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# Recombinant Pregnancy-Specific Glycoprotein 1 Has a Protective Role in a Murine Model of Acute Graft-versus-Host Disease

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Acute graft-versus-host disease (aGVHD) is an immune-mediated reaction that can occur after hematopoietic stem cell transplantation in which donor T cells recognize the host antigens as foreign, destroying host tissues. Establishment of a tolerogenic immune environment while preserving the immune response to infectious agents is required for successful bone marrow transplantation. Pregnancy-specific glycoprotein 1 (PSG1), which is secreted by the human placenta into the maternal circulation throughout pregnancy, likely plays a role in maintaining immunotolerance to prevent rejection of the fetus by the maternal immune system. We have previously shown that PSG1 activates the latent form of transforming growth factor $\beta$1 (TGF-$\beta$), a cytokine essential for the differentiation of tolerance-inducing $CD4^+FoxP3^+$ regulatory T cells (Tregs). Consistent with this observation, treatment of naïve murine T cells with PSG1 resulted in a significant increase in $FoxP3^+$ cells that was blocked by a TGF-$\beta$ receptor I inhibitor. We also show here that PSG1 can increase the availability of active TGF-$\beta$ in vivo. As the role of $CD4^+FoxP3^+$ cells in the prevention of aGVHD is well established, we tested whether PSG1 has beneficial effects in a murine aGHVD transplantation model. PSG1-treated mice had reduced numbers of tissue-infiltrating inflammatory $CD3^+$ T cells and had increased expression of FoxP3 in T cells compared with vehicle-treated mice. In addition, administration of PSG1 significantly inhibited aGVHD-associated weight loss and mortality. On the other hand, administration of PSG1 was less effective in managing aGVHD in the presence of an alloimmune reaction against a malignancy in a graft-versus-leukemia experimental model. Combined, this data strongly suggests that PSG1 could be a promising treatment option for patients with aGVHD following bone marrow transplantation for a nonmalignant condition, such as an autoimmune disorder or a genetic immunodeficiency.

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## INTRODUCTION

Hematopoietic stem cell transplantation is curative for many disorders, including autoimmune disorders, genetic immunodeficiencies and malignancies; however, it can be associated with significant morbidity and mortality, often as a result of graft-versus-host disease (GVHD) [1]. Characterized by an immune reaction of donor cells to host tissues, GVHD occurs in approximately 40% of allogeneic stem cell transplantation recipients [2]. Currently, GVHD treatment and prevention involves the use of immunosuppressants, a treatment that has been shown to be effective in only approximately 50% of patients and that can cause significant side effects [1].

Pregnancy-specific glycoprotein 1, also known as pregnancy-specific $\beta$-1 glycoprotein 1 (PSG1), is the most highly expressed protein of the family of 10 closely related pregnancy- specific glycoproteins [3], reaching concentrations of ~200 $\mu$g/mL in the serum of pregnant women at term [4,5]. During pregnancy, changes in the immune system are required to maintain the fetal semi-allograft, resulting in a general reduction in the clinical disease activity of several autoimmune-based pathologies, including multiple sclerosis, rheumatoid arthritis, and uveitis [6–11]. These tolerogenic changes are believed to be mediated, at least in part, by factors secreted by the placenta, including PSGs [12,13].

PSG1 activates latent transforming growth factor beta 1 (TGF-$\beta$1), a cytokine essential for the differentiation of tolerance-inducing $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) and suppression of inflammatory T cells [14–16]. Because PSG1 has previously been shown to have the potential to induce immune tolerance and has a significant effect on activation of

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TGF-$\beta$1, we hypothesize that PSG1 might function as a treatment option for patients suffering from GVHD. Our results in an acute GVHD (aGVHD) mouse model suggest that PSG1 is effective in preventing aGVHD and has the therapeutic potential for patients suffering from this disease.

## METHODS

### Mice

C57BL/6J (H2-$K^b$); B6.PL-Thy1$^a$/CyJ (H2-$K^b$) and B6D2$F_1$/J (H2-$K^{b/d}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FoxP3-IRES-GFP knockin mice were provided by W. Strober (National Institutes of Health). All animal studies were approved by the National Institute of Allergy and Infectious Diseases' Animal Care and Use Committee.

### PSG1 Production and Purification

PSG1-Fc and the control FLAG-Fc were generated as described previously and were used at equimolar concentrations [17]. For most in vivo experiments we used PSG1-His (R&D Systems, Minneapolis, MN).

### Murine Transplantation Models

Male and female 8- to 12-week-old mice were used as transplant recipients and as cell donors. Bone marrow cells were collected from the femurs and tibias of donor mice and depleted of T cells using mouse pan-t (Thy1.2) Dynabeads (Thermo Fisher Scientific, Grand Island, NY) to produce $CD3^+$ T cell-depleted bone marrow (TCD-BM). Spleen $CD3^+$ T cells were purified from donor mice using mouse $CD3^+$ T cell enrichment columns (R&D Systems).

C57BL/6J or B6.PL-Thy1$^a$/CyJ donor cells were transplanted into B6D2$F_1$/J recipients (H2-$K^b$ into H20$K^{b/d}$). Mice were first irradiated with 850 rad on day -1. Then 24 hours later, on day 0, mice underwent transplantation with donor TCD-BM ($1 \times 10^7$ cells) and $CD3^+$ T lymphocytes ($1 \times 10^7$ cells) via tail vein injection to induce GVHD. A negative control group of B6D2$F_1$/J mice received TCD-BM only from B6D2$F_1$/J donor mice (designated BM-only).

Mice were injected i.p. every other day with 100 $\mu$g of PSG1, vehicle (PBS), or rapamycin (Sigma-Aldrich, St. Louis, MO) beginning on day -2 and continuing through to day +14 or day +26 as indicated. This dosage was chosen based on previous experiments [15]. Weights were monitored daily, and any mice exceeding a 20% weight loss from starting weight or exhibiting a prostrate posture matching a high GVHD clinical score were sacrificed. Clinical scores were assigned following the criteria described by Naserian et al [18].

To study the effects of PSG1 on T cell activation against leukemic cells (graft-versus-leukemia [GVL]), 8- to 12-week-old female mice were used as transplant recipients and cell donors. C57BL/6 donor cells were transplanted into B6D2$F_1$ recipients. Mice were irradiated on day -1 as described previously, and 24 hours later underwent transplantation with donor TCD-BM cells ($1 \times 10^7$), $CD3^+$ T lymphocytes ($1 \times 10^7$) and $E_{2A}$ $PB_X$ BL6 leukemia cells ($5 \times 10^4$) via tail vein injection [19]. A positive control group of B6D2$F_1$ mice received TCD-BM only from B6D2$F_1$ donor mice along with $E_{2A}$ $PB_X$ BL6 leukemia cells. Mice were injected i.p. every other day with 100 $\mu$g of PSG1, rapamycin, or vehicle (PBS) beginning on day -2. Weights were monitored as described previously.

### Flow Cytometry Analysis

For in vitro FoxP3 expression analysis, cells were washed and resuspended in serum- and azide-free PBS for 72 hours after plating and then stained for viability with eFluor 780 viability dye (Affymetrix, Santa Clara, CA) for 30 minutes at 4°C, followed by washing and incubation with anti-mouse CD4-FITC (RM4-5) and CD25-APC (PC61.5) or with anti-human CD4-FITC (OKT4) (eBioscience, San Diego, CA) and CD25-PE (BD Biosciences, San Jose, CA). Cells were fixed and permeabilized using the FoxP3 Staining Buffer Set (eBioscience), and then stained with anti-mouse FoxP3-PE (FJK-16s) or anti-human FoxP3-APC (236A/E7) (eBioscience).

SMAD2/3 phosphorylation was determined in cells treated with 100 $\mu$g/mL of recombinant proteins for 1 hour at 37° C, followed by fixation in BD Phosflow Lyse/Fix Buffer (BD Biosciences). Cells were then permeabilized using Perm Buffer III (BD Biosciences) and stained with pSMAD2/3-PE (BD Biosciences).

