Regulating T-cell differentiation through the polyamine spermidine

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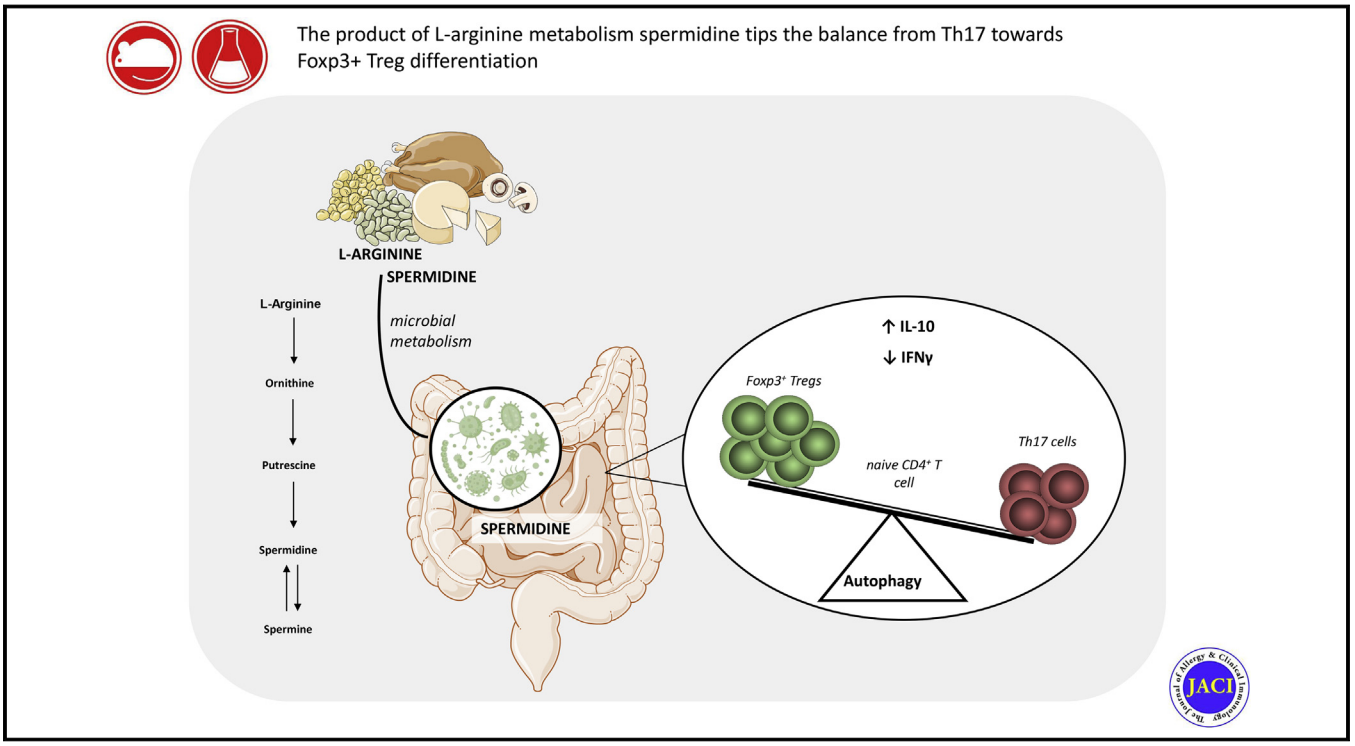
GRAPHICAL ABSTRACT



Background: The cross-talk between the host and its microbiota plays a key role in the promotion of health. The production of metabolites such as polyamines by intestinal-resident bacteria is part of this symbiosis shaping host immunity. The polyamines putrescine, spermine, and spermidine are abundant within the gastrointestinal tract and might substantially contribute to gut immunity.

Objective: We aimed to characterize the polyamine spermidine as a modulator of T-cell differentiation and function.

Methods: Naive T cells were isolated from wild-type mice or cord blood from healthy donors and submitted to polarizing cytokines, with and without spermidine treatment, to evaluate $CD4^+$ T-cell differentiation *in vitro*. Moreover, mice were subjected to oral supplementation of spermidine, or its

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precursor L-arginine, to assess the frequency and total numbers of regulatory T (Treg) cells *in vivo*.
Results: Spermidine modulates CD4$^+$ T-cell differentiation *in vitro*, preferentially committing naive T cells to a regulatory phenotype. After spermidine treatment, activated T cells lacking the autophagy gene *Atg5* fail to upregulate Foxp3 to the same extent as wild-type cells. These results indicate that spermidine's polarizing effect requires an intact autophagic machinery. Furthermore, dietary supplementation with spermidine promotes homeostatic differentiation of Treg cells within the gut and reduces pathology in a model of T-cell transfer-induced colitis.
Conclusion: Altogether, our results highlight the beneficial effects of spermidine, or L-arginine, on gut immunity by promoting Treg cell development. (J Allergy Clin Immunol 2021;147:335-48.)

Key words: *Polyamines, spermidine, gut immunity, Treg cells, T_H17 cells*

Abbreviations used
Atg: Autophagy related
DC: Dendritic cell
FACS: Fluorescence-activated cell sorting
GC-MS/MS: Gas chromatography–tandem mass spectrometry
IBD: Inflammatory bowel disease
IMDM: Iscove modified Dulbecco medium
iTreg: Induced regulatory T
MLN: Mesenteric lymph node
mTor: Mechanistic target of rapamycin kinase
ODC: Ornithine decarboxylase
SFB: Segmented filamentous bacterium
SMO: Spermine oxidase
Teff: Effector T
Treg: Regulatory T
WT: Wild-type

The human body is under constant exposure to foreign antigens, with the mucosal surfaces acting as major physical barriers for host protection. In the context of the intestinal tract, maintenance of a homeostatic environment is essential to stave off pathogens while remaining tolerogenic toward commensal bacteria and dietary antigens.[1] The gut microbiota itself can contribute to intestinal homeostasis by producing metabolites that shape immunity both locally and systemically. IL-17–producing T_H17 cells, which are often induced by signals from the microbiota, are crucial for maintaining the integrity of epithelial cell function via IL-17 and IL-22 production and for surveillance against pathogens through inflammation.[2] Additionally, the induction of Foxp3$^+$ regulatory T (Treg) cells is of particular importance to enforce intestinal homeostasis.[2-4] Treg cells establish tolerance by suppressing proinflammatory responses of not only effector T (Teff) cells but also dendritic cells (DCs) and macrophages.[5,6] They do so by constitutively expressing coinhibitory receptors and secreting anti-inflammatory cytokines, such as IL-10, that prevent or downregulate aberrant immune responses.[5,7] Several microbiota-derived molecules, including polysaccharide A[8,9] and short-chain fatty acids,[10] can promote Treg cell differentiation, thus orchestrating a cross-talk between microbiota and host.

Polyamines are small polycationic molecules derived from the metabolism of L-arginine.[11] Recently, polyamines have been suggested as a class of metabolites with anti-inflammatory properties[12-14] promoting M2 macrophage polarization[15] and the suppressive activity of tolerogenic DCs.[16] Although all eukaryotic cells produce polyamines,[17] microbiota and food intake constitute their major sources.[14,18,19] Spermidine, the best characterized polyamine, is found in micromolar concentrations within the gastrointestinal tract,[20] and its absorption and availability vary over time according to food consumption,[18] fasting periods,[18,21] and microbiota composition.[12] A recently published clinical trial assessing all-cause and cause-specific mortality throughout 20 years reported epidemiologic data supporting a positive association between spermidine-rich diets and human longevity.[22] A plethora of biologic effects have been described for spermidine, such as cell growth and proliferation, antioxidative effects, stabilization of DNA and RNA, and enzymatic modulation.[23,24] Importantly, spermidine is shown to be an autophagy inducer in cardiomyocytes, neurons, satellite cells, CD8$^+$ T cells, and B cells.[25,26] However, there is yet no report on the direct effect of spermidine on CD4$^+$ T-cell differentiation and function. Because the intestinal tract is rich in polyamines as end products of microbial metabolism, we postulated that spermidine could be a biomolecule contributing to the establishment of tolerance by promoting Treg differentiation. Previous studies have reported the beneficial role of L-arginine in regulating intestinal homeostasis during inflammation and pathology.[27-29] We propose that the anti-inflammatory properties of L-arginine can also be due to a concomitant increase in metabolized polyamines. In this study, we show that spermidine induces Foxp3 expression both under T_H17 cell– and Treg cell–inducing conditions *in vitro* in an autophagy-associated manner. Furthermore, spermidine administration promotes a tolerogenic environment in the gut and ameliorates inflammation-induced tissue pathology in a model of adoptive T-cell transfer-induced colitis. Altogether, this report provides further evidence that dietary products can modulate the immune system, in particular, the ability of polyamines to promote a regulatory milieu, which may serve as a noninvasive tool to limit excessive inflammation.

METHODS

Mice

C57BL/6, BALB/cJ, *Rag2*$^{-/-}$, and DO11.10 TCR transgenic *Rag2*$^{-/-}$ mice (on a BALB/cJ background) mice were purchase from Jackson Laboratories or Charles River Laboratories. *Atg5*floxOX40cre mice were generated by crossing *Atg5*flox with OX40cre mice.[30] An in-depth characterization of these mice will be described elsewhere. All mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Helmholtz Centre for Infection Research (Braunschweig, Germany) or at TWINCORE (Hannover, Germany). Animal experiments at TWINCORE were performed in compliance with German animal protection law and were approved by the Lower Saxony Committee on the Ethics of Animal Experiments as well as by the responsible state office (Lower Saxony State Office of Consumer Protection and Food Safety under permit 33.9-42502-04-15/1851).

For all experiments, male and female mice with ages ranging from 5 to 20 weeks were used. No influence of the mouse sex on the results was observed. For all *in vivo* and *in vitro* experimental procedures littermate controls were used. The mice were caged in groups of 6 or fewer and had access to a pellet-based food and autoclaved reverse-osmosis water. The mice were

kept in a temperature- (20°C-24°C) and humidity-controlled environment with a 16-hour light cycle.

For antibiotic treatment, mice were treated with 0.5 g/L of metronidazole (Sigma-Aldrich), 0.5 g/mL of vancomycin (Roth), 1 g/L of ampicillin (Sigma-Aldrich), and 1 g/L of kanamycin (Fisher Scientific) dissolved in drinking water.

Administration of spermidine (Sigma-Aldrich) was performed by adding 30 mM spermidine to the drinking water.

Mouse T-cell cultures

For isolation of naive $CD4^+$ lymphocytes, cells were collected from spleens and lymph nodes of mice and enriched by negative selection using the EasySep Mouse $CD4^+$ T-cell Isolation Kit (StemCell Technologies) and biotinylated antibodies against CD25 (clone PC61.5 [eBioscience]) and CD44 (clone IM7 [eBioscience]). The purity of the isolated naive cells was greater than 95%. All cell culturing was carried out at 37C in a humidified atmosphere containing 5% CO_2 in Iscove modified Dulbecco medium (IMDM) or RPMI cell culture media supplemented with 10% heat-inactivated FCS (Biochrom), 500 units of penicillin-streptomycin (PAA Laboratories or Invitrogen), and 50 μM β-mercaptoethanol (Life Technologies or Sigma-Aldrich). RPMI was used for Treg, T_H1, and T_H2 cell cultures, and IMDM was used for T_H17 cell cultures. For T_H17 cell induction, 2.5×10^5 naive T cells were cultured for 4 days with plate-bound anti-CD3ε (10 μg/mL of clone 145-2C11 [Bio X Cell]) and soluble anti-CD28 (1 μg/mL of clone 37.51 [Bio X Cell]), anti–IFN-γ (5 μg/mL of clone XMG1.2 [Bio X Cell]), anti–IL-4 (5 μg/mL of clone 11B11 [Bio X Cell]), rhTGF-β1 (2 ng/mL [Peprotech]), rmIL-6 (7.5 ng/mL [Peprotech]), and rmIL-1β (50 ng/mL [Peprotech]) in IMDM GlutaMAX complete medium (Thermo Fischer). For T_H1 cell polarization, 1×10^5 naive T cells per well were cultured for 4 days with plate-bound anti-CD3ε (10 μg/mL of clone 145-2C11 [Bio X Cell]), anti-CD28 (1 μg/mL of clone 37.51 [Bio X Cell]), anti–IL-4 (5 μg/mL of clone 11B11 [Bio X Cell]), and rmIL-12 (20 ng/mL [Peprotech]). T_H2 cells were induced by culturing 1×10^5 naive T cells per well for 4 days with plate-bound anti-CD3ε (10 μg/mL of clone 145-2C11 [Bio X Cell]), anti-CD28 (1 μg/mL of clone 37.51 [Bio X Cell]), anti–IFN-γ (5 μg/mL of clone XMG1.2 [Bio X Cell]), anti–IL-12 (5 μg/mL of clone 17.8 [Bio X Cell]), and rIL-4 (50 ng/mL [Peprotech]).

For Treg cell induction, 2.5×10^4 naive T cells were cultured for 4 days in the presence of plate-bound anti-CD3 (10 μg/mL), anti-CD28 (1 μg/mL), rhIL-2 (200 U/mL [Roche Applied Science]), and rhTGF-β1 (1 ng/mL) in RPMI GlutaMAX complete medium (Thermo Fischer). On day 2, cells were again supplemented with rhIL-2 (200 U/mL [Roche Applied Science]). Optimal Treg cell cultures were performed in round-bottom plates, whereas suboptimal cultures were carried out in flat-bottom plates.

For proliferation analysis, naive T cells were labeled by using CellTrace Violet Cell Proliferation Kit (CTV [Life Technologies]) according to the manufacturer's instructions and analyzed after 4 days of activation.

Putrescine, spermine, and spermidine were purchased from Sigma-Aldrich. and stock solutions of 1 M were prepared in 1 × PBS. To ensure the bioactivity of the spermidine, new compound was reconstituted monthly.

In vivo induction of ovalbumin-specific Treg cells

BALB/cJ recipient mice received drinking water supplemented with 30 mM spermidine for 5 days before cell transfer or given nonsupplemented control water. The recipient mice were then injected intravenously with 1×10^6 $CD4^+CD25^-$ naive T cells isolated from DO11.10 TCR-transgenic *Rag2*$^{-/-}$ mice and treated with ovalbumin by oral gavage (160 mg per day) for another 6 days. The water containing spermidine was changed every 2 days. On day 7, mice were humanely sacrificed and their organs (small intestine, colon, spleen, and mesenteric lymph nodes [MLNs]) were processed.

