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ORIGINAL RESEARCH

Bendamustine with total body irradiation conditioning yields tolerant T-cells while preserving T-cell-dependent graft-versus-leukemia

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ABSTRACT

Graft-versus-host disease (GvHD) remains a significant impediment to allogeneic hematopoietic cell transplantation (HCT) success, necessitating studies focused on alleviating GvHD, while preserving the graft-versus-leukemia (GvL) effect. Based on our previous studies showing bendamustine with total body irradiation (BEN-TBI) conditioning reduces GvHD compared to the current clinical standard of care cyclophosphamide (CY)-TBI in a murine MHC-mismatched bone marrow transplantation (BMT) model, this study aimed to evaluate the role and fate of donor T-cells following BEN-TBI conditioning. We demonstrate that BEN-TBI reduces GvHD compared to CY-TBI independently of T regulatory cells (Tregs). BEN-TBI conditioned mice have a smaller proportion and less activated donor T-cells, with lower CD47 expression, early post-transplant, but no sustained phenotypic differences in T-cells. In BEN-TBI conditioned mice, donor T-cells gain tolerance specific to host MHC antigens. Though these T-cells are tolerant to host antigens, we demonstrate that BEN-TBI preserves a T-cell-dependent GvL effect. These findings indicate that BEN-TBI conditioning reduces GvHD without compromising GvL, warranting its further investigation as a potentially safer and more efficacious clinical alternative to CY-TBI.

Introduction

The success of allogeneic hematopoietic cell transplantation (HCT) as a treatment for hematological disorders and malignancies is limited by prevalent and often lethal graft-versus-host disease (GvHD). While GvHD prophylaxis and treatment is necessary, it is associated with suppression of graft-versus-leukemia (GvL).[1] This necessitates investigating novel approaches that can optimally reduce GvHD while preserving GvL. We have previously shown in a murine model that administration of bendamustine (BEN) post-haploidentical bone marrow transplantation (BMT) can reduce GvHD and preserve GvL.[2] Furthermore, we have shown that post-transplant (PT)-BEN administration results in protection from GvHD comparable to and GvL effects superior to that of PT-cyclophosphamide (CY),[2] the current clinical standard of care following haploidentical BMT.[3-5]

Based on our data indicating BEN performs favorable immunomodulatory activities when given post-transplant to reduce GvHD, we explored its effects when employed as part of the conditioning regimen. BEN has previously been shown to be a safe and effective component of chemotherapy-based conditioning regimens for autologous[6-8] and allogeneic[9,10] transplants, but it has not been studied in combination with total body irradiation (TBI). CY with TBI is the most widely applied conditioning regimen for acute lymphoblastic leukemia (ALL)[11] and is also used to condition for unrelated HCT to treat severe aplastic anemia.[12-16] However, it is associated with severe tissue damage and GvHD.[17,18] We previously reported that substituting BEN for CY, followed by TBI, as pre-transplant conditioning in an MHC-mismatched murine model significantly reduces GvHD morbidity and mortality.[19] Our previous study showed that this effect was at least partially dependent on myeloid-derived suppressor cells (MDSCs),[19] leading us to, in this study, explore the impact of BEN conditioning on the function and phenotype of donor T-cells post-transplant. We examined changes in T-cell phenotype to explain the suppression of GvHD, as Th-1/Th-2 skewing,[20,21] T regulatory cell (Treg) frequency,[22-24] and co-stimulatory/co-inhibitory molecule expression,[25-29] among other factors, have been shown to strongly impact GvHD. We found that BEN-TBI conditioning reduces GvHD compared to CY-TBI, independently of Tregs, and without appreciable differences in T-cell numbers, proportions, and phenotype in our murine model of MHC-mismatched BMT. We demonstrate further that donor-derived T-cells develop tolerance to host MHC antigens, which is observed both

in vitro and *in vivo*. Finally, despite the induction of tolerance to host MHC antigens, we demonstrate that BEN-TBI conditioning preserves a T-cell-dependent GvL effect against A20 B-cell leukemia.

Materials and methods

Mice

Age-matched 6–10 week-old female C57BL/6, BALB/c, BoyJ, and FVB/N mice were purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free conditions and cared for according to the guidelines of the University of Arizona Institutional Animal Care and Use Committee (**IACUC**). All murine experiments were conducted within IACUC approval and guidelines.