For the Nrp1 studies, flow cytometry analysis was performed in spleen cell suspensions and peripheral blood collected from tail veins following removal of red blood cells with ACK lysis buffer (Quality Biological, Gaithersburg, MD). Cells were then incubated with anti-mouse CD4-FITC (RM4-5), CD25-APC (PC61.5), or NrpI-PE-Cy7 (3DS304M) (Affymetrix), followed by fixation and permeabilization with the FoxP3 Staining Buffer Set and addition of anti-mouse Foxp3-PE (FJK-16s) (Affymetrix).

Isotype-matched control antibodies were used in all experiments. All samples were run on a benchtop FACSCanto flow cytometer (BD Biosciences), and analysis was performed using FlowJo FACS analysis software (FlowJo, Ashland, OR).

### Cytokine and Chemokine Measurements

$CD4^+$ T cells were isolated from C57BL/6 mouse spleens using CD4 T cell biotin antibody cocktail and anti-biotin microbeads (Miltenyi Biotec, Gaithersburg, MD). Cells were plated in 96-well plates at a concentration of $1 \times 10^5$ cells/well and then activated with mouse CD3/CD28 T cell activator Dynabeads (Thermo Fisher Scientific). IL-2 secretion was examined as described previously [20].

Cytokines were measured using the Luminex cytokine/chemokine multiplex immunoassay kit (R&D Systems) with the BioPlex System (Bio-Rad, Hercules, CA) in serum collected from mouse peripheral blood after induction of aGVHD by adoptive transfer of T cells. Serum was collected on day +27 to measure cytokine expression after cessation of treatment on day +14.

### Mouse and Human Cell Culture

Spleens were collected from 12-week-old FoxP3-IRES-GFP knockin mice on a C57BL/6 background or from 12-week-old C57BL/6 mice (The Jackson Laboratory). Cell suspensions were prepared from spleens following treatment with ACK lysing buffer. $CD4^+CD25^-$ and $CD4^+CD25^+$ splenic T cells were isolated using the mouse $CD4^+CD25^+$ Regulatory T-Cell Isolation Kit (Miltenyi Biotec). For FoxP3 expression studies, cells were cultured in 24-well plates ($3 \times 10^5$ cells per well), stimulated with CD3/CD28 T cell activator Dynabeads, and incubated with 50 ng/mL of recombinant human IL-2 (Peprotech, Rocky Hill, NJ) in Iscove's modified Dulbecco's medium (Thermo Fisher Scientific) with 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 100 $\mu$g/mL penicillin/streptomycin (Quality Biological) in the presence of the indicated recombinant proteins. To study the ability of PSG1 to phosphorylate SMAD2/3, cells were cultured in 24-well plates ($1 \times 10^6$ cells per well) and incubated overnight in Iscove's modified Dulbecco's medium with no added supplements.

Human peripheral blood was collected from healthy volunteers who provided informed consent on an Institutional Review Board-approved protocol. Isolation of peripheral blood mononuclear cells (PBMCs) was performed by gradient centrifugation using lymphocyte separation medium (MP Biomedicals, Solon, OH), followed by removal of red blood cells. $CD4^+CD25^-$ T cells were isolated from PBMCs using a human $CD4^+CD25^+$ Regulatory T-cell Isolation Kit (Miltenyi Biotec). Cells were cultured in 24-well plates ($3 \times 10^5$ cells per well) in RPMI (Thermo Fisher Scientific) with 10% FBS and 100 $\mu$g/mL penicillin/streptomycin. Cells were treated with 50 ng/mL human IL-2 (Peprotech) and then stimulated with CD3/CD28 T cell activator Dynabeads in the presence of the indicated proteins.

### In Vivo Bioluminescence Imaging

Bioluminescence emitted from the SBE-luc mice was detected with the IVIS Spectrum in vivo imaging system (PerkinElmer, Waltham, MA). Mice were injected i.p. with 150 mg/kg D-luciferin at 10 min before imaging and were anesthetized during imaging. Images were obtained from the same mice in dorsal and ventral positions at 24 hours before PSG1 or control protein (FLAG-Fc) injection (as baseline) and then at 6 hours and 24 hours after injection. For bioluminescence signal quantification, regions of interest were manually selected over the head, abdomen, and back areas on the surface of the mice, and photons emitted from each region of interest were acquired as photons/s/cm$^2$/steridian (sr) using LivingImage 4.0 software (PerkinElmer). For data analysis, bioluminescence was expressed as fold induction over baseline for each mouse.

### Histology and Immunostaining

Colon and small intestine were fixed in 4% paraformaldehyde and embedded in paraffin. Some sections were stained with hematoxylin and eosin. The numbers of $CD3^+$ intraepithelial lymphocytes were determined after staining with anti-CD3 Ab (2GV6) (Ventana Medical Systems, Tucson, AZ) by counting 3 high-power fields per section per mouse (n = 5) per treatment. We also stained the formalin-fixed colon tissues of the negative control, positive control, and PSG treatment groups with Trichrome II Blue (Ventana Medical Systems) following the manufacturer's recommendations. This stain is specific for collagen fibers and is routinely used as an indicator of fibrosis, which was scored by a pathologist blinded to the treatments.

FoxP3 expression was determined in deparaffinized slides, followed by heat-induced epitope retrieval using sodium citrate buffer. After blocking with 10% normal serum, 1% BSA, and 0.025% Triton X-100, slides were incubated overnight at 4°C with FoxP3-FITC (FJK-16s; eBioscience). Coverslips were applied with DAPI mounting medium (Sigma-Aldrich). Blinded cell counts were performed over 3 areas chosen at random in at least 2 different tissue sections each from a minimum of 5 mice. To assess for the presence or absence of GVHD and/or leukemia, slides of femur, spleen, colon, and large intestine from all animals were reviewed by a pathologist.

### Statistics

Prism software (GraphPad Software, La Jolla, CA) was used for all statistical analyses. The Student *t* test with Welch's correction or Mann-Whitney *U* test, 1-way ANOVA with Bonferroni correction, or 2-way ANOVA with Tukey's multiple comparisons test and Fisher's exact test were used to compare groups.

## RESULTS

### *PSG1 Induces the Differentiation of FoxP3⁺ Tregs and Inhibits the Secretion of IL-2 in Vitro in a TGF-β1-Dependent Manner*

FoxP3 regulatory T cells are important for immune tolerance and are induced by the presence of active TGF-β1 [21,22]. Because PSG1 is involved in the activation of latent TGF-β1, we tested whether PSG1 was able to convert $CD4^+$ naïve mouse T cells into Tregs. Cells were collected from C57BL/6 mouse spleens, and $CD4^+CD25^-$ ($CD4^+$ naïve) cells were isolated and activated with anti-CD3/CD28 Abs in the presence of PSG1 or vehicle (0.2% DMSO). Cells treated with PSG1 showed an increase in FoxP3 expression by day +3, with even higher expression by day +7 (Figure 1A). When cells were treated with PSG1 in conjunction with 5 μM of the TGF-β receptor I (ALK5) inhibitor SB431542, there was no increase in FoxP3 expression (Figure 1A).

To determine whether PSG1 has the same effect on human cells, naïve $CD4^+$ T cells were isolated from peripheral blood and activated with anti-CD3/CD28 Abs. We observed an increase in the percentage of cells expressing FoxP3 on PSG1 treatment (Figure 1B). As observed for mouse cells, human T cells treated with PSG1 and 5 μM of SB431542 showed a reduction in FoxP3 expression down to untreated levels (Figure 1B, right). These results indicate that PSG1 induces the differentiation of mouse and human naïve $CD4^+$ T cells into regulatory T cells, and that this process requires a functional TGF-β receptor on the cell surface.

