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Bendamustine with Total Body Irradiation Limits Murine Graft-versus-Host Disease in Part Through Effects on Myeloid-Derived Suppressor Cells

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A B S T R A C T

Graft-versus-host disease (GVHD) remains a significant challenge in allogeneic hematopoietic cell transplantation (HCT). An underinvestigated strategy to reduce GVHD is the modification of the preparative conditioning regimen. In the present study, we aimed to evaluate GVHD associated with bendamustine (BEN) conditioning in conjunction with total body irradiation (TBI) as an alternative to the standard myeloablative regimen of cyclophosphamide (CY) and TBI. We demonstrate that BEN-TBI conditioning, although facilitating complete donor chimerism, results in significantly less GVHD compared with CY-TBI. In BEN-TBI-conditioned mice, suppressive $CD11b^{+}Gr\text{-}1^{high}$ myeloid cells are increased in the blood, bone marrow, spleen, and intestines. When $Gr\text{-}1^{high}$ cells are depleted before transplantation, the beneficial effects of BEN-TBI are partially lost. Alternatively, administration of granulocyte colony-stimulating factor, which promotes $CD11b^{+}Gr\text{-}1^{+}$ myeloid cell expansion, is associated with a trend toward increased survival in BEN-TBI-conditioned mice. These findings indicate a potential role of myeloid-derived suppressor cells in the mechanism by which BEN allows engraftment with reduced GVHD. BEN-TBI conditioning may present a safer alternative to CY-TBI conditioning for allogeneic HCT.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) can be curative for many patients with hematologic disorders and malignancies, but graft-versus-host disease (GVHD) remains a significant barrier to its success. It is well documented that the dose intensity and the specific agents used in pretransplantation conditioning have an impact on the incidence and severity of GVHD [1]. Total body irradiation (TBI)-based myeloablative conditioning (MAC) may be associated with a higher incidence of acute GVHD compared with chemotherapy-based preparative regimens [2]. However, TBI is widely used in HCT owing to its antileukemic and immunosuppressive effects and its ability to treat extramedullary sanctuary sites of disease. Although other agents, such as etoposide [3] and cytarabine [4], have been evaluated in combination with TBI, the combination of cyclophosphamide (CY) and TBI has remained the most widely applied conditioning regimen for acute lymphoblastic leukemia (ALL) for almost half a century [5] and is also a frequently used regimen (albeit at lower doses) for nonmalignant conditions, such as severe aplastic anemia [6–10]. There is little information on how GVHD may be altered by replacing CY with other agents in TBI-based conditioning regimens.

Bendamustine (BEN), an alkylating agent and purine analog, has been used as treatment for lymphomas [11–16] and chronic lymphocytic leukemia (CLL) [17–19] and has been shown to be safe and effective as a conditioning agent for autologous HCT [20,21]. In addition, when replacing CY and given in combination with fludarabine and rituximab as allogeneic reduced-intensity conditioning for CLL, BEN was associated with reduced myelosuppression and GVHD [22], decreased treatment-related mortality, and superior survival [23].

We recently reported that BEN can safely replace post-transplantation CY (PT-CY) following murine haploidentical

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bone marrow (BM) transplantation (BMT), resulting in comparable protection from GVHD and superior graft-versus-leukemia effects. We observed that post-transplantation bendamustine (PT-BEN) preserved the myeloid compartment and resulted in an increased number of $CD11b^{+}Gr\text{-}1^{high}$ granulocytic myeloid-derived suppressor cells (MDSCs) compared with PT-CY. In addition, BEN treatment in vitro enhanced MDSC function [24]. It is well documented that MDSCs play an important role in limiting GVHD and can be modulated by chemotherapy treatment [25]. Adoptive transfer of MDSCs generated in vivo or in vitro can attenuate GVHD in an allogeneic murine BMT model [26–29]. Furthermore, greater MDSC content in donor grafts is correlated with a reduced incidence of acute GVHD in humans [30,31].

Based on these data, we hypothesized that BEN could effectively replace CY in the traditional CY-TBI myeloablative conditioning regimen, reducing GVHD through its effects on MDSCs. Here we demonstrate that BEN-TBI conditioning results in significantly less GVHD than the standard CY-TBI, in part through its effects on $CD11b^{+}Gr\text{-}1^{high}$ MDSCs.

MATERIALS AND METHODS

Mice

All strains of mice used were age-matched 6- to 10-week-old females purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in specific pathogen-free conditions and cared for according to the guidelines of the University of Arizona's Institutional Animal Care and Use Committee.

BMT Models

For the MHC-mismatched model used throughout, recipient BALB/c ($H\text{-}2^{d}$) mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day -2 and 400 cGy TBI on day -1 using a Cesium 137 irradiator. Based on the literature, it is expected that the drugs will be cleared by 24 hours postadministration [32–34]. On day 0, mice received 10^7 C57BL/6 ($H\text{-}2^{b}$) BM cells with 3×10^6 spleen cells (SCs) or 10^7 T cell depleted BM (TCD-BM) with 3×10^6 isolated total T cells (tT) i.v. In some experiments, tT were isolated from congenic $CD45.1^{+}$ BoyJ mice. Moribund mice were euthanized according to Institutional Animal Care and Use Committee-approved criteria and procedures, and survival was monitored daily. Mice were weighed every 3 to 4 days, and the percentage of starting weight was calculated. Mice were also scored clinically on skin integrity, fur texture, posture, and activity, and cumulative GVHD scores were calculated [35]. Mice with a cumulative score of 8 at day +8 were euthanized. A veterinary pathologist evaluated tissues for histological evidence of GVHD [36]. For the haploidentical BMT model used, CB6F1 ($H\text{-}2^{b/d}$) mice received 50 mg/kg BEN or 225 mg/kg CY, 300 cGy TBI, and 10^7 B6AF1 ($H\text{-}2^{b/k}$) BM cells with 3×10^7 SCs.

Preparation of Total T Cells and T Cell-Depleted BM

Total T cells were isolated from naïve C57BL/6 spleens by negative selection using mouse Pan T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA), with a purity of >97%. T cells were depleted from BM cells using the CD3ε MicroBead Kit (Miltenyi Biotec), with <.3% $CD3\varepsilon^{+}$ cells remaining.

Drug Preparation and Administration

CY and BEN were reconstituted and diluted as described previously [24]. Anti-Gr-1 depleting antibody (clone RB6-8C5; Thermo Fisher Scientific, Waltham, MA) and granulocyte colony-stimulating factor (G-CSF) (Amgen, Thousand Oaks, CA) were diluted in sterile saline for injection. Then 200 μg of anti-Gr-1 was administered i.p. on days -3, -1, and +5, and 250 μg/kg of G-CSF was administered s.c. on days -2 through +11.

Flow Cytometry

Before analysis by flow cytometry, blood was collected by cardiac puncture or tail tipping, and red blood cells were lysed (BD Biosciences, San Jose, CA). Spleens were processed to single cell suspension, and red blood cells were lysed. Intestines were digested as described below. Flow cytometry was performed as described previously [37]. Fluorescence data were collected with an LSRFortessa cell analyzer (BD Biosciences) and analyzed using FlowJo 2 (Tree Star, Ashland, OR). Antibodies included anti-mouse Gr-1 FITC (RB6-8C5), CD11b eFluor450 (M1/70), H2kb PerCP-eFluor710 (AF6-88.5.5.3), H2kd PE (SF1-1.1.1), and CD45.1 APC (A20) (all from Thermo Fisher Scientific), and CD45.1 PE-CF594 (A20) (from BD Biosciences). Of note, anti-mouse Gr-1 clone RB6-8C5 has been shown to react strongly with Ly6G (a marker for granulocytic MDSCs) and more weakly with Ly6C (a marker for monocytic MDSCs), resulting in a delineated $Gr\text{-}1^{high}$ population, representing the $Ly6G^{+}$ granulocytic MDSCs, and a $Gr\text{-}1^{mid}$ population, representing the $Ly6C^{+}$ monocytic MDSCs [38]. To determine absolute cell numbers in blood, white blood cell counts were determined using a HemaVet 950 (Drew Scientific, Miami Lakes, FL) [39]. Cells were analyzed for reactive oxygen species (ROS) using a Cellular ROS Detection Assay Kit (Abcam, Cambridge, UK).

Intestine Digestion and Immunofluorescence

The immune cells present in the intestines were analyzed by flow cytometry and immunofluorescence. For flow cytometry, intestines were first digested to single-cell suspensions using a modification of a previously published protocol [40]. In brief, intestines were flushed and incubated at 37°C in a shaker with Hank's balanced salt solution (HBSS; Thermo Fisher Scientific) with 5% FBS (Atlanta Biologicals, Flowery Branch, GA), 10 mM Hepes (Thermo Fisher Scientific), and 1 mM dithiothreitol (DTT; Bio-Rad Laboratories, Hercules, CA) for 15 minutes. Intestines were then sequentially incubated in digestion solution (HBSS with 5% FBS and 10 mM Hepes, 100 U/mL type I collagenase, and 40 μg/mL DNase I, grade II (MilliporeSigma, St. Louis, MO)) for 5 and 10 minutes. For immunofluorescence, intestines were fixed in formalin and embedded in paraffin, and slides were prepared by the University of Arizona Animal Care Pathology Services Laboratory. Slides were dewaxed using xylenes, antigen retrieval was performed by steaming in sodium citrate buffer (MilliporeSigma), and slides were incubated with anti-Gr-1 (1:125) and goat anti-rat NL 637 (1:500; R&D Systems, Minneapolis, MN) and mounted in Fluoroshield mounting medium with DAPI (Abcam). Staining was analyzed using a digital fluorescence microscope (BZ-X700; Keyence, Itasca, IL) and quantified using ImageJ software.