BMT model

Recipient BALB/c (H-2^d) mice received 40 mg/kg of BEN (Selleckchem S1212) intravenously (**iv**) or 200 mg/kg of CY (Sigma-Aldrich C0768) intraperitoneally (**ip**) on day −2 and 400 cGy TBI on day −1 using a Cesium 137 irradiator.[19] CY and BEN were reconstituted and diluted as previously reported.[2] Studies indicate that the drugs are cleared by 24 hours post-administration,[30-32] negating direct effects on the donor graft. On day 0, mice received 10^7 C57BL/6 (H-2^b) bone marrow (**BM**) cells with 3 × 10^6 spleen cells (**SC**) or 10^7 T-cell depleted BM cells (**TCD-BM**) with 3 × 10^6 isolated CellTrace Violet-stained (Invitrogen; C34557) total T-cells (**tT**) iv. In some experiments, donor tT or SC were isolated from congenic CD45.1+ BoyJ mice. Moribund mice were euthanized according to IACUC-approved criteria and procedures and survival was monitored daily. Mice were weighed and scored clinically every three to four days on skin integrity, fur texture, posture, and activity and cumulative GvHD scores were calculated.[33] Mice given a cumulative score of 8 following day +8 were euthanized. We additionally evaluated graft-versus-host disease free, relapse free survival (**GRFS**). This is a composite end-point used frequently in clinical studies[34-36] where incidents are defined as acute grade III–IV GvHD, chronic GvHD, relapse, or death.[37] We adapted this for our mouse model, defining incidents as a total GvHD score of 6 or more, analogous to grade III–IV GvHD in humans, quantifiable tumor burden, both following day +8, or death.

Isolations and depletions

Total T-cells were isolated from naïve BoyJ spleens by negative selection (mouse Pan T-Cell Isolation Kit II, Miltenyi Biotec; 130-095-130) with a purity of >97%. T-cells were depleted from BM using Miltenyi Biotec's CD3ε MicroBead Kit (130-094-973), with less than 0.3% CD3ε+ cells remaining. CD25+ cells were depleted from tT cells using CD25 Microbeads (Miltenyi Biotec; 130-091-072), resulting in less than 0.4% remaining CD25+ FoxP3+ cells (data not shown).

Treg generation, isolation, and suppression assays

Tregs were generated as previously reported.[38] Briefly, splenic CD4+ CD62L+ cells were isolated from BALB/c mice (Miltenyi Biotec; 130-106-643) and cultured with anti-CD3 /CD28 beads (Gibco; 11453D) and TGF-β (Peprotech; 100–21) for 3 days. Viability was determined by Trypan Blue staining (Hyclone GE Healthcare Life Sciences; SV3008401). Tregs were isolated from conditioned spleens using Miltenyi Biotec's Treg Isolation Kit (130-091-041). Suppression assays were conducted and analyzed as previously described.[2] T-cells were isolated from spleens of naïve C57BL/6 mice, CellTrace Violet stained, and stimulated with anti-CD3/CD28 beads. Tregs were co-incubated with stimulated T-cells at various ratios for 3 days. Flow cytometry was performed, followed by Modfit (Verity Software House) analysis to determine the proliferation index (**PI**) of the T-cells and calculate % proliferation.