TGF-β1 plays a significant role in immune regulation by inducing the phosphorylation of Smad2/3, resulting in increased expression of FoxP3 and inhibition of IL-2 secretion in T cells [23,24]. PSG1 treatment of murine naïve $CD4^+$ T cells inhibited IL-2 secretion without affecting T cell proliferation measured at 72 hours poststimulation (Figure 2A and data not shown). This IL-2 inhibition was reversed when cells were treated with the ALK5 inhibitor (Figure 2B). In addition, treatment with PSG1 increased the level of Smad2/3 phosphorylation in these cells (Figure 2C).

To study Smad2/3-dependent signaling in vivo as a result of PSG1 administration, we used transgenic reporter mice that express luciferase in response to activation of Smad2/3 (SBE-luc mice) [25]. After these SBE-luc mice were injected with a single dose of PSG1 and a luciferase substrate, we observed

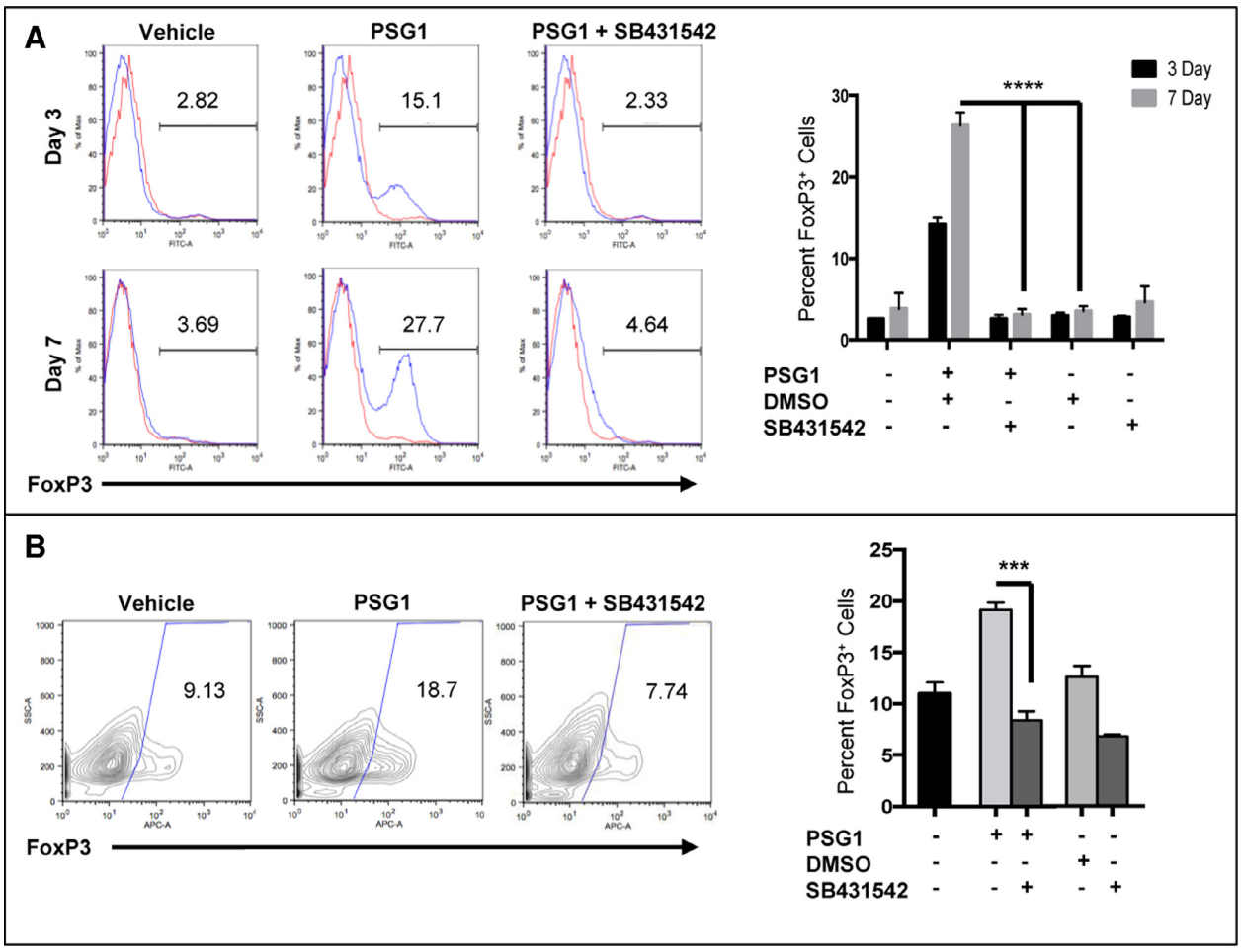


**Figure 1.** Conversion of mouse and human naïve T cells into FoxP3 Tregs by PSG1 is dependent on TGF-β. (A) Naïve $CD4^+$ mouse T cells isolated from the spleens of FoxP3-GFP transgenic mice were stimulated with T cell activator Dynabeads in the presence of IL-2 and then treated with PSG1 (100 μg/mL), 0.2% DMSO vehicle control, or PSG1 plus 5 μM SB431542 . Cells were analyzed for FoxP3-GFP expression on days +3 and +7. (Left) Representative flow cytometry data of FoxP3 expression in murine cells on days +3 and +7 (red, isotype control; blue, treated cells). (Right) Graphical representation of the flow cytometry data. $^{****}P < .0001$). (B) Naïve $CD4^+$ human T cells isolated from peripheral blood were stimulated with T cell activator Dynabeads in the presence of IL-2 and treated with PSG1 (100 μg/mL), vehicle (PBS), or PSG1 plus SB431542 (5 μM). Cells were analyzed on day +3. (Left) Representative flow cytometry data for FoxP3 expression in human cells. Cell populations were gated on viable $CD4^+CD25^+$ cells and stained with APC-labeled anti-human FoxP3 antibody. (Right) Graphical representation of flow cytometry data. $^{***}P < .0005$. The data shown are representative of at least 3 independent experiments performed in triplicate. Data are mean ± SEM. *P* values were calculated using 1-way ANOVA.