Suppression Assays

Suppression assays were conducted and analyzed as reported previously [24]. In brief, T cells were isolated from spleens of naïve C57BL/6 mice, stained, and stimulated. $Gr\text{-}1^{high}$ MDSCs were isolated from spleens using a mouse Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec), with >95% purity. MDSCs were coincubated with T cells at various ratios for 3 days. Flow cytometry was followed by analysis with Modfit (Verity Software House, Topsham, ME) to determine the proliferation index of the T cells, to calculate proliferation.

Quantitative Real-Time Polymerase Chain Reaction

Cells were frozen in dry pellets before mRNA isolation using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was generated, and quantitative real-time polymerase chain reaction (qRT-PCR) was performed and analyzed as described previously [39,41].

Statistics

Kaplan-Meier survival curves were generated, and the log-rank statistic was used to evaluate differences between groups [42,43]. The Mann-Whitney *U* test was used to determine other differences between groups.

RESULTS

BEN-TBI Conditioning Results in Improved Survival Compared with CY-TBI

Using a fully MHC-mismatched murine BMT model (C57BL/6, $H2^{b} \rightarrow$BALB/c, $H2^{d}$), we compared BEN-TBI with traditional CY-TBI conditioning. Equivalent doses, ~50% of the maximum tolerated dose, of BEN and CY were used (Supplementary Figure S1). With no post-transplantation GVHD prophylaxis, BEN-TBI resulted in significantly increased survival compared with CY-TBI in both a severe GVHD model (3×10^6 T cells) (Figure 1A) and a milder model (3×10^6 SCs) (Figure 1B). To further confirm these findings, SCs (3×10^6) were given alongside a comparable number of purified T cells (10^6), with BEN-TBI-conditioned mice demonstrating similarly increased survival compared with CY-TBI- conditioned mice regardless of the inoculum (Figure 1C). This indicates that the presence of other donor immune cells in the graft is not required for the protective effects of BEN-TBI, and that this difference in survival holds true over a range of T cell doses. Moreover, BEN-TBI was associated with increased survival compared with CY-TBI over a range of drug doses (Figure 1D).

To verify that this difference in GVHD was not due to graft rejection, donor cell engraftment was evaluated in the blood at various intervals. Both BEN-TBI and CY-TBI conditioning resulted in full donor chimerism, with no apparent difference

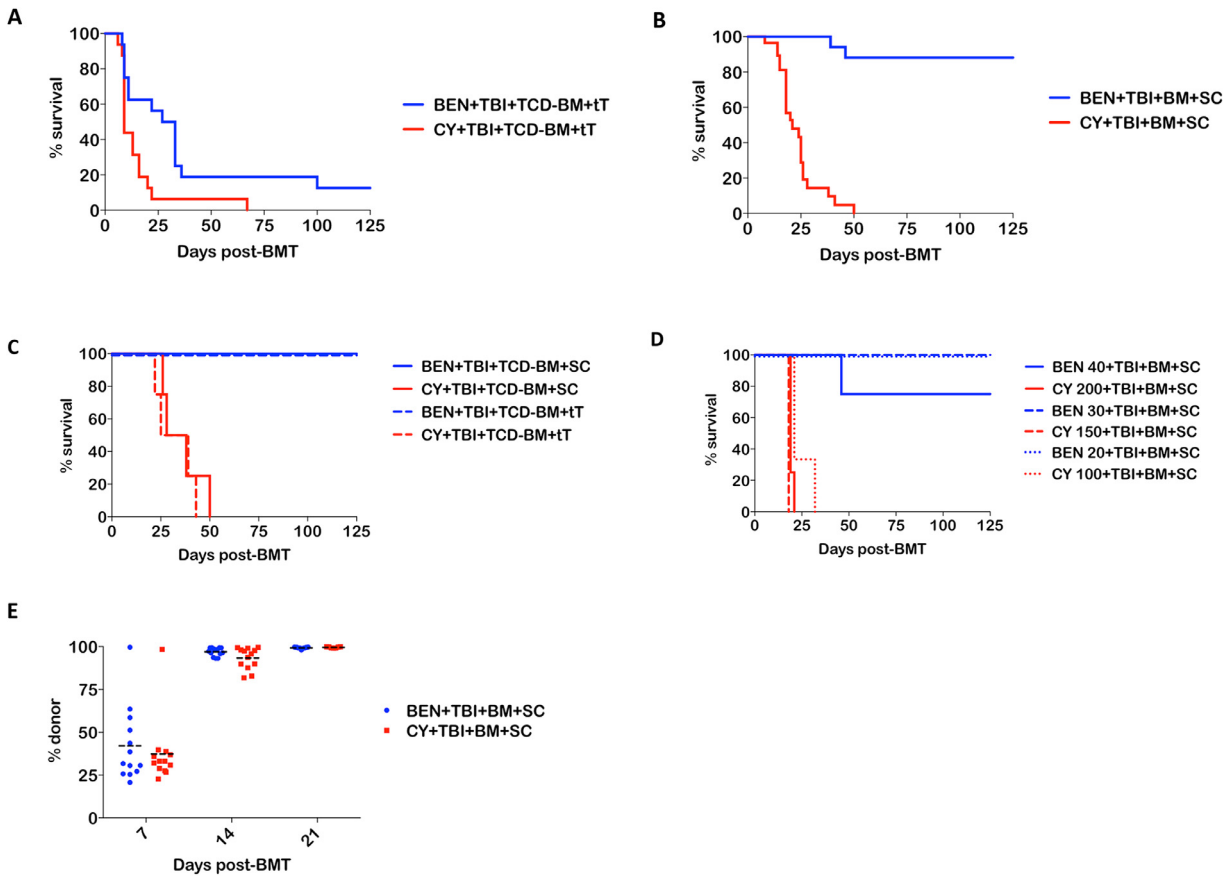

Figure 1. BEN-TBI conditioning is associated with significantly increased survival compared with CY-TBI. BALB/c recipient mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day -2, 400 cGy TBI on day -1, and 10^7 TCD-BM cells with 3×10^6 purified T cells (tT) (A), 10^7 BM with 3×10^6 SCs (B and D), or 10^7 TCD-BM with 3×10^6 SCs or 10^6 purified T cells (C) from naïve C57BL/6 mice on day 0. (A to C) Survival data. (A) Pooled data from 4 experiments, n = 16 mice per group; $P = .0072$. (B) Pooled data from 6 experiments, n = 31 mice per group; $P < .0001$. (C) Representative data from 2 experiments, n = 4 mice per group; BEN versus CY, $P = .0067$; SCs versus T cells, P = not significant. (D) BALB/c mice were given BEN or CY at various doses, followed by 400 cGy TBI and 10^7 BM cells with 3×10^6 SCs. Pooled data from 2 experiments are shown, n = 6 to 8 mice per group. For all doses, BEN versus CY, $P < .01$. (E) Peripheral blood was collected on days +7, +14, and +21 and analyzed by flow cytometry. H2kb$^+$ cells were considered to be of donor origin. Pooled data from 3 experiments are shown, with n = 7 to 13 mice per group per time point.

in engraftment kinetics or myeloid/lymphoid subset chimerism (Figure 1E and Supplementary Figure S2). Greater than 90% donor chimerism was observed in the BM on days +7 and +14, confirming a lack of graft failure (data not shown). Donor cell engraftment was confirmed in all experiments and for all donor T cell inoculums and conditioning drug doses used. In addition, complete donor cell engraftment in the blood and spleen was confirmed at the conclusion of the experiments (data not shown). Importantly, using an F1→F1 haploidentical murine BMT model, we demonstrated that the effects of BEN-TBI on GVHD are not model-specific. In CB6F1 ($H2^{b/d}$) mice transplanted with B6AF1 ($H2^{b/a}$) BM and SCs, BEN-TBI resulted in significantly increased survival (Supplementary Figure S3) and equivalent full donor chimerism compared with CY-TBI (data not shown).

BEN-TBI Results in Decreased GVHD Morbidity

To further evaluate differences in GVHD between the 2 conditioning regimens, mouse weights and clinical GVHD scores were monitored over time [35]. Although the BEN-TBI- conditioned mice initially showed weight loss and GVHD scores comparable to those of CY-TBI-conditioned mice, they began to improve by approximately 2 weeks post-BMT, whereas the CY-TBI-conditioned mice continued to deteriorate (Figure 2A and B). In agreement with the clinical GVHD scores seen early after BMT, pathological examination of liver, skin, and intestines demonstrated comparable histological evidence of GVHD (Supplementary Figure S4). Because mice receiving CY conditioning die from GVHD by 3 to 4 weeks after BMT, we were not able to compare differences in histological GVHD between the 2 groups at later time points. To confirm that the clinical signs used to evaluate morbidity actually were due to GVHD rather than to conditioning regimen-related toxicity, additional mice received BEN-TBI or CY-TBI and syngeneic BM cells and SCs, and these mice did not exhibit clinical or histological evidence of GVHD (Supplementary Figure S5).

