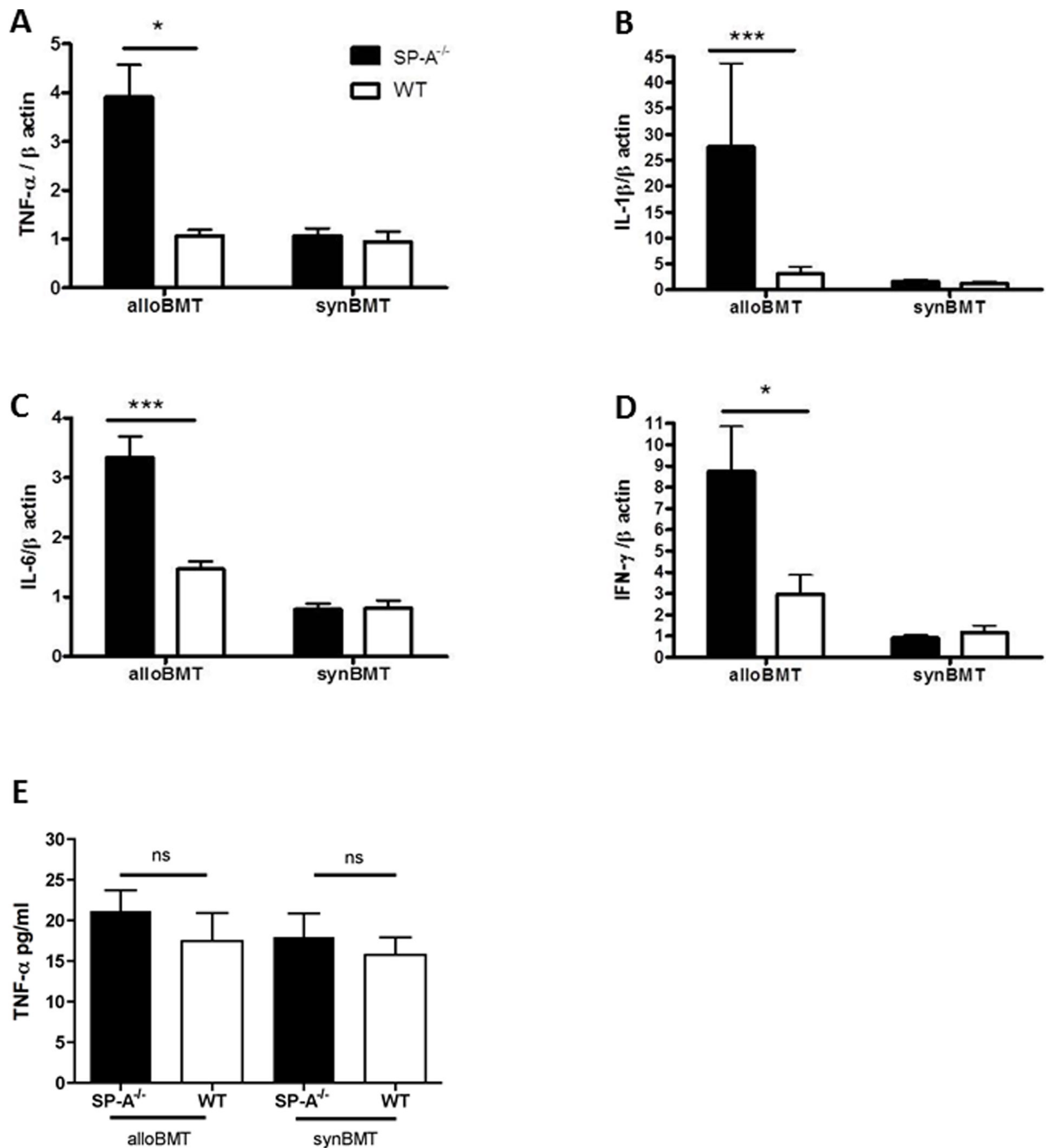

FIGURE 5. Surfactant Protein A (SP-A) deficiency promotes the upregulation of proinflammatory cytokine RNA in the colon after allogeneic BMT

5 weeks after BMT, colon tissue was analyzed by real time PCR for cytokine expression. (A) TNF-α (B) IL-1β (C) IL-6 and (D) IFN-γ transcripts. (* = p-value less than 0.05, *** = p-value less than 0.0001) (graph is representative of 1 experiment n=5–8/group, data was replicated in 5 independent experiments).