Flow cytometry

Prior to analysis by flow cytometry, blood was collected by tail tipping or cardiac puncture and spleens were processed to single cell suspension. Red blood cells were lysed (BD Pharm Lyse, BD Biosciences; 555899) and flow cytometry was performed as previously reported.[39] Flow cytometric data were collected using an LSRFortessa cell analyzer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo 2 (Tree Star). Antibodies used were anti-mouse H2kb PerCP-eFluor710 (clone AF6-88.5.5.3; 46–5958), CD8α PE-Cy7 (53–6.7; 25–0081), CD4 APC (RM4-5; 50-148-54), FoxP3 APC (FJK-16s; 17–5773), CD45.1 APC (A20; 17–0453), GATA3 PE-Cy7 (TWAJ; 25–9966), CD134 PE-Cy7 (OX-86; 25–1341), FoxP3 PE (150D/E4; 12–4774), CD69 PE-Cy5 (H1.2F3; 15–0691), RORγt APC (AFKJS-9; 17–6988), IFN gamma PE (XMG1.2, 12–7311) (Thermo Fisher Scientific), CD45.1 PE-CF594 (A20; 562452), CD3ε PE-CF594 (145–2 C11; 562286), CD44 BB515 (IM7; 565941), CD44 BV510 (IM7; 563114) (BD Biosciences), CD47 PE-Vio770 (REA170; 130-102-383), TIM-3 VioBright FITC (REA602; 130-109-449), TIM-3 PE (REA602; 130-118-563), CD278 VioGreen (REA192; 130-100-739), CD272 PE (REA224; 130-102-689), CD4 APC-Vio770 (GK1.5; 130-102-786), CD4 VioGreen (GK1.5; 130-102-444) (Miltenyi Biotec), Tbet PE-Dazzle594 (4B10; 644828), CTLA-4 PE-Dazzle594 (UC10-4B9; 106318), CD8α Brilliant Violet 421 (53–6.7; 100738), CCR7 PE-Cy5 (4B12; 120114), PD-1 APC (29F.1A12; 135210), CD25 AlexaFluor700 (PC61; 102024), and TNF-α Brilliant Violet 510 (MP6-XT22, 506339) (Biolegend). To determine absolute cell numbers in blood, white blood cell counts were determined using a HemaVet 950 (Drew Scientific, Miami Lakes, FL, USA).[40]

Plasma and DC stimulation

BALB/c mice received BEN or CY on day −2, TBI on day −1, and were sacrificed on day 0. Blood was collected by cardiac puncture for plasma isolation and splenic dendritic cells (**DCs**) were isolated using a Pan DC Isolation Kit (Miltenyi Biotec; 130-100-875). T-cells were isolated from the spleens of naïve C57BL/6 mice and plated with 50% plasma for four

hours or plated with pan-DCs (1 DC:10 T-cells) for 16 hours. Assays were incubated at 37°C and 5% CO_2.

Mixed Leukocyte Reaction (MLR)

Recipient BALB/c (H-2^d) mice received BEN iv on day −2, TBI on day −1, and 10^6 BM cells with 3×10^6 SC from naïve C57BL/6 (H-2^b) mice on day 0. Survival was monitored and moribund mice were euthanized. Between day +100 and day +150, mice were sacrificed, splenic T-cells isolated, and their donor origin (H2kb+) was confirmed by flow cytometry. These T-cells were plated at 100,000 cells/well in a 96-well U-bottom plate. Splenocytes from naïve C57BL/6 (H-2^b), BALB/c (H-2^d), and FVB/N (H-2^q) mice were used as stimulators. Spleens were processed to single cell suspensions and red blood cells were lysed. Splenocytes then received 1500 cGy irradiation using a Cesium 137 irradiator and were plated 800,000 cells/well with the T-cells. The cells were co-incubated in DMEM high glucose media (Corning; 10-013-CV) with 10% FBS (Hyclone; SH30071), MEM nonessential amino acids (Hyclone; SH30598), 2 mM L-glutamine (Gibco; 25030081), 55 μM β-mercaptoethanol (Gibco; 21985023), and penicillin-streptomycin (Gibco; 15140122) at 37°C and 7.5% CO_2 for 3 days.[41] 0.5 μCi of tritiated-thymidine (PerkinElmer; NET027001MC) was then added for an additional 18 hours of co-culture. Plates were harvested using a Brandel Wash Pump (CH-696; Gaithersburg, MD, USA), allowed to dry overnight, and counts per minute (CPM) were determined using a MicroBeta^2 2450 Microplate Counter (PerkinElmer, Waltham, MA, USA).