BEN-TBI Conditioning Results in Fewer Donor T Cells and More Gr-1$^+$ Cells in the Intestines Early Post-BMT

Increased intestinal T cell infiltration has been correlated with more severe GVHD [44,45]. In addition, increased MDSC frequency in the intestines has been associated with reduced GVHD [46]. BEN-TBI was associated with a trend toward more Gr-1$^+$ cells in the small and large intestines compared with CY-TBI early after conditioning (Figure 3A). We also found a significantly higher frequency of donor T cells in the large and small intestines of CY-TBI-conditioned mice early post-BMT (Figure 3B and C). A negative control for CD45.1$^+$ intestine infiltration is shown in Supplementary Figure S6. Conversely, early post-BMT, significantly more Gr-1$^+$ cells were present in the

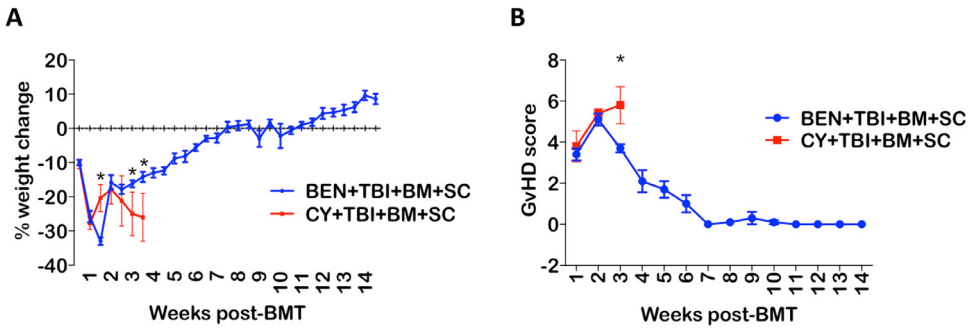

Figure 2. BEN-TBI results in decreased GvHD morbidity. BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2, 400 cGy TBI on day -1, and 10^7 BM cells with 3×10^6 SCs from C57BL/6 mice on day 0. Mice were weighed and clinically scored twice weekly. (A) Mean % weight change from the starting weight with SEM. (B) Weekly average of the mean clinical GVHD score per group with SEM. Representative data from 6 experiments are shown, n = 5 mice per group.

intestines of BEN-TBI-conditioned mice. Compared with intestines from naïve mice, both BEN-TBI- and CY-TBI-treated mice showed increased Gr-1$^+$ cells, including both Ly6G$^+$ and Ly6C$^+$ cells, consistent with reports that allogeneic BMT leads to increased numbers of Ly6G$^+$ cells in the gut [47] (Figure 3D). In summary, early post-BMT, BEN-TBI-conditioned mice have a lower T cell-to-MDSC ratio, which is consistent with less GVHD. In addition, on day +14, more Gr-1$^+$ cells were detected in the intestines of BEN-TBI-conditioned mice, indicating an enduring effect from the conditioning regimen (Figure 3E).

BEN-TBI Conditioning Leads to More Granulocytic MDSCs in the BM, Blood, and Spleen

MDSCs are also very important when present in blood and spleen post-BMT and have been shown to suppress T cell activation [29], reduce T cell infiltration of the intestines [48], and attenuate the overall impact of GVHD [29]. At 5 days after conditioning, BEN-TBI resulted in a higher number of host CD11b$^+$Gr-1^{high} cells (granulocytic MDSCs; gating shown in Figure 4A) in the BM, blood, and spleen compared with CY-TBI (Figure 4 B to D). More CD11b$^+$Gr-1^{high} cells were detected in the spleen on day 0, before transplantation (Figure 4E). Although the BEN-TBI-conditioned mice had more granulocytic MDSCs than the CY-TBI-conditioned mice, both groups had reduced numbers in the BM, blood, and spleen compared with the number of CD11b$^+$Gr-1^{high} cells found in naïve mice (Figure 4B, C, and E). This indicates that BEN-TBI preserves the myeloid compartment, specifically the granulocytic MDSCs, more so than CY-TBI. The same difference was not seen in the monocytic subset (data not shown). In addition, on day +7 post-BMT, host MDSCs in the blood were more proliferative by Ki-67 expression and donor MDSCs were more abundant in the spleens of BEN-TBI-conditioned mice (Figure 4F and G), indicating enduring differential effects of BEN on MDSCs. Of note, the CY-TBI-conditioned mice had more neutrophils in the blood on day +7, coinciding with a higher white blood cell count, with no differences at later time points, as determined by complete blood count analysis (Supplementary Figure S7).

BEN-TBI Conditioning Does Not Result in Altered Granulocytic MDSC Function Compared with CY-TBI

Given the differences in numbers of MDSCs between BEN-TBI-conditioned and CY-TBI-conditioned mice, we next sought to evaluate potential differences in MDSC function. Splenic CD11b$^+$Gr-1^{high} cells from each group suppressed T cell proliferation, confirming their identity as MDSCs (Supplementary Figure S8). No between-group difference in suppressive function was observed on day 0 before transplantation (host MDSCs) or days +7 (mixed chimerism MDSCs) and +14 (donor MDSCs) post-BMT (Figure 5A). We next investigated the mechanisms underlying MDSCs' suppressive function. MDSCs are able to suppress T cell proliferation using various mechanisms, including the expression of arginase-1 (arg-1) [49], inducible nitric oxide synthase (iNOS) [50,51], and indoleamine 2,3-dioxygenase (IDO) [52] and ROS production [53]. No difference was detected in ROS production by MDSCs isolated in BEN-TBI-conditioned and CY-TBI-conditioned mice on day 0 (Figure 5B). On the day of transplantation and days +7 and +14 post-BMT, no significant differences in mRNA levels of iNOS were observed between the BEN-TBI-conditioned and CY-TBI-conditioned mice (Figure 5C). Arg-1 was not detectable in naïve CD11b$^+$Gr-1^{high} cells or in most day 0 MDSC samples (data not shown). On days +7 and +14, differences in arg-1 levels, although trending, were not significant (Figure 5D). IDO was consistently detectable in MDSC samples only on day +7 post-BMT, when BEN-TBI-conditioned and CY-TBI-conditioned mice demonstrated similarly elevated levels of IDO mRNA compared with naïve mice (Figure 5E). In summary, we were unable to provide clear evidence of differences in the cell-intrinsic suppressive function of MDSCs between BEN-TBI-conditioned and CY-TBI-conditioned mice.

The Beneficial Effects of BEN-TBI Rely on Gr-1^{high} Cells

To further evaluate the role of MDSCs in GVHD protection following BEN-TBI conditioning, we depleted Gr-1^{high} cells pre-BMT and early post-BMT, eliminating residual host MDSCs as well as early donor MDSCs. Depletion was confirmed in the blood and spleen on the day of BEN or CY administration and transplantation (data not shown). As shown in Figure 6A, on day +7, >99% of CD11b$^+$Gr-1^{high} cells (granulocytic MDSCs) were depleted in the blood, whereas monocytic MDSCs (CD11b$^+$Gr-1^{mid} cells) were spared. Gr-1^{high} cell depletion significantly decreased survival from GVHD in the BEN-TBI-conditioned mice. No significant change in survival was seen in CY-TBI-conditioned mice (Figure 6B). This indicates that pre-BMT and early post-BMT Gr-1^{high} cells are required for BEN-TBI conditioning to suppress GHD.

G-CSF Administration Accentuates the Difference in GVHD between BEN-TBI and CY-TBI

G-CSF administration has been shown to expand the Gr-1$^+$ myeloid cell compartment [28,54,55]. Given that Gr-1 depletion exacerbates GVHD, we sought to determine whether G-CSF administration would further alleviate GVHD. G-CSF administration resulted in an overall increase in CD11b$^+$Gr-1$^+$ cells in the blood on day +7. Both monocytic MDSCs (Gr-1^{mid}) and granulocytic MDSCs (Gr-1^{high}) appeared to increase in number, but only the difference in monocytic MDSCs was