Suppression assay

Treg cells that had been *ex vivo*–differentiated in the presence of spermidine were cocultured at ratios of 1:1 to 1:10 with CellTrace Violet–labeled $CD45.1^+CD4^+CD25^-$ T cells along with 5000 GMCSF-DCs and 10 μg/mL of anti-CD3 in round-bottom 96-well plates. As a positive control, cells were plated in the absence of Treg cells, and as a negative control, cells were seeded without Treg cells, and DCs as antigen-presenting cells. Suppression of Teff cell proliferation was determined at 96 hours by dilution of the CellTrace Violet proliferation dye gated in the CD45.1 population.

Gene expression analysis

Total RNA was isolated from T cells by using the Qiagen RNeasy Kit (Qiagen). cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions by using Oligo(dT) 12-18 Primers (Invitrogen). Quantitative PCR was performed by using SYBR Green quantitative PCR Bioline SensiFAST and measured in a LightCycler 480 High-Throughput Real-Time PCR system (Roche). The primer pairs used were as follows: for *Foxp3* primer, (forward) 5′-AGAAGCTGGGAGCTATG CAG-3′ and (reverse) 5′- GCTACGATGCAGCAAGAGC-3′ and for *IL-17A,* (forward) 5′-CTCCAGAAGGCCCTCAGACTAC-3′ and (reverse) 5′-AGCT TTCCCTCCGCATTGACACAG-3′.

Human T-cell cultures

Human cord blood samples were obtained from the Department of Prenatal Medicine and Midwifery of the Medical School Hannover. All work with human blood samples was approved by the local ethics committee (ethical committee of the Medical School Hannover under permit 6287), and informed consent was obtained from all subjects. Blood was subjected to Biocoll (Biochrom) separating solution, and $CD4^+$ T cells were enriched by magnetic separation using an EasySep Human naive $CD4^+$ T-Cell Enrichment Kit (Stem Cell). $CD4^+CD45RO^-$ cells were seeded at a cell density of 1×10^6 cells per mL in a *U*-bottom 96-well plate previously coated with plate-bound anti-CD3 (Ultra-LEAF Purified anti-human CD3; 5 μg/mL of clone OKT3 [BioLegend]). For human induced Treg (iTreg) cell differentiation, cells were cultured with soluble anti-CD28 (Ultra-LEAF purified anti-human, 1 μg/mL [BioLegend]) IL-2 (100 U/mL [Roche]), rhTGF-β1 (5 ng/mL [Peprotech]), and all-trans retinoic acid (10 nM [Sigma-Aldrich]) in X-VIVO 15 medium (Lonza), serum-free, supplemented with penicillin/streptomycin. T_H17 cell cultures were established according to a previously described protocol for differentiation of pathogenic T_H17 cells.[31] Cells were cultured in X-VIVO 15 medium (Lonza) supplemented with 40 mM NaCl and soluble anti-CD28 (Ultra-LEAF purified anti-human, 1 μg/mL [BioLegend]), rhTGF-β1 (5 ng/mL [Peprotech]), rhIL-1β (10 ng/mL [R&D]), rhIL-6 (25 ng/mL [Peprotech]), rIL-21 (25 ng/mL [R&D]), and rIL-23 (25 ng/mL [R&D]). For both Treg cell and T_H17 cell cultures, cells were incubated for 5 days at 37 °C and 5% CO_2.

Flow cytometry

For flow cytometry, mAbs specific to the following mouse antigens (and labeled with the indicated fluorochrome) were purchased from Affymetrix/eBioscience: CD25-PE (PC61), CD4-Alexa488 (GK1.5), CD4-PE (GK1.5), CD4-eFluor450 (RM4-5), Foxp3-eFluor450, Foxp3-eFluor660 (FJK-16s), IL-17A–PE-Cy7 (eBio17B7), DO11.10 TCR-APC (KJ1-26), CD3-APC (145-2C11), Thy1.1-APC (HIS51), IL-10–PE (JES5-16E3), IFN-γ–PE (XMG1.2), RORγt-PE (B2D), T-bet–eFluor660 (eBio4B10), and IL-13–PE (eBio13A). For human T_H17 cells, the antibodies CD4-FITC (SK3) and IL-17–PE–Cy7 (eBio64DEC17) were used. For human Treg cultures, the following antibodies were used: CD3ε-eFluor780 (SK7), CD4-FITC (SK3), and Foxp3-eFluor450 (236A/E7).

For analysis of surface markers, cells were stained in PBS containing 0.25% BSA and 0.02% azide. Live cells were identified by using a LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies) according to the manufacturer's protocol. For intracellular cytokine staining, cells were stimulated with phorbol 12–myristate 13–acetate (0.1 μg/mL [Sigma-Aldrich]) and ionomycin (1 μg/mL [Sigma-Aldrich]) for 2 hours followed by another 2 hours of stimulation with BrefeldinA (5 μg/mL).

Detection of autophagosome formation was performed by using the FlowCellect Autophagy LC3 Antibody-bead Assay Kit (FCCH100171 [Merck-Millipore]) according to the manufacturer's instructions.

For mechanistic target of rapamycin kinase (mTOR) (clone 7C10 [Cell Signaling Technology]) and p-S6 (clone D57.2.2E [Cell Signaling Technology]) staining, after LIVE/DEAD staining, cells were permeabilized with ice-cold 100% methanol for 10 minutes, stained at room temperature for 1 hour with the primary antibody for mTOR, and stained for 30 minutes with the secondary antibody Alexa488 goat anti-rabbit (F(ab')2 fragment [Cell Signaling Technology]).

Cells were acquired on a CyAn ADP (Beckman Coulter) or LSR II (Becton Dickinson) flow cytometer, and data were analyzed with FlowJo software.

Western blot

Whole-cell lysates were prepared by using lysis buffer (Pierce RIPA buffer [Thermo Scientific]) supplemented with complete EASYpack Mini Protease Inhibitor cocktail and PhosSTOP Phosphatase Inhibitor (both from Roche Applied Science). Cell lysates were separated by SDS gel electrophoresis and transferred to polyvinylidene fluoride membranes (Merck Millipore). Immunoblotting was performed by using antibodies against LC3A/B (clone D3U4C, 1:1000 [Cell Signaling Technology]), Atg5 (clone D5F5U [Cell Signaling Technology]), mTOR (clone 7C10 [Cell Signaling Technology]), p-mTOR (Ser2448 [Cell Signaling Technology]), p70 S6 kinase (clone 49D7 [Cell Signaling Technology]), p-p70 S6 kinase (Ser371 [Cell Signaling Technology]), β-actin (clone AC-15 [Sigma-Aldrich]) anti-GAPDH (1: 1000 [Cell Signaling Technology]), and goat anti-rabbit horseradish peroxidase (7074; 1:2000 [Cell Signaling Technology]) and detected by using ECL prime (GE Healthcare).

Metabolomic quantification by HPLC-MS/MS

Fecal samples were collected directly into Eppendorf tubes and snap-frozen before preparation of material for polyamide quantification by HPLC/MS.

For quantification of the polyamines, 11 calibrators were prepared in PBS solution at a concentration range of 96.66 nM to 100000 nM by spiking a PBS solution with putrescine, spermidine, and spermine standards (Sigma-Aldrich, St Louis, Mo).

Hexamethylendiamine (Sigma-Aldrich) served as the internal standard for putrescine. For spermidine and spermine, spermidine-(butyl-d8) trihydrochloride (Sigma-Aldrich) was used. An internal standard mix solution was prepared in 0.1 M aqueous hydrochloric acid containing 100 μM of each standard.

For sample preparation, a 250-μL aliquot of sample and calibrator was transferred into a flat glass vial. Next, 25 μL of the internal standard mix solution, 10 μL of a 5 M aqueous sodium hydroxide solution, and 500 μL of ethyl-chloroformate solution (1:20 dilution in diethyl ether) were added. Vials were capped and vortexed for 30 minutes at 30°C. Afterward, samples were centrifuged for 5 minutes at 5445 relative centrifugal force at 4°C. The ethereal phase (upper layer) was carefully transferred into a conic vial. The remaining aqueous phase was reextracted with 500 μL of ethyl-chloroformate solution, and the combined ether extracts were evaporated to dryness under a gentle nitrogen stream. For the subsequent derivatization, 300 μL of trifluoroacetic anhydride solution (2 parts trifluoroacetic anhydride and 1 part ethyl acetate) were added to the dried residues. Vials were capped and incubated for 60 minutes at 75°C. Afterward, the mixture was taken to dryness under a nitrogen stream. The pellet was resuspended in 200 μL of ethyl acetate and transferred to an amber glass vial for gas chromatography–tandem mass spectrometry (GC-MS/MS) analysis.

GC-MS/MS analysis of polyamines was performed on an Agilent 7890B GC system equipped with an Agilent 7693 auto sampler and coupled to a 7000C GC-MS/MS Triple Quadrupole mass spectrometer. The electron energy was 150 eV, and the ion source temperature was kept at 150°C. The collision energy was set to 5 V. Chromatography was performed by using an MN OPTIMA 17 MS column (30 m × 0.25 mm internal diameter; 0.25 film thickness [Macherey-Nagel, Düren, Germany]); 1 μL was injected at 280°C. The oven temperature was raised from 60°C to 320°C within 16 minutes (20°C per minute). Helium was used as the carrier gas.

Polyamines were detected in multiple reaction monitoring mode. The mass transitions were as follows: putrescine, m/z 424.3 → 351; spermidine, m/z 553.5 → 480; spermine, m/z 682.6 → 609; hexamethyldiamine (internal standard), m/z 452.4 → 379; and spermidine-(butyl-d8) trihydrochloride (internal standard), m/z 561.4 → 488.

Calibration curves were created by plotting peak area ratios of the polyamines, and the corresponding internal standard versus the nominal concentration of the calibrators. The calibration curve was calculated by using quadratic regression and 1/x weighing.

T-cell transfer colitis model

$CD4^+CD25^-CD45RB^{high}$ T cells were isolated from spleens and peripheral lymph nodes of congenic Thy1.1 mice via fluorescence-activated cell sorting (FACS) sorting (BD FACS Aria II or FACS Aria Fusion) and injected intraperitoneally into *Rag2*$^{-/-}$ mice (0.4×10^6 cells in PBS per mouse) to induce colitis. Throughout the disease development process, the drinking water of the experimental mice was supplemented with 30 mM spermidine. The weight of the mice was monitored, and the supplemented water was changed every 2 days. On day 27 after transfer, mice were humanely sacrificed and their tissues were harvested for histopathologic and immunologic analyses.

For histopathology, at the end point date, colon tissue was fixed in paraformaldehyde. Samples were further processed to paraffin blocks, sectioned, and stained with hematoxylin and eosin at the Mouse Pathology Department of the Hannover Medical School. Samples were scored as described previously.[32] In brief, the presence of rare inflammatory cells in the lamina propria were counted as follows: 0, increased numbers of inflammatory cells; 1, confluence of inflammatory cells; 2, extending into the submucosa; and 3, transmural extension of the inflammatory cell infiltrate. For epithelial damage, absence of mucosal damage was counted as 0, discrete focal lymphoepithelial lesions were counted as 1, mucosal erosion/ulceration was counted as 2, and a score of 3 was given for extensive mucosal damage and extension through deeper structures of the bowel wall. The 2 subscores were added, and the combined histologic score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

Cell isolation from small intestine and colon

Small intestine and colon were collected from mice and washed with PBS, and the remaining feces were removed. The organs were cut into several pieces and incubated in 20 mL of PBS supplemented with 0.5 mM EDTA for 30 minutes at 37°C on a shaker. The tissues were then detached from the mucus, rinsed with ice-cold PBS once, and submitted to additional mechanical dissociation with a scalpel. Afterward, the tissue was subjected to enzymatic digestion by incubation in Dulbecco modified Eagle medium (Life/Gibco, Darmstadt, Germany) supplemented with 2% FCS and containing 1 mg/mL of collagenase D (Roche) and 0.1 mg/mL of DNase I (Roche) for 30 minutes at 37 °C. Tissue suspensions were then passed through 100-μm mesh, pelleted, resuspended in a 40% isotonic Percoll solution (GE Healthcare), and underlain with an 80% isotonic Percoll solution. After centrifugation at 900 *g* for 25 minutes at room temperature, cells were yielded from the interface of 40% to 80% Percoll solution. The cells were washed with complete medium and then used for analyses.

Statistical analysis

The statistical parameters applied can be found in the figure legends. Data were analyzed by using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, Calif). Statistical analyses were performed as follows: 2-way ANOVA followed by Sidak multiple comparison was used to analyze experiments with 2 variables and 3 or more groups, and 1-way ANOVA followed to Duvett comparison with a control was used for experiments with 1 variable and 3 or more groups. The *in vitro* experiments with 2 groups were analyzed with the

Student *t* test, and the *in vivo* experiments with 2 groups were analyzed with the Mann-Whitney test owing to small sample size. In all cases, *P* values less than .05 were considered statistically significant. In the figure legends, graphs show mean values and error bars represent SDs unless otherwise specified. *P* values are represented as less than .05, less than .01, less than .001, and less than .0001.