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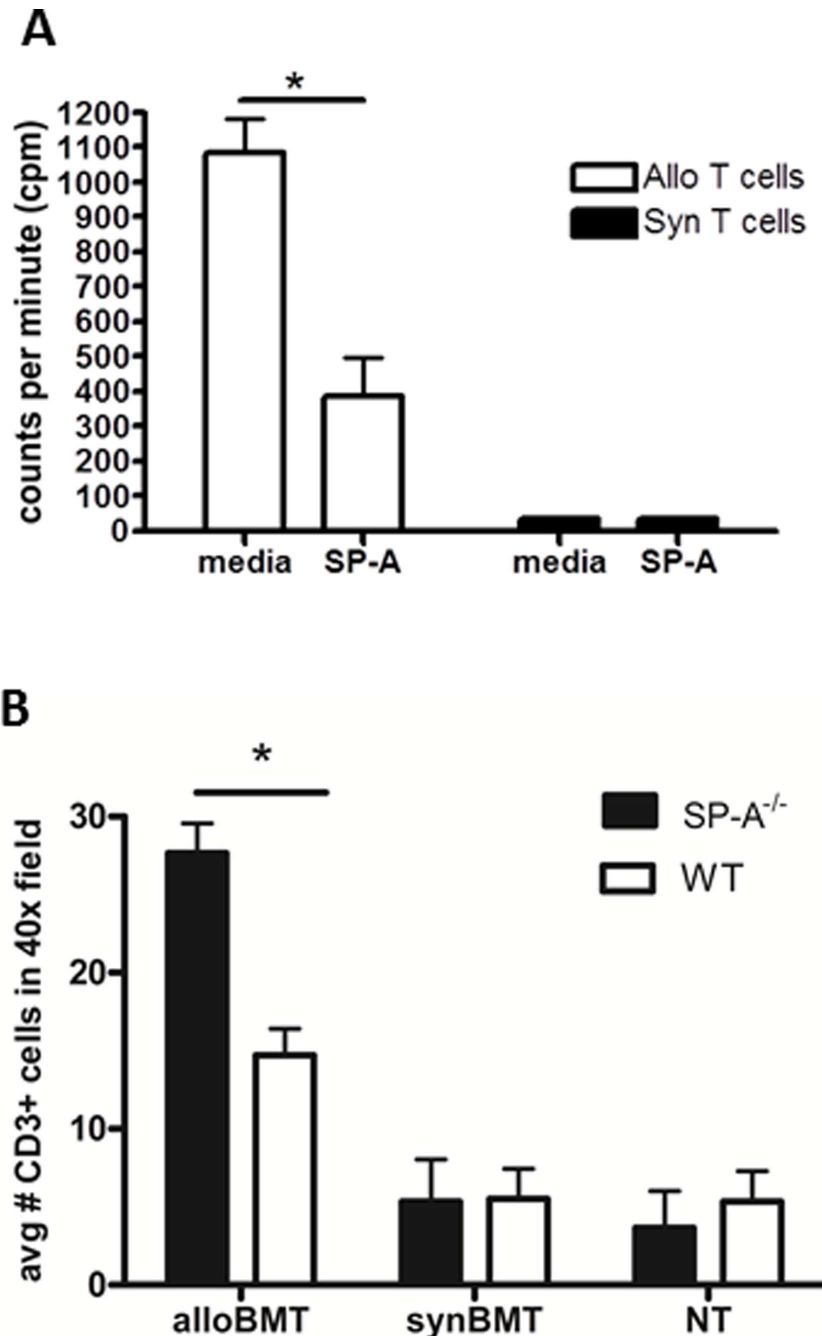

FIGURE 6. SP-A deficiency leads to excessive T cell proliferation in the colon after allogeneic BMT

(A) Mixed lymphocyte reaction with T cells isolated from spleens of C3BFeJ 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 ug/ml of exogenous human SP-A. Proliferation was assessed by ^{3}H incorporation and expressed as counts per minute (cpm). SP-A deficient (SP-A$^{-/-}$) or sufficient (WT) mice were reconstituted with cells from allogeneic (alloBMT) or syngeneic (synBMT) mice and sacrificed 5 weeks 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; (C–F) Representative $CD3^+$ staining of colon sections are shown for alloBMT groups ($CD3^+$ stain, 40×) (C) SP-$A^{-/-}$alloBMT; (D) WTalloBMT; ($CD3^+$ stain, 400×) (E) SP-$A^{-/-}$alloBMT; (F) WTalloBMT. (* = p-value less than 0.05) (graph is representative of 1 experiment n=5–8/group, data was replicated in 5 independent experiments).

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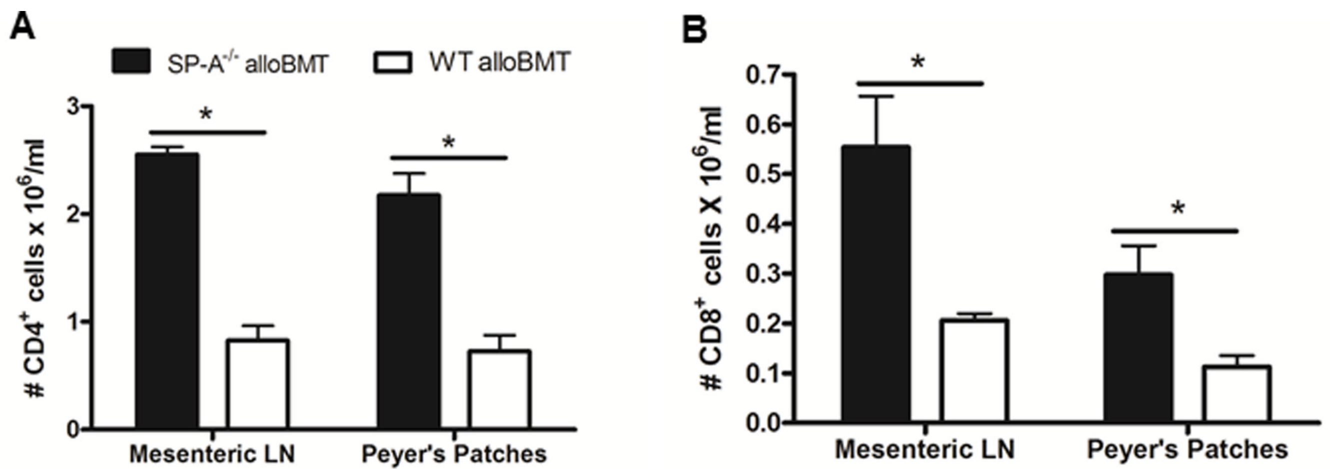

FIGURE 7. SP-A deficiency increases the number of CD4 and CD8 T cells in mesenteric lymph nodes and Peyer's patches after allogeneic BMT

5 weeks after BMT, mesenteric lymph nodes and Peyer's patches were isolated and analyzed by flow cytometry for T cell phenotypes. (A) Absolute numbers of $CD4^+$ T cells in MLNs and PPs; (B) Absolute numbers of $CD8^+$ T cells in MLNs and PPs; (* = p-value less than 0.05) (graph is representative of 1 experiment n=5–8/group, data was replicated in 3 independent experiments).

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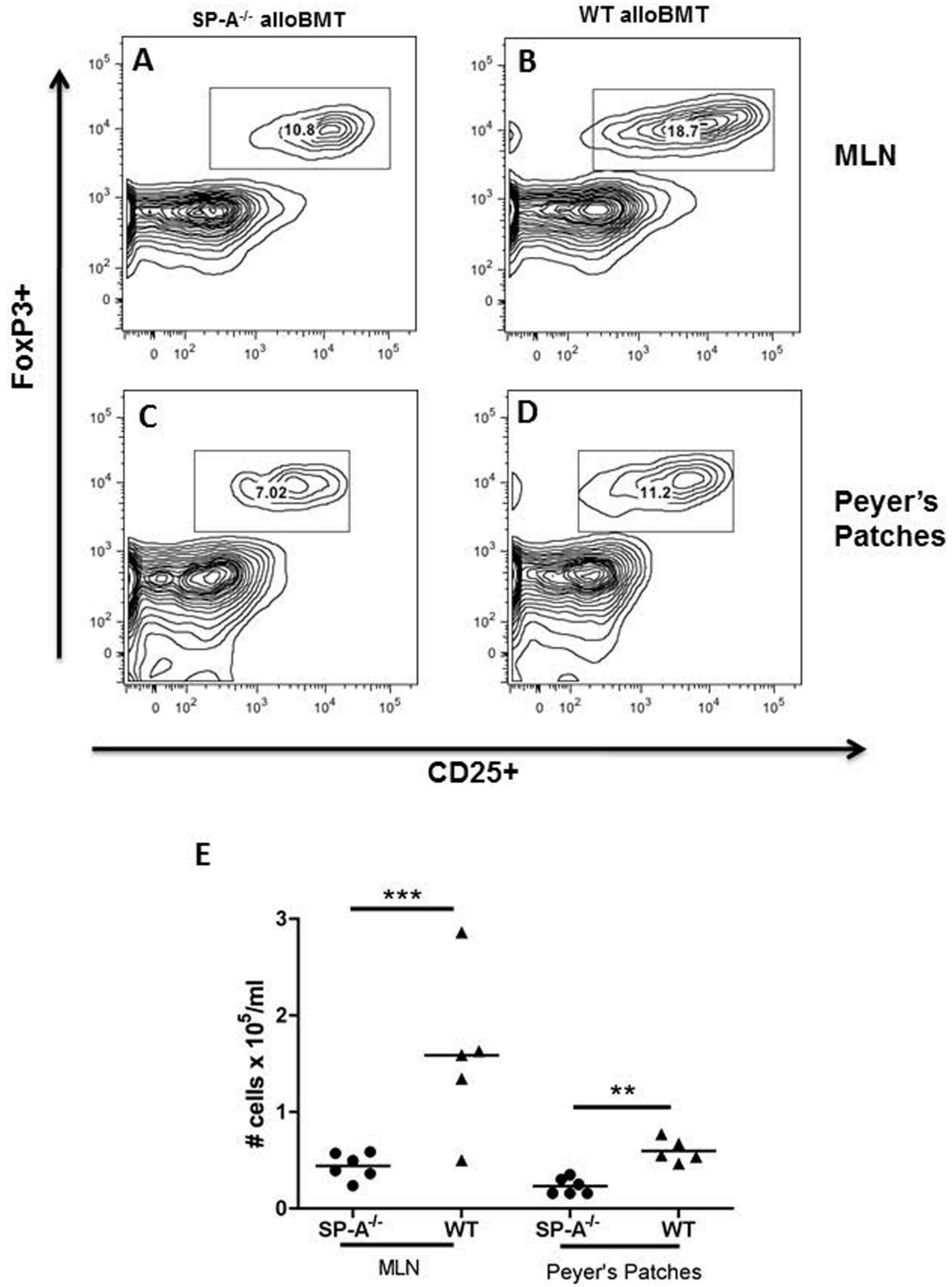

FIGURE 8. SP-A deficiency decreases regulatory T cell differentiation in the gastrointestinal tract after allogeneic BMT

After BMT, mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) cells were isolated and cells were analyzed by flow cytometry. (A–D) Representative flow cytometric plots show $CD3^+CD4^+CD25^+FoxP3^+$ (Treg) populations for each experimental group (A) SP-$A^{-/-}$alloBMT; (B) WTalloBMT; (PPs) (C) SP-$A^{-/-}$alloBMT; (D) WTalloBMT; (E) Absolute numbers of Treg cells in MLNs and PPs. (** = p-value less than 0.001, *** = p-value less than 0.0001) (graph is representative of 1 experiment n=5–8/group, data was replicated in 3 independent experiments).

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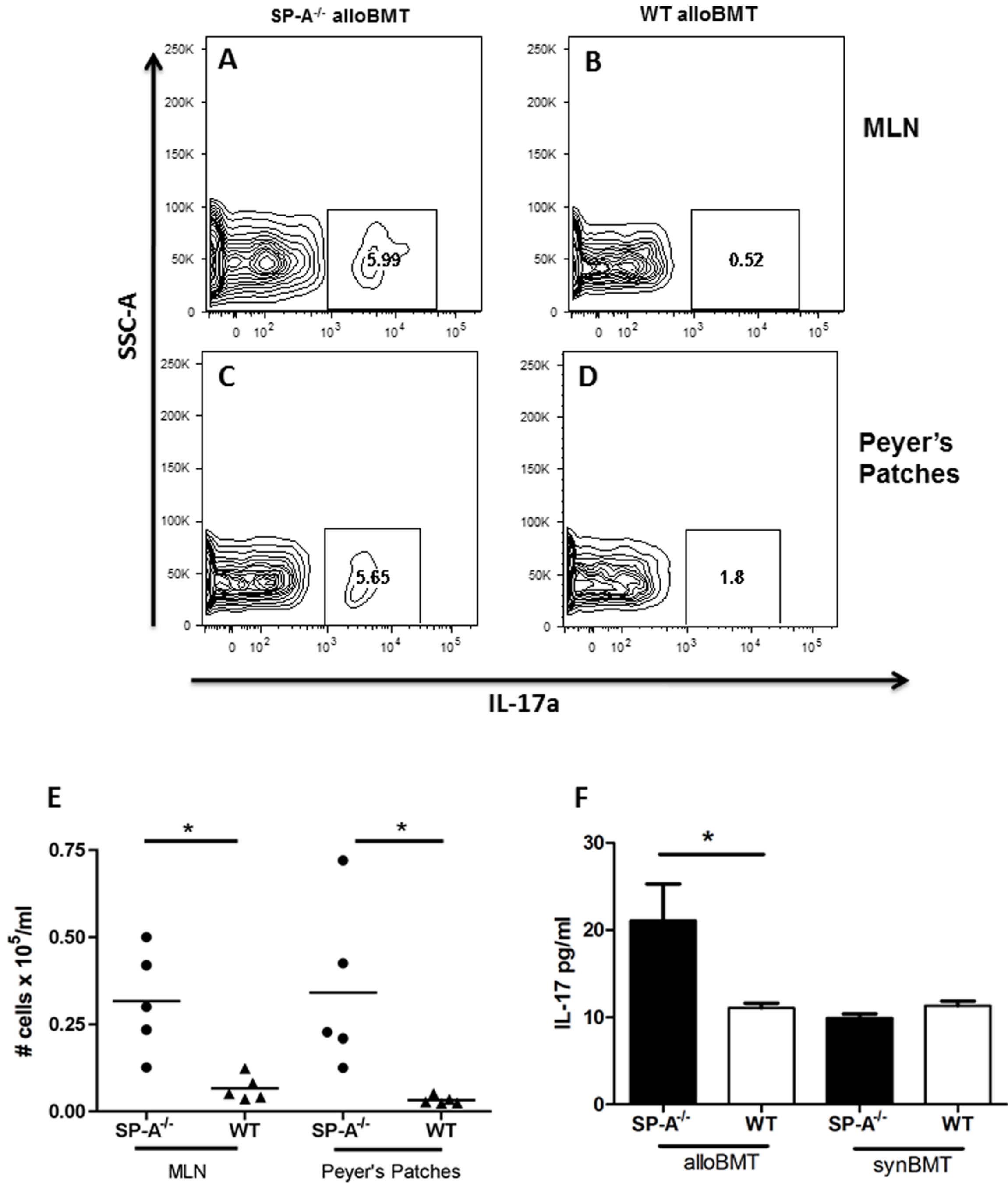

FIGURE 9. SP-A deficiency promotes Th17 cell polarization in the gastrointestinal tract after allogeneic BMT

After BMT, mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) cells were isolated and cells were analyzed by flow cytometry. (A–D) Representative flow cytometric plots show $CD3^{+}CD4^{+}IL\text{-}17^{+}$ (Th17) populations for each experimental group (MLNs) (A) SP-$A^{-/-}$alloBMT; (B) WTalloBMT; (PPs) (C) SP-$A^{-/-}$alloBMT; (D) WTalloBMT; (E) Absolute numbers of Th17 cells in MLNs and PPs. (F) IL-17a production by colon explant cultures were measured by ELISA at 24hrs post-harvest. (* = p-value less than 0.05) (graph is representative of 1 experiment n=5–8/group, data was replicated in 3 independent experiments).

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Table I

A semiquantitative scoring system was used to document the presence and severity of various GVHD associated morphologic features.

LIVER	SMALL AND LARGE BOWEL
Portal Triads	*Architecture*
Portal tract expansion	Villus blunting
Bile Ducts/Ductules	Crypt regeneration
Mononuclear infiltrate of epithelium	Ulceration
Pyknotic/apoptotic duct cells	Lamina propria inflammation
Intraluminal epithelial cells	Crypt loss
Vascular	*Epithelial Cytology*
Endothelialitis	Apoptosis
Mononuclear cells around CV	Sloughing into lumen
Hepatocellular Damage	Lymphocytic infiltrate
Confluent necrosis	Neutrophilic infiltrate
Acidophilic bodies	*Vascular*
Mitotic figures	Edema
Neutrophil accumulations	
Macrophage aggregates	
Cholestasis	
Steatosis	