Secondary transplant

Primary Transplant: Recipient BALB/c (H-2^d) mice received 40 mg/kg of BEN iv on day −2, 400 cGy TBI on day −1, and 10^6 BM cells with 3×10^6 SC from naïve BoyJ (H-2^b) mice on day 0. Survival was monitored and moribund mice were euthanized. Between day +100 and day +150, mice were sacrificed and splenic T-cells were isolated. Secondary transplant: Recipient BALB/c mice (H-2^d) received 850 cGy TBI on day −1 and 10^6 TCD-BM from naïve C57BL/6 mice (H-2^b) on day 0. These recipient mice additionally received 10^6 T-cells of confirmed donor BoyJ origin (H-2^b; H2kb +CD45.1+) isolated from the surviving primary transplant recipients. Survival was monitored and moribund mice were euthanized. Engraftment of the T-cells (H2kb+CD45.1+) and BM (H2kb+CD45.2+) were monitored over time.

Tumor cells, imaging, and in vivo depletions

A20-luciferase (A20-luc), a BALB/c B-cell lymphoblastic leukemia cell line, was generously provided by Dr. Xue-Zhong Yu, MD (Medical University of South Carolina) and has been used previously in murine BMT studies.[2] A20-luc was cultured in RPMI 1640 (Hyclone SH30027) with 10% FBS, MEM, and sodium pyruvate (Hyclone SH30239) at 37°C and 5% CO_2 and administered iv (0.1x10^6) on day 0, with the BMT. To image tumor burden, A20-luc bearing mice were given luciferin (GoldBio; LUCK) ip 0.15 mg/g, anesthetized with isoflurane (Piramal Critical Care; 440532079), and imaged

using a LagoX (Spectral Instruments Imaging, Tucson, AZ, USA). Luminescence was quantified using AmiView software (Spectral Instruments Imaging) and presented as ln(photons/second). For depletion experiments, injections of depletion antibodies (NK1.1, BE0036; GK1.5, BE0003-1; 2.43, BE0061) were given ip 200 μg weekly beginning on day +3 (Bio X Cell).

Statistics

Kaplan–Meier survival curves were generated and the log-rank statistic was used to evaluate differences between conditions.[42,43] Mann–Whitney tests were used to determine other differences between groups. P values less than 0.05 were considered statistically significant and are indicated in the graph or figure legend. If statistics are not indicated, the differences were nonsignificant.

Results

BEN-TBI conditioning improves survival and decreases morbidity from GvHD

Using a fully MHC-mismatched murine BMT model (C57BL/6 → BALB/c), comparing BEN-TBI to CY-TBI conditioning, we confirmed that BEN-TBI conditioning significantly protects recipients from GvHD lethality and morbidity, demonstrated by reduced GvHD score and weight loss (Figure 1(a–c)). We have previously reported that the BEN and CY doses used are comparable, comprising ~50% of the maximum tolerated dose in BALB/c mice, and that BEN-TBI and CY-TBI show comparable rates of complete engraftment.[19] Additionally, when applied in a syngeneic transplant setting, BEN-TBI and CY-TBI conditioning do not result in clinical toxicity or lethality, confirming that the difference in morbidity and mortality in this MHC-mismatched setting is due to GvHD.[19]

BEN-TBI conditioning improves GvHD independently of donor Tregs

Having previously correlated the reduction of GvHD with BEN-TBI to an increase in MDSCs,[19] we sought to evaluate the effect of BEN on Treg number and function, as well as the necessity of Tregs for the reduction of GvHD seen with BEN-TBI. We found no difference in number (Figure 2(a)) or proportion (data not shown) of Tregs between BEN-TBI and CY-TBI conditioned mice. Representative flow from day +35 is shown. We further investigated the effect of BEN-TBI on this subset of cells by isolating splenic Tregs from BEN-TBI or CY-TBI conditioned mice just prior to transplant and on days +7 and +14 post-BMT. We plated isolated Tregs with CD3/CD28 stimulated, CellTrace Violet-stained T-cells from naïve C57BL/6 mice to evaluate the suppressive function of the conditioned Tregs. While we observed increased suppressive function post-conditioning compared to Tregs from untreated naïve mice, there was no difference in suppressive function between BEN-TBI and CY-TBI conditioning (Figure 2(b)).