RESULTS

Spermidine shifts the *in vitro* polarization of T_H17 cells toward Foxp3$^+$ Treg cells

Because of the prominent role that T_H17 cells have in gut immune surveillance, coupled with the fine balance between T_H17 cells and Treg cells, we hypothesized that polyamines (see Fig E1, *A* in this article's Online Repository at www.jacionline.org) might influence T_H17 cell differentiation. To test this hypothesis, we differentiated murine naive CD4$^+$CD25$^-$ T cells under T_H17 cell–polarizing conditions, in the presence of titrated amounts of each polyamine (see Fig E1, *A*). We observed that spermidine and spermine reduced IL-17 production while concomitantly increasing the percentage of Foxp3$^+$ cells (Fig 1, *A* and see Fig E1, *B*). In contrast, putrescine did not affect T_H17 cell differentiation in the concentrations tested (see Fig E1, *C*). Next, to confirm the presence of spermidine in the intestinal tract of wild-type (WT) C57BL/6 mice, we quantified the relative amount of polyamines present in murine fecal samples (see Fig E2, *A* and *B* in this article's Online Repository at www.jacionline.org). Among all of the polyamines tested, spermidine was found in highest concentrations, followed by putrescine, whereas spermine levels were below the detection limits (see Fig E2, *B*). Although both spermidine and spermine showed immunomodulatory effects, spermine proved to be cytotoxic for the cells at lower doses in comparison with spermidine (Fig 1, *A* and see Fig E1, *B*). Thus, we focused exclusively on spermidine for further mechanistic studies. T_H17 cell cultures performed in the presence of TGF-β can generate both T_H17 cells and Treg cells.[33,34] To test whether the lower IL-17 production mediated by spermidine occurred as a result of increased suppression by a higher number of Foxp3$^+$ cells, we cultured naive T cells under T_H17 cell–polarizing conditions without TGF-β in the presence of spermidine. In the absence of TGF-β, T_H17 cells could still differentiate, but Treg cells were unable to do so. Importantly, we showed that the reduction of IL 17 cell–producing cells still took place, even without a concomitant upregulation of Foxp3 (see Fig E3, *A* in this article's Online Repository at www.jacionline.org). Therefore, we aimed to evaluate whether the suppressive role of spermidine in T_H17 cell development regulates the master transcription factor RORγt. In the presence of spermidine, however, T_H17 cells maintained RORγt expression, leading to a double-positive RORγt$^+$ Foxp3$^+$ population (see Fig E3, *B*). Next, we assessed the kinetics of the Treg cell–polarizing effect of spermidine. From day 3 to day 4, we observed that spermidine constrained IL-17 production during differentiation while promoting and maintaining Foxp3$^+$ expression (Fig 1, *B*). Moreover, these results were further corroborated at the transcriptional level, where we could discern an increase in the relative expression of the *Foxp3* transcripts (Fig 1, *C*). However, no differences were observed for *IL17a* expression (Fig 1, *C*). We next addressed whether the effect of spermidine on the reduction of IL-17 protein secretion was a result of decreased proliferation of cytokine-producing Teff cells. By labeling naive T cells with the CellTrace Violet proliferation dye, we showed that spermidine-treated cells do not exhibit impaired proliferation (Fig 1, *D*). Furthermore, we observed an IL-17 reduction and Foxp3 increase in cells from each individual proliferation cycle (Fig 1, *E*). Next, we aimed to investigate whether spermidine could also downregulate T_H17 cell differentiation in human cells. Blood mononuclear cells were isolated from the cord blood of healthy donors, enriched for CD4$^+$ naive cells and cultured under T_H17 cell–polarizing conditions for 5 days (Fig 1, *F*). Spermidine successfully impaired IL-17 production in the cells of all donors tested (Fig 1, *F*), with no detrimental effect on cell viability (see Fig E4, *A* in this article's Online Repository at www.jacionline.org).

In a setting of infection or allergy, T_H1 and T_H2 cells orchestrate the immune response, respectively. Hence, we addressed whether spermidine also downmodulates T_H1 and T_H2 cell differentiation. When stimulated with spermidine, murine naive CD4$^+$ T cells differentiated in T_H1 cell–polarizing conditions showed slightly reduced frequencies of T-bet$^+$ CD4$^+$ T cells (see Fig E5, *A* in this article's Online Repository at www.jacionline.org); however, neither the production of IFN-γ nor cell proliferation was affected (see Fig E5, *A* and *B*). Several reports have demonstrated increased commitment to T_H2 immunity in BALB/c mice when compared with C57BL/6 mice.[35] Therefore, cells from BALB/c and C57BL/6 mice were isolated and treated with a T_H2-inducing cytokine milieu; in both experimental systems spermidine did not affect the production of IL-13 on day 4 of the cell culture (see Fig E5, *C* and *D*).

Overall, our results show that spermidine stimulation is able to shift the *in vitro* differentiation of T_H17 cells polarized toward Treg cells, with no effect on cell proliferation.

Spermidine potentiates Foxp3$^+$ Treg cell differentiation, both *in vitro* and *in vivo*

To evaluate the increase of Foxp3$^+$ Treg cells independently of T_H17 cell–polarizing cytokines, enriched naive CD4$^+$CD25$^-$ T cells were cultured with rhIL-2 and increasing amounts of TGF-β and spermidine. *In vitro* stimulation of iTreg cell cultures with spermidine, confirmed the ability of this polyamine to augment the expression of Foxp3 in a TGF-β dose–dependent manner (Fig 2, *A-C*), without affecting cell proliferation (see Fig E6, *A* in this article's Online Repository at www.jacionline.org). Moreover, we investigated whether spermidine treatment promoted increased frequencies of Foxp3$^+$ cells in human iTreg cell cultures. Similar to murine cultures, human CD4$^+$ CD45RO$^-$ T cells enriched from peripheral blood cultured in iTreg cell–skewing cytokines, when exposed to spermidine, boosted Foxp3 frequency (Fig 2, *D*). Next, we aimed to investigate the suppressive function of differentiated Treg cells by coculturing CellTrace Violet–labeled naive CD4$^+$CD25$^-$ T cells (Teff cells), GM-CSF–derived DCs, and Treg cells that were previously treated with or without spermidine. We observed that spermidine-treated Treg cells decreased the proliferation of Teff cells to the same extent as control Treg cells did (see Fig E6, *B*). We next addressed whether spermidine can increase the frequency of Foxp3$^+$ cells in an antigen-specific manner *in vivo*. The induction of oral tolerance in ovalbumin-specific T-cell receptor transgenic mice (DO11.10 mice) resulted in an increase in Treg cells not only in the lamina propria but also systemically.[36,37] Hence, we

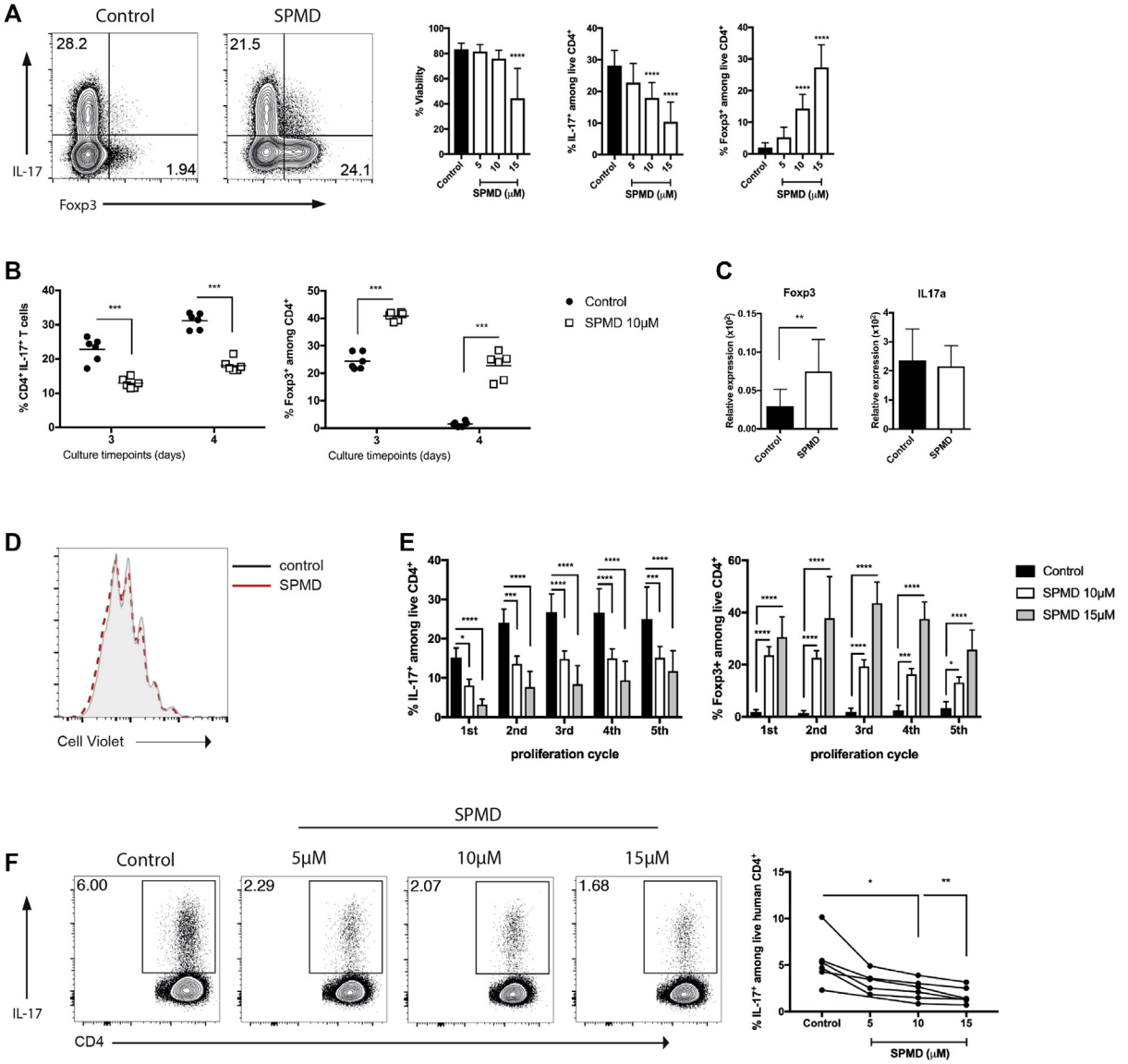

FIG 1. Under T_H17 cell–polarizing conditions, spermidine (SPMD)-stimulated $CD4^+$ T cells differentiate toward regulatory $Foxp3^+$ cells. $CD4^+CD25^-$ T cells enriched from spleen and lymph nodes of C57BL/6 WT mice were cultured under T_H17 cell–polarizing conditions and increasing doses of SPMD for 4 days. **A,** Cells were stained for intracellular IL-17 and Foxp3 and gated on live $CD4^+$ T cells. Pooled data are from 4 experiments. **B,** Cells were stained at day 3 and day 4 for intracellular IL-17 and Foxp3. Pooled data are from 2 experiments. **C,** Expression of *IL17a,* and *Foxp3* mRNA relative to β-*actin* was determined in T_H17 cells differentiated with SPMD, after 96 hours. Pooled data are from 2 independent experiments. **D,** $CD4^+CD25^-$ T cells were labeled with CellTrace Violet proliferation dye and cultured under T_H17 cell–polarizing conditions. Cells were gated on live $CD4^+$ T cells; proliferation after 96 hours of 1 of 3 independent experiments is shown. **E,** Frequency of IL-17^+ cells and transcription factor $Foxp3^+$ were gated on each proliferation cycle after incorporation of the CellTrace Violet proliferation dye. Data are from 2 pooled experiments. **F,** Naive $CD4^+$ $CD45RO^-$ T cells isolated from human cord blood were cultured under T_H17 cell–polarizing conditions and in the presence of increasing concentrations of SPMD. At day 5 cells were stained intracellularly for IL-17. Each dot represents an independent donor. Graphs show mean values, and error bars represent SDs unless otherwise specified. $*P < .05$; $**P < .01$; $***P < .001$; $****P < .0001$.

postulated that naive TCR-transgenic cells could react to ovalbumin and have an enhanced generation of $Foxp3^+$ cells when treated with spermidine. Accordingly, BALB/c mice were treated with 30 mM spermidine in drinking water for 5 days, followed by adoptive transfer of naive $CD4^+CD25^-DO11.10^+$ ovalbumin-specific T cells and daily oral treatment of ovalbumin (see Fig E7, *A* in this article's Online Repository at www.jacionline.org). After 1 week of treatment, we observed that spermidine treatment led to a significantly increased frequency of $Foxp3^+$ Treg cells in the small intestine (Fig 2, *E* and *F*), together with a tendency toward increased Treg cell frequency and numbers in the colon (see Fig E7, *B*). These findings support the hypothesis

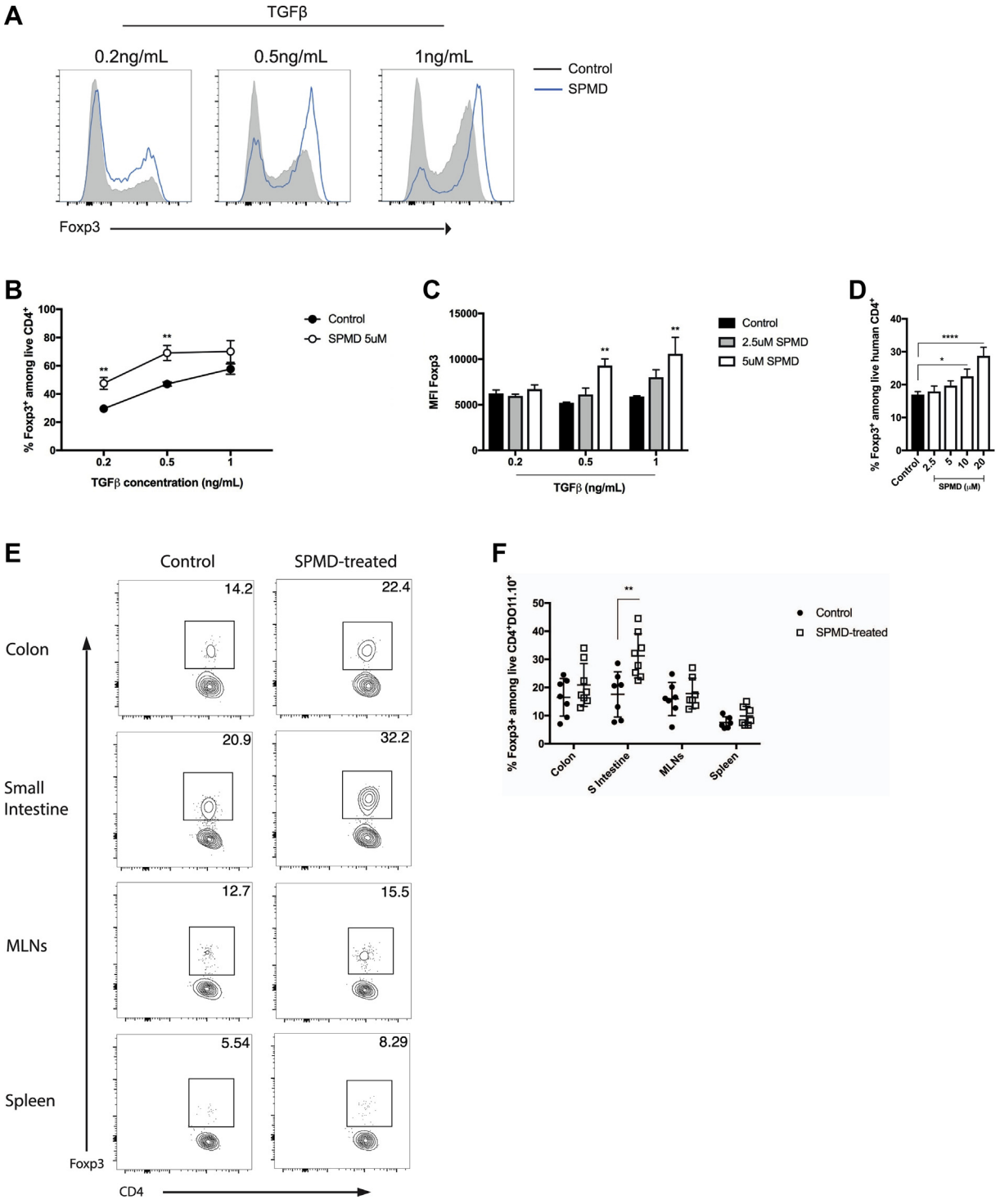

FIG 2. Spermidine (SPMD) potentiates *in vitro* and *in vivo* Treg cell differentiation. **A-C,** $CD4^+CD25^-$ T cells stimulated with 5 μM SPMD were differentiated under Treg cell–polarizing conditions with increasing concentrations of TGF-β. After 4 days of culture, the frequency of $Foxp3^+$ T cells was quantified by flow cytometry (**B**), and the mean fluorescence intensity was calculated (**C**). Data are from 1 of 3 independent experiments. **D,** Naive $CD4^+$ $CD45RO^-$ cells, enriched from human cord blood cells, were cultured in Treg cell–polarizing conditions in the presence of SPMD; at day 5 cells were stained for intracellular Foxp3. Representative data from 1 of 2 experiments are shown. **E** and **F,** $CD4^+CD25^-$ naive T cells from DO11.10 TCR-transgenic *Rag2*$^{-/-}$ mice were adoptively transferred into BALB/cJ mice receiving 30 mM SPMD in drinking water. **E,** Representative FACS plots and (**F**) frequency of transferred live $CD4^+CD3^+Foxp3^+$ Treg cells in the indicated organs, after 5 days of ovalbumin gavage and SPMD treatment. Symbols represent data from individual mice. Pooled data are from 2 independent experiments. Graphs show mean values, and error bars represent SDs unless otherwise specified. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

that polyamines, in particular spermidine, might have a role in establishing a regulatory environment in the gut in response to antigens present in the intestinal lumen.

