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Flagellin, a TLR5 Agonist, Reduces Graft-versus-Host Disease in Allogeneic Hematopoietic Stem Cell Transplantation Recipients While Enhancing Antiviral Immunity

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Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality in patients treated with allogeneic hematopoietic stem cell transplantation (HSCT). Posttransplant immunosuppressive drugs incompletely control GVHD and increase susceptibility to opportunistic infections. In this study, we used flagellin, a TLR5 agonist protein (∼50 kDa) extracted from bacterial flagella, as a novel experimental treatment strategy to reduce both acute and chronic GVHD in allogeneic HSCT recipients. On the basis of the radioprotective effects of flagellin, we hypothesized that flagellin could ameliorate GVHD in lethally irradiated murine models of allogeneic HSCT. Two doses of highly purified flagellin (administered 3 h before irradiation and 24 h after HSCT) reduced GVHD and led to better survival in both H-2b→ CB6F1 and H-2K→ B6 allogeneic HSCT models while preserving >99% donor T cell chimerism. Flagellin treatment preserved long-term posttransplant immune reconstitution characterized by more donor thymic-derived CD4+CD25+Foxp3+ regulatory T cells and significantly enhanced antiviral immunity after murine CMV infection. The proliferation index and activation status of donor spleen-derived T cells and serum concentration of proinflammatory cytokines in flagellin-treated recipients were reduced significantly within 4 d posttransplant compared with those of the PBS-treated control recipients. Allogeneic transplantation of radiation chimeras previously engrafted with TLR5 knockout hematopoietic cells showed that interactions between flagellin and TLR5 expressed on both donor hematopoietic and host nonhematopoietic cells were required to reduce GVHD. Thus, the peritransplant administration of flagellin is a novel therapeutic approach to control GVHD while preserving posttransplant donor immunity. *The Journal of Immunology, 2011, 187: 000–000.

The major causes of mortality in cancer patients treated with allogeneic hematopoietic stem cell transplantation (HSCT) are graft-versus-host disease (GVHD), relapse, and opportunistic infections (1, 2). GVHD is caused by alloreactive activated donor T cells, in part, through the elaboration of inflammatory cytokines released by host tissues in response to pretransplant conditioning with radiation and/or high-dose chemotherapy (3). Recent data suggest that, beside the donor T cells, host epithelial cells also have a role in the initial presentation of the alloantigen to donor T cells during the initiation of GVHD (4–6). Approximately 30–40% and 50–60% of long-term survivors of allogeneic HSCT develop acute GVHD and/or chronic GVHD, respectively, despite prophylactic administration of immunosuppressive drugs (7). GVHD causes host tissue damage and suppresses the function of lymphoid organs, leading to increased susceptibility to opportunistic infections in human and in animal model systems (8, 9). Moreover, the long-term impairment of immune function in transplant recipients may contribute to disease relapse (10).

Flagellin, a TLR5 agonist protein extracted from the flagella of Salmonella typhimurium, can modulate both innate and adaptive immunity in mice and humans (11–15). In nontransplant settings, flagellin has been shown to regulate human CD4+ T cell immune function by increasing the generation of regulatory T cells (Tregs) (16) and by decreasing LPS-induced ERK1/2 activation in macrophages (17, 18). In addition to its immunomodulatory properties, flagellin protects epithelial cells from radiation-induced toxicity. Published literature indicates that single doses of flagellin [50 μg per mouse i.p. (19) and 200 μg/kg i.p. (20)] administered before total body irradiation protect mice from lethal radiation-induced toxicities and prevent host cell apoptosis. Moreover, flagellin has been shown to help maintain gut immune homeostasis (21) and has been found to be safe for use in humans as a vaccine adjuvant in clinical trials against influenza infection and diarrheal illnesses. However, no previous reports have described the use of flagellin in the setting of allogeneic HSCT.

We hypothesized that treatment with flagellin in allogeneic HSCT recipients might reduce T cell activation, production of inflammatory cytokines, and epithelial cell damage that contribute

Abbreviations used in this article: B6, C57BL/6; BM, bone marrow; DC, dendritic cell; GI, gastrointestinal; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; ICOS-1, inducible costimulation molecule 1; KO, TLR5 knockout; mCMV, murine cytomegalovirus; PD-1, program death 1; TCD, T cell depleted; Treg, regulatory T cell.
to the initiation of GVHD. To test this hypothesis, we administered flagellin to allogeneic HSCT recipients and measured acute and chronic GVHD as well as posttransplant immune reconstitution in established murine GVHD models. Our results show that flagellin treatment had a striking effect on reducing the incidence and severities of GVHD in transplant recipients while preserving immunity against murine CMV (mCMV) infection.

Materials and Methods

Mice

C57BL/6 (B6), CB6F1 (C57BL/6 × BALB/c) (H-2b/d, CD45.2/Thy1.2), B10.BR (H-2d, CD45.2, Thy1.2), and PepBoy (B6.129-Ptg/Pep587/BoyJ) (H-2d, CD45.1, Thy1.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BA mice (H-2d, CD45.2, Thy1.1) on the B6 background (a gift from Dr. Miriam Lieberman; Stanford University, Stanford, CA) and BAB10 mice (H-2d, CD45.2, Thy1.1) on the H-2d background were bred at Emory University. TLR5−/− mice were generated as described previously (22) and were bred in the Emory University animal facility. Procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Emory University Institutional Animal Care and Use Committee.

Preparation of lymphocytes obtained from donor mice

Donor splenocytes from 8- to 10-wk-old naive PepBoy or B10.BR mice were harvested as described previously (23, 24). Bone marrow (BM) was flushed from femurs and tibiae of 8- to 10-wk-old naive BA or BA.B10 donor mice, and CD3− T cells were depleted (23). Numbers of live cells were counted by fluorescent microscopy (23).