We additionally investigated whether Tregs are required for the reduction of GvHD observed with BEN-TBI. It is well-
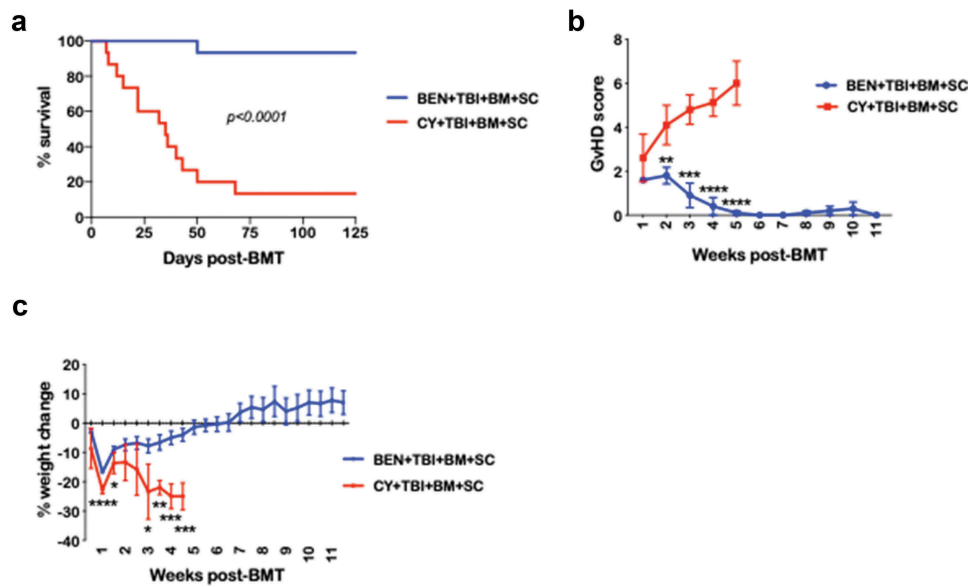
Figure 1. BEN-TBI conditioning improves survival and decreases morbidity from GvHD. BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve C57BL/6 mice on day 0. (a) Survival is shown. Pooled data from 3 experiments are shown, n = 15 mice/group, $p < .0001$. (b) Average weekly GvHD score with SEM is shown. Representative data from 3 experiments is shown, n = 5 mice/group. (c) Average % weight change from starting weight with SEM is shown. Representative data from 3 experiments is shown, n = 5 mice/group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

established that depletion of CD25+ cells, and thus, Tregs, from the donor graft exacerbates GvHD.[22,44-46] However, in our model, we demonstrate that depleting CD25+ cells from the donor T-cell graft does not significantly impact GvHD survival in BEN-TBI conditioned mice (Figure 2(c)). This indicates that Tregs are not necessary for the reduction of GvHD observed with BEN-TBI conditioning. In fact, though not significant, with BEN-TBI conditioning, we see a trend towards an increase in survival following depletion of CD25 + cells from the graft. CD25+ depletion successfully eliminates FoxP3+ cells, as well as a portion of the FoxP3- conventional CD4+ T-cell population, resulting in an increase in the ratio of CD8 to CD4 T-cells infused (data not shown). We have found that, in this model, GvHD is less severe when isolated CD8+ T-cells are transplanted in the absence of CD4+ T-cells compared to an equal number of total T-cells (Supplemental Figure 1), potentially explaining the decreased GvHD lethality observed with CD25+ depletion.

Lastly, we investigated the effect of BEN on the *in vitro* generation of Tregs by generating Tregs in the presence of various BEN concentrations. Following a three-day culture, we observed no difference in percentage of CD4 + CD25+ FoxP3+ cells (Figure 2(d); left) or cell viability (Figure 2(d); middle) regardless of BEN concentration. To evaluate Treg function, we washed the BEN out and plated the Tregs with CD3/CD28 activated, CellTrace Violet-stained T-cells from naïve mice. The generated Tregs were, in fact, suppressive and we saw no difference in suppressive function with the addition of BEN (Figure 2(d); right). Representative CellTrace Violet dilution by flow cytometry and PIs, indicating comparable suppression, are shown (Figure 2(d); bottom). These

data indicate that *in vitro* exposure to BEN does not affect Treg development or function.