The immunomodulatory effect of spermidine in T cells is mediated through autophagy

The ability of spermidine to trigger autophagy has previously been described for $CD8^+$ memory cells.[38] To test whether the effect of spermidine on Treg cell induction is likewise mediated via autophagy, we assessed the conversion of LC3-I to LC3-II protein, a marker for autophagosomes. Considering that on day 3 of a T_H17 cell culture spermidine had already shifted the proinflammatory toward a regulatory phenotype, we evaluated autophagy on day 1 and day 2. Using Western blot, we detected higher LC3-II and Atg5 protein levels in spermidine-treated cells compared to control cells (Fig 3, *A*). This finding was confirmed by flow cytometry with use of the FlowCellect Autophagy Kit, a method that enables the quantification of autophagosome formation by monitoring lipidated LC3-II. Before the staining, cells were treated with chloroquine, which increases the lysosomal pH, to inhibit lysosome-autophagosome fusion and consequent degradation. We observed that spermidine-treated cells display a significantly increased LC3 frequency (Fig 3, *B*) and mean fluorescence intensity level (see Fig E8, *A* in this article's Online Repository at www.jacionline.org) in a concentration-dependent fashion compared with that in an untreated control. Autophagy is controlled by the mechanistic target of rapamycin (mTOR), which is a protein kinase complex that can determine the differentiation and function of T cells in agreement with their metabolic context[39]; often, the inhibition of mTOR signaling is associated with $Foxp3^+$ Treg cell induction.[40] Hence, we intended to evaluate whether spermidine was compromising mTOR signaling, similarly to rapamycin. $CD4^+$ T cells were stimulated with spermidine or rapamycin for 1 or 2 days and stained intracellularly for S6 phosphorylation, a marker for activation of the mTOR pathway, and mTOR. Rapamycin promptly inhibited mTOR, whereas spermidine decreased mTOR signaling solely on day 1, followed by a return to control levels at day 2 (Fig 3, *C* and D and see Fig E8, *B*). This slight decrease in mTOR signaling was further observed by Western blot (see Fig E8, *C*).

Treg cell stability and function are known to require the activation of autophagic pathways regulated by Atg7 and Atg5.[41] Accordingly, T_H17 cell cultures established with naive $CD4^+$ T cells isolated from *Atg5*floxOX40cre mice, in which Atg5 is conditionally deleted in activated T cells, no longer reproduced the immunomodulatory phenotype of spermidine detected by the increase in $Foxp3^+$ cells (Fig 3, *E*) and decrease of $IL\text{-}17^+$ cells (see Fig E8, *D*). Moreover, in iTreg cell cultures, spermidine-treated cells lacking Atg5 did not display enhanced $Foxp3^+$ frequencies compared with treated control cells (Fig 3, *F*). Altogether, these results suggest that spermidine requires a functional autophagic machinery to induce its anti-inflammatory role, a phenotype partially modulated by mTOR.

The intestinal T-cell compartment can be influenced by administration of spermidine

Bacteria present in the gut are among the major producers of polyamines.[17] To discriminate the effect of spermidine against other microbiota-derived metabolites on the induction of $Foxp3^+$ Treg cells, we first reduced the presence of bacterial metabolites by establishing dysbiosis in WT mice. Previous studies have shown that supplementation of drinking water by adding broad-spectrum antibiotics reduces the levels of immunomodulatory metabolites (ie, short-chain fatty acids) within the gut by depleting the microbiota.[4] Thus, after 1 week of antibiotic treatment, we administered spermidine in the drinking water (Fig 4, *A*). We observed a significant increase in the frequency and number of $Foxp3^+$ cells exclusively in the colonic lamina propria (Fig 5, *B-D*). Although the relative numbers of Treg cells were also increased in both the small intestine and MLNs) (Fig 4, *B* and *C*), the total cell number was not statistically different from that in the controls (Fig 4, *D*).

Even though spermidine is present in several dietary products, it can also be produced via the L-arginine pathway (see Fig E1, *A*) by host cells and especially by the microbiota.[24] Therefore, we next investigated whether administration of L-arginine would also be reflected in changes in the $Foxp3^+$ Treg cell compartment. Following 2 weeks of 2% (wt/vol) L-arginine supplementation, mice showed significantly increased frequencies of Treg cells in the small intestine (Fig 4, *E*), together with higher total cell numbers of $CD4^+Foxp3^+$ cells in the colon and a non–statistically significant increase in cell numbers within the small intestine (Fig 4, F). Importantly, mice subjected to L-arginine treatment displayed significantly elevated relative concentrations of spermidine in the serum (Fig 4, *G*), suggesting that the polyamines metabolized from L-arginine were distributed systemically.

Our *in vitro* results showed that spermidine decreases the percentage of IL-17–producing $CD4^+$ T cells. We therefore assessed whether increased T_H17 cell differentiation by colonizing mice with segmented filamentous bacterium (SFB)[42] could be mitigated by consecutive L-arginine treatment (see Fig E9, *A* in this article's Online Repository at www.jacionline.org). Under these conditions, we indeed found an increase in IL-17–producing T cells; however, we did not observe any changes in the percentages of $IL\text{-}17^+$ cells following spermidine treatment (see Fig E7, *B*). Interestingly, the increase in $Foxp3^+$ Treg cells induced by L-arginine feeding was recapitulated independently of the presence or absence of SFB (see Fig E7, *B*). Altogether, our findings indicate that spermidine alone induces the generation of $Foxp3^+$ Treg cells, a process that can also be mediated by the microbiota, through their metabolization of L-arginine.

Administration of spermidine ameliorates gut pathology in a model of T-cell transfer colitis

After we had established that spermidine promotes Treg cell differentiation, our next aim was to test the immunosuppressive capacity of spermidine *in vivo* by using a disease model that relies on Treg cells to suppress inflammation. For this, we chose the T-cell transfer model of colitis in which Treg cells are critical to control the response of Teff cells.[43] We transferred naive $CD4^+CD25^-$ T cells into lymphopenic $Rag2^{-/-}$ mice and administered 30 mM spermidine in their drinking water throughout the course of the experiment (see Fig E10, *A* in this article's Online Repository at www.jacionline.org). The spermidine-treated mice experienced a less severe decrease in body weight (Fig 5, *A*) when compared with the control mice in addition to a decrease in the characteristic shortening of the colon size (Fig 5, *B*). Hematoxylin and eosin staining of the colons unveiled that spermidine treatment was able to preserve the

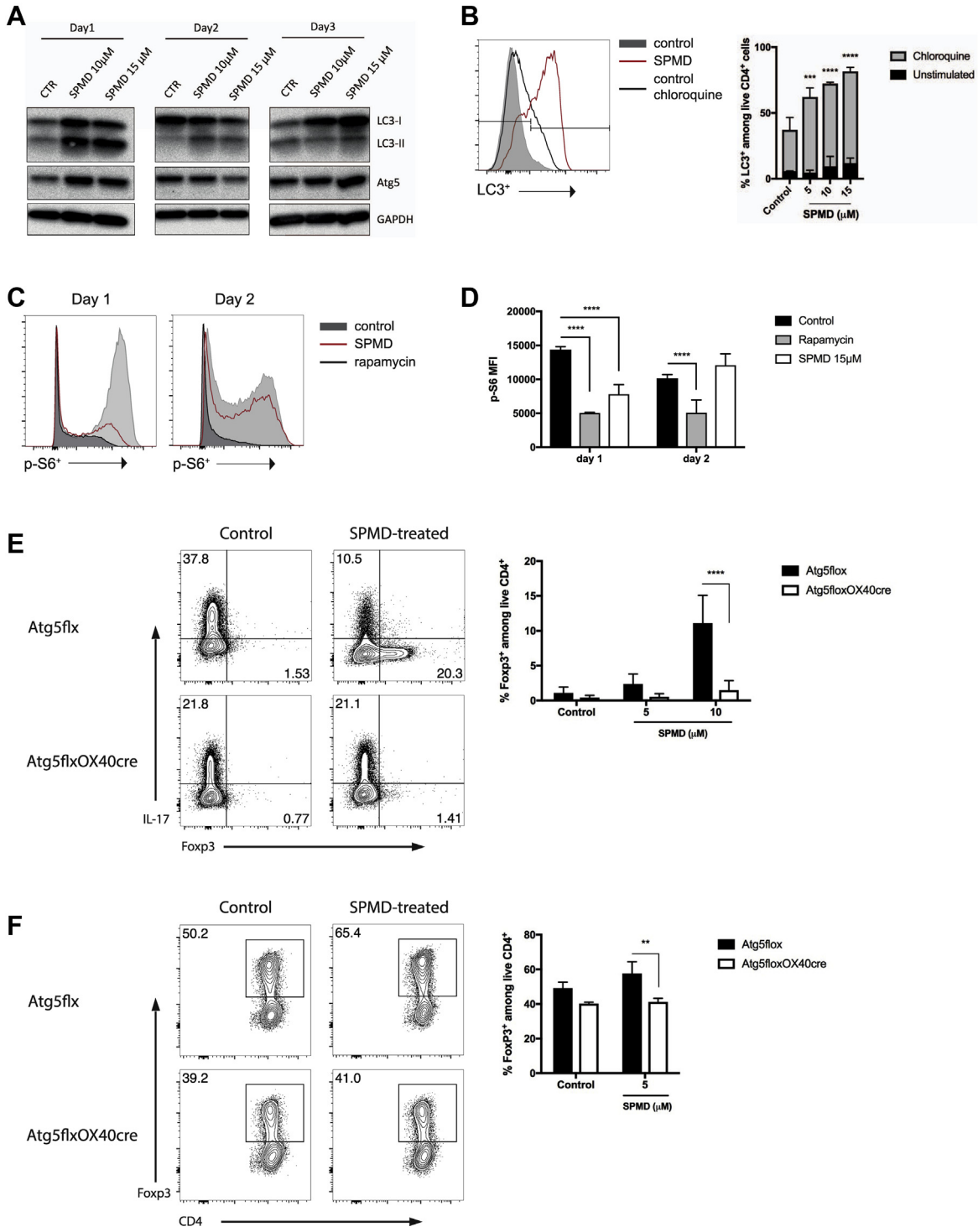

FIG 3. The effect of spermidine (SPMD) on $CD4^+$ T cells is autophagy-related. **A,** Protein expression of LC3 lipidation and Atg5 in $CD4^+CD25^-$ T cells, differentiated in the presence of T_H17 cytokines, for 1, 2, or 3 days with or without SPMD was evaluated by Western blot at the indicated time points; data are from 1 of 2 independent experiments. **B-D,** $CD4^+CD25^-$ T cells were cultured for 2 days under T_H17 cell–polarizing conditions and increasing concentrations of SPMD. **B,** $LC3^+$ autophagosome formation in cells that were pretreated for 2 hours with chloroquine, to inhibit lysosomal degradation. Representative FACS histogram of untreated $CD4^+$ cells (*gray*), chloroquine-treated control cells (*black-lined*), and SPMD-treated cells (*red-lined*); frequency of live $CD4^+LC3^+$ T cells was determined by using the FlowCellect Autophagy LC3 Antibody-based Assay Kit. **C,** Cells were stained for p-S6 protein. Representative FACS histogram of untreated $CD4^+$ cells untreated cells (*gray*), SPMD-treated cells (*red*), and rapamycin-treated cells (*black-lined*) is depicted and the corresponding mean fluorescence intensity is quantified (**D**). Pooled data are from 2 experiments. **E,** Naive $CD4^+CD25^-$ T cells from *Atg5*floxOX40cre mice and corresponding *Atg5*flox controls were cultured with T_H17 cell–polarizing cytokines and under Treg cell conditions for the analysis of $Foxp3^+$ cell frequency at day 4 (**F**). Data are representative of 3 independent experiments. Graphs show mean values, and error bars represent SDs unless otherwise specified. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