Flagellin preparation and administration to HSCT recipients

Highly purified endotoxin-free native flagellin was extracted from S. typhimurium (SL3201) as described previously (25, 26). Routine SDS-PAGE analysis revealed no contaminating proteins accompanying the expected single band around 50-kDa flagellin determined by using Coomassie brilliant blue staining (Supplemental Fig. 1A). LPS contamination was checked by administering 50 μg flagellin i.p. in B6 mice, and expansion of splenic CD3− B220− B cells was measured on day 3 postinfection (Supplemental Fig. 1B). Moreover, isolated flagellin protein also was checked routinely in vitro for the presence of endotoxin using the gel clot Limulus amoebocyte lysate assay (Lonza), and the results were generally negative for endotoxin. Purified flagellin was stored at −80°C at a concentration of 1 mg/ml in PBS. Flagellin was reconstituted with ice-cold PBS at a concentration of 50 μg/0.2 ml and injected into recipient mice i.p. 3 h before irradiation and 24 h after HSCT. To determine the therapeutic effect of flagellin against established GVHD, two 50 μg per mouse flagellin doses were administered i.p. on days 5 and 7 or days 14 and 16 post-HSCT.

Irradiation of recipient mice and HSCT

On day −1, CB6F1 or B6 recipient mice received a total of 11 Gy irradiation divided into two doses, 5.5 Gy, 3 h apart (9). On the next day, spleenocytes and T cell depleted (TCD) BM cells from naive congenic donors were transplanted through the tail vein as described previously (9, 24, 27).

mCMV infection of allogeneic HSCT recipients

CB6F1 recipients were infected i.p. with 2.5 × 108 PFU salivary gland passed Smith strain mCMV (a gift from Professor H. Yushida, Saga University, Japan) or vehicle on 100+ posttransplant.

Liver viral load determination

mCMV loads in the liver were determined on day 10 postinfection as described previously (28). Briefly, liver was harvested aseptically, homogenized, and centrifuged. Serially diluted supernatants were added to a confluent monolayer of 3T3 cells in 24-well tissue culture plates and incubated for 90 min at 37°C. Approximately 1 ml of 2.5% methylocellulose in DMEM (10% PBS) was added to each treated well and incubated for 4 d at 37°C. mCMV PFU were counted directly under a light microscope (Nikon, Melville, NY) after removing the methylocellulose and staining the 3T3 cells with methylene blue.

Lymphocyte isolation from organs of HSCT recipients

Blood was collected in tubes containing 0.05% heparin, and PBMCs were separated from RBCs as described previously (9). Splenocytes, BM cells from femurs and tibias, and thymocytes were harvested from transplant recipients as described previously (29).

Assessment of acute and chronic GVHD

Acute GVHD scores were determined independently from intestinal tissue sections by experienced pathologists (D.L.J. and A.B.F.) (30, 31). Chronic GVHD in small intestine was scored based on nonspecific inflammation, crypt apoptosis, and nonspecific relative increases in lamina propria chronic inflammatory cells (lymphocytes, plasma cells, histiocytes, etc.). Chronic GVHD from recipients’ skin was scored based on the thickness of dermal collagen by an experienced dermatopathologist (B.P.P.). Microscopic images from skin were taken at 200× using an Eclipse E400 microscope (Nikon) using a SPOT Flex 15.2 64 MP shifting pixel camera and SPOT software (Diagnostic Instruments, Sterling Heights, MI).

Flow cytometry

The origin of CD8+ and CD4+ T cells in HSCT recipients was determined by staining the cells with mAbs specific for donor BM (9). To determine the activation status of donor spleen-derived T cells, mAbs for CD62L, inducible costimulation molecule 1 (ICOS-1), program death 1 (PD-1), CD69, and CD25 were used. Stem cells from the BM of normal B6 mice and HSCT recipients were determined by staining cells with the mAbs to lineage markers (CD3, B220, NK1.1, and DX5), CD11b, CD11c, Sca-1, and c-Kit. Abs were used primarily from BD Pharmingen (San Jose, CA) and eBioscience (San Diego, CA). Anti-mCMV–specific CD8+ T cells were counted using an allophycocyanin-conjugated HGRNASFI-H-2Dc tetramer (National Institute of Allergy and Infectious Diseases Tetramer Core Facility, Atlanta, GA) (32). The CD4+CD25−Foxp3− Tregs were determined by intracellular staining using an anti-mouse Foxp3 kit purchased from eBioscience. Granzyme B and IFN-γ-producing donor spleen- and donor BM-derived CD8+ T cells were determined by intracellular cytokine staining after 4–6 h of in vitro stimulation with PMA/calciumpantothenate with GolgiPlug mixture (BD Pharmingen). Proliferation of donor spleen-derived CD4+ and CD8+ T cells in the recipients’ spleens on day 4 posttransplant were measured by transplanting CFSE-labeled (CellTrace CFSE Cell Proliferation Kit; Invitrogen) donor B10.BR splenocytes and by intracellular staining with PE mouse anti-human (cross-reactive) Ki67 kit purchased from BD Pharmingen. A specific Ab isotype was used to avoid nonspecific Ab staining. The proliferation index of the CFSE-stained donor spleen-derived CD4+ and CD8+ T cells were calculated as described by Wallace and Muirhead (33) as follows: the number of events (A) in each generation (k) are added together and divided by the total number of precursors, which is calculated for each generation by dividing the number of events in that generation by 2k.