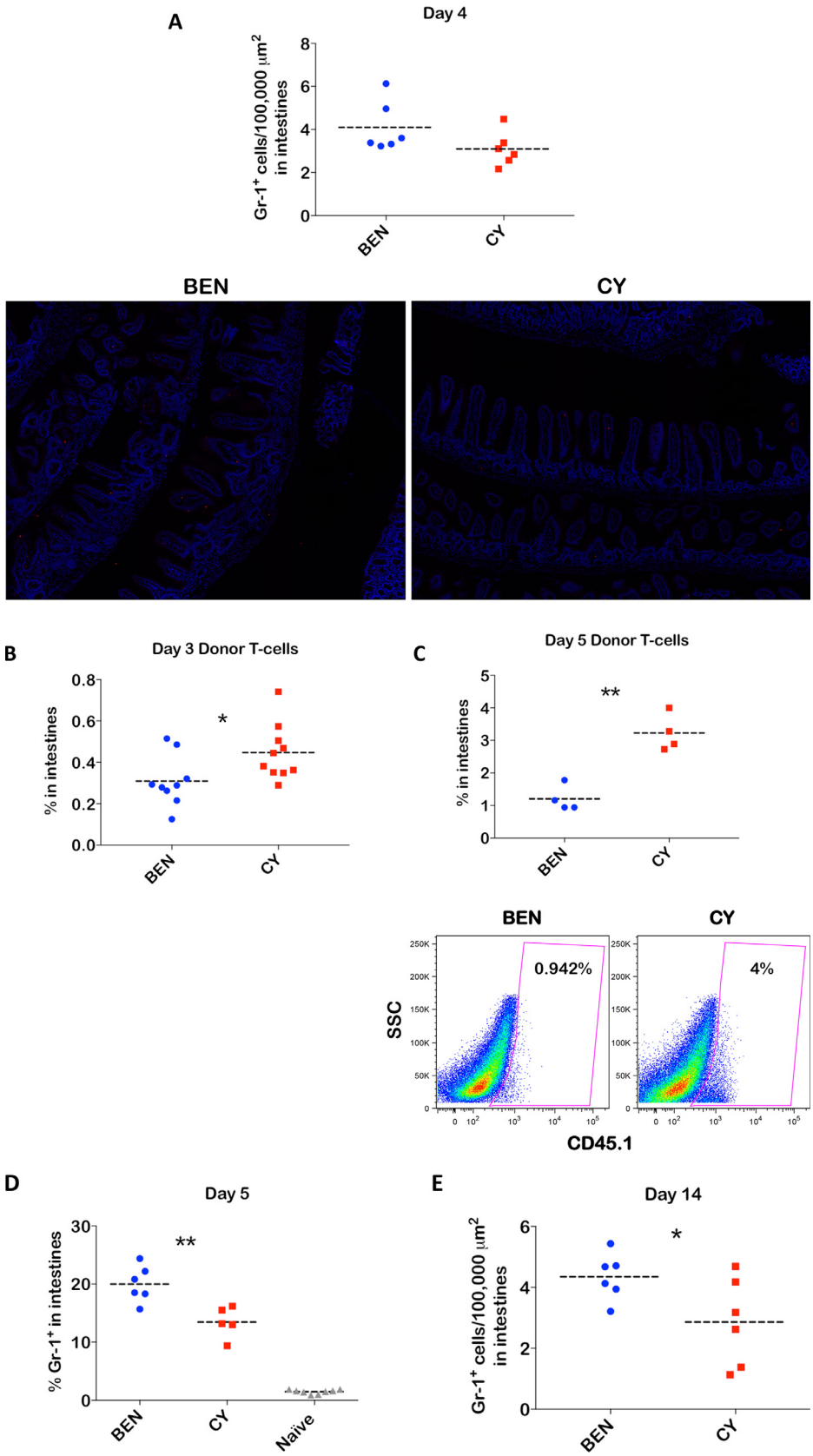

Figure 3. BEN-TBI conditioning results in fewer donor T cells and more Gr-1$^+$ cells in the intestines early post-BMT. (A) BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2 and 400 cGy TBI on day -1. On day +4, intestines were collected for analysis by immunofluorescence. Intestines were stained for Gr-1, and the number of Gr-1$^+$ cells per area of intestines was quantified. Representative images obtained at 20× are shown (blue, DAPI; red, Gr-1). Pooled data from 2 experiments are shown, n = 7 to 8 mice per group. (B to D) BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2, 400 cGy TBI on day -1, and 10^7 TCD-BM cells from C57BL/6 mice with 3×10^6 purified T cells stained with CellTrace Violet from BoyJ (CD45.1$^+$) mice on day 0. Large and small intestines were analyzed by flow

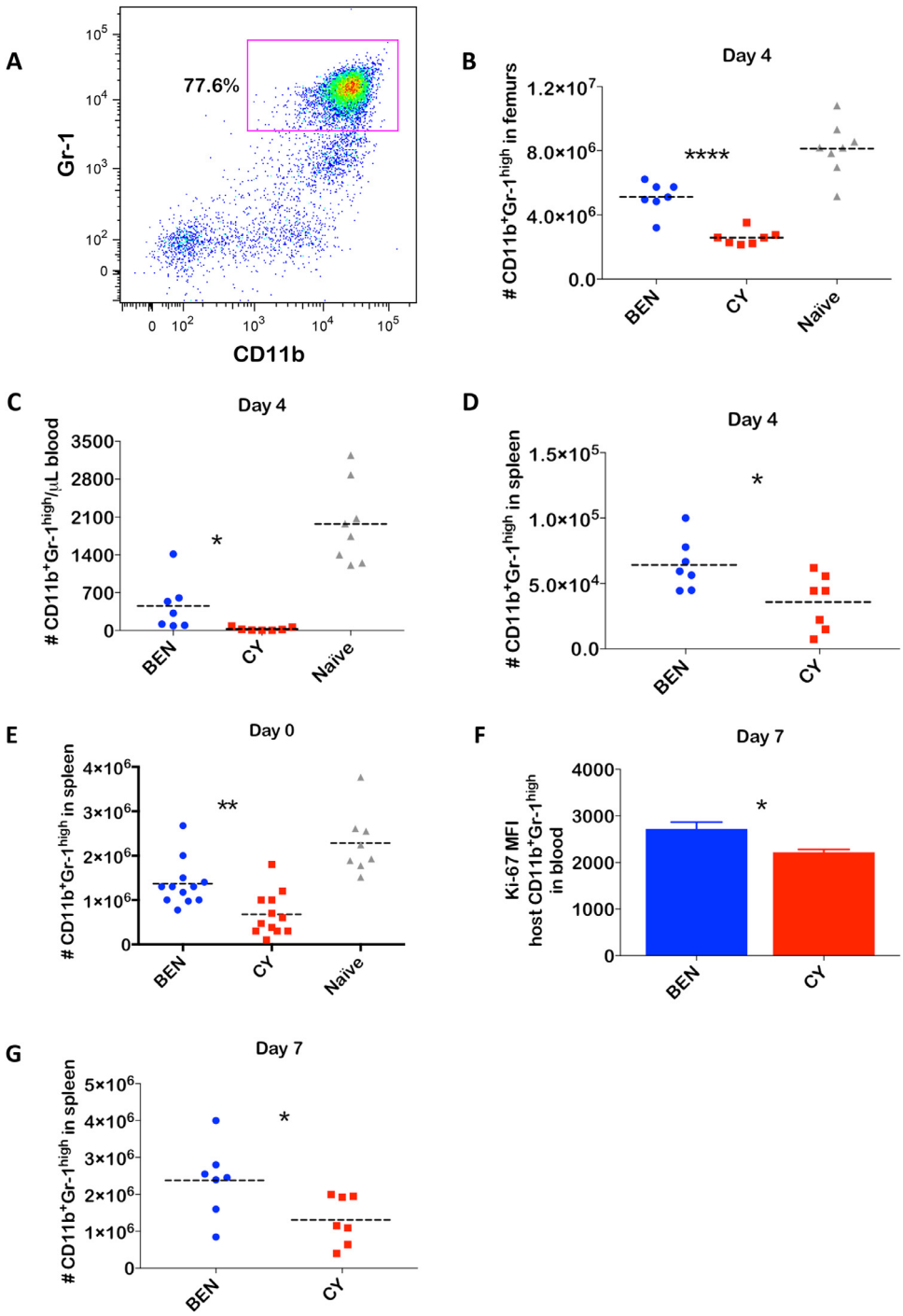

Figure 4. BEN-TBI conditioning leads to more granulocytic MDSCs in the BM, blood, and spleen. (A) Representative flow cytometry gating of the $CD11b^{+}Gr\text{-}1^{high}$ cell population. (B to D) BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2 and 400 cGy TBI on day -1. BM, blood, and spleen tissues were collected on day +4. Naïve mice were used as a control. Bone marrow (B), blood (C), and spleen (D) were analyzed by flow cytometry, and complete blood counts and percentages of $CD11b^{+}Gr\text{-}1^{high}$ cells were used to calculate absolute numbers. Pooled data from 2 experiments are shown, n = 7 to 8 mice per group. (E) Spleens were also collected on day 0, and $CD11b^{+}Gr\text{-}1^{high}$ cells were isolated using the Miltenyi kit. The number of cells isolated are shown. Pooled data from 3 experiments are shown, n = 11 to 12 mice per group. (F and G) BALB/c mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day -2, 400 cGy TBI on day -1, and 10^7 BM cells and 3×10^6 SCs from C57BL/6 mice on day 0. On day +7, blood and spleen cells were analyzed by flow cytometry. (F) Average Ki-67 expression by mean fluorescence intensity with SEM for host $CD11b^{+}Gr\text{-}1^{high}$ cells in the blood. Pooled data from 2 experiments are shown, n = 7 mice/group. (G) Numbers of splenic $CD11b^{+}Gr\text{-}1^{high}$ cells. Pooled data from 2 experiments are shown, n = 9 mice per group. $^{*}P < .05$; $^{**}P < .01$.

cytometry. (B) Percentages of donor T cells on day +3 in the leukocytes recovered from intestine digestion. Pooled data from 2 experiments are shown, n = 9 to 10 mice per group. (C) Percentages of donor T cells on day +5 in the leukocytes recovered from intestine digestion are shown. Representative data from 2 experiments are shown, n = 4 mice per group. Representative flow plots are shown as SSC versus CD45.1 (used to identify donor T cells). (D) Percentages of $Gr\text{-}1^{+}$ cells on day +5 in the leukocytes recovered from intestine digestion are shown. Naïve mice were used as a control. Pooled data from 2 experiments are shown, n = 5 to 8 mice per group. (E) BALB/c mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day -2, 400 cGy TBI on day -1, and 10^7 BM cells and 3×10^6 SCs from C57BL/6 mice on day 0. Intestines were harvested on day +14 and stained for Gr-1. Pooled data from 2 experiments are shown, n = 7 to 8 mice per group. $^{*}P < .05$; $^{**}P < .01$.