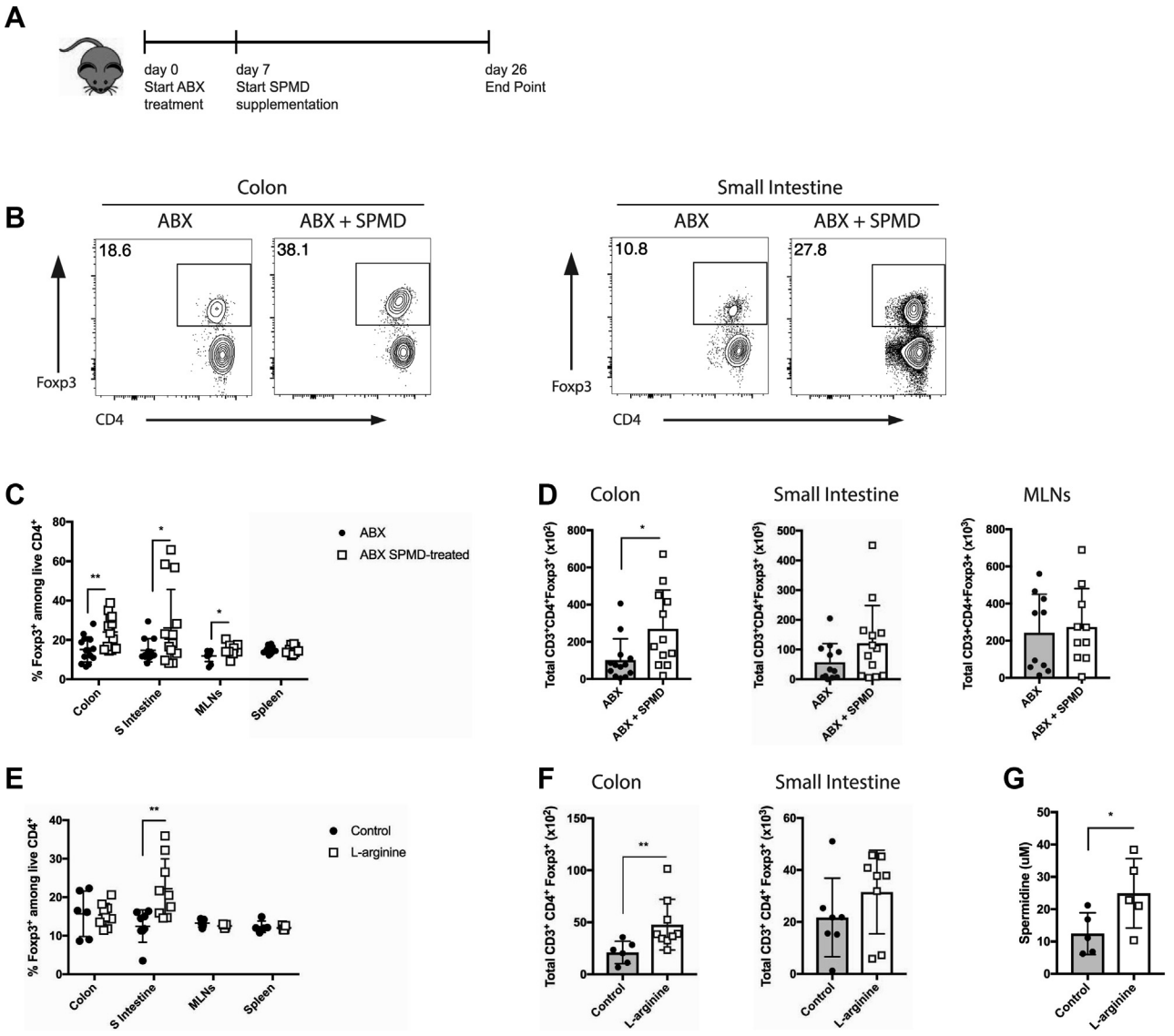

FIG 4. Supplementation of spermidine (SPMD) or its precursor L-arginine can modulate gut T-cell compartment. **A,** An antibiotic cocktail comprising ampicillin, kanamycin, vancomycin, and metronidazole was administered to the mice 1 week before supplementation with 30 mM SPMD in drinking water for 2 weeks, both treatments. **B**, Representative FACS plots of the gating strategy for $CD3^+CD4^+Foxp3^+$ cells in the colon and small intestine. The frequency (**C**) and total numbers (**D**) of live $CD3^+CD4^+$ $Foxp3^+$ Treg cells in the indicated organs of antibiotic-treated or antibiotic- and spermidine-treated mice were analyzed. The data were pooled from 3 independent experiments. Percentage (**E**) and total numbers (**F**) of live $CD3^+CD4^+$ $Foxp3^+$ Treg cells in the indicated organs of mice that were supplemented with 2% (wt/vol) of L-arginine in drinking water for 26 days. Symbols represent the data from individual mice. Pooled data are from 2 independent experiments. **G,** Serum was collected at the end point of the experiment, and the relative amount of SPMD was quantified via gas chromatography–HPLC. Each dot represents a mouse. Graphs show mean values, and error bars represent SDs unless otherwise specified. $*P < .05$; $**P < .01$.

epithelial structure, with minor diffuse cell infiltrates when compared with that in control mice (Fig 5, *C*). The scoring of the histology sections substantiated the improved colonic architecture in spermidine-treated mice (Fig 5, *D*). Analysis of the T-cell compartment (Fig 5, *E*) within the lamina propria of the colon and small intestine showed a significant increase in the frequencies of $Foxp3^+$ Treg cells and total $Foxp3^+$ cell numbers (Fig 5, *F* and *G*, respectively). Treg cells can exert their suppressive role by secreting anti-inflammatory cytokines, such as IL-10.[44] In the colon, Treg cells induced by spermidine displayed a significantly increased production of IL-10 (Fig 5, *H*), whereas in the small intestine we noted a nonsignificant increase in frequency and cell numbers of IL-10–producing Treg cells (Fig 5, *I*).

We additionally quantified the amount of IL-17–producing cells; however, no significant change was observed (see Fig E10, *B*). Moreover, the frequency and cell number of $Foxp3^+$ and IL-17–producing $CD4^+$ T cells in the MLNs (see Fig E10, *C*) and spleen (see Fig E10, *D*) were not significantly altered after spermidine treatment. Often, in the model of T-cell transfer colitis, the inflammation is associated with an IFN-γ response.[45] Thus, we further analyzed whether treatment with spermidine

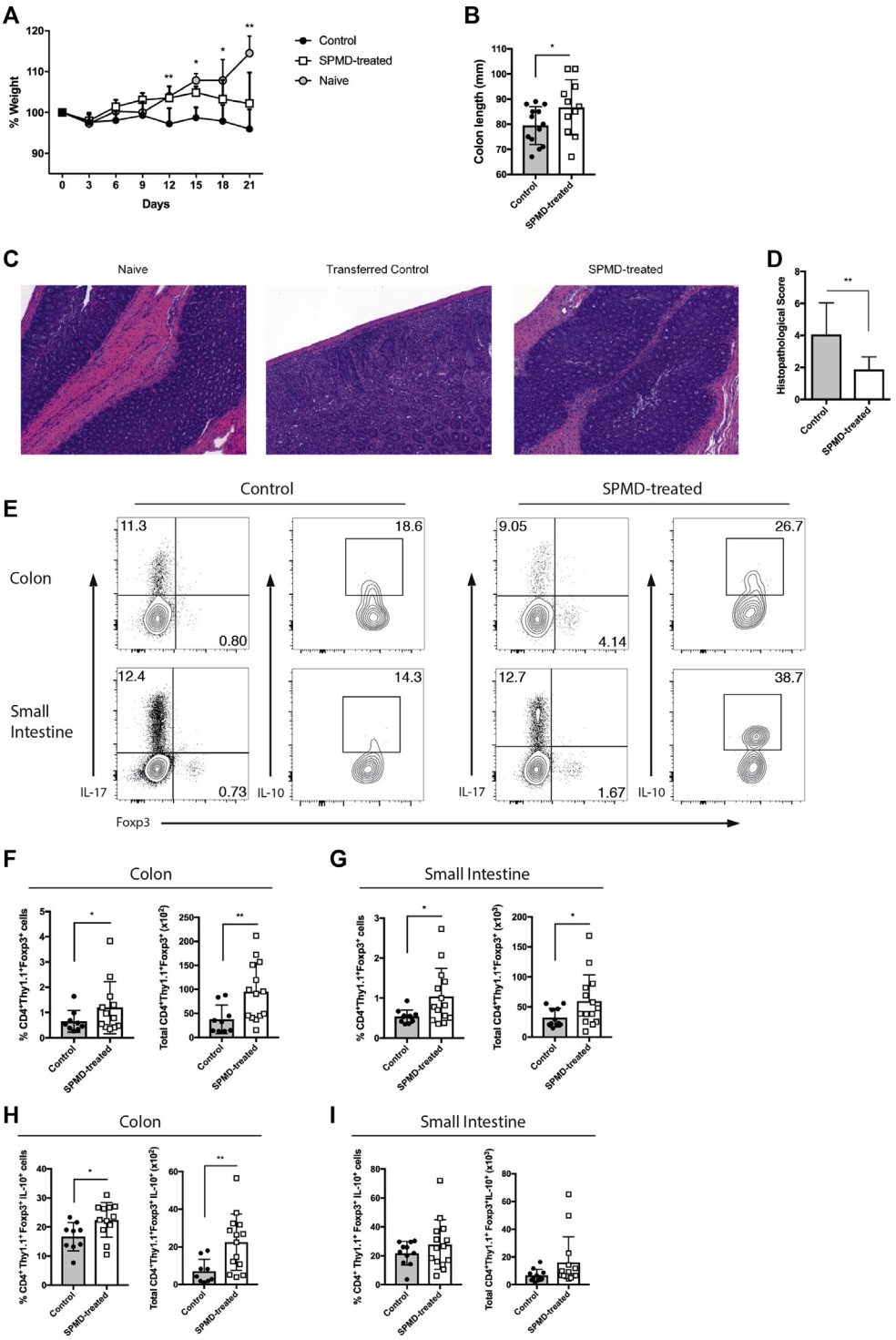

FIG 5. Spermidine (SPMD) ameliorates transfer colitis pathology by increasing the gut Treg cell population. *Rag2*$^{-/-}$ mice were injected with sorted Thy1.1^{+}CD4^{+}CD45RB^{hi}CD25^{-} T cells and supplemented, or not, with 30 mM SPMD in drinking water for minimum of 21 days. **A,** The weight of the mice was monitored regularly and is presented as a percentage of initial weight across the experimental groups. **B,** The colon length was measured at the end point. **C,** Representative figure for each experimental group of the colon sections analyzed by hematoxylin and eosin staining. **D,** Quantification of histologic scores for cell infiltration and epithelial damage of individual mice. Pooled data are from 3 independent experiments. **E,** Representative FACS plots of the gating strategy for transferred CD4^{+}Thy1.1^{+} cells in the colon and small intestine of control and SPMD-treated mice. The cells from the lamina propria were isolated and stained for intracellular Foxp3, IL-17, and IL-10. The percentages and cell numbers of live Thy1.1^{+}CD4^{+}Foxp3^{+} cells from the (**F**) colon and (**G**) small intestine are shown. Among the live Thy1.1^{+}CD4^{+}Foxp3^{+} population, the frequency and number of IL-10–producing cells were assessed in the colon (**H**) and small intestine (**I**). Symbols represent the data from each individual mouse; horizontal lines show the mean. Pooled data are from 4 independent experiments. Graphs show mean values, and error bars represent SDs unless otherwise specified. **P* < .05; ***P* < .01; ****P* < .001.

would affect IFN-γ–producing T cells in the gut. Mice treated with spermidine displayed fewer $CD4^+Thy1.1^+IFN\text{-}\gamma^+$ cells in the colon (see Fig E11, *A* in this article's Online Repository at www.jacionline.org) and in the small intestine (see Fig E11, *B*), although no significant decrease was observed in the MLNs (see Fig E11, *C*) nor spleen (see Fig E11, *D*).

Together, these results demonstrate that spermidine can prevent the induction of intestinal inflammation and suggest that its immunosuppressive function can be explained mechanistically by potentiating Treg cell differentiation. Accordingly, ingestion of polyamine-rich diets could serve as a prophylactic measure to attenuate inflammatory gut pathologies.

Discussion

A plethora of metabolites are secreted by intestinal bacteria, depending on the host diet and lifestyle.[1,46] Understanding of the biologic mechanisms that sustain this symbiotic relationship is important for the development of prophylactic treatments and novel immunotherapies. Spermidine is an end product of L-arginine metabolism[11] that is mainly produced by the microbiota and has recently been studied for its immunologic properties.[24] Yet, little is known about the role of spermidine in $CD4^+$ T-cell biology. Here, we report that spermidine modulates T-cell development, potentiating the differentiation of both murine and human naive T cells toward Treg cells, in an autophagy-associated manner.

Previous literature has highlighted the role of polyamine metabolism in proliferative cells. A great number of reports associated aberrant polyamine concentrations with skin, breast, lung, colon, and prostate cancers[47] mostly linked to uncontrolled activity of the enzymes ornithine decarboxylase (ODC) and spermine oxidase, part of the polyamine synthesis pathway. Hence, the ODC inhibitor α-difluoromethylornithine appears to suppress tumor growth[48] and boost IFN-γ production by $CD8^+$ T cells.[49] Additionally, *in vivo* cotreatment with α-difluoromethylornithine and a polyamine transporter inhibitor resulted in a decrease of $CD45^+CD4^+CD25^+$ Treg cells within the tumor.[50] Potentially, the elevated polyamine synthesis by tumor cells leads to the secretion of spermidine, which in turn might impair antitumor immunity by promoting Treg cells. Nonetheless, future studies are required to address the role of secreted polyamines in tumor immunity.

The immune system of the gut regulates the balance between an effective response against foreign antigens while avoiding tissue damage.[51] This complex system relies on the activity of T cells. In this regard, T_H17 and Treg cells stand out for their abundance in the intestinal lamina propria.[52,53] Calibrating the balance between T_H17 and Treg cells is pivotal for the outcome of health versus disease, by protecting against infections and preventing unrestrained inflammation, respectively. Therefore, we first evaluated whether spermidine induces $Foxp3^+$ Treg cells when cells are cultured under T_H17 cell–polarizing conditions *in vitro*. Our results show that spermidine not only downregulates the production of IL-17 but also triggers Treg cell differentiation. This is possible because both T-cell subsets require TGF-β stimulation to differentiate,[54,55] as TGF-β signaling induces both RORγt and Foxp3 expression. Indeed, we observed that spermidine is dependent on TGF-β to potentiate Treg cell commitment. Additionally, in the T_H17 cell *in vitro* setting, the resulting $Foxp3^+$ T cells also expressed RORγt, showing that spermidine possibly suppresses IL-17 at a posttranslational level.

We aimed to additionally characterize the immunomodulatory role of spermidine in other T_H cell subsets. Spermidine does not impair cell proliferation or cytokine production of either T_H1 or T_H2 cells. However, we observed a slight reduction of T-bet^+ T cells when stimulated with higher amounts of spermidine. This suggests that spermidine may interfere with the T_H1 cell commitment program. Recently, a number of reports have demonstrated that the pathogenesis associated with colitis is due to exacerbated activity of T_H1 cells.[45,56] In the *in vivo* model of T-cell transfer–induced colitis, we showed that $Thy1.1^+CD4^+$ transferred cells—when mice were subjected to spermidine treatment—had a decreased ability to become IFN-γ producers. Alternatively, the impaired number of T_H1-like cells in the intestine may be a result of the suppressive capacity of the spermidine-induced Treg cells, instead of a direct effect on T_H1 cell differentiation. However, further studies are required to assess how spermidine downregulates IFN-γ production *in vivo*.