Stained cells were acquired by either FACSCanto or FACSaria (BD Biosciences, San Jose, CA) and analyzed by FlowJo software.

Serum IFN-γ, TNF-α, and IL-6 determination by ELISA

Flagellin- or PBS-treated B6 recipients of 3 × 106 B10.BR splenocytes and 5 × 106 BA.B10 TCD BM cells were sacrificed on days 4 and 10 posttransplant, serum was collected, and IFN-γ, TNF-α, and IL-6 levels were determined by using ELISA kits purchased from eBioscience and BD Pharmingen, respectively.

Generation of TLR5 knockout radiation chimera

The B6 → TLR5 knockout (KO) and KO → B6 radiation chimeras were generated by transplanting 10 × 106 BM cells from wild-type B6 or TLR5 KO donors into the congenic CD45.1+ B6 or KO recipients conditioned with 11 Gy (5.5 Gy × 2) total body irradiation. Four sets of radiation chimeras were created: 1) B6 → KO, 2) KO → B6, 3) B6 → B6, and 4) KO → KO. All of the groups of radiation chimeras were irradiated again with 9.0 Gy (4.5 Gy × 2) 60 d after the first transplant and then received a second infusion of 3 × 106 splenocytes and 5 × 106 TCD BM cells from H-2k congenic donors (H-2k → H-2h HSCT model).

Statistical analyses

The Student t test and log–rank test were applied as described in the text. Differences were considered significant when p values were <0.05.
Results
Flagellin protected allogeneic HSCT recipients from GVHD

We have shown previously that allogeneic B6 recipients (H-2^k → H-2^b HSCT model) transplanted with 5 × 10^6 splenocytes and 5 × 10^6 TCD BM cells developed severe acute GVHD (27). To investigate the protective effect of flagellin against acute GVHD, allogeneic HSCT recipients were treated with two doses of flagellin as described in Materials and Methods. Surprisingly, 80% of flagellin-treated recipients survived until 132 d posttransplant with 15% weight loss, whereas all of the control recipients had severe and uniformly lethal acute GVHD and died within 10 d posttransplant (Fig. 1A). The survival data were statistically significant because the p value was equal to 0.025 (log-rank test).

We also have shown previously that allogeneic CB6F1 recipients (H-2^b → H-2^bd HSCT model) transplanted with 5 × 10^6 splenocytes and 5 × 10^6 TCD BM cells developed chronic GVHD within 2 mo posttransplant (9). The presence of chronic GVHD in this model is characterized by ∼15% weight loss and 10–20% mortality, fur loss, skin and tail rashes, histological evidence of lymphocyte infiltration in the liver perportal region, the presence of apoptotic cells in the intestinal epithelium along with foci of epithelial ulceration, and the presence of sclerodermoid sclerosis or fibrosis in the skin (9, 24). To investigate the protective effect of flagellin against chronic GVHD, we administered flagellin to CB6F1 recipients of congenic B6 splenocytes and TCD BM as described in Materials and Methods. All of the flagellin-treated recipients survived, whereas only 60% of PBS-treated control recipients survived until day 66 (Fig. 1B, p = 0.067, log-rank test). Although both groups had a similar pattern of weight loss until day 30 posttransplant, flagellin-treated recipients steadily gained weight without any clinical signs of GVHD from 30 d onward. However, PBS-treated recipients continuously lost weight and had clinical signs of chronic GVHD. Next, to test the effect of flagellin on acute GVHD after the transplantation of a lethal dose of donor splenocytes in the H-2^b → H-2^bd HSCT model, CB6F1 recipients were transplanted with 10 × 10^6 splenocytes and 5 × 10^6 TCD BM cells. Sixty percent of flagellin-treated recipients survived until day 75 posttransplant with ∼20% weight loss and signs of chronic GVHD. In contrast, all of the PBS-treated control recipients had severe acute GVHD with >25% weight loss within 10 d post-HSCT and were sacrificed (Supplemental Fig. 2). These data suggest that flagellin can protect allogeneic HSCT recipients from both acute and chronic GVHD.

Flagellin-treated recipients had improved lymphoid immune reconstitution with complete donor T cell chimerism

To investigate the immunology of donor T cells in flagellin-treated allogeneic HSCT recipients, we next studied donor spleen- and donor BM-derived T cells isolated from the blood, spleen, and thymus of flagellin- and PBS-treated recipients on day 66 post-HSCT. Although the numbers of nucleated cells and donor spleen-derived T cells per milliliter of blood were equivalent between flagellin- and PBS-treated recipients (Fig. 2A, 2B), the numbers of nucleated cells and donor spleen-derived T cells per spleen were significantly higher in the flagellin-treated recipients compared with those in the PBS-treated recipients (Fig. 2C, 2D, p < 0.05). Interestingly, the flagellin-treated recipients had >99% donor T cell chimerism (Fig. 2E) despite reduced clinical GVHD. The numbers of donor spleen- and donor BM-derived CD4^+ and CD8^+ T cells per spleen were also significantly higher in flagellin-treated recipients compared with those in PBS-treated recipients (Fig. 2F, p < 0.05). Although the numbers of donor BM-derived naïve CD62L^+CD4^+ T cells were significantly higher in flagellin-treated recipients compared with those in PBS-treated recipients (Fig. 2G, p = 0.008), the numbers of donor spleen-derived CD62L^+CD4^+ T cells per spleen were not significantly different between the two groups (Fig. 2G, p = 0.2). Because the function of alloreactive donor T cells can be controlled through Tregs (34), we hypothesized that the protective effect of flagellin on chronic GVHD might be due to increased thymic generation of donor-derived Tregs. To address this hypothesis, we measured numbers of donor BM-derived Tregs in the thymus of transplant recipients. Significantly more thymocytes (Fig. 2H, p < 0.005) and thymic Tregs (Fig. 2I, p < 0.05) were found in flagellin-treated recipients compared with those in PBS-treated recipients on day 66 post-transplant. These data show that flagellin-treated recipients had improved immune reconstitution and that posttransplant immune homeostasis was maintained by the generation of BM-derived naïve T cells and Tregs through the preservation of a functional thymus.