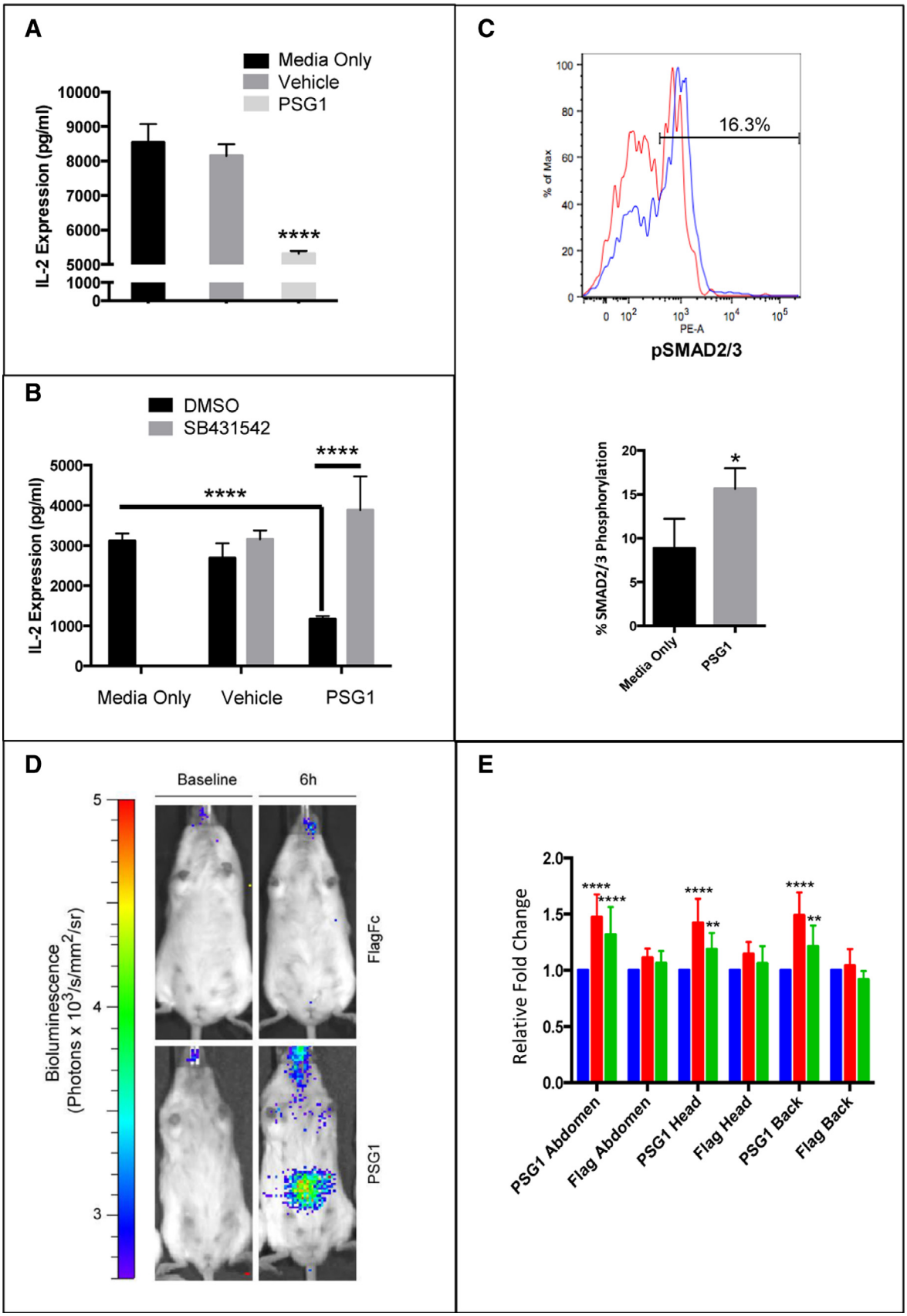


**Figure 2.** Treatment of naïve mouse CD4$^+$ T cells with PSG1 inhibits IL-2 expression and activation of the pSMAD2/3 pathway by PSG1 in vitro and in vivo. (A) Naïve CD4$^+$ T cells were purified from C57BL/6 mouse spleens and activated with T cell activator Dynabeads. Cells were treated with vehicle (PBS) or 40 $\mu$g/mL PSG1-Fc. IL-2 levels were determined by ELISA at 72 hours after activation. ****$P < .0001$. (B) Cells were purified as described previously and treated as in (A) in the presence of 5 $\mu$M SB431542 or vehicle (0.2% DMSO). IL-2 levels were determined by ELISA at 72 hours after activation. ****$P < .0001$. (C) CD4$^+$CD25$^-$ T cells were treated with

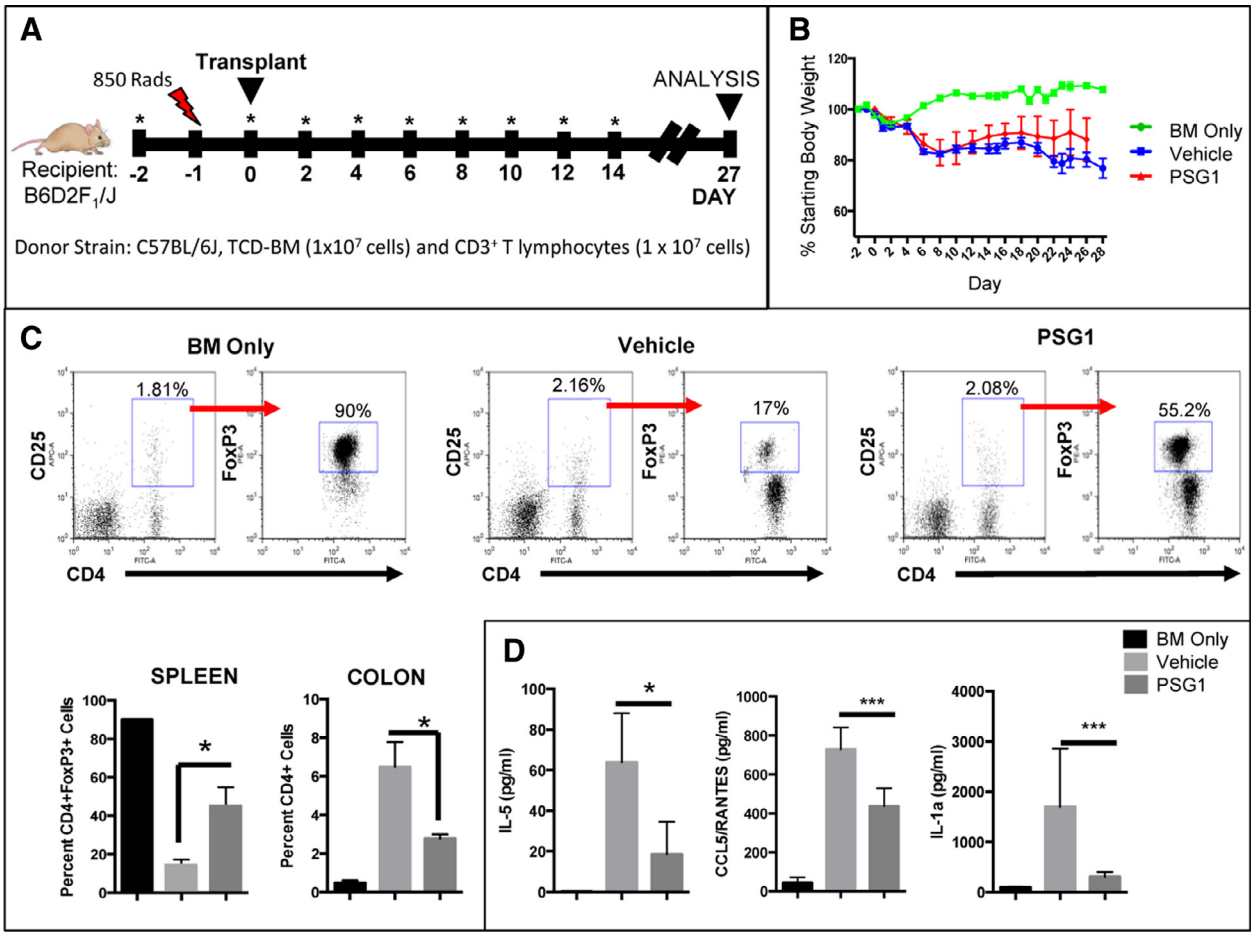


**Figure 3.** PSG1 treatment reduces mortality and increases FoxP3 Treg expression in an aGVHD model. (A) aGVHD mice were injected with 100 $\mu$g of PSG1 or PBS on day -2. On day -1, the mice were irradiated with 850 rads (red bolt), and GVHD was induced 24 hours later on day 0 via tail vein injection of donor TCD-BM ($1 \times 10^7$ cells) and $CD3^+$ T lymphocytes ($1 \times 10^7$ cells). PSG1 or vehicle (PBS) injections were repeated on day 0 and then every other day until day +14 (as indicated by the stars). On day +27, mice were analyzed. (B) Weight loss in mice from day -2 to day +27 indicated as percentage of total starting body weight. (C) Representative flow cytometry results showing the percentage of BM-only cells and of PBS vehicle-treated or PSG1-treated cells expressing FoxP3 following the induction of aGVHD by adoptive transfer of T cells. Splenic cells were gated on CD4- and CD25-expressing cells, followed by FoxP3 expression. (Bottom Left) Graphical representation of the flow cytometry data for $CD4^+$ spleen cells expressing Foxp3. (Bottom Right) Graphical representation of colon cells expressing CD4 cells. $^*P < .05$. (D) IL-5, CCL5/RANTES, and IL-1a cytokine analysis of mouse serum collected on day +27. Cytokines were measured using multiplex immunoassay kits with the Bioplex system. $^*P < .05$; $^{***}P < .0005$) All experiments were performed in triplicate in at least 2 independent experiments (n = 4 to 5 mice per treatment group for each experiment). Data are mean ± SEM. All *P* values were calculated using 1-way ANOVA.

significant increases in luciferase expression in a temporal manner in the abdomen, head, and back (Figure 2D). Increased luciferase expression over that seen in the vehicle-injected mice was most evident at 6 hours and could be still observed at 24 hours post-PSG1 injection, albeit at lower levels (Figure 2E). These results support the role of the TGF$\beta$-SMAD pathway in the mouse immune response to PSG1.