Spermidine has been characterized in several systems as an inducer of autophagy.[25] Autophagy is broadly known as the process by which cells recycle their components, being upregulated as a cell survival mechanism when cells experience stress, such as starvation of nutrients.[57] Moreover, autophagy is required for T-cell activation, function, and survival.[58,59] T cells lacking autophagy-related genes show enhanced apoptosis.[60] Accordingly, the conditional deletion of the autophagy genes *Atg7* or *Atg5* in $Foxp3^+$ Treg cells leads to impaired lineage stability, loss of functional integrity, and decreased survival of Treg cells.[41] In that context, *Atg7* appears to be important to restrict mTOR activity,[41] which is known to control the cellular lineage commitment. mTOR responds to the cellular metabolic status and dictates T-cell differentiation accordingly.[61] The inhibition of mTOR signaling (eg, by rapamycin) inhibits T_H17 and T_H1 cell differentiation while favoring the differentiation of $Foxp3^+$ T cells.[40,62] Moreover, mTOR signaling is also sensitive to autophagy.[63] Here, we show that spermidine triggers autophagy during early stages of T-cell differentiation. When exposed to spermidine, Atg5-deficient T cells fail to upregulate Foxp3 as efficiently as WT cells do, both in T_H17 cell– and iTreg cell–inducing conditions. Importantly, spermidine lessens mTOR signaling at day 1 of cell differentiation, which might be the molecular signal that precedes the induction of autophagy. Spermidine was previously shown to induce autophagy in an mTOR-dependent manner in cardiomyocytes,[64] but not in human colon carcinoma cell lines[65] or in B cells.[26] As mTOR is a central metabolic regulator, it is plausible that contrasting reports are due to different extracellular environments—such as nutrient conditioning—and time points of experimental analysis. In the context of autoimmunity, autophagy appears to be a crucial immune regulator, as SNPs in the ATG16L1 loci are associated with inflammatory bowel disease (IBD) incidence.[66] Interestingly, it is not only mice with T-cell–specific *Atg16l1* deletion that develop intestinal inflammation; the conditional deletion of *Atg16l1* in Treg cells also deeply affects Treg cell homeostasis.[59] Likewise, an unbalanced T_H17 cell/Treg cell regulation is a characteristic of forms of IBD.[67] Taken together, these reports provided the grounds for the link between autophagy and tolerogenic gut immunity. Spermidine, as a Treg cell inducer triggering autophagy, could be an appealing therapeutic candidate to promote protection against gastrointestinal diseases.

Spermidine, whether produced by commensal microbiota or orally ingested, is quickly absorbed by the lamina propria.[18] Therefore, we aimed to evaluate whether supplementation of spermidine in the drinking water would promote Treg cell differentiation within the gastrointestinal tract. To circumvent the effect of other bacteria-derived metabolites, we first pretreated the mice with a broad-spectrum antibiotic cocktail for 1 week, an already established protocol to deplete microbiota.[4] Oral treatment with spermidine was able to change the T-cell compartment of mice, as we observed increased frequencies and total cell numbers of Foxp3$^+$ T cells. Apart from their uptake, polyamines can also be synthesized via the L-arginine pathway through a series of enzymatic reactions.[11] L-arginine is the substrate for arginase giving rise to ornithine, which then initiates the polyamine pathway through the ODC. We hypothesized that fueling the polyamine biosynthetic pathway by administrating L-arginine would recapitulate the results obtained with spermidine administration. Indeed, we observed an increase in the frequency of Foxp3$^+$ cells in the small intestine. Interestingly, previous reports on the production of amines by human microbiota had determined that the major producers of polyamines belong to the genera *Bifidobacterium*, *Bacteroides,* *Clostridium*, *Enterococcus*, *Lactobacillus*, *Enterobacter*, *Streptococcus,* and *Escherichia*.[68] Among these, *Bacteroides*, *Clostridium,* and *Streptococcus* are particularly found in higher numbers both in the small intestine and colon.[69] Colonization with SFB induces T_H17 cell differentiation in the lamina propria of the small intestine.[42] We hypothesized that the administration of L-arginine would stimulate polyamine production by the microbiota, in turn affecting the SFB cell–polarizing capacity. However, we did not observe any change in IL-17–producing T cells in the lamina propria, which indicates that polyamines could not overcome the effect mediated by SFB. Interestingly, we did observe an increase in Foxp3$^+$ Treg cells in the intestinal lamina propria, which was independent of the concurrent induction of T_H17 cells. Taken together, these findings strengthen the idea that an adequate diet could modulate the metabolism of gut bacteria to stimulate the production of immune regulatory metabolites.

Transfer colitis is a mouse model that mimics the intestinal inflammation observed in IBD.[70] In a previous clinical study, the quantification of amino acids from the colonic tissue of patients with ulcerative colitis showed a significant decrease in L-arginine levels.[71] It is then plausible to hypothesize that the decreased L-arginine availability might consequently result in unbalanced levels of polyamines. Here, we observed that after 3 to 4 weeks of T-cell transfer–induced colitis in mice, administration of spermidine was able to dampen the severity of the inflammation-induced tissue damage, associated with an increase in Treg cell numbers in both small intestine and colon. Importantly, the Treg cell population induced by spermidine displayed enhanced production of the anti-inflammatory cytokine IL-10. Further studies will be necessary to investigate not only the availability of L-arginine but also its downstream polyamine metabolites in patients with IBD.

In conclusion, polyamines are microbiota-derived metabolites critical for cellular fitness. Here we sought to further characterize the role of spermidine in T-cell differentiation and function. Our data show that spermidine potentiates Foxp3$^+$ T-cell differentiation *in vitro* in an autophagy-associated manner. Additionally, spermidine appears to have immunomodulatory effects *in vivo*, driven by an increase in the Treg cell gut compartment. Given that many dietary products (such as dairy products, soybeans, vegetables, meat, and seafood[72]) contain polyamines or their precursor L-arginine, our results help to elucidate their significance for the immune system, highlighting the beneficial role of a polyamine-rich diet in the context of gut inflammation.

We thank the members of the Sparwasser laboratory for the technical support and scientific discussions and Dr Luciana Berod for critical reading of the article. We also thank the Research Core Unit Metabolomics at the Hannover Medical School for performing the measurements of the polyamines via HPLC-MS.

Key messages

- **Spermidine shifts T_H17 cell polarization toward Treg cells.**
- **Spermidine induces autophagy in CD4$^+$ T cells.**
- **Spermidine potentiates the development of Treg cells within the lamina propria.**

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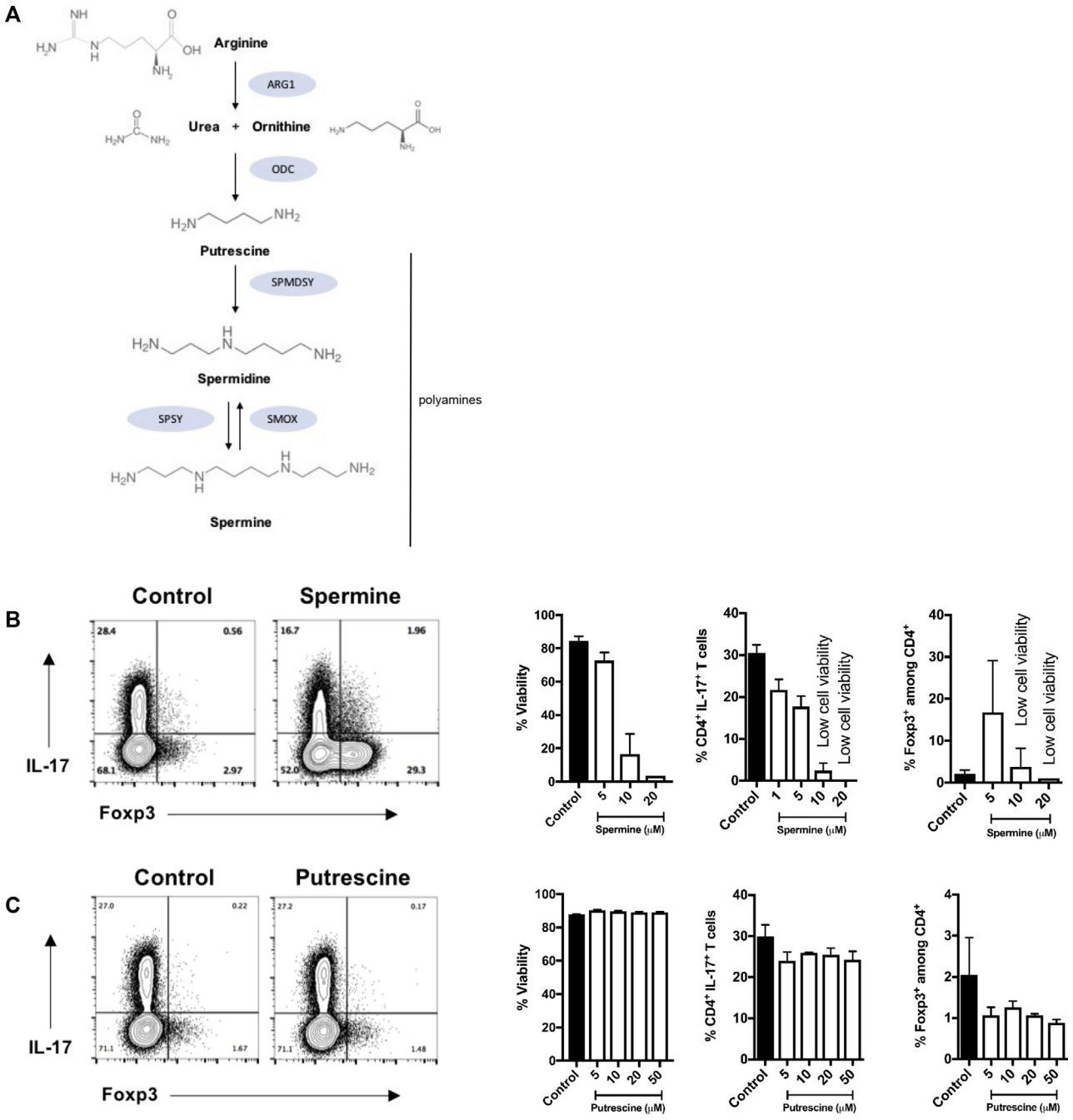

FIG E1. The effect of the polyamines spermine and putrescine on T_H17 cell cultures *in vitro*. **A,** Schematic depiction of polyamine biosynthesis pathway. **B** and **C,** Representative FACS plot, gated on live $CD4^+IL\text{-}17^+$ and $CD4^+Foxp3^+$ cells, for T_H17 cell cultures differentiated without (control) or with stimulation of spermine or putrescine, respectively. $CD4^+CD25^-$ T cells enriched from spleen and lymph nodes of WT mice were cultured in T_H17 cell–polarizing conditions and increasing doses of spermine (**B**) or putrescine (**C**) for 4 days and stained for viability, IL-17 cytokine production, and transcription factor Foxp3. Pooled data are from 2 independent experiments. *ARG1*, Arginase 1; *SMO*, spermine oxidase; *SPMDSY*, spermidine synthase; *SPSY*, spermine synthase.

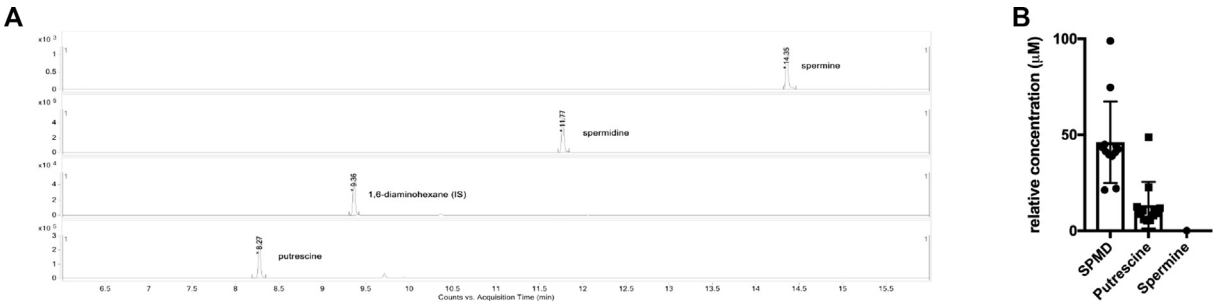

FIG E2. Polyamines are readily found in murine fecal samples. **A,** Stool samples from C57BL/6 mice were collected and snap-frozen for polyamine quantification via gas chromatography–HPLC. The area of the peak for putrescine, SPMD, and spermine was normalized to the area of 1,6-diaminohexane. **B,** Relative amount of SPMD and putrescine isolated from individual 6- to 12-week-old WT mice. The values of spermine were lower than the detection limit.

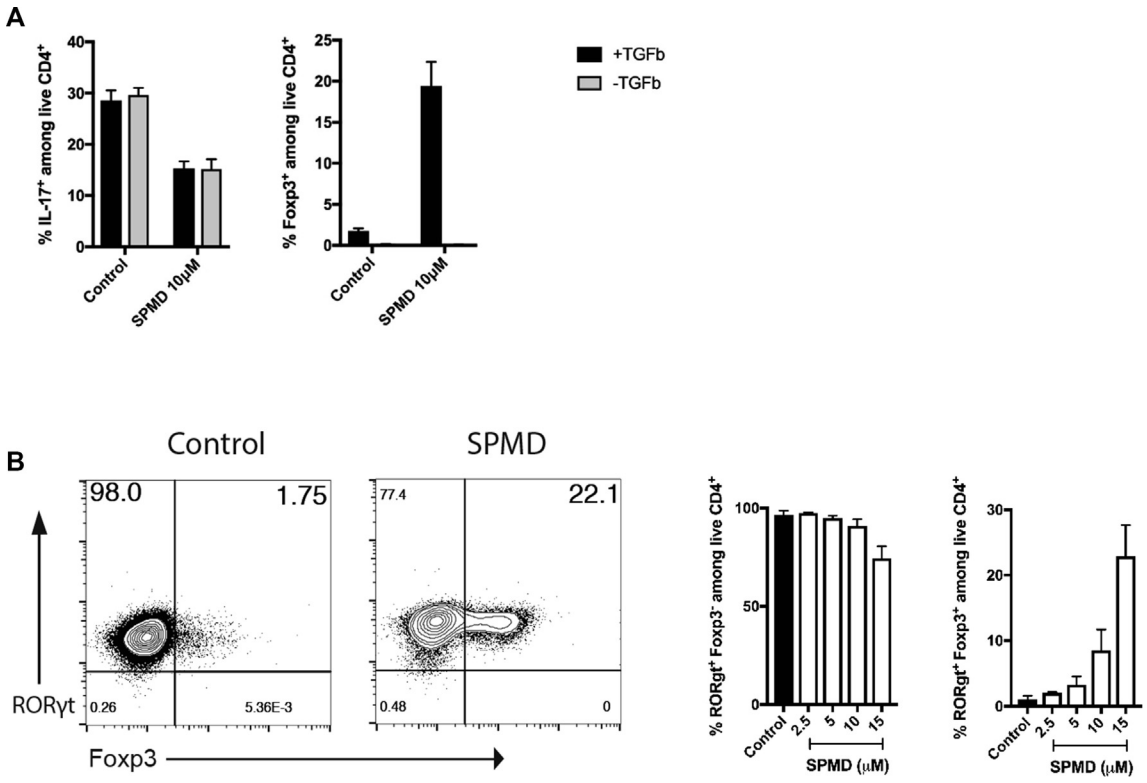

FIG E3. Spermidine (SPMD) shifts T_H17 cell differentiation toward Foxp3$^+$ Treg cells in a TGF-β–dependent fashion. **A,** CD4$^+$CD25$^-$ T cells enriched from spleen and lymph nodes of WT mice that were cultured under T_H17 cell–polarizing conditions. Cell were differentiated with or without stimulation using 10 μM SPMD and with or without TGF-β for 4 days. Frequency of CD4$^+$IL-17A$^+$ and CD4$^+$Foxp3$^+$ cells among live cells. Pooled data are from 2 independent experiments. **B,** Representative FACS plots and quantification of live CD4$^+$RORγ$^+$, CD4$^+$Foxp3$^+$, and CD4$^+$IL-17$^+$ cells from T_H17 cell cultures differentiated without (control) or with SPMD treatment. Representative data are from 3 experiments.