Beside donor T cells, B cells also have been implicated in the initiation and development of chronic GVHD (35, 36). We next determined the numbers of CD3^+ B220^+ B cells in the blood and spleen of both flagellin- and PBS-treated HSCT recipients. The percentages of donor spleen-derived B cells (CD45^- gated cells) were >4-fold less (10.4 versus 42.7%) in the blood (Fig. 2J) and 2-fold less (10.1 versus 19.5%) in the spleen (Fig. 2K) of flagellin-treated recipients compared with those of PBS-treated recipients.

FIGURE 1. Flagellin protected allogeneic HSCT recipients from GVHD. A, Survival and percentage weight loss for flagellin- and PBS-treated B6 recipients transplanted with 5 × 10^6 splenocytes and 5 × 10^6 TCD BM cells from B10.BR and BA.B10 congenic donors, respectively, in the H-2^k → H-2^b model. B, Survival and percentage weight loss of the flagellin- and PBS-treated CB6F1 recipients transplanted with 5 × 10^6 splenocytes and 5 × 10^6 TCD BM cells from PepBoy and BA congenic donors, respectively, in the H-2^b → H-2^bd model. Survival data were derived from daily monitoring of HSCT recipients for 3 wk after transplant and twice per week thereafter. Body weights of individual recipients were measured twice per week during the first 3 wk after HSCT and once per week after that. Six to 10 mice were used per group. The data are the representative of three similar experiments.
The percentages of donor BM-derived B cells (CD45.2+ gated cells) were less (18.9 versus 44.4%) in the blood of flagellin-treated recipients compared with those of PBS-treated recipients (Fig. 2J), but not in the spleen (55.9 versus 41.6%) (Fig. 2K).

Flagellin-treated recipients had improved immune reconstitution with complete donor chimerism. Flagellin- and PBS-treated control recipients of the H-2b→H-2b/d HSCT model were sacrificed on day 66 after HSCT. PBMCs, splenocytes and thymocytes were harvested from the blood, spleen, and thymus, respectively. Nucleated cells per organ were counted microscopically and donor spleen- and donor BM-derived T cells were determined by FACS analysis as described in Materials and Methods. A. Total nucleated cells per milliliter of blood obtained by FACS analysis. B. Total nucleated cells per milliliter of blood. C. Total donor spleen-derived T cells per milliliter of blood. D. Total donor BM-derived T cells per milliliter of blood. E. FACS data for T cell chimerism obtained from the CD3-gated population of the spleen of one mouse per group. F. Donor spleen- and donor BM-derived CD4+ and CD8+ T cells per spleen. G. Donor spleen- and donor BM-derived CD62L CD4+ T cells per spleen. H. Absolute numbers of thymocytes obtained from the thymus of flagellin- and PBS-treated recipients. I. Absolute numbers of total Tregs per thymus. J and K. FACS data from blood and spleen, respectively, of one mouse from each group of flagellin- and PBS-treated recipients. Side scatter data of CD45.2+ cells are for the donor BM-derived population, and CD45.2− cells are for the donor spleen-derived population. B220−CD3+ cells from the donor BM- and donor spleen-derived gated cells are presented as B cells. Four to five mice were used per group. *p < 0.5, **p < 0.005, Student t test.

The percentages of donor BM-derived B cells (CD45.2+ gated cells) were less (18.9 versus 44.4%) in the blood of flagellin-treated recipients compared with those of PBS-treated recipients (Fig. 2J), but not in the spleen (55.9 versus 41.6%) (Fig. 2K).

Flagellin-treated recipients had immune reconstitution in the blood and BM similar to that of normal B6 mice

To investigate the long-term immune reconstitution in another established model for acute GVHD, recipients of H-2k→H-2b (B10.BR→B6) (Fig. 1A) were sacrificed on day 132 posttransplant, and cells from the blood, spleen, BM, and thymus were collected. Because all of the PBS-treated HSCT recipients died of GVHD within 10 d posttransplant, we compared cell numbers harvested from the blood and BM of flagellin-treated recipients to cell numbers of the blood and BM in normal B6 mice of the same age. The total cell numbers per milliliter of blood and in BM of two femurs and two tibias in the flagellin-treated mice were similar to those of normal B6 mice. Interestingly, the numbers of CD3+, CD4+, and CD8+ T cells, CD3−B220−B cells, and NK1.1+ NK cells per milliliter of blood of flagellin-treated recipients were also similar to those of normal mice, except NK1.1+ T cells, which were significantly higher in flagellin-treated recipients (Table I, p < 0.05). However, significantly higher numbers of CD3+, CD4+, and CD8+ T cells and Lin−CD11b−CD11c−Sca-1−Kit+ stem cells and fewer B cells were detected in the BM of flagellin-treated recipients compared with those of normal mice (Table I). The numbers of splenocytes and thymocytes were significantly lower in the flagellin-treated HSCT recipients compared with those of normal mice (data are not shown).