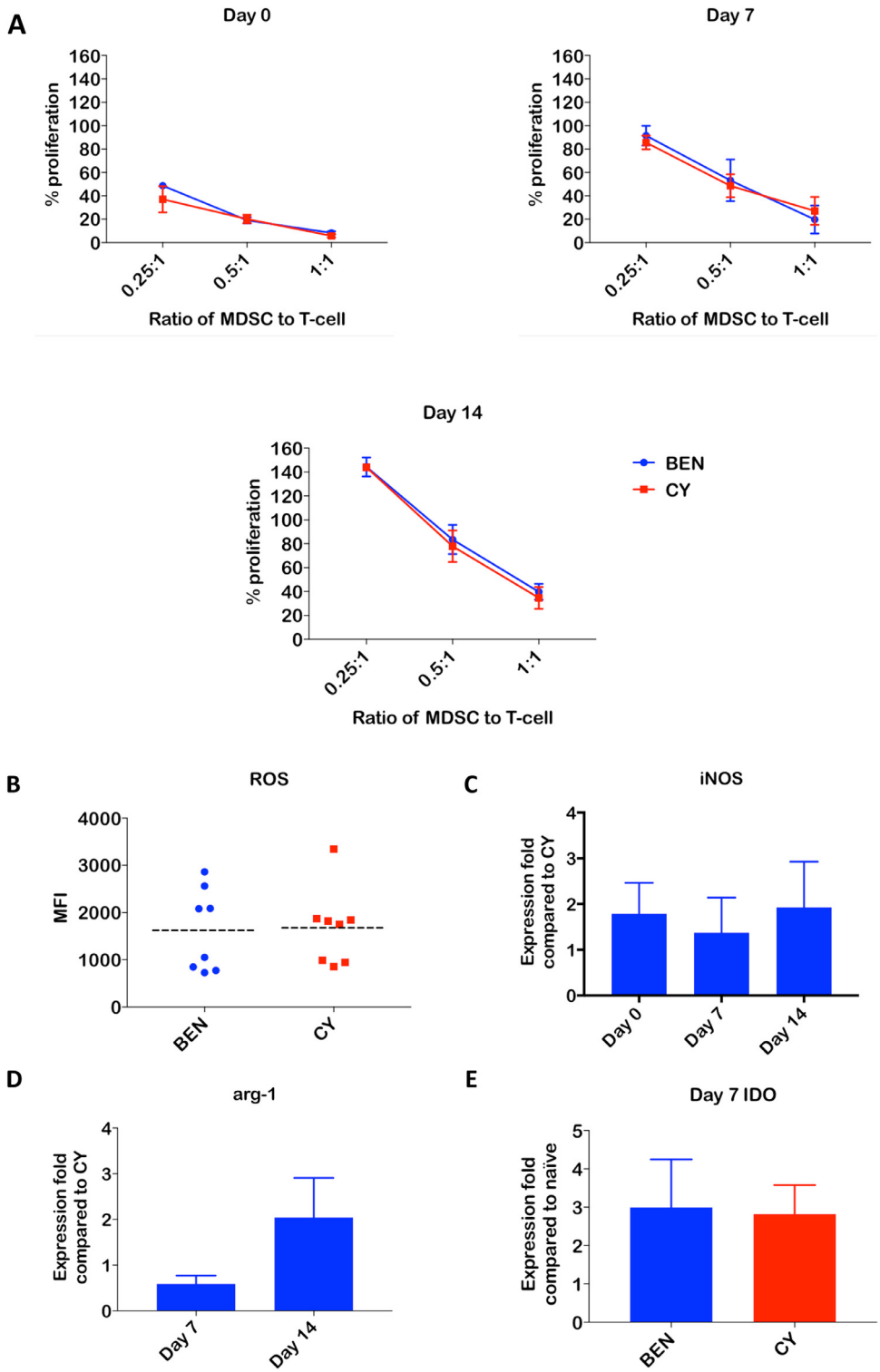

Figure 5. BEN-TBI conditioning does not result in altered granulocytic MDSC function compared with CY-TBI. BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2, 400 cGy TBI on day -1, and 10^7 BM cells with 3×10^6 SCs from C57BL/6 mice on day 0. (A) Splenic MDSCs were isolated and plated at various ratios in a suppression assay with CellTrace Violet-stained CD3/CD28 bead-activated C57BL/6 T cells. Proliferation was assessed by flow cytometry after 3 days of coculture. The average percent proliferation with SEM is shown compared with the control of no MDSCs. Representative data from 2 experiments are shown, n = 4 mice per group. (B) ROS production by day 0 MDSCs measured by flow cytometry. Pooled data from 2 experiments are shown, n = 8 mice per group. (C to E) Expression of iNOS, arg-1, and IDO in cDNA generated from MDSCs isolated on day 0 assessed by qRT-PCR. Average expression fold compared with CY-TBI $CD11b^+Gr\text{-}1^{high}$ cells (C and D) or $CD11b^+Gr\text{-}1^{high}$ cells from naïve mice (E) is shown with SEM. Representative data from 2 experiments are shown, n = 4 mice per group.

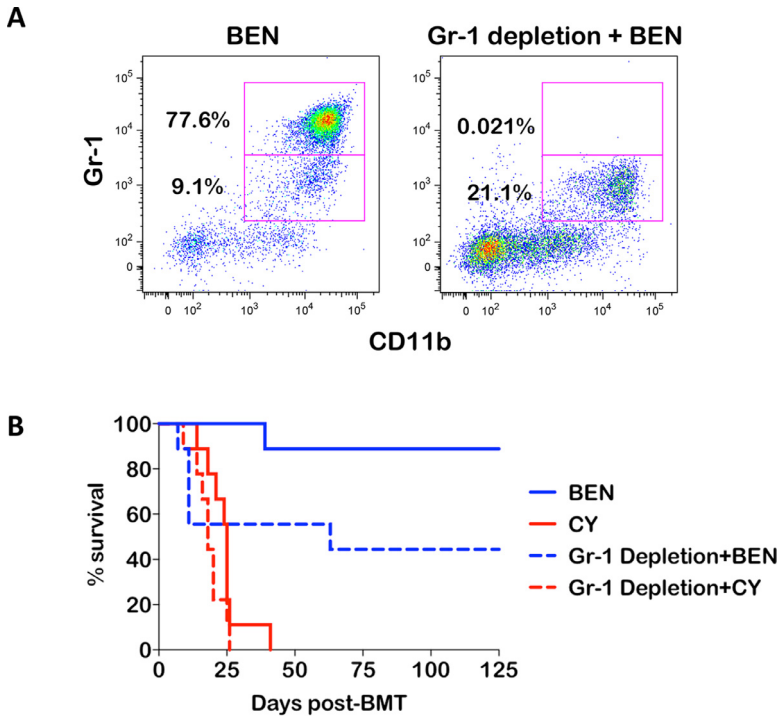

Figure 6. The beneficial effects of BEN-TBI rely on Gr-1^{high} cells. BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2, 400 cGy TBI on day -1, and 10^7 BM cells and 3×10^6 SCs from C57BL/6 mice on day 0. On days -3, -1, and +5, the appropriate groups received 200 μg of anti-Gr-1 monoclonal antibody i.p. (A) Successful depletion of CD11b$^+$Gr-1^{high} cells in blood was confirmed by flow cytometry on day +7. Representative flow plots are shown. (B) Survival data. Pooled data from 2 experiments, n = 8 mice per group. BEN versus CY, $P < .0001$; BEN versus Gr-1 dep + BEN, $P = .045$; Gr-1 dep + BEN versus Gr-1 dep + CY, $P = .09$; CY versus Gr-1 dep + CY, $P = .106$; Gr-1 dep + BEN versus CY, $P = .1234$.

significant (Figure 7A). G-CSF administration resulted in a trend toward increased survival in BEN-TBI-conditioned mice, no observed effect in CY-TBI-conditioned mice, and a widening of the survival gap between BEN-TBI and CY-TBI conditioning (Figure 7B). This points to a synergistic effect of BEN and G-CSF and supports the role of MDSCs in improved GVHD survival in the BEN-TBI-conditioned mice. Levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF, both of which can contribute to expansion or survival of these cells [28,54,56], were evaluated in plasma. GM-CSF was not detectable by our methods, and higher levels of G-CSF were not seen in the BEN-TBI-conditioned mice (data not shown).