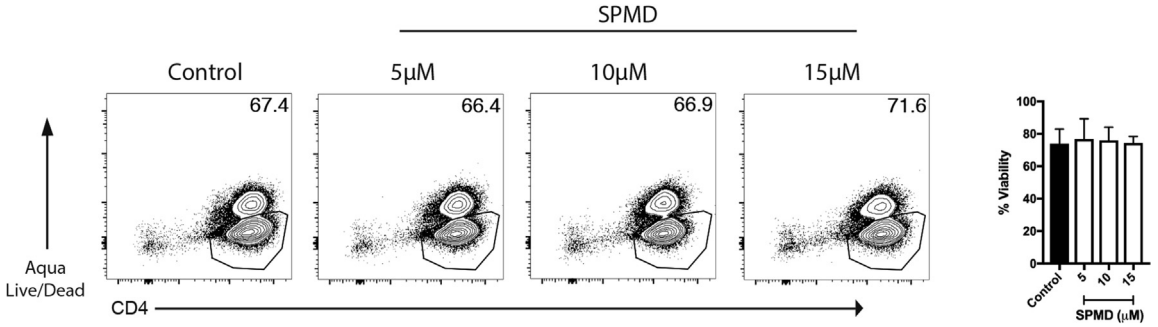

FIG E4. Spermidine (SPMD) treatment does not impair human T_H17 cell viability. Representative FACS plots and quantification of $CD4^+$ cell viability. Naive $CD4^+$ T cells isolated from human cord blood cells were cultured for 5 days under T_H17 cell–polarizing conditions and in the presence of increasing concentrations of SPMD. At day 5, cells were stained by using a LIVE/DEAD Fixable Aqua Dead Cell Stain Kit. Each dot represents an independent donor.

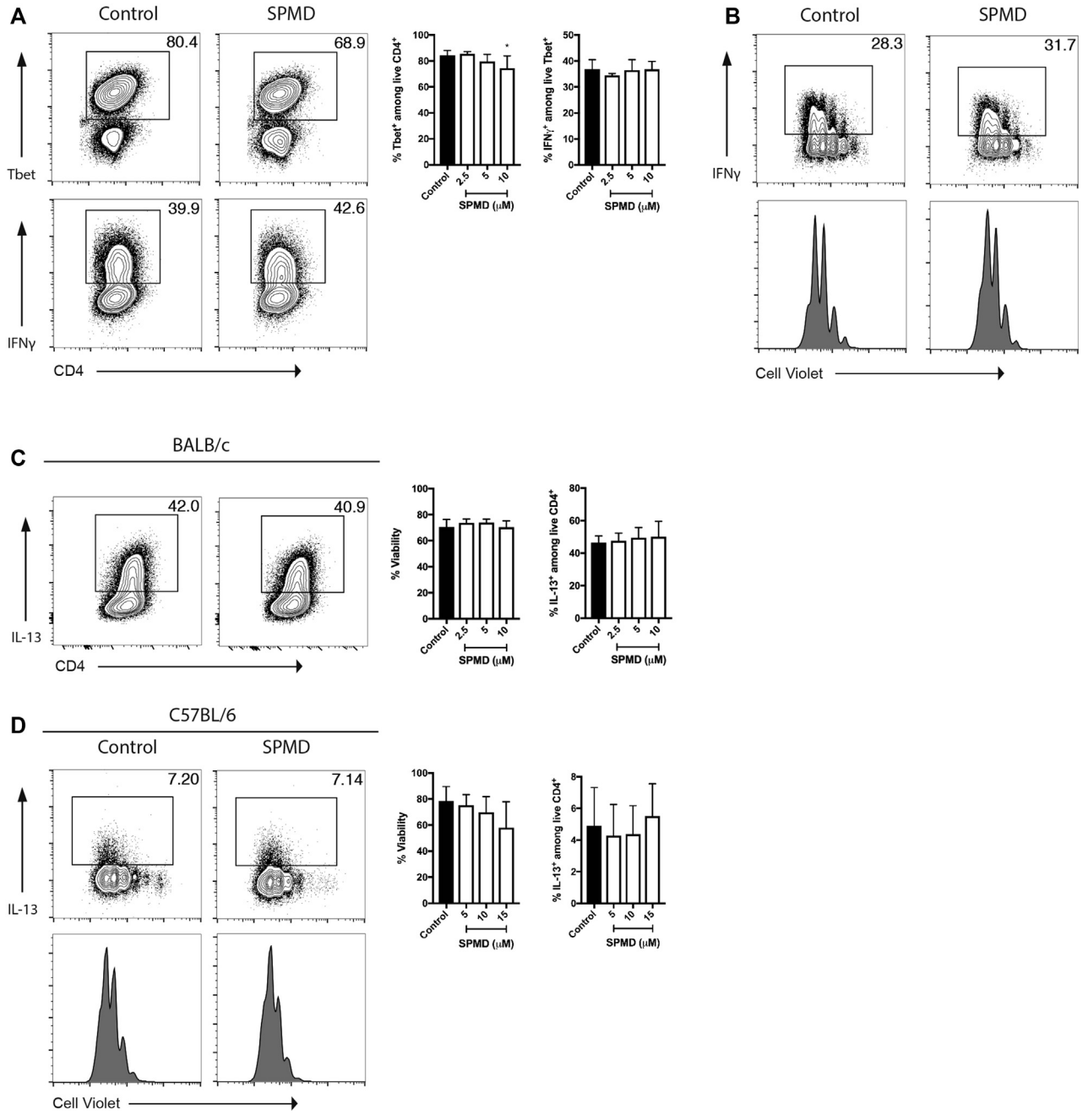

FIG E5. Spermidine (SPMD) does not compromise differentiation to T_H1 or T_H2 cells. **A** and **B,** Murine naive $CD4^+$ T cells were cultured under T_H1 cell–polarizing conditions in the presence of increasing concentrations of SPMD. Representative FACS plots of differentiated T_H1 cells. **A,** At day 4, cells were stained intracellularly for T-bet and IFN-γ. Pooled data are from 3 independent experiments. **B,** Proliferation was assessed by labeling $CD4^+CD25^-$ T cells with CellTrace Violet proliferation dye. Representative plot from 2 independent experiments is shown. **C** and **D,** Representative FACS plots and quantification of differentiated T_H2 cells. Naive $CD4^+$ T cells from BALB/c (**C**) or C57BL/6 (**D**) mice were cultured under T_H2 cell–polarizing conditions in the presence of increasing concentrations of SPMD. At day 4, cells were stained intracellularly for IL-13. Proliferation was assessed by labeling $CD4^+CD25^-$ T cells with CellTrace Violet proliferation dye. Pooled data are from 2 independent experiments.

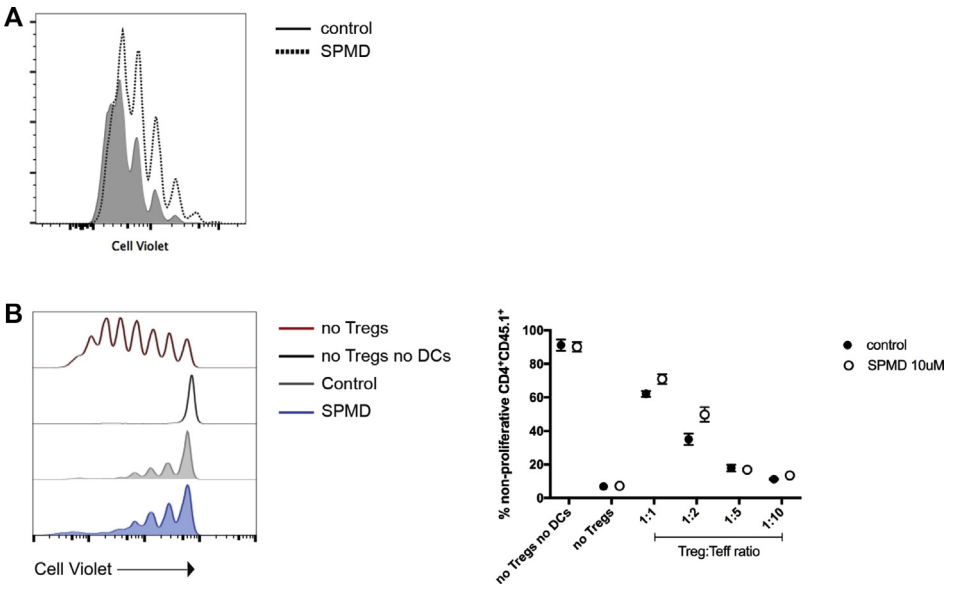

FIG E6. Treg cells differentiated under spermidine (SPMD) stimulation preserve their suppressive function. **A,** Enriched $CD4^{+}CD25^{-}$ T cells were labeled with CellTrace Violet proliferation dye and cultured under iTreg cell–polarizing conditions, with or without treatment with 5 μM SPMD. Proliferation after 96 hours from 1 of 2 independent experiments. **B,** Treg cells were differentiated with or without SPMD stimulation and cocultured with CellTrace Violet–labeled Teff cells and DCs for 96 hours. Representative histogram depicting the proliferation cycles of Teff cells cocultured with Treg cells in a 1:2 ratio, without Treg cells as a positive control, or without costimulatory DCs as a negative control. The graph shows the percentage of suppression for different Treg cell–to–Teff cell ratios. Representative data are from 2 independent experiments.

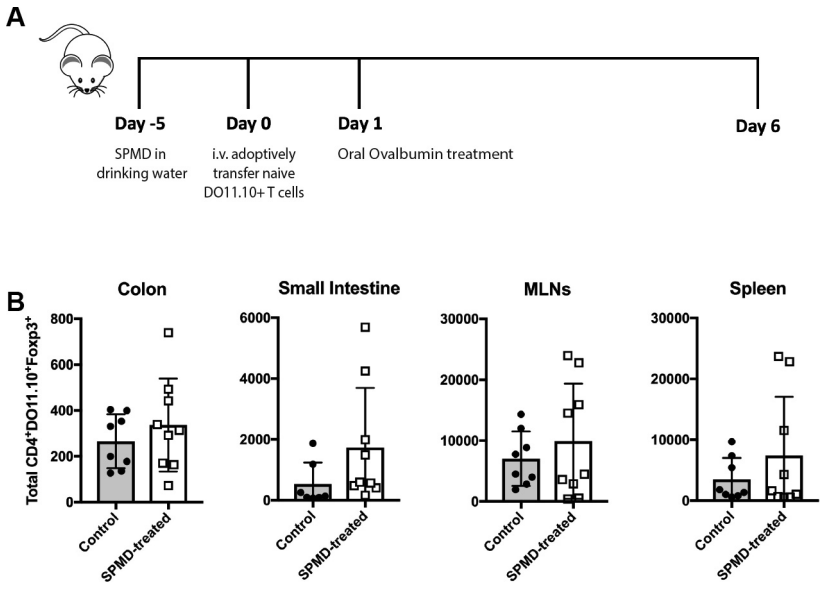

FIG E7. Ovalbumin-specific TCR-transgenic mice show increased Foxp3$^+$ cell numbers after treatment with spermidine (SPMD). **A,** CD4$^+$CD25$^-$ naive cells from spleens and lymph nodes of DO11.10 TCR-transgenic Rag2$^{-/-}$ mice were adoptively transferred into BALB/cJ mice receiving 30 mM SPMD in their drinking water. After CD4$^+$CD25$^-$ naive T-cell transfer, mice received daily ovalbumin (160 mg per day) treatments via oral gavage. **B,** Absolute numbers of CD4$^+$Foxp3$^+$ live cells were assessed in the indicated organs after 1 week of oral treatment with ovalbumin. Symbols represent the data from individual mice. Pooled data are from 2 independent experiments.