Flagellin-treated recipients preserved increased antiviral immunity and had reduced chronic GVHD

To investigate the effect of preventing chronic GVHD using flagellin prophylaxis on posttransplant antiviral immunity, flagellin- or PBS-treated CB6F1 recipients (H-2b→H-2b/d HSCT model) using a nonlethal dose of 7 × 10^7 splenocytes per mouse and infected with a nonlethal dose of mCMV on 100+ d posttransplant.
Recipients were sacrificed on day 10 after mCMV infection, anti-mCMV T cells were counted in blood and spleen, and viral load per liver was determined. The numbers of nucleated cells and donor spleen-derived T cells per milliliter of blood were significantly higher in flagellin-treated recipients compared with those of the PBS-treated recipients (Fig. 3, C and Fig. 3G, respectively). Flagellin-treated recipients had higher frequencies of donor spleen-derived mCMV peptide MHC tetramer+ CD8+ T cells (Fig. 3C) and significantly higher numbers of both donor spleen- (p < 0.005) and BM-derived (p < 0.05) mCMV peptide-specific tetramer+ CD8+ T cells per milliliter of blood compared with those of the PBS-treated recipients (Fig. 3D). After in vitro stimulation of splenocytes with PMA/ionomycin and GolgiPlug, flagellin-treated recipients showed higher frequencies of Granulocyte B- and IFN-γ-producing donor spleen- and donor BM-derived CD8+ T cells (Fig. 3E). We next determined the viral load in liver on day 10 post-mCMV infection. Significantly lower mCMV PFUs were detected in the livers of flagellin-treated recipients compared with those of the PBS-treated recipients (Fig. 3F, p = 0.02). We next determined chronic GVHD pathology scores in formalin-fixed histological tissue sections of small intestine and skin collected from flagellin-treated and PBS-treated B6 → F1 transplant recipients on day 10 post-mCMV infection. Flagellin-treated recipients had significantly lower GVHD scores in small intestine (Fig. 3G, p = 0.002) and significantly thinner dermis (Fig. 3H, p = 0.008) compared with those of PBS-treated recipients. Representative pictures of the dermal thickness (micrometer) with the measurement line (arrow) of one mouse each from five flagellin- and five PBS-treated recipients were shown in Fig. 3I.

**Flagellin reduced acute GVHD by reducing early donor T cell activation and proliferation**

To investigate the effect of flagellin on acute GVHD, we next studied the immune responses in recipients of a nonlethal dose of donor T cells (3 × 10^6 splenocytes) in the H-2^k → H-2^b acute GVHD HSCT model. Both flagellin- and PBS-treated recipients were sacrificed on days 4 and 10 posttransplant, and total nucleated cells and different subsets of donor spleen-derived T cells per spleen were determined as described in Materials and Methods. The numbers of total nucleated cells and donor spleen-derived T cells in the spleens of transplant recipients were significantly lower in flagellin-treated recipients compared with those in PBS-treated recipients on day 4 posttransplant (Fig. 4A, 4B, p = 0.003 and p = 0.04, respectively), but not different on day 10 post-transplant (data not shown). Next, we determined the GVHD scores from formalin-fixed small intestine histological tissue sections on day 4 post-HSCT. Although acute GVHD scores in flagellin-treated recipients (mean 1.5 ± 1.5) were lower compared with those in PBS-treated recipients (mean 2.9 ± 0.9), the differences were not statistically significant (Fig. 4C, p = 0.1). Next, to investigate the activation status of the donor spleen-derived T cells, we determined the expression of CD62L, ICOS-1, PD-1, CD69, and CD25 by the donor spleen-derived CD4+ and CD8+ T cells on days 4 and 10 posttransplant. Interestingly, numbers of donor spleen-derived CD4+ and CD8+ T cells expressing all of the activation markers, including PD-1, were significantly lower in the spleens of flagellin-treated recipients compared with those of PBS-treated recipients (Fig. 4D, p < 0.05, **p < 0.005) on day 4 but not on day 10 post-HSCT. Next, we investigated the effect of flagellin on the proliferation of donor spleen-derived CD4+ and CD8+ T cells and CD11b+ myloid cells in recipients’ spleens on day 4 posttransplant by measuring CFSE dilution in labeled cells and expression of Ki67. Donor spleen-derived CD4+ and CD8+ T cells and CD11b+ myloid cells in the spleens of flagellin-treated recipients had significantly less Ki67 expression and less dilution of CFSE (less proliferation) on day 4 posttransplant compared with those of PBS-treated recipients as shown by both FACS data (Fig. 4E) and average percentages with the SEs (Fig. 4F). Accordingly, the proliferation indexes of donor spleen-derived CD4+ T cells but not donor spleen-derived CD8+ T cells were decreased significantly in flagellin-treated recipients compared with those of PBS-treated recipients (Fig. 4G). We also determined the percentages of myloid cells based upon scatter gates in FACS analysis of the spleens of flagellin- and PBS-treated control recipients on days 4 and 10 posttransplant. Myloid cells significantly increased from days 4 to 10 posttransplant in the spleens of both flagellin- and PBS-treated recipients (flagellin, day 4, 2.2 ± 0.2%, day 10, 25.9 ± 4.1%; PBS, day 4, 2.5 ± 1.1%, day 10, 24.2 ± 2.0%, p < 0.0005 for day 4 versus day 10). These data suggest that flagellin treatment selectively reduced activation and proliferation of donor spleen-derived T cells and myloid cells during the very early phase posttransplant.