### *Administration of PSG1 in a GVHD Mouse Model Ameliorates GVHD Severity*

PSG1 has been shown to have a protective effect in Dextran Sulfate Sodium (DDS)-induced colitis and in collagen-induced arthritis [15,26]. Owing to the protective effect of Tregs in aGVHD, we hypothesized that PSG1 could ameliorate aGVHD and constitute a novel therapeutic strategy. To test this hypothesis, we induced aGVHD in an MHC-mismatched mouse model and administered PSG1 or vehicle (PBS) as outlined in Figure 3A. A third group of BM-only mice served as a control for the induction of aGVHD by adoptive transfer of T cells. In brief, beginning on day -2, mice were injected with PSG1 at 24 hours before irradiation. At 24 hours after irradiation (day 0), aGVHD was induced by administration of TCD-BM cells and T cells from the spleen. Mice were injected every other day from day -2 to day +14. On day +27, expression of FoxP3was analyzed in $CD4^+$ cells isolated from the spleen and colon. There was a significant difference in survival between PBS-treated and PSG1-treated mice ($P = .0002$). Although both the vehicle-treated and PSG1-treated mice lost weight (Figure 3B), 50% of

100 $\mu$g/mL PSG1 for 1 hour and then stained with pSMAD2/3-PE. (Top) Representative flow cytometry of pSMAD2/3 expression (red, media only; blue, PSG1). (Bottom) Graphical representation of pSMAD2/3 expression. $^*P < .05$. All treatments were performed in triplicate, and 3 independent experiments were completed. Data are mean ± SEM. *P* values were calculated using Student's *t* test (A and C) or 2-way ANOVA (B). (D) Luciferase expression by bioluminescence imaging in the abdomen, head, and back of SBE-luc mice at 6 hours after i.p. injection with a single dose of PSG1 (100 $\mu$g). (E) Luciferase expression in the head, neck, and back of SBE-luc mice at 0 hours (baseline), 6 hours, and 24 hours after PSG1 or Flag control injection. $^{**}P < .005$; $^{****}P < .0001$. Data are mean ± SEM. All *P* values were obtained using 2-way ANOVA. Blue, baseline; red, 6 hours after injection; green, 24 hours after injection. n = 8 mice (4 females and 4 males).

the mice in the PBS-treated control group died or had to be euthanized before day +27. Mice in the PBS-treated GVHD group had clinical GVHD scores of 4 to 5 and were hunched, moribund, and suffering from diarrhea. Conversely, mice in the PSG1-treated GVHD group were alert and had clinical GVHD scores of 1 to 2. The BM-only mice had a clinical GVHD score of 0.

Flow cytometry analysis showed an increase in the percentage of cells expressing FoxP3 in the spleen of PSG1-treated mice, with an average of 45 ± 10% of T cells expressing FoxP3, compared with only 14 ± 3.6% of T cells from vehicle-treated mice. FoxP3 expression was seen in 90 ± 0.1% of T cells in healthy BM-only controls. There was also a reduction in the percentage of total viable $CD4^+$ helper T cells in the colons of PSG1-treated mice compared with vehicle-treated mice (Figure 3C). We did not observe any significant difference in fibrosis in the colon between PSG1-treated and vehicle-treated mice, as determined by staining of collagen fibers (data not shown).

Analysis of cytokine expression in serum performed on day +27 revealed PSG1's ability to inhibit the expression of some inflammatory cytokines. IL-5, an inflammatory cytokine known to trigger activated B cells for terminal differentiation into antibody-secreting plasma cells [27], was significantly lower in PSG1-treated mice compared with vehicle-treated mice (Figure 3D). Two additional potent inflammatory chemokines, CCR5/RANTES and IL-1$\alpha$, were also significantly reduced in the mice treated with PSG1. No differences in expression of TNF-$\alpha$, IL-10, CCL3, CCL11, CXCL1, IL-4, IL-13, G-CSF, or IL-9 were observed between the PSG1 and vehicle-treated mice, and the expression levels of GM-CSF, CCL2, IL-2, IL-17a, CCL4, IL-12p70, IL-1$\beta$, IL-6, and IFN-$\gamma$ were below the limit of detection (data not shown).

Small intestine and colon, 2 important aGVHD target organs, were collected from all mice for analysis [28] (Figure 4A). Significant tissue erosion was seen in the intestines of vehicle-injected mice, whereas the intestines of PSG1-treated mice resembled those of the healthy BM-only controls. Quantitation of the intraepithelial T cells in the colon showed a significant decrease in $CD3^+$ cells in the mice treated with PSG1 (Figure 4B). Finally, we observed significantly higher numbers of $FoxP3^+$ cells in the colons of PSG1-treated mice compared with vehicle-treated mice (Figure 4C). Overall, our physiological and molecular data indicate that PSG1 is able to prevent inflammatory responses and tissue damage in a mouse model of aGVHD.