BEN-TBI does not result in appreciable donor T-cell phenotypic differences post-transplant when compared to CY-TBI

Following the exclusion of Tregs as the mechanism by which BEN-TBI results in suppression of GvHD, we focused our studies on assessing differences in donor T-cell phenotype and effector function following transplant. We initially sought to investigate the fate of adoptively transferred donor T-cells in the early post-transplant period, as we hypothesized that the host environment of BEN-TBI conditioned mice might skew the donor T-cells toward phenotypes that minimize GvHD. Prior to infusion, we stained CD45.1+ donor T-cells with CellTrace Violet to monitor their proliferation *in vivo*, then collected spleen and blood on day +3. In both the blood and spleen, BEN-TBI conditioned mice had a lower proportion of donor T-cells, with no difference in absolute donor T-cell number compared to CY-TBI (Figure 3(a)). We analyzed CellTrace Violet dilution to evaluate proliferation and noted no significant difference in the proliferation of donor T-cells (Figure 3(b)). We further phenotyped the T-cells, grouping them into CellTrace^low cells (those that had proliferated) and CellTrace^high cells (those that had not proliferated) and observed a lower percentage of CD25+ cells among the proliferating T-cells in BEN-TBI mice in the blood on day +3 (Figure 3(c)). This difference persisted through day +5 (data not shown). Representative stratification of CellTrace Violet populations is shown in Supplemental Figure 2. This shows that although T-cells from BEN-TBI conditioned mice

proliferate similarly to those from CY-TBI conditioned mice, they are less activated, shown by CD25 expression. We also evaluated intracellular TNFα and IFNγ, both pro-inflammatory cytokines, and saw reduced intracellular TNFα in the proliferated donor T-cells in the blood of BEN-TBI conditioned mice compared to CY-TBI (Supplemental

Figure 3). Lastly, we evaluated CD47 expression, as it has been shown to exacerbate GvHD by preventing the clearance of alloreactive T-cells,[47] and observed a lower expression of CD47 on donor T-cells in the blood on day +3 in BEN-TBI conditioned mice (Figure 3(c)). In summary, in the early post-transplant period, we observed a lower proportion
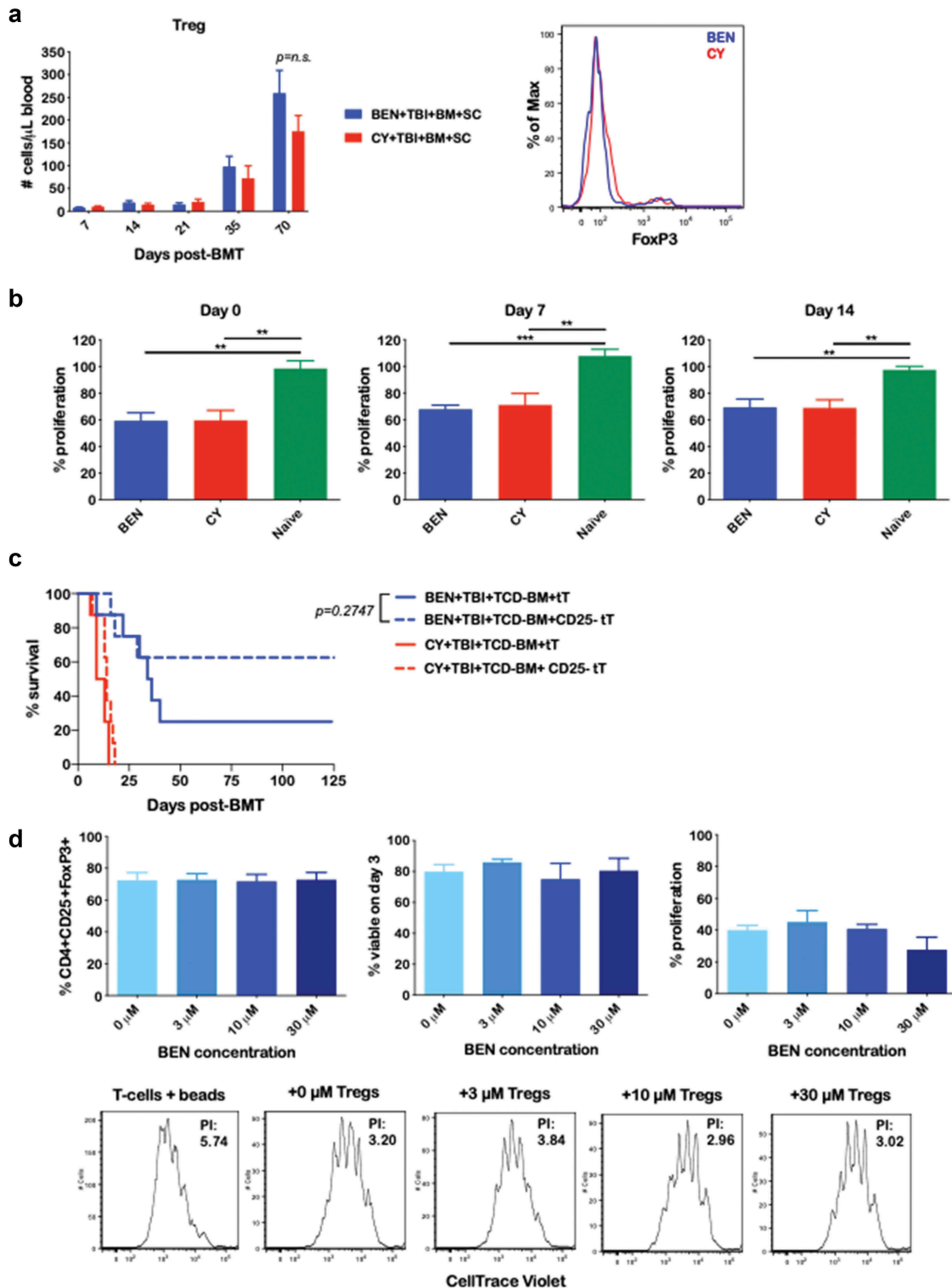
Figure 2. BEN-TBI conditioning improves GvHD independently of donor Tregs. (a) BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve C57BL/6 mice on day 0. Peripheral blood was collected on days +7, +14, +21, +35, and +70 and stained for CD4, CD25, and