**Reduction of flagellin-induced GVHD was due to a decrease in proinflammatory cytokine production by donor T cells**

Proinflammatory cytokines such as IFN-γ, TNF-α, and IL-6 are produced mostly by activated donor T cells in the early days of allogeneic HSCT (31, 37–39). Next, we measured serum IFN-γ, TNF-α, and IL-6 on days 4 and 10 posttransplant. Significantly reduced levels of serum IFN-γ were determined only on day 4 but not on day 10 posttransplant in flagellin-treated recipients compared with those in PBS-treated recipients (Fig. 5, left panel). Although the serum concentration of TNF-α was not detectable on day 4 posttransplant, a significantly decreased level was determined on day 10 posttransplant in recipients of flagellin-treated recipients compared with that of PBS-treated recipients (Fig. 5, middle panel). Moreover, a significantly lower serum concentration of IL-6 was measured on day 10 posttransplant, but equivalent levels of serum IL-6 were found on day 4 posttransplant in both flagellin- and PBS-treated recipients (Fig. 5, right panel).

### Table I. Effect of flagellin on immune reconstitution in H-2^k → H-2^b allogeneic HSCT recipients

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<th>Cells/BM (×10^6)</th>
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<th>CD4^+ T Cells</th>
<th>CD8^+ T Cells</th>
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<th>NK Cells</th>
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<td>1.1±0.2</td>
<td>0.5±0.1</td>
<td>0.6±0.2</td>
<td>5.4±1.1</td>
<td>0.2±0.05</td>
<td>0.04±0.01</td>
<td></td>
</tr>
</tbody>
</table>

Recipients of Fig. 1A were sacrificed on day 132 posttransplant. BM (from two legs’ femurs and tibias) was harvested and analyzed by flow cytometry for the presence of T, B, NK1.1*, NKt, and stem cells. Absolute numbers of each cell type per BM and per milliliter of blood were calculated and compared with the similar cells harvested from four normal B6 mice with a similar age to the HSCT recipients.

*p < 0.05, **p < 0.005, ***p < 0.001, Student’s t test.

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Flagellin–TLR5 interactions are required to reduce GVHD in allogeneic HSCT recipients

Flagellin has been shown to have diverse immunomodulatory activities that are mediated mostly through binding TLR5 expressed on epithelial cells of the gastrointestinal (GI) tract, macrophages, and dendritic cells (DCs) of hematopoietic origin (40–42). To explore the mechanism and involvement of flagellin–TLR5 immune interactions in the reduction of GVHD in allogeneic HSCT recipients, we first generated four sets of radiation chimeras (B6 → KO, KO → B6, B6 → B6, and KO → KO described in Materials and Methods). Sixty days after the initial B6 congenic transplant, radiation chimera recipients were treated with flagellin and/or PBS, and all of the radiation chimera recipients were transplanted again with 3 × 10^6 splenocytes and 5 × 10^6 TCD BM cells from H-2^K allogeneic donors (H-2^K → H-2^b acute GVHD model) as described in Materials and Methods. All of the recipients of flagellin-treated B6 → B6 radiation chimeras survived with only 12% weight loss by day 80 posttransplant compared with 50% survival among recipients of flagellin-treated B6 → KO and 40% survival among KO → B6 radiation chimeras. Notably, all of the flagellin-treated KO → KO and PBS-treated radiation chimeras died within 65 d posttransplant (Fig. 6).
These data suggested that the interaction of flagellin with the TLR5-expressing hematopoietic cells in the recipient and TLR5+ GI epithelium are both required to maximize the effect of flagellin in reducing GVHD in allogeneic HSCT recipients.

**Discussion**

Toxicity after total body irradiation conditioning regimens is seen primarily in the hematopoietic system and GI tract (43). Radiation-induced damage of the GI epithelium produces a number of inflammatory cytokines and chemokines that stimulate donor alloreactive T cells to initiate GVHD (44). It has been reported that prophylactic administration of flagellin in mice reduced radiation-induced GI toxicity in nontransplant settings (19, 20). On the basis of these published reports, our initial hypothesis was that administration of flagellin in lethally irradiated allogeneic HSCT recipients would reduce radiation-induced toxicity in GI epithelium and would reduce production of inflammatory mediators that activate donor T cells and contribute to acute GVHD. The increased survival and decreased weight loss seen in flagellin-treated recipients compared with those in PBS-treated recipients (Fig. 1A, 1B) support this initial hypothesis. Although administration of a potent TLR agonist might be expected to enhance T cell activation, we found significantly less early activation of donor spleen-derived CD4+ and CD8+ T cells in flagellin-treated recipients compared with that in PBS-treated recipients (Fig. 4B, 4D). Moreover, the decrease in serum concentrations of proinflammatory cytokines IFN-γ, TNF-α, and IL-6 (produced by activated donor T cells and