We also investigated the transcriptional expression of G-CSF and GM-CSF receptors in the bone marrow and splenic MDSCs, to determine whether increased receptor expression in BEN-TBI mice could explain the increased numbers of MDSCs. No difference was seen in receptor expression in bone marrow (Supplementary Figure S9A) or in splenic MDSCs isolated on day +7 or +14 (data not shown). On day 0, BEN-TBI-conditioned MDSCs showed increased mRNA levels of Csf2rb, indicating potential increased sensitivity to GM-CSF (Supplementary Figure S9B). This could contribute to the survival or expansion of MDSCs. BM cells were also evaluated for levels of IFR8 (a negative regulator of MDSC development) [57–59] and PU.1 (a transcription factor up-regulated during myeloid lineage commitment) [60,61] mRNA, and no difference between the groups was noted (data not shown).

DISCUSSION

Despite the application of numerous prophylactic and therapeutic immunosuppressive approaches post-BMT, GVHD remains a serious, often fatal complication of HCT. Pretransplantation conditioning damages host tissues, resulting in the release of inflammatory stimuli that promote activation of antigen-presenting cells, setting the stage for the development of GVHD [62]. CY-TBI is a commonly used myeloablative regimen associated with significant tissue damage and GVHD [1,63]. Despite the importance of conditioning in the pathophysiology of GVHD, research on the effects of specific preparative chemotherapeutic agents on GVHD induction has been limited. Therefore, we sought to examine whether replacing CY with BEN in a TBI-based conditioning regimen would affect GVHD. We chose to focus on BEN based on our previous report revealing its immunomodulatory effects on MDSCs when given post-transplantation [24]. Although BEN has been successfully incorporated into chemotherapy-based preparative regimens previously, we provide the first experimental evidence that substituting BEN for CY in conjunction with TBI may have significant advantages in reducing GVHD morbidity and mortality. This approach may provide a safer alternative for patients requiring TBI as part of conditioning.

MDSCs, including the Gr-1^{high} granulocytic and the Gr-1^{mid} monocytic subsets, are recognized as important immunosuppressive cell populations in the control of GVHD [29,48]. In allogeneic murine BMT models, adoptive transfer of MDSCs generated in vivo or in vitro can reduce GVHD mortality and morbidity [26–29]. Furthermore, donor grafts with a higher proportion of MDSCs are correlated with reduced acute GVHD in human allogeneic HCT [30,31]. We show that BEN-TBI results in increased numbers of MDSCs, particularly the granulocytic subset, in the spleen, BM, blood, and intestines compared with CY-TBI (Figure 4). We also found decreased donor T cells in the intestines of BEN-TBI-conditioned mice compared with CY-TBI-conditioned mice (Figure 3). This increased number of suppressive CD11b$^+$Gr-1$^+$ cells, particularly in target

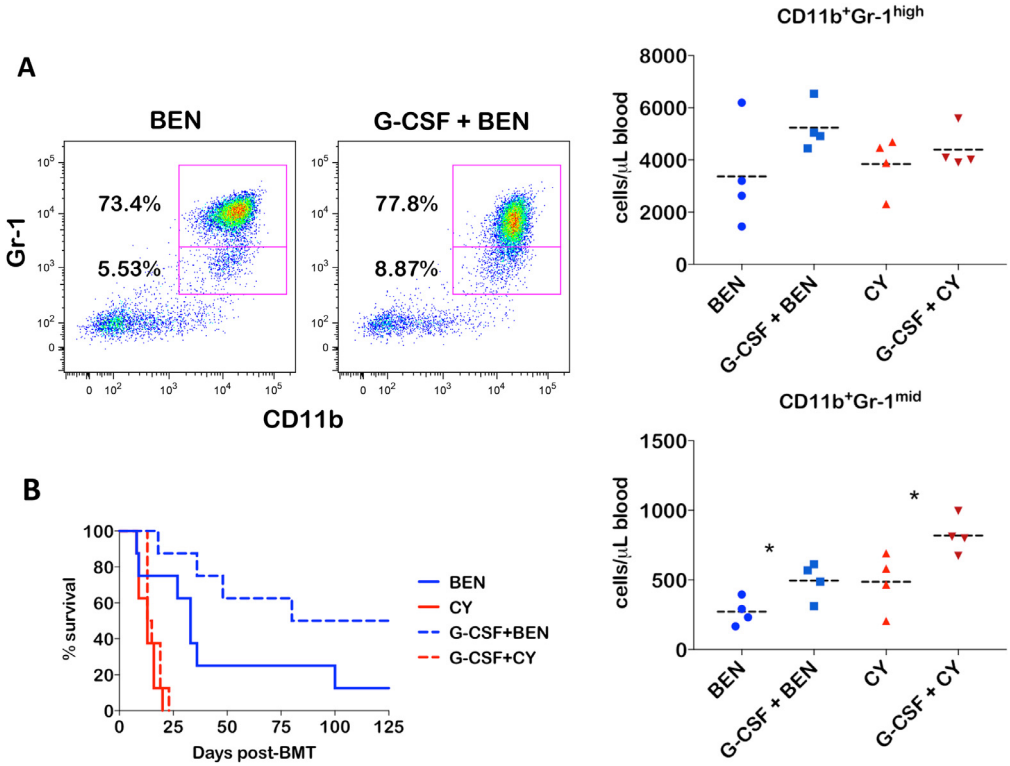

Figure 7. G-CSF administration accentuates the difference in GVHD between BEN-TBI and CY-TBI. BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day -2, 400 cGy TBI on day -1, and 10^7 TCD-BM cells with 3×10^6 purified T cells (tT) from C57BL/6 mice on day 0. Appropriate groups received 250 μg/kg G-CSF s.c. on days -2 through +11. (A) Expansion of the $CD11b^+Gr\text{-}1^+$ population was confirmed by flow cytometry. Representative flow plots and gating of high and mid populations are shown, as well as average absolute numbers of these cells in the blood on day +7. (B) Survival. Pooled data from 2 experiments are shown, n = 9 mice per group. BEN versus CY, $P = .0084$; BEN versus G-CSF + BEN, $P = .068$; G-CSF + BEN versus G-CSF + CY, $P = .0003$; CY versus G-CSF + CY, $P = .26$.

GVHD organs, may play at least a partial role in the superior survival of BEN-TBI-conditioned mice. Although we found no difference in the suppressive activity of MDSCs between the 2 groups on a per cell basis, the increased number of MDSCs in multiple tissues following BEN-TBI conditioning may account for the decrease in GVHD. The MDSC-to-T cell ratio at the time of engraftment has been shown to predict the development of GVHD, particularly in the gut [48], which is where we found a clear increase in the MDSC-to-T cell ratio in BEN-TBI-conditioned mice (~20 $Gr\text{-}1^+$ cells to 1 donor T cell, compared with 4 to 1 with CY-TBI) (Figure 3).

The importance of MDSCs in BEN-TBI mice was further confirmed using Gr-1-depleting antibodies. When $Gr\text{-}1^{high}$ cells were depleted, the survival of BEN-TBI-conditioned mice significantly decreased (Figure 6). This suggests that granulocytic MDSCs are required for the beneficial effects of BEN-TBI conditioning, and, importantly, that the monocytic MDSCs alone, which were not depleted, are not sufficient for the BEN-induced suppression of GVHD. These results should be interpreted with some caution, considering that MDSC expansion via G-CSF administration resulted in a trend toward increased survival in BEN-TBI mice and widened the survival gap between BEN and CY conditioning (Figure 7). Importantly, G-CSF expanded both the monocytic and granulocytic subsets of MDSCs, with a greater effect on the monocytic subset, whereas our data indicate that the MDSC-related effects of BEN are attributed primarily to the granulocytic subset. G-CSF is commonly given to patients following HCT to increase neutrophil counts [64]. Although retrospective clinical studies have not shown a reduction in GVHD with G-CSF administration [65,66], the synergistic beneficial effect of G-CSF and BEN-TBI conditioning in our murine model warrants further investigation as another potential clinical advantage of BEN-TBI conditioning.

We observed a moderate increase in the GM-CSF receptors in BEN-TBI-conditioned MDSCs, which may contribute to the preservation of these cells (Supplementary Figure S9B). Given that certain cytokines, including IL-6, VEGF, Flt3L, and M-CSF [67–69], also have been shown to promote MDSC expansion or survival, additional studies are needed to further elucidate how BEN-TBI is affecting these factors and promoting increased MDSC numbers.

We saw no difference in the suppressive functions of granulocytic MDSCs by T cell suppression assays, ROS production, and mRNA levels of arg-1, iNOS, and IDO. However, our investigation of MDSC function was not exhaustive and other functional aspects need further investigation in the context of BEN-TBI. In addition to overall suppression of T cell proliferation, MDSCs can confer antigen-specific tolerance [70,71]. MDSCs have been shown to modify tyrosine residues in the T cell receptors of CD8 T cells, resulting in their inability to bind peptide-MHC but allowing them to retain their ability to respond to nonspecific stimulation [70]. In addition, MDSCs have been shown to engage in bidirectional crosstalk with dendritic cells and macrophages, resulting in increased immunosuppression [72,73], partially through increased IL-10 production. BEN has been shown to increase IL-10 production specifically in B cells [74], supporting the notion that BEN-TBI creates an anti-inflammatory cytokine milieu. We have not yet explored how BEN-TBI may be impacting the induction of antigen-specific tolerance by MDSCs or the crosstalk of MDSCs

with other cell types. Because MDSCs have complex, multifaceted functions and means of expansion and survival, much remains to be elucidated on how BEN-TBI is affecting $CD11b^{+}Gr\text{-}1^{high}$ granulocytic MDSCs and their interactions with other immune cells. Although our data indicate that MDSCs have an important role in the mechanism by which BEN-TBI limits GVHD, it is likely that other immune cells have salient roles as well, warranting further investigation.