and less activated donor T-cells in BEN-TBI mice, with decreased expression of CD47, compared to CY-TBI, all of which favors a reduction of GvHD.

We then examined the peripheral blood T-cell phenotype through day +70. We observed no difference in absolute numbers of CD8+ and CD4+ T-cells in the blood (Figure 3(d)). We evaluated the absolute numbers of Th-1, Th-2, and Th-17 cells, by Tbet, GATA3, and RORγt expression, respectively. We found more Th-2 cells, which have been associated with less GvHD,[20,48] in BEN-TBI mice compared to CY-TBI mice on day +7 (Figure 3(e)), a significant difference that was not sustained. We also found fewer Th-17 cells in BEN-TBI conditioned mice on day 14 (Figure 3(e)), a modest but significant difference. Th-17 cells are generally thought to contribute to GvHD pathogenesis, though some groups have concluded they can ameliorate GvHD.[49] Representative histograms from day +7 are shown (Figure 3(e)). We additionally evaluated co-stimulatory molecule expression, typically associated with increases in GvHD, including CD278 (ICOS)[29] and CD134 (OX40),[50] and co-inhibitory molecule expression, typically associated with decreases in GvHD, including CTLA-4,[26] PD-1,[27,51] CD272[52] (BTLA), and TIM3.[25,53] We saw no differences in absolute numbers of cells expressing these markers in peripheral blood (Figure 3(f–g)). Representative CD4+ flow plots from day +35 are shown in Supplemental Figure 4. We also saw no lasting differences in the percentage of T-cells expressing these markers (data not shown). We assessed effector memory, central memory, and naïve T-cells, as well as CD25 and CD47 expression, and again found no sustained differences in absolute cell numbers in peripheral blood (Supplemental Figure 5). These data fail to demonstrate any reliable trends in T-cell phenotypic differences between mice conditioned with BEN-TBI and those receiving CY-TBI.

Given the critical roles of cytokine milieu[54,55] and host antigen presenting cells[56-58] in the pathophysiology of GvHD, we evaluated the effect of each on T-cell phenotype. On day 0, we collected plasma (Supplemental Figure 6a) and isolated splenic pan-DCs (Supplemental Figure 6b-D) from BEN-TBI and CY-TBI conditioned mice. We co-cultured each with T-cells from naïve C57BL/6 mice to examine potential effects on T-cell phenotype. We saw a modest increase in CD4 PD-1 expression, consistent with decreases in GvHD, as well as CD8 CD134 expression, when T-cells were plated with DCs from BEN-TBI conditioned mice, but no other differences.