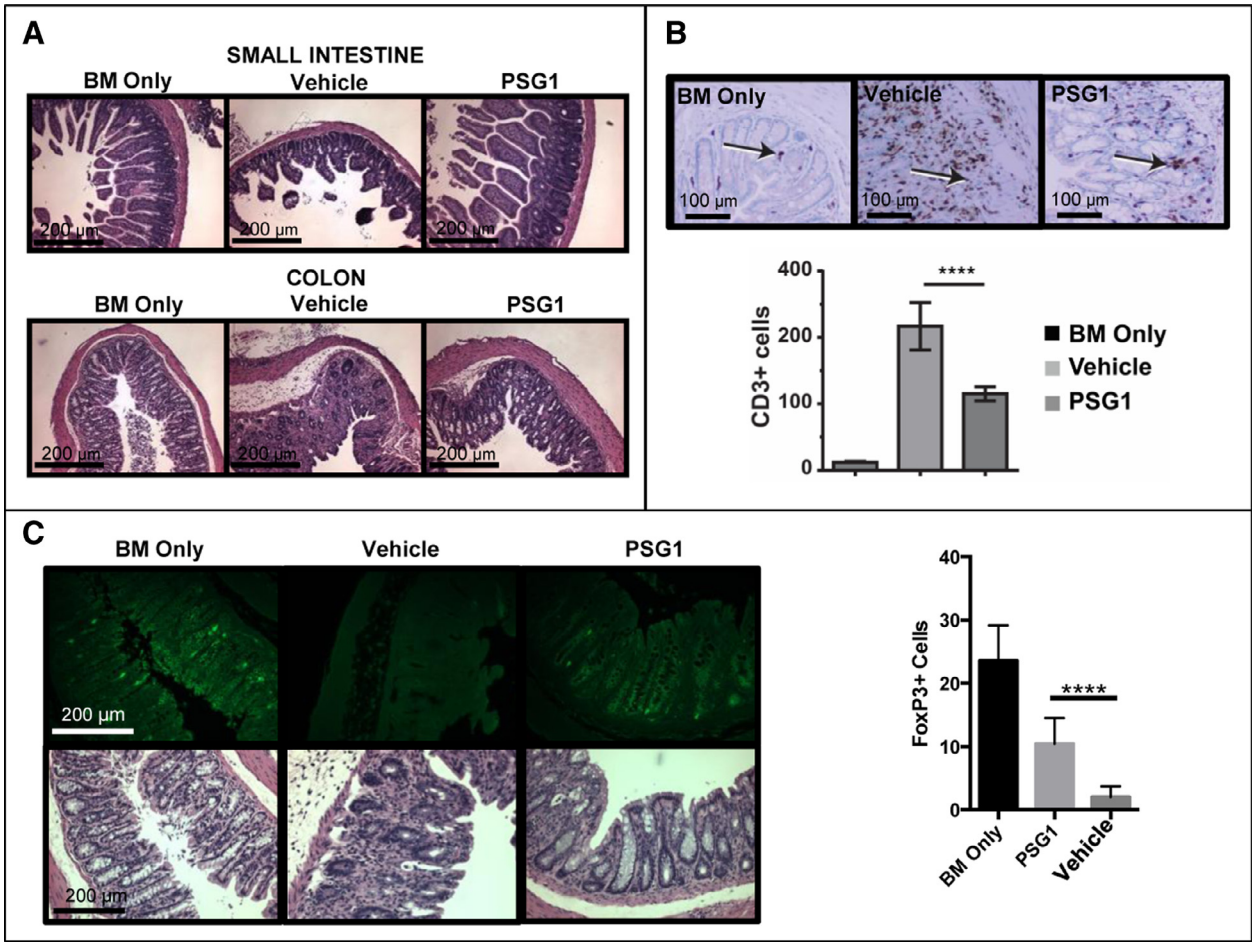


**Figure 4.** Beneficial effects of PSG1 administration seen in the colon and small intestine of mice receiving TCD-BM plus T cells. (A) Colon and small intestine were collected from BM-only control mice, vehicle (PBS)-treated aGVHD mice, and PSG1-treated aGVHD mice on day +27 for analysis. Hematoxylin and eosin-stained sections from these tissues were photographed on a Zeiss AxioImager Z1 microscope with Zeiss 1026.548 PI 10X/23 lens and Plan-Apochromat 10X/0.45 (1063-139) aperture (Carl Zeiss, Oberkochen, Germany). Representative images from each treatment group are shown. (B) Colon sections were stained with an anti-CD3 mAb, and intraepithelial cells expressing CD3 (arrow) were enumerated as described in Methods. $^{****}P < .0001$. (C) Colon sections were stained with anti-FoxP3-FITC mAb, and expression was quantified as described in Methods. $^{****}P < .0001$. All images were obtained with either a Zeiss AxioCam ICC1 white light camera or an AxioCamMRm fluorescent camera with AxioVision 4.8 imaging software at the same exposure and magnification. All counts were performed in triplicate in at least 3 independent experiments (n = 4 to 5 mice per treatment group for each experiment). Data are mean ± SEM. All *P* values were obtained using 1-way ANOVA.

### *Extended Administration of PSG1 Results in More Favorable Outcomes Following Bone Marrow Transplantation*

Although PSG1 was able to improve the symptoms of aGVHD when administered for 14 days, the mice lost weight compared with the BM-only group (Figure 3B). To determine whether an extended course of PSG1 would further improve outcomes, mice were treated with PSG1 from day -2 until the termination of the experiment on day +26. In addition, efficacy was compared between PSG1 and rapamycin (sirolimus), a current standard of care immunosuppressant for treating of aGVHD that is associated with significant side effects [29,30]. Because PSG1 is a natural human protein, it carries lower risks of side effects and toxicity than rapamycin.

Mice treated with an extended course of PSG1 showed marked improvement in weight gain over vehicle-treated mice (Figure 5A). This weight gain was comparable to that observed in mice receiving rapamycin. As we observed in the short duration treatment, there were marked differences in

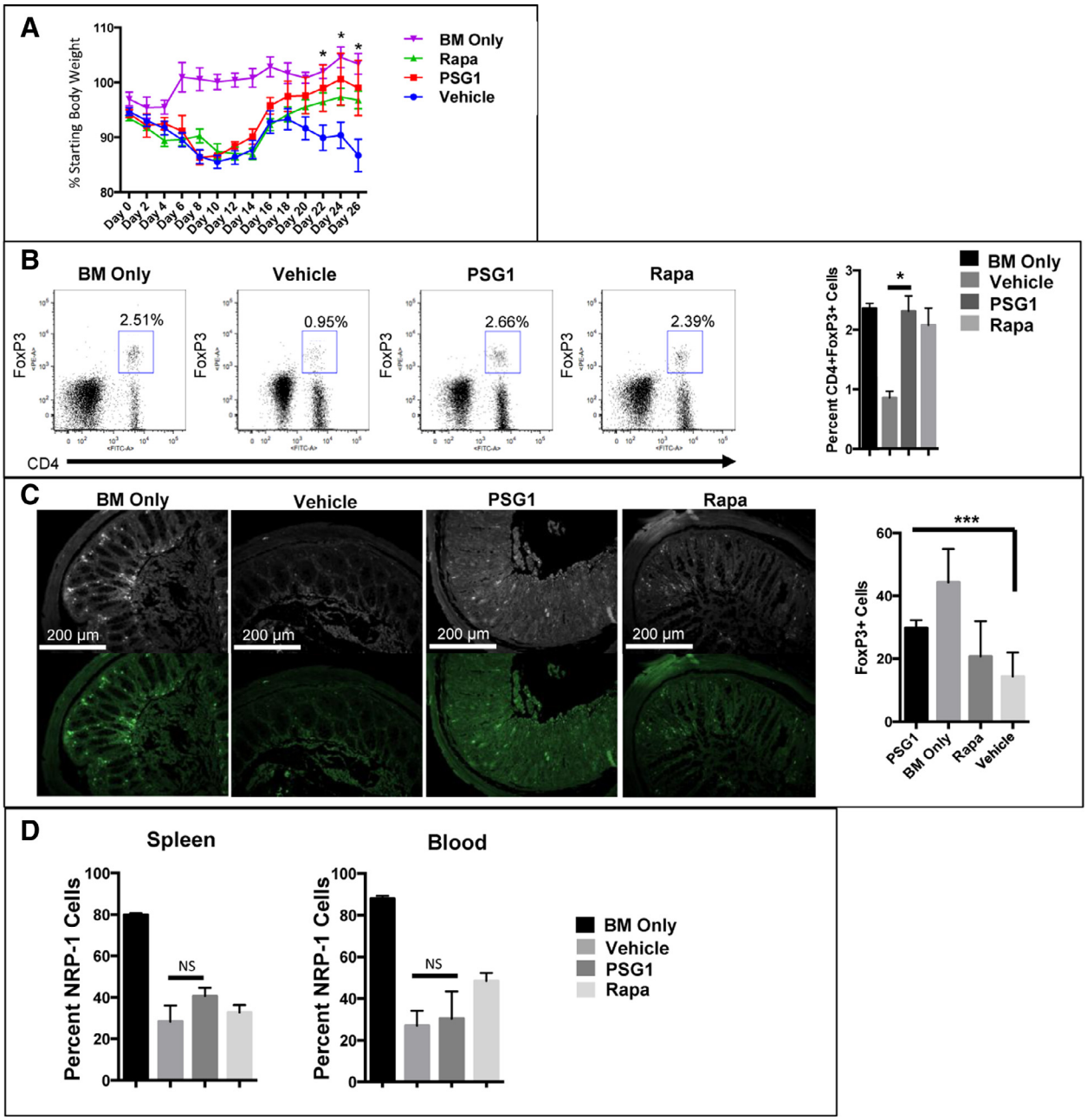


**Figure 5.** An extended course of PSG1 in aGVHD increases FoxP3 expression, decreases weight loss, and induces comparable or improved outcomes compared with treatment with rapamycin. (A) aGVHD mice were injected with 100 $\mu$g of PSG1, PBS, or rapamycin every other day from day -2 to day +26. The percentage of total starting body weight from day 0 to day +26 is shown. Rapa, rapamycin-treated GVHD mice. *$P < .05$. (B) Representative flow cytometry data showing FoxP3 expression in splenic CD4$^+$ T cells from BM-only, vehicle (PBS)-treated GVHD mice, PSG1-treated GVHD mice, and rapamycin-treated GVHD mice. *$P < .05$. (C) Colon sections from all experimental mouse groups were stained for FoxP3 expression and imaged on a Zeiss AxioImager Z1 microscope with a 1026.548 PI 10X/23 lens and Plan-Apochromat 10X/0.45 (1063-139) aperture. All images were obtained with either an AxioCam ICC1 white light camera or an AxioCamMRm fluorescent camera with the AxioVision 4.8 imaging software at the same exposure and magnification. All counts were performed in triplicate in at least 2 independent experiments (n = 4 to 5 mice per treatment group for each experiment). Data are mean ± SEM, and all *P* values were obtained using 2-way ANOVA (A) or 1-way ANOVA (B and C). (D) FoxP3$^+$ T cells were collected from the spleen and blood of all experimental groups on day +27, and Nrp-1 expression was analyzed by flow cytometry.