Interestingly, recent publications by Zeiser et al. have highlighted the important role of $Gr\text{-}1^{+}$ granulocytes in GVHD, particularly in the gut. These studies demonstrated that $Ly6G^{+}$ cell depletion can mitigate GVHD in murine models [47,75]. Granulocytes (defined by Gr-1 or Ly6G expression) are a functionally heterogeneous population comprising both proinflammatory (as seen in these reports) and anti-inflammatory (as demonstrated by our data) cells. Our research findings add to the literature regarding this dynamic, complex subset of cells. Our data indicate that $Gr\text{-}1^{high}$ cell depletion, comparable to $Ly6G^{+}$ cell depletion, exacerbates GVHD when BEN-TBI is used as conditioning and has no effect on survival when CY-TBI is used (Figure 6). It is important to note that Zeiser et al. used conditioning regimens of TBI, CY and busulfan, and CY and fludarabine and did not combine chemotherapy with TBI, providing a potential explanation for these differing results, given that the combination of CY and TBI has been shown to have synergistic tissue-damaging effects [76]. They also did not use BEN, which we believe differentially affects myeloid cells. In addition, our 2 groups used different timing of depletion antibody administration. Moreover, Zeiser et al. observed that the effect of $Ly6G^{+}$ granulocytes on GVHD is dependent on intestinal microbiota [47,75]. How BEN-TBI may affect intestinal microbiota differently than CY-TBI and other conditioning regimens, thereby modifying the function and role of $Ly6G^{+}$ cells, remains to be determined.

In summary, BEN-TBI is associated with reduced GVHD, owing in part to differential effects of BEN compared with CY on $CD11b^{+}Gr\text{-}1^{high}$ myeloid cells. This conditioning regimen warrants further investigation as an alternative for patients requiring TBI-based conditioning for HCT, and studies evaluating the effects of BEN-TBI on the graft-versus-leukemia effect are ongoing in our laboratory.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at https://doi.org/10.1016/j.bbmt.2018.10.009.

REFERENCES

1. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood*. 2014;124:344–353.
2. Nakasone H, Fukuda T, Kanda J, et al. Impact of conditioning intensity and TBI on acute GVHD after hematopoietic cell transplantation. *Bone Marrow Transplant*. 2015;50:559–565.
3. Marks DI, Forman SJ, Blume KG, et al. A comparison of cyclophosphamide and total body irradiation with etoposide and total body irradiation as conditioning regimens for patients undergoing sibling allografting for acute lymphoblastic leukemia in first or second complete remission. *Biol Blood Marrow Transplant*. 2006;12:438–453.
4. Woods WG, Ramsay NK, Weisdorf DJ, et al. Bone marrow transplantation for acute lymphocytic leukemia utilizing total body irradiation followed by high doses of cytosine arabinoside: lack of superiority over cyclophosphamide-containing conditioning regimens. *Bone Marrow Transplant*. 1990;6:9–16.
5. Holter-Chakrabarty JL, Pierson N, Zhang MJ, et al. The sequence of cyclophosphamide and myeloablative total body irradiation in hematopoietic cell transplantation for patients with acute leukemia. *Biol Blood Marrow Transplant*. 2015;21:1251–1257.
6. Deeg HJ, O'Donnell M, Tolar J, et al. Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. *Blood*. 2006;108:1485–1491.
7. Svenberg P, Remberger M, Svennilson J, et al. Allogenic stem cell transplantation for nonmalignant disorders using matched unrelated donors. *Biol Blood Marrow Transplant*. 2004;10:877–882.
8. Bacigalupo A, Socie G, Lanino E, et al. Fludarabine, cyclophosphamide, antithymocyte globulin, with or without low-dose total body irradiation, for alternative donor transplants, in acquired severe aplastic anemia: a retrospective study from the EBMT-SAA Working Party. *Haematologica*. 2010;95:976–982.
9. Deeg HJ, Amylon ID, Harris RE, et al. Marrow transplants from unrelated donors for patients with aplastic anemia: minimum effective dose of total body irradiation. *Biol Blood Marrow Transplant*. 2001;7:208–215.
10. Brodsky RA, Luznik L, Bolaños-Meade J, Leffell MS, Jones RJ, Fuchs EJ. Reduced-intensity HLA-haploidentical BMT with post-transplantation cyclophosphamide in nonmalignant hematologic diseases. *Bone Marrow Transplant*. 2008;42:523–527.
11. Robinson KS, Williams ME, van der Jagt RH, et al. Phase II multicenter study of bendamustine plus rituximab in patients with relapsed indolent B-cell and mantle cell non-Hodgkin's lymphoma. *J Clin Oncol*. 2008;26:4473–4479.
12. Kahl BS, Bartlett NL, Leonard JP, et al. Bendamustine is effective therapy in patients with rituximab-refractory, indolent B-cell non-Hodgkin lymphoma: results from a multicenter study. *Cancer*. 2010;116:106–114.
13. Rigacci L, Puccini B, Cortelazzo S, et al. Bendamustine with or without rituximab for the treatment of heavily pretreated non-Hodgkin's lymphoma patients: a multicenter retrospective study on behalf of the Italian Lymphoma Foundation (FIL). *Ann Hematol*. 2012;91:1013–1022.
14. Corazzelli G, Angrilli F, D'Arco A, et al. Efficacy and safety of bendamustine for the treatment of patients with recurring Hodgkin lymphoma. *Br J Haematol*. 2013;160:207–215.
15. Castelli R, Bergamaschini L, Deliliers GL. First-line treatment with bendamustine and rituximab, in patients with intermediate-/high-risk splenic marginal zone lymphomas. *Med Oncol*. 2017;35:15.
16. Mondello P, Steiner N, Willenbacher W, et al. Bendamustine plus rituximab versus R-CHOP as first-line treatment for patients with follicular lymphoma grade 3A: evidence from a multicenter, retrospective study. *Oncologist*. 2018;23:454–460.
17. Bergmann MA, Goebeler ME, Herold M, et al. Efficacy of bendamustine in patients with relapsed or refractory chronic lymphocytic leukemia: results of a phase I/II study of the German CLL Study Group. *Haematologica*. 2005;90:1357–1364.
18. Quinquenel A, Willekens C, Dupuis J, et al. Bendamustine and rituximab combination in the management of chronic lymphocytic leukemia-associated autoimmune hemolytic anemia: a multicentric retrospective study of the French CLL intergroup (GCFLLC/MW and GOELAMS). *Am J Hematol*. 2015;90:204–207.
19. Penne M, Sarraf Yazdy M, Nair KS, Cheson BD. Extended follow-up of patients treated with bendamustine for lymphoid malignancies. *Clin Lymphoma Myeloma Leuk*. 2017;17:637–644.