## BEN-TBI results in T-cell tolerance to host, but not third party, MHC antigens *in vitro*

Though we did not find clear phenotypic differences in donor T-cells post-transplant between the two conditioning regimens, we proceeded to evaluate their function. As shown in Figure 1, the vast majority of BEN-TBI conditioned mice survive and have little to no remaining GvHD beyond five weeks post-BMT. Insufficient numbers of CY-TBI conditioned mice survive, precluding their use for comparison. We euthanized surviving BEN-TBI conditioned mice after day +100, isolated splenic total T-cells (H-2^b, of C57BL/6 donor origin), and co-cultured them with C57BL/6 (syngeneic control), BALB/c (H-2^d, representing MHC-mismatched host cells), and FVB/N (H-2^q, third-party MHC-mismatch) irradiated splenocytes as stimulators. We used tritiated-thymidine to measure T-cell proliferation. As expected, reconstituted donor (C57BL/6, H-2^b) T-cells from surviving BEN-TBI conditioned mice showed no proliferative response to syngeneic C57BL/6 irradiated spleen cells (Figure 4(a)). Interestingly, reconstituted T-cells demonstrated significantly suppressed proliferation in response to splenocytes expressing host MHC (BALB/c, H-2^d) when compared to the proliferation in response to third party splenocytes from FVB/N mice (H-2^q) (Figure 4(a)). This ~3-fold difference indicates that the T-cells retain the ability to respond to MHC-disparate antigens, but develop tolerance specifically to recipient host MHC antigens. We also compared these post-BEN-TBI conditioned BMT T-cells to T-cells taken from naïve, healthy age-matched C57BL/6 mice. When stimulated with FVB/N splenocytes, we saw comparable levels of proliferation between the BEN-TBI conditioned post-BMT T-cells and the T-cells from naïve mice. However, in contrast to the BEN-TBI T-cells, T-cells from naïve mice responded equally to stimulation by FVB/N and BALB/c splenocytes (Figure 4(a)). To further confirm that the T-cells taken from BEN-TBI conditioned mice were not simply dysfunctional, we stimulated them with anti-CD3/CD28 beads and saw a significant proliferative response (Figure 4(b)).

BEN-TBI results in T-cell tolerance to host MHC antigens *in vivo*

We next evaluated whether donor-derived T-cells following BEN-TBI conditioning remained tolerant to BALB/c MHC antigens *in vivo* when infused in a secondary BMT. We isolated splenic T-cells from surviving BEN-TBI conditioned mice and

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FoxP3 for flow cytometric analysis. CD4+ CD25+ FoxP3+ cells were considered Tregs. Representative flow cytometry histograms from day +35 are shown. CBCs were determined and used to calculate absolute cell numbers. Average absolute numbers of cells per μL of blood are shown with SEM. Pooled data from 4 experiments are shown, n = 19 mice/group. (b) BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3 × 10^6 SC from naïve C57BL/6 mice on day 0. Splenic Tregs were isolated on day 0 (prior to transplant), +7, and +14. Tregs were plated with anti-CD3/CD28 bead activated, CellTrace Violet-stained T-cells from naïve C57BL/6 mice in a suppression assay. T-cells were plated at 100,000 cells per well in a 96-well U-bottom plate. Tregs were plated at a ratio of 1 Treg to 2 T-cells. Proliferation was assessed by flow after 3 days of co-culture. Average % proliferation with SEM is shown, compared to a control with no Tregs. Pooled data from 2 experiments are shown, n = 4–7 mice/group. (c) BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 TCD-BM with 3 × 10^6 tT with or without CD25+ cell depletion from naïve C57BL/6 mice on day 0. Survival is shown. CY+TBI+TCD-BM+tT vs. CY+TBI+TCD-BM+CD25- tT *p = .0857*, BEN+TBI+TCD-BM+CD25- tT vs. CY +TBI+TCD-BM+CD25- tT *p = .0005*. (d) CD4+ CD62L+ T-cells were isolated from naïve BALB/c mouse spleens and cultured with 5 ng/mL TGF-β and a 1:1 ratio of anti-CD3/CD28 beads for 3 