clinical scores and survival between the mice treated with vehicle (scores of 4 to 5) and PSG1 treated mice (scores of 0 to 1) ($P = .003$). During these experiments, Nutrigel was added in cages at day 0 in an attempt to prolong survival of the PBS-treated mice [31]. Although no early death was observed in these experiments, by day +26, all vehicle-treated mice had to be sacrificed because they were moribund, with an approximate 20% loss of body weight. The PBS-treated mice showed severe hunching, diarrhea, ruffled fur, and lethargy, whereas the PSG1- and rapamycin-treated mice were active and alert, with no obvious signs of distress.

Both PSG1 and rapamycin were able to induce FoxP3 expression to the levels observed in the BM-only control mice, something that was only partially achieved with a shorter duration of PSG1 treatment (Figure 5B). Analysis of sections of colon from all treatment groups for cells expressing FoxP3 revealed had significantly more FoxP3$^+$ cells in the PSG1-treated mice compared with the rapamycin- and vehicle-treated mice (Figure 5C). Based on these results, we conclude that PSG1 is at least as effective for GVHD prevention as other immunosuppressants.

Neuropilin-1 (Nrp-1) is expressed at high levels on natural Tregs and can be used to distinguish natural Tregs that arise in the thymus from Tregs induced in the periphery [32]. To determine the origin of the FoxP3$^+$ cells found in PSG1- and rapamycin-treated mice, we analyzed Nrp-1 expression on cells from the spleen and the circulation (Figure 5D). We did not find significantly higher Nrp-1 expression in FoxP3$^+$ cells in the PSG1- and rapamycin-treated mice compared with the vehicle-treated mice, indicating that these cells are induced in the periphery rather than originating in the thymus.

### *Regulatory T Cells in Mice Treated with PSG1 Are of Donor Origin*

To determine the origin of the FoxP3$^+$ Tregs observed following PSG1 treatment, we substituted B6 donor mouse cells with donor cells from B6-Thy1.1 mice. These Thy1.1$^+$ donor cells allowed us to determine the origin of FoxP3$^+$ Tregs in the recipient by tracking expression of Thy1.1 on Tregs appearing in mice that underwent transplantation. This B6-Thy1.1 into B6-D2 mouse model successfully replicated the aGVHD conditions observed in the B6 into B6-D2 mouse model and responded equally well to PSG1 treatment. Analysis of the Thy1.1 donor marker versus the Thy1.2 recipient marker showed that in the BM-only control group, all Tregs were of recipient origin, which was expected given that donor T cells were depleted. FoxP3$^+$ Tregs in mice treated with PSG1 were found to be mostly Thy1.1$^+$, indicating that almost all Tregs arising in PSG1-treated mice are of donor origin (Figure 6). These data support the requirement for a successful donor graft for functional Tregs to develop and provide protection against aGVHD.

### *The GVL Effect Is Impaired Following PSG1 Treatment*

To determine whether PSG1 is able to ameliorate aGVHD and maintain the GVL effect, we treated mice with leukemia and aGVHD with PSG1 [19]. The mice received PSG1, rapamycin, or vehicle (PBS) every other day for 14 days. After treatment, mice were subjected to pathological evaluation for the presence of leukemic cells and signs of aGVHD. As expected, control mice treated with leukemic cells and bone marrow but no T cells showed evidence of diffuse leukemia, as demonstrated by infiltration of the leukemic cells and, in some cases, disruption of splenic architecture (arrows, Figure 7). The rapamycin-treated mice showed evidence of leukemia but no aGVHD, and the PSG1-treated mice showed only focal leukemia in addition to aGVHD. These findings suggest that PSG1 does not completely abrogate the GVL effect of the T cells, but that it is less effective in managing GVHD in the presence of an alloimmune reaction against a malignancy.

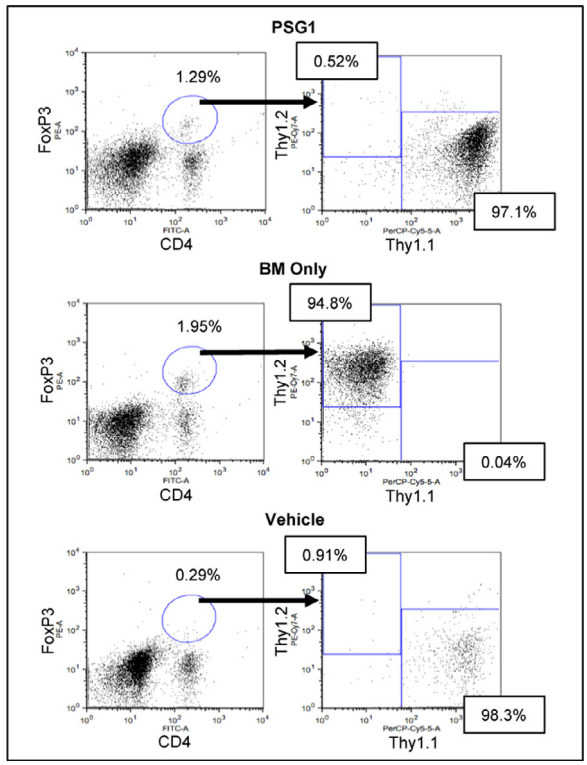


**Figure 6.** GVHD mice treated with PSG1 show an increase in regulatory T cells of donor origin. Shown are the percentages of CD4, FoxP3$^+$ found in B6-D2 recipient spleens after administration of cells from B6-Thy1.1 donors and treated with either vehicle (PBS) or PSG1. The BM-only mice received B6-D2 donor cells without T cells . Flow cytometry was performed on cells obtained from 5 mice per treatment group.

## DISCUSSION

Allogeneic transplantation is currently the sole curative option for a number of diseases, both malignant and nonmalignant [33–40]. However, bone marrow transplantation is associated with GVHD, which is the leading cause of the most significant adverse effects associated with this procedure [41]. Current treatments for aGVHD involve the use of immunosuppressants, including corticosteroids, increasing the patient's susceptibility to opportunistic infections [42,43]. In addition, a small subset of patients with GVHD are steroid-refractory and have a very poor prognosis [44,45]. Given the limitations of currently available treatment options for GVHD, there is a need for novel therapeutic strategies to treat this disease.

PSG1 differs in its mechanism of action from all available therapies currently used for prevention or treatment of aGVHD and likely lacks the toxicity and immunogenicity seen with other treatments, a hypothesis supported by the high levels of this protein found during human pregnancy and its expression in the normal epithelium of the colon and esophagus [46]. The experiments presented herein were performed in both male and female mice, indicating that PSG1 likely will be effective in humans of both sexes.

Treatment of mouse naïve and human T cells with PSG1 resulted in a significant increase in their expression of FoxP3.