**FIGURE 4.** Flagellin reduced acute GVHD by reducing early donor T cell proliferation and activation. Flagellin- and PBS-treated recipients transplanted with $3 \times 10^6$ splenocytes from naïve B10.BR and $5 \times 10^6$ TCD BM cells from naïve BA.B10 congenic donors of the H-2^k → H-2^b allogeneic HSCT model were sacrificed on days 4 and 10 post-HSCT, and splenocytes were analyzed. A. Absolute numbers of nucleated cells per spleen determined microscopically. B. Absolute numbers of donor spleen-derived T cells per spleen determined by FACS. C. Acute GVHD scores obtained from the small intestine histological tissue sections of flagellin- and PBS-treated recipients on day 4 post-HSCT. D. Activation statuses of donor spleen-derived CD4+ and CD8+ T cells were determined by costaining the cells with the Abs to CD62L, ICOS-1, PD-1, CD25, and CD69 along with the Abs to CD4, CD8, CD45.2, and Thy1.2. Closed circles represent the data obtained from flagellin-treated recipients; open circles represent PBS-treated recipients. E. FACS data representing the Ki67+ and CFSE-stained donor spleen-derived CD4+ and CD8+ T cells in the spleen of flagellin- and PBS-treated recipients on day 4 posttransplant. F. Average percentages of Ki67+ donor spleen-derived CD4+ and CD8+ T cells and Thy1.2+CD11b+ myeloid populations in the spleen of flagellin- and PBS-treated allogeneic HSCT recipients on day 4 posttransplant. G. Proliferation index calculated from the CFSE-stained donor spleen-derived CD4+ and CD8+ T cells in the spleen of flagellin- and PBS-treated recipients on day 4 posttransplant. Five to six mice were used per group in each time point. *p < 0.05, **p < 0.005, Student t test.
Radiation chimeras were generated by transplanting 10 naive BA.B10 congenic donors of the H-2K^b → H-2b allogeneic HSCT model were sacrificed on days 4 and 10 post-HSCT. Serum was harvested, and concentrations of IFN-γ, TNF-α, and IL-6 per milliliter of serum were determined by ELISA as described in Materials and Methods. Serum concentrations of IFN-γ (left panel), TNF-α (middle panel), and IL-6 (right panel) are presented. Five to six mice were used per group at each time point. *p < 0.05, Student t test. ND, nondetectable serum cytokine concentrations.

GVHD (31, 37–39) further supports the hypothesis that protecting GI epithelium with a TLR5 agonist can have global effects on donor T cell activation and GVHD. To the best of our knowledge, this is the first report of this paradoxical immunomodulatory activity of flagellin in allogeneic HSCT recipients. Our observations are consistent with published data that increased expression of ICOS-1 on donor spleen-derived CD4^+ and CD8^+ T cells is associated with acute GVHD (45, 46). Along with lower levels of inflammatory cytokines, we found lower levels of ICOS-1 expression on donor T cells in the spleens of flagellin-treated recipients compared with those of PBS-treated recipients (Fig. 5C, 5D) and ICOS-1 levels proportional to the degree of acute GVHD. This finding is significant, because B7RP-1–ICOS costimulatory signals play an important role in the activation of alloantigen-reactive donor T cells, particularly on CD8^+ T cells in murine models of acute GVHD (46). Blockade of the B7RP-1–ICOS interaction reduced the immune response of alloreactive T cells and reduced acute GVHD in allogeneic HSCT recipients (45, 46). Moreover, significantly less expression of CD69 and CD25 activation markers was seen on donor CD4^+ and CD8^+ T cells in the spleen of flagellin-treated recipients compared with that of PBS-treated recipients, further supporting the role of flagellin in reducing the activation of donor T cells. Of interest, expression of PD-1 by donor CD4^+ and CD8^+ T cells also was significantly lower in flagellin-treated recipients compared with that in PBS-treated recipients (Fig. 4D). Although the exact role of PD-1 expression on donor T cells and on the induction of acute GVHD in allogeneic HSCT is not yet known, recently published data claimed that upregulation of PD-1 expression on activated donor T cells is required to initiate acute GVHD and that blockade of PD-1/PD-L1 interactions by their Abs reduced GVHD with partially restored T cell effector functions and improved GVL (47).

Results from transplanting an allogeneic B10.BR (H-2^k) graft to B6 BM → TLR5 KO and TLR5 KO BM → B6 radiation chimeras indicate that the mechanism by which flagellin reduces GVHD requires TLR5 expression on both host hematopoietic and host GI epithelial cells. This finding is unexpected, because activation of host APCs through a TLR ligand might be predicted to increase GVHD in allogeneic HSCT recipients (44, 48). Previously published reports by us and others showed that flagellin selectively binds only TLR5 (49), which is expressed abundantly on the cells of various mucosal organs, including the GI tract, lung, and uterus, as well as CD11c^+CD11b^+ hematopoietic cells isolated from the lamina propria of the small intestine, macrophages, and DCs (11, 25, 40–42, 50–52). The effect of flagellin on TLR5-expressing APCs is primarily to promote stimulatory immune responses and generation of inflammatory cytokines in vitro. Flagellin treatment of TLR5-expressing DCs increased production of IL-12, decreased production of IL-10, and stimulated tolerogenic DCs to promote Th1-type responses (17, 18). Honko and Mizel (53) showed that flagellin treatment transiently enhanced innate immune responses in the respiratory system through increased infiltration of neutrophils and increased production of TNF-α, IL-6, G-CSF, MIP-1α, and MIP-2. However, it also was reported that in vitro flagellin treatment of murine immature DCs did not enhance secretion of cytokines or chemokines (42). In contrast to these studies, we report in this article a new observation that prophylactic flagellin–TLR5 interaction in irradiated allogeneic HSCT recipients had a paradoxical effect of significantly reducing inflammatory cytokines and activation of donor spleen-derived T cells during the early phase of transplantation (Figs. 4, 5). Moreover, therapeutic posttransplant administration of flagellin did not reduce GVHD that already had been initiated (Supplemental Fig. 3). Thus, prophylactic administration of flagellin to prevent GVHD in allogeneic HSCT recipients is consistent with its radiation-protective effect in mice (20).
The mechanism by which prophylactically administered flagellin regulates donor T cells and reduces the development and severity of chronic GVHD is less clear. Because flagellin has a very short half-life (54), any direct pharmacological effect cannot persist >30 d posttransplant (Fig. 1), so the observed reduction in chronic GVHD in flagellin-treated recipients thus may be due to a combination of multiple indirect effects. 1) For example, because acute GVHD is followed generally by chronic GVHD (55–57), prophylactically administered flagellin could decrease chronic GVHD indirectly through its effect on reducing acute GVHD (Fig. 1A). 2) The ability of flagellin to facilitate donor cell engraftment and full chimerism (Fig. 2, Table I) could reduce chronic GVHD by enhancing immune homeostasis and donor immune reconstitution (58). 3) Flagellin treatment may directly promote generation of significantly increased numbers of donor BM-derived natural Tregs from donor hematopoietic stem cells that mature and differentiate in the recipient’s thymus (Fig. 2G, 2I) and limit development of chronic GVHD in murine HSCT recipients (59, 60). Moreover, it is possible that, in addition to the increased numbers of natural thymic-derived Tregs in flagellin-treated recipients, the increased numbers of donor NKT cells seen in flagellin-treated recipients (Table I) may play an important role in reducing chronic GVHD (61). Finally, 4) the effect of flagellin on reducing the numbers of donor B cells also plays an important role in reducing chronic GVHD (35, 62). Using a minor histocompatibility-mismatched murine model, Zhang et al. (63) showed that B cells (in addition to donor T cells) were required to induce chronic GVHD. Moreover, autoantibodies are detected frequently in patients with chronic GVHD (64). Hence, the persistence of fewer donor spleen-derived CD3+ B220+ B cells in the spleen and blood of flagellin-treated recipients compared with PBS-treated control recipients (Fig. 2J, 2K) may play an important role in the reduction chronic GVHD. However, it also has been reported that patients with both acute and chronic GVHD had lower numbers of B cells and B cell progenitors in their BM (65).