20. Gilli S, Novak U, Taleghani BM, et al. BeEAM conditioning with bendamustine-replacing BCNU before autologous transplantation is safe and effective in lymphoma patients. *Ann Hematol*. 2017;96:421–429.
21. Martino M, Tripepi G, Messina G, et al. A phase II, single-arm, prospective study of bendamustine plus melphalan conditioning for second autologous stem cell transplantation in de novo multiple myeloma patients through a tandem transplant strategy. *Bone Marrow Transplant*. 2016;51:1197–1203.
22. Khouri IF, Wei W, Korbling M, et al. BFR (bendamustine, fludarabine, and rituximab) allogeneic conditioning for chronic lymphocytic leukemia/lymphoma: reduced myelosuppression and GVHD. *Blood*. 2014;124:2306–2312.
23. Khouri IF, Sui D, Jabbour EJ, et al. Bendamustine added to allogeneic conditioning improves long-term outcomes in patients with CLL. *Bone Marrow Transplant*. 2017;52:28–33.
24. Stokes J, Hoffman EA, Zeng Y, Larmonier N, Katsanis E. Post-transplant bendamustine reduces GvHD while preserving GvL in experimental haploidentical bone marrow transplantation. *Br J Haematol*. 2016;174:102–116.
25. Alizadeh D, Trad M, Hanke NT, et al. Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. *Cancer Res*. 2014;74:104–118.
26. Wang D, Yu Y, Haarberg K, et al. Dynamic change and impact of myeloid-derived suppressor cells in allogeneic bone marrow transplantation in mice. *Biol Blood Marrow Transplant*. 2013;19:692–702.
27. Messmann JJ, Reisser T, Leithäuser F, Lutz MB, Debatin KM, Strauss G. In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling antitumor cytotoxicity. *Blood*. 2015;126:1138–1148.
28. Joo YD, Lee SM, Lee SW, et al. Granulocyte colony-stimulating factor-induced immature myeloid cells inhibit acute graft-versus-host disease lethality through an indoleamine dioxygenase-independent mechanism. *Immunology*. 2009;128(1 suppl):e632–e640.
29. Highfill SL, Rodriguez PC, Zhou Q, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood*. 2010;116:5738–5747.
30. Fan Q, Liu H, Liang X, et al. Superior GVHD-free, relapse-free survival for G-BM to G-PBSC grafts is associated with higher MDSCs content in allografting for patients with acute leukemia. *J Hematol Oncol*. 2017;10:135.
31. Vendramin A, Gimondi S, Bermema A, et al. Graft monocytic myeloid-derived suppressor cell content predicts the risk of acute graft-versus-host disease after allogeneic transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood stem cells. *Biol Blood Marrow Transplant*. 2014;20:2049–2055.
32. Chandrashekar DV, Suresh PS, Kumar R, et al. Sensitive LC-MS/MS method for the simultaneous determination of bendamustine and its active metabolite, γ-hydroxybendamustine in small volume mice and dog plasma and its application to a pharmacokinetic study in mice and dogs. *Drug Res (Stuttg)*. 2017;67:497–508.
33. Sadagopan N, Cohen L, Roberts B, Collard W, Omer C. Liquid chromatography-tandem mass spectrometric quantitation of cyclophosphamide and its hydroxy metabolite in plasma and tissue for determination of tissue distribution. *J Chromatogr B Biomed Sci Appl*. 2001;759:277–284.
34. Srinivas NR, Richter W, Devaraj VC, Suresh PS, Bhamdipati RK, Mullangi R. Infusion rate-dependent pharmacokinetics of bendamustine with altered formation of γ-hydroxybendamustine (M3) metabolite following 30- and 60-min infusion of bendamustine in rats. *Drug Res (Stuttg)*. 2016;66:351–356.
35. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation, I: the roles of minor H antigens and endotoxin. *Blood*. 1996;88:3230–3239.
36. Kaplan DH, Anderson BE, McNiff JM, Jain D, Shlomchik MJ, Shlomchik WD. Target antigens determine graft-versus-host disease phenotype. *J Immunol*. 2004;173:5467–5475.
37. Zeng Y, Stokes J, Hahn S, Hoffman E, Katsanis E. Activated MHC-mismatched T helper-1 lymphocyte infusion enhances GvL with limited GvHD. *Bone Marrow Transplant*. 2014;49:1076–1083.
38. Ribechini E, Leenen PJ, Lutz MB. Gr-1 antibody induces STAT signaling, macrophage marker expression and abrogation of myeloid-derived suppressor cell activity in BM cells. *Eur J Immunol*. 2009;39:3538–3551.
39. Zeng Y, Hahn S, Stokes J, et al. Pak2 regulates myeloid-derived suppressor cell development in mice. *Blood Adv*. 2017;1:1923–1933.
40. Couter CJ, Surana NK. Isolation and flow cytometric characterization of murine small intestinal lymphocytes. *J Vis Exp*. 2016;111:54114.
41. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402–408.
42. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc*. 1958;53:457–481.
43. Peto R, Peto J. Asymptotically efficient rank invariant test procedures. *J R Stat Soc Ser A*. 1972;135:185–207.
44. Kawakami K, Minami N, Matsuura M, et al. Osteopontin attenuates acute gastrointestinal graft-versus-host disease by preventing apoptosis of intestinal epithelial cells. *Biochem Biophys Res Commun*. 2017;485:468–475.
45. Beilhack A, Schulz S, Baker J, et al. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood*. 2005;106:1113–1122.
46. Zhang J, Ramadan AM, Griesenauer B, et al. ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease. *Sci Transl Med*. 2015;7:308ra160.
47. Hülsdünker J, Ottmüller KJ, Neeff HP, et al. Neutrophils provide cellular communication between ileum and mesenteric lymph nodes at graft-versus-host disease onset. *Blood*. 2018;131:1858–1869.
48. Lim JY, Lee YK, Lee SE, et al. MyD88 in donor bone marrow cells is critical for protection from acute intestinal graft-vs.-host disease. *Mucosal Immunol*. 2016;9:730–743.
49. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. Regulation of T cell receptor CD3zeta chain expression by L-arginine. *J Biol Chem*. 2002;277:21123–21129.
50. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood*. 2007;109:1568–1573.
51. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9:162–174.
52. Muller AJ, Prendergast GC. Indoleamine 2,3-dioxygenase in immune suppression and cancer. *Curr Cancer Drug Targets*. 2007;7:31–40.
53. Corzo CA, Cotter MJ, Cheng P, et al. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol*. 2009;182:5693–5701.
54. Luyckx A, Schouppe E, Rutgeerts O, et al. G-CSF stem cell mobilization in human donors induces polymorphonuclear and mononuclear myeloid-derived suppressor cells. *Clin Immunol*. 2012;143:83–87.
55. Mielcarek M, Martin PJ, Torok-Storb B. Suppression of alloantigen-induced T-cell proliferation by $CD14^+$ cells derived from granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells. *Blood*. 1997;89:1629–1634.
56. Morales JK, Kmieciak M, Knutson KL, Bear HD, Manjili MH. GM-CSF is one of the main breast tumor-derived soluble factors involved in the differentiation of $CD11b^-Gr1^-$ bone marrow progenitor cells into myeloid-derived suppressor cells. *Breast Cancer Res Treat*. 2010;123:39–49.
57. Valanparambil RM, Tam M, Gros PP, et al. IRF-8 regulates expansion of myeloid-derived suppressor cells and $Foxp3^+$ regulatory T cells and modulates Th2 immune responses to gastrointestinal nematode infection. *PLoS Pathog*. 2017;13 e1006647.
58. Netherby CS, Messmer MN, Burkard-Mandel L, et al. The granulocyte progenitor stage is a key target of IRF8-mediated regulation of myeloid-derived suppressor cell production. *J Immunol*. 2017;198:4129–4139.
59. Waight JD, Netherby C, Hensen ML, et al. Myeloid-derived suppressor cell development is regulated by a STAT/IRF-8 axis. *J Clin Invest*. 2013;123:4464–4478.
60. Chen H, Ray-Gallet D, Zhang P, et al. PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene*. 1995;11:1549–1560.
61. Chen HM, Zhang P, Voso MT, et al. Neutrophils and monocytes express high levels of PU.1 (Spi-1) but not Spi-B. *Blood*. 1995;85:2918–2928.
62. Reddy P. Pathophysiology of acute graft-versus-host disease. *Hematol Oncol*. 2003;21:149–161.
63. Nagler A, Rocha V, Labopin M, et al. Allogeneic hematopoietic stem-cell transplantation for acute myeloid leukemia in remission: comparison of intravenous busulfan plus cyclophosphamide (Cy) versus total-body irradiation plus Cy as conditioning regimen: a report from the Acute Leukemia Working Party of the European Group For Blood and Marrow Transplantation. *J Clin Oncol*. 2013;31:3549–3556.
64. Khoury HJ, Loberiza Jr FR, Ringdén O, et al. Impact of posttransplantation G-CSF on outcomes of allogeneic hematopoietic stem cell transplantation. *Blood*. 2006;107:1712–1716.
65. Ernst P, Bacigalupo A, Ringdén O, et al. A phase 3, randomized, placebo-controlled trial of filgrastim in patients with haematological malignancies undergoing matched-related allogeneic bone marrow transplantation. *Arch Drug Inf*. 2008;1:89–96.
66. Battiwalla M, McCarthy PL. Filgrastim support in allogeneic HSCT for myeloid malignancies: a review of the role of G-CSF and the implications for current practice. *Bone Marrow Transplant*. 2009;43:351–356.
67. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res*. 2007;67:10019–10026.
68. Gabrilovich D, Ishida T, Oyama T, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood*. 1998;92:4150–4166.
69. Rosborough BR, Mathews LR, Matta BM, et al. Cutting edge: Flt3 ligand mediates STAT3-independent expansion but STAT3-dependent activation of myeloid-derived suppressor cells. *J Immunol*. 2014;192:3470–3473.
70. Nagaraj S, Gupta K, Pisarev V, et al. Altered recognition of antigen is a mechanism of $CD8^+$ T cell tolerance in cancer. *Nat Med*. 2007;13:828–835.
71. Yang F, Li Y, Wu T, et al. TNFα-induced M-MDSCs promote transplant immune tolerance via nitric oxide. *J Mol Med (Berl)*. 2016;94:911–920.
72. Ostrand-Rosenberg S, Sinha P, Beury DW, Clements VK. Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and

dendritic cells enhances tumor-induced immune suppression. *Semin Cancer Biol*. 2012;22:275–281.
73. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol*. 2007;179:977–983.
74. Lu L, Yoshimoto K, Morita A, Kameda H, Takeuchi T. Bendamustine increases interleukin-10 secretion from B cells via p38 MAP kinase activation. *Int Immunopharmacol*. 2016;39:273–279.
75. Schwab L, Goroncy L, Palaniyandi S, et al. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. *Nat Med*. 2014;20:648–654.
76. Bodge MN, Culos KA, Haider SN, Thompson MS, Savani BN. Preparative regimen dosing for hematopoietic stem cell transplantation in patients with chronic hepatic impairment: analysis of the literature and recommendations. *Biol Blood Marrow Transplant*. 2014; 20:622–629.