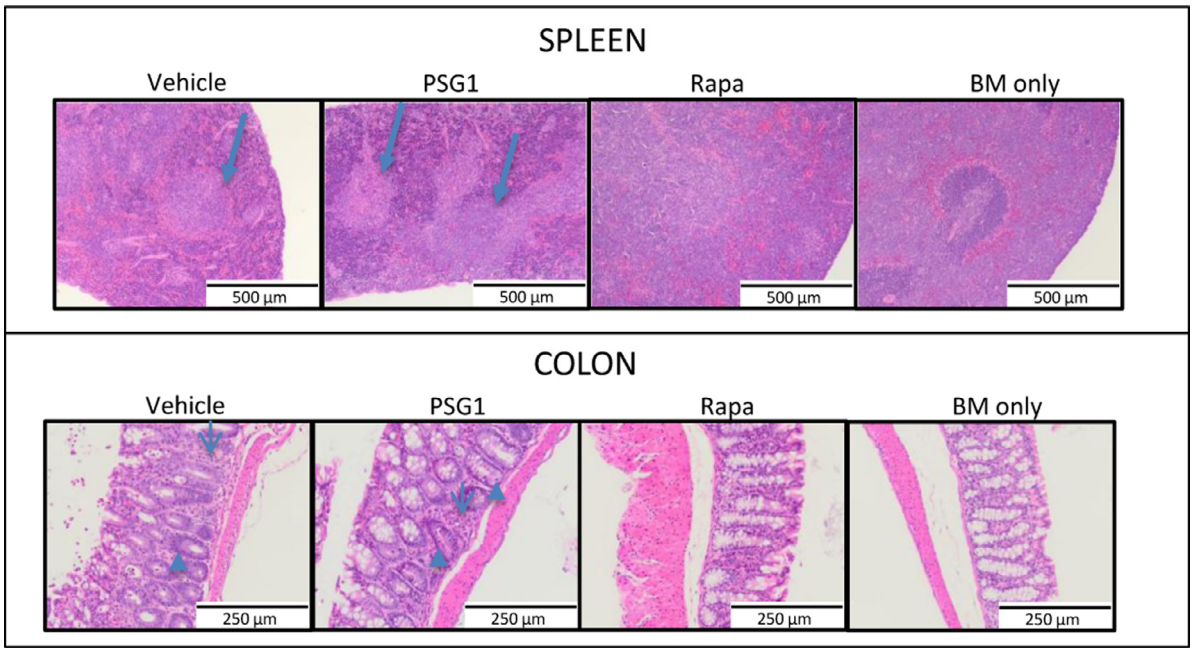


**Figure 7.** Signs of GVHD and leukemia in mice treated with PSG1 or rapamycin. Colons and spleens were collected from mice infused with BM and T cells along with $E_{2A}$ $PB_X$ BL6 leukemic cells and then treated with PBS, PSG1, or rapamycin. Mice receiving BM and leukemia cells without T cells (BM-only) were also analyzed. Hematoxylin and eosin-stained sections from mouse colon and spleen were reviewed by the pathologist for evidence of GVHD, in particular apoptotic cells (arrowheads), disruption of crypt architecture and T cell infiltration (thin arrows), presence of leukemia (foci of leukemic cells or diffusely spread cell groups; arrows), and disruption of splenic architecture. Representative slides were imaged on a Zeiss AxioImager Z1 microscope with a 1026.548 Pl 10X/23 lens and Plan-Apochromat 20X/0.75 440649 (1101-957) aperture. All images were obtained with an AxioCam ICC1 white light camera with AxioVision 4.8 imaging software at the same exposure and 20× magnification.

We further observed, both in vitro and in vivo, an increase in pSMAD2/3, an important mediator of TGF-$\beta$ signaling, after treatment with PSG1 [47]. The results presented here support the potential benefits of PSG1 in treating aGVHD. We found that after administration of PSG1 in an aGVHD mouse model, tissue damage and molecular changes associated with aGVHD were minimized or prevented entirely. In addition, PSG1-treated mice showed reductions in some proinflammatory cytokines with no increased signs of fibrosis.

When the course of PSG1 treatment was extended, the percentage of weight loss in mice treated with PSG1 improved by day +26, almost reaching the levels of healthy controls. PSG1- and rapamycin-treated mice showed increased numbers of Foxp3-expressing cells in the spleen, but only PSG1 was associated with increased FoxP3 expression in the colon, in agreement with previous results from our laboratory [15].

PSG1 has been shown to induce the expression of an alternative phenotype in macrophages marked by induction of arginase I and also to affect the maturation of dendritic cells [48,49]. Whether the effects of PSG1 in the phenotype of these cells is related to the increased availability of active TGF-$\beta$, which has been shown to induce arginase I and affect dendritic cell maturation, has not yet been explored [50,51]. As such, additional mechanisms of action in aGVHD protection by PSG1 independent of TGF-$\beta$ or increased FoxP3 expression in T cells cannot be ruled out.

We previously reported that mice receiving i.p. injections of PSG1 are protected from dextran sulfate-induced colitis [15]. In these mice, mesenteric lymph node cells from PSG1-treated mice secreted less IL-6, TNF-$\alpha$, and IFN-$\gamma$ when stimulated with anti-CD3/anti CD28 Abs compared with controls. These same cells expressed increased IL-10 mRNA levels while exhibiting reduced expression of TNF-$\alpha$ and IFN-$\gamma$ and IL-17 mRNA in the colon. In addition, and in agreement with these results, splenocytes from mice infected with a vaccinia virus expressing PSG1 expressed lower levels of IL-2 and IFN-$\gamma$ when challenged with mitogens (Con A), anti-CD3, or specific antigen (OVA) [49]. Motran et al [49] have reported that PSG1 does not inhibit the proliferation of purified $CD3^+$ murine T cells when treated with PMA and ionomycin or anti-CD3/CD28 antibodies. In agreement with this report, although we observed inhibited IL-2 secretion, we found no significant effect on the proliferation (measured at 72 hours) of murine $CD4^+$ T cells in response to anti-CD3/CD28 Ab stimulation (data not shown).

Although our group and others have previously measured cytokines in cells isolated from PSG1-treated mice, to our knowledge this is the first study examining the changes in serum cytokines following i.p. injection of PSG1. Our results show reduced concentrations of RANTES and IL-1$\alpha$ in the PSG1-treated mice. Changes in the production of other chemokines, which may result from the proposed regulation of monocyte/macrophage phenotype by PSG1, should be further explored, given that many of the chemokines that we tested were present only at levels below the limit of detection of the Luminex assay.

We also explored whether PSG1 could be used for treatment or prevention of aGVHD in recipients of hematopoietic stem cell transplantation performed to treat a hematologic malignancy. The main mediators of the beneficial GVL activity of allogeneic bone marrow transplantation are T cells within the donor grafts, though NK cells also have been shown to play a role [52]. Tregs may be detrimental in cancer, and treatments that minimize Treg function may augment antitumor immune responses [53]. The partial antileukemic effect observed in our PSG1-treated mice compared with the vehicle-treated mice may be explained by the inhibitory effects of PSG1 on natural killer cells, which play roles in both antileukemic effects and engraftment [52], and this pathway warrants future

exploration. As noted above, PSG1 regulates the function of other cells, such as antigen-presenting cells, which play a role in aGVHD initiation but not in the GVL effect [54].

The data collected in this study strongly suggest that PSG1 could be a promising treatment option for patients with aGVHD. Although no significant GVL effect was observed in our cohort, such parameters as tumor burden and the characteristics of the malignant cells used in the study likely determine whether GVL activity is lost on PSG1 treatment, and these factors will be explored in future studies. Nonetheless, the results of this preclinical study support the use of PSG1 to prevent aGVHD in patients undergoing bone marrow transplantation to treat nonmalignant monogeneic hematopoietic disorders, such as primary immunodeficiencies, in which the graft-versus-tumor effect is not a consideration [55].

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