In this study, we specifically addressed one of the major clinical complications of allogeneic HSCT, namely, posttransplant opportunistic CMV infection (66). Using an established mCMV model, which has similarities to the pathogenicity of human CMV infection (67, 68), we observed that flagellin-treated recipients successfully recovered from a sublethal dose of mCMV infection with faster viral clearance and higher antiviral cellular immunity compared with those of PBS-treated control recipients. In addition to opportunistic infections, disease relapse is another major clinical problem in allogeneic HSCT (69), and it is possible that full donor chimerism with improved immune reconstitution (Fig. 2, Table I) will protect recipients from disease relapse. Although we did not test the antitumor activity of flagellin in allogeneic HSCT recipients in the current study, we are testing currently whether flagellin also may enhance the antitumor immunity of donor T cells. Of note, a published report by Sfondrini et al. (70) showed that flagellin significantly inhibited in vivo growth of an antigenic variant tumor in normal mice.

In summary, our data demonstrate that peritransplant administration of flagellin reduced early activation of donor T cells and reduced production of proinflammatory cytokines in irradiated HSCT recipient with associated reductions in the severity of acute and chronic GVHD. By reduction of early donor T cell activation, flagellin treatment preserved the immune function of lymphoid organs while permitting full donor T cell chimerism. With improved posttransplant immune reconstitution, flagellin-treated recipients had an improved immune response to CMV infection with significantly increased numbers of anti-CMV–specific donor CD8+ T cells compared with those of PBS-treated control recipients. Furthermore, transplanting radiation chimeras showed that TLR5 expression on both GI epithelium and recipients’ hematopoietic cells was needed for the full therapeutic effect of flagellin to reduce the severity of GVHD. The beneficial effect of flagellin was dependent upon flagellin–TLR5 interactions, and only prophylactic administration of flagellin before transplantation conferred protection against GVHD. Because flagellin has been found to be safe to use in human as a vaccine adjuvant in a number of clinical trials, our data indicate that the use of flagellin to control GVHD may have potential clinical impact.

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Disclosures
The authors have no financial conflicts of interest.

References

FLAGELLIN–TLR5 IMMUNE RESPONSE REDUCES GVHD


Days after transplant

% Weight loss

-30.0 -25.0 -20.0 -15.0 -10.0 -5.0 0.0

Fla
PBS
Supplemental Legends

**Figure S1. Flagellin was highly pure and LPS free.** A. Purity of the flagellin protein was verified by running SDS-PAGE followed by Coomassie Blue staining as described in materials and methods. The gel was then photographed using an illuminated light box. B. 50 μg flagellin reconstituted in 0.2ml ice-cold PBS and was administered i.p to normal B6 mice. On 3 days post flagellintreatment mice were sacrificed and CD3-B220+ B cells were determined from harvested splenocytes by FACS. The FACS data is the representative of one mouse from each group out of 3. Control mice received only 0.2 ml ice-cold PBS i.p.

**Figure S2. Flagellin can protect H-2b→H-2bd recipients transplanted with high dose donor splenocytes from acute GvHD.** CB6F1 recipients of 10 x 10⁶ donor splenocytes along with 5 x 10⁶ TCD BM cells from naïve congenic B6 donors and two 50 μg/mouse flagellin doses were administered i.p 3 hours before irradiation and 24 hours after HSCT. PBS-treated Control recipients were treated with 0.2 ml PBS/mouse i.p. For survival data, recipients were monitored every day within 3 weeks of HSCT and twice per week after that. The body weights of individual recipients were measured twice/week within 3 weeks and once a week after that. 6 mice were used per group.

**Figure S3. Therapeutic administration of flagellin did not protect HSCT recipients from GvHD.** CB6F1 recipients of 7 x 10⁶ donor splenocytes along with 5 x 10⁶ TCD BM cells from naïve congenic B6 donors were treated with two 50 μg/mouse flagellin doses on
day 5 and 7 post transplant. Another group received two 50μg/mouse flagellin doses on day 14 and 16 post HSCT. Control recipients were injected with 0.2 ml PBS/mouse i.p. The body weights of individual recipients were measured twice/week within 3 weeks and once a week after that. 6 mice were used per group.