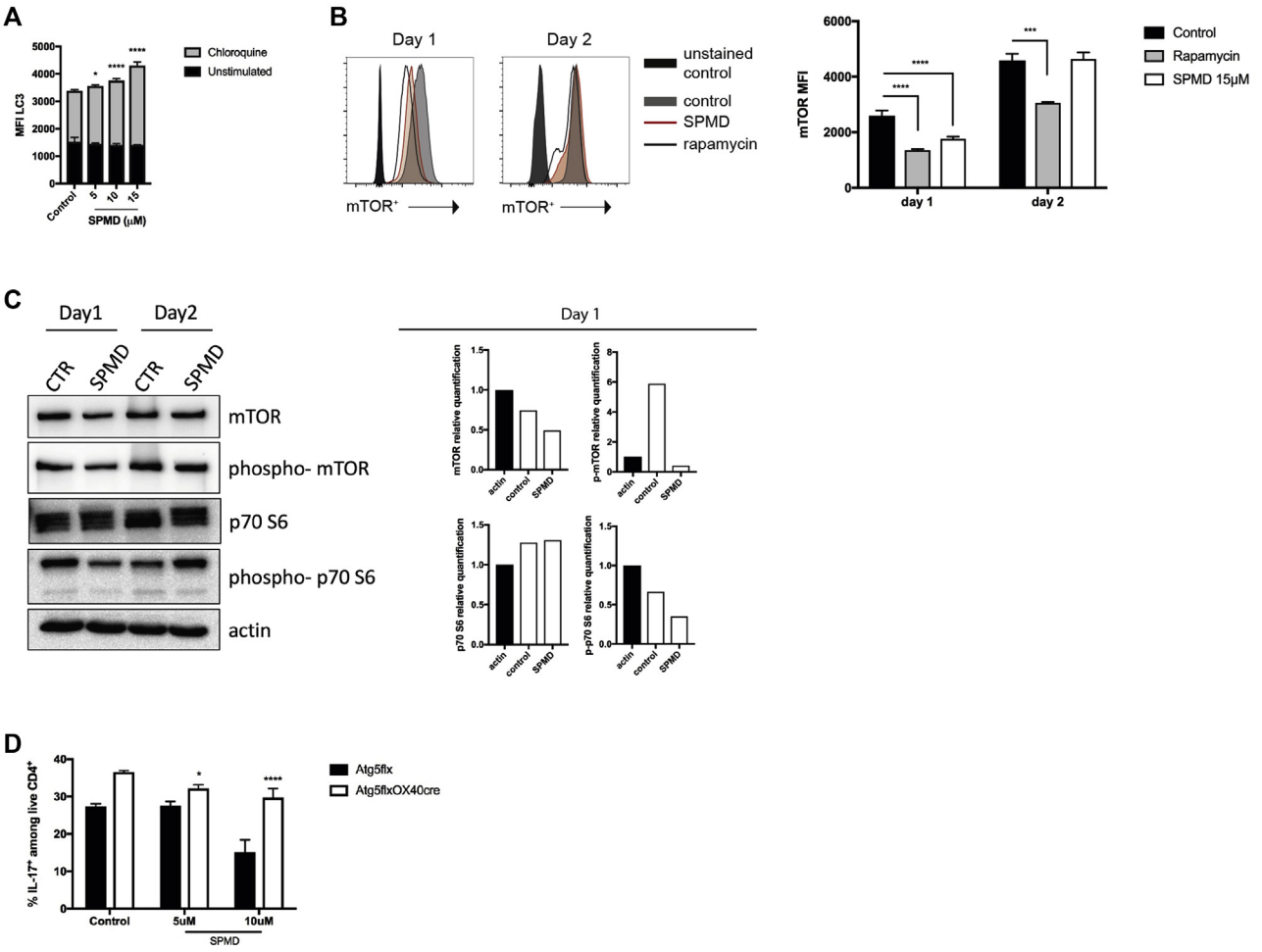

FIG E8. Autophagy is required for the decrease in IL-17 production of spermidine (SPMD)-stimulated cells *in vitro*. **A,** $CD4^+CD25^-$ T cells were cultured with increasing concentrations of SPMD for 2 days. Cells were pretreated for 2 hours with chloroquine to inhibit lysosomal degradation and stained by using a FlowCellect Autophagy LC3 Antibody-based Assay Kit. Mean fluorescence intensity quantification of $LC3^+$ autophagosome formation. **B,** Naive $CD4^+CD25^-$ cells were stained for mTOR protein after treatment with SPMD or rapamycin. Representative FACS histogram of untreated control cells (*gray*), SPMD-treated cells, (*red*), and rapamycin-treated cells (*black-lined*) is depicted, and the corresponding mean fluorescence intensity is quantified. Pooled data are from 2 independent experiments. **C,** Protein expression evaluated by Western blot of mTOR, phospho-mTOR, p70 S6, phospho-p70 S6, and β-actin in $CD4^+$ T cells at days 1 and 2 of T_H17 cell differentiation with or without 10 μM SPMD. One of 2 experiments is shown. **D,** Naive $CD4^+CD25^-$ cells from Atg5flxOX40cre mice and from corresponding Atg5flx controls were cultured under T_H17 cell–polarizing cytokines and analyzed for the frequency of $CD4^+IL\text{-}17^+$ cells at day 4. Data are representative of 3 independent experiments. Graphs show mean values, and error bars represent SDs unless otherwise specified. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

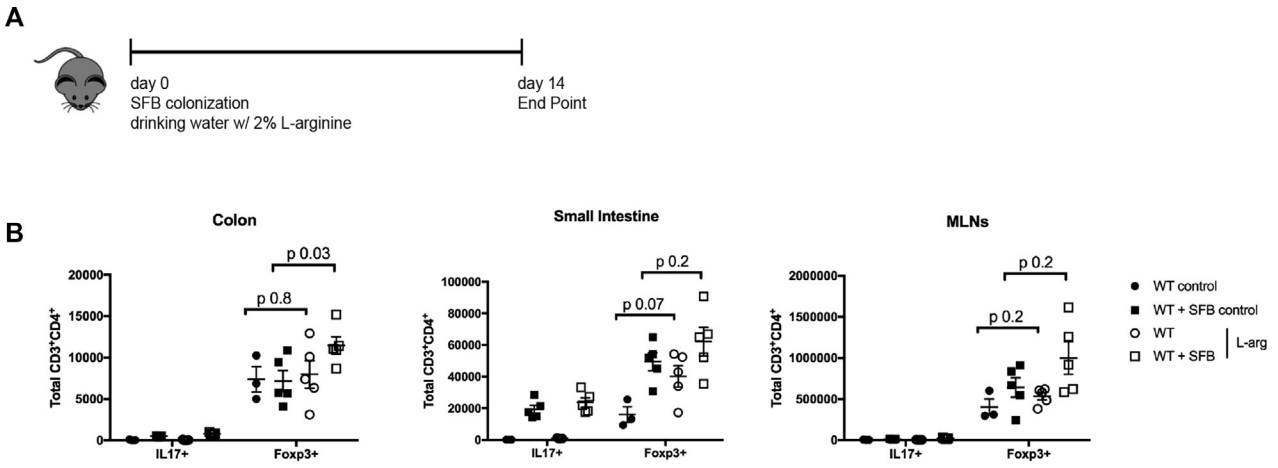

FIG E9. Supplementation of L-arginine increases Foxp3$^+$ cell numbers independently of SFB colonization. **A,** WT Mice were colonized with SFB and submitted to supplementation with 2% (wt/vol) of L-arginine in the drinking water for 2 weeks. **B,** Absolute numbers of live CD3$^+$CD4$^+$ cells of the colon, small intestine, and MLNs of WT control mice and SFB-colonized mice after the cells were stained for intracellular IL-17 and Foxp3. Symbols represent the data from each individual mouse. Representative data of 2 independent experiments. The *P* value is depicted in the figure.

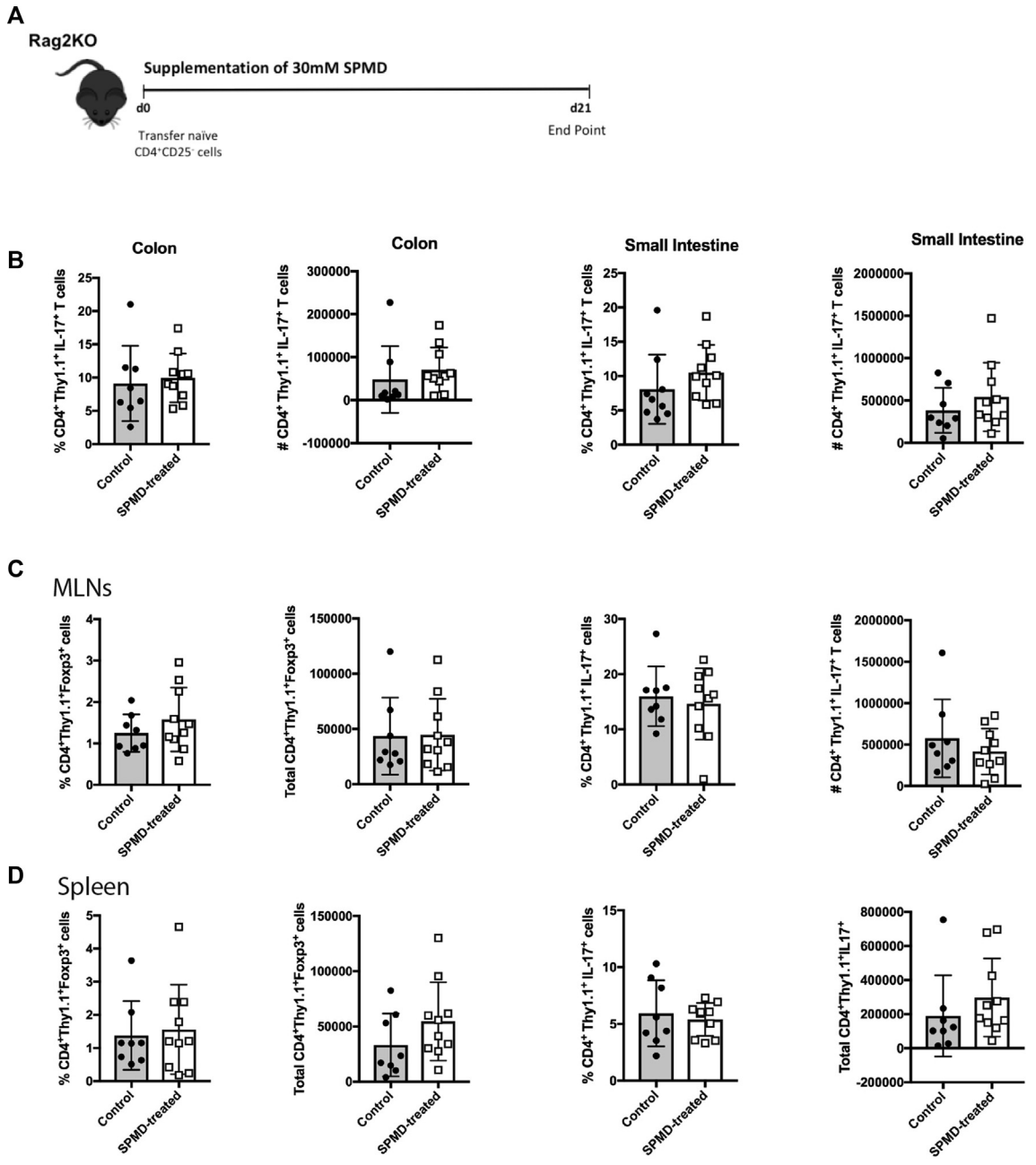

FIG E10. Spermidine (SPMD) administration does not alter the frequency of IL-17–producing $CD4^+$ cells or number of cells in the model of T-cell transfer colitis. **A,** *Rag2*$^{-/-}$ mice were injected with $Thy1.1^+CD4^+CD45RB^{high}CD25^-$ naive T cells in the peritoneum. After the injection, the mice received water supplemented with 30 mM SPMD throughout the disease. **B,** Cells from the colon and small intestine were isolated and stained for intracellular IL-17. Cells from the MLNs (**C**) and spleens (**D**) of control and SPMD-treated mice were isolated and stained intracellularly for Foxp3 and IL-17. The percentages and total cell numbers among live $Thy1.1^+CD4^+$ cells are shown. Symbols represent the data from each individual mouse. Pooled data are from 3 independent experiments.

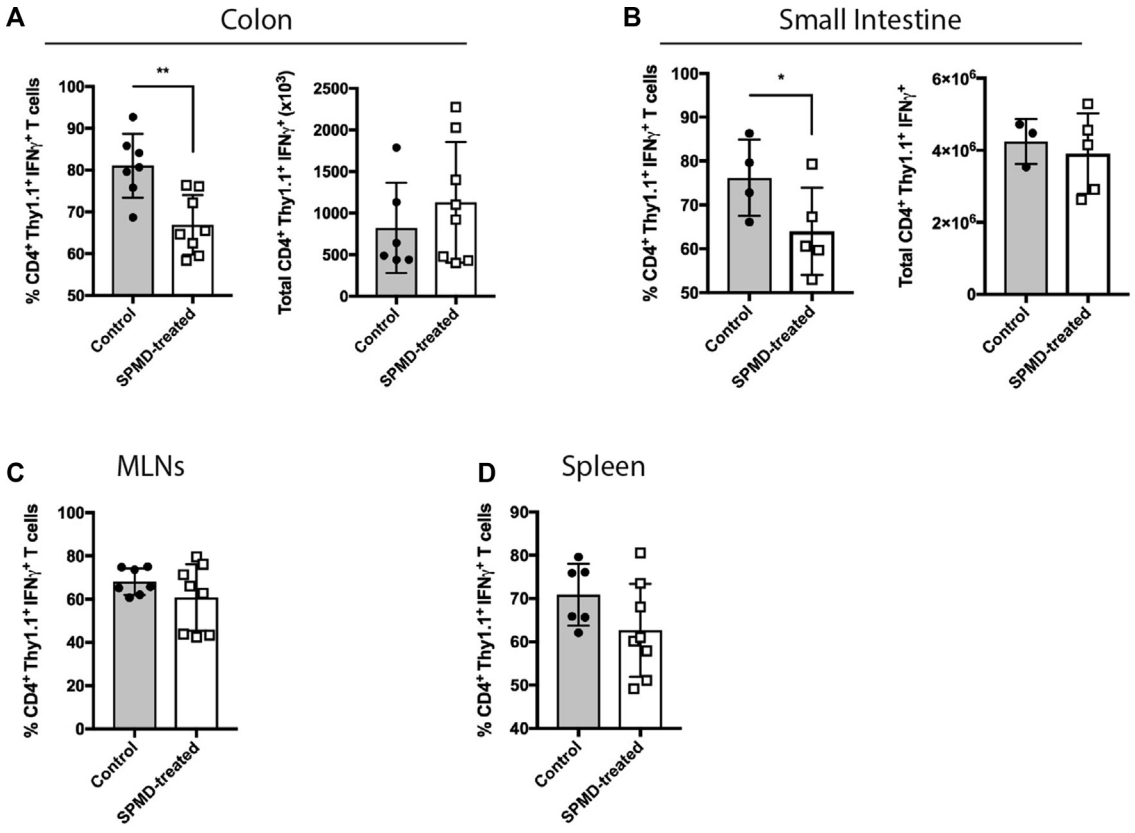

FIG E11. Spermidine (SPMD) administration reduces the frequency of IFN-γ–producing cells in the model of T-cell transfer colitis. **A**-**D**, Cells from the colon (**A**), small intestine (**B**), MLNs (**C**), and spleen (**D**) were isolated from mice with T-cell transfer–induced colitis that were subjected to SPMD treatment or left untreated. Cells were stained intracellularly for IFN-γ, among the live $Thy1.1^+$ $CD4^+$ population. Frequencies and total cell numbers of $CD4^+Thy1.1^+IFN\text{-}\gamma^+$ cells are shown. Symbols represent the data from each individual mouse. Pooled data are from 2 independent experiments. Graphs show mean values, and error bars represent SDs unless specified otherwise. $^*P < .05$; $^{**}P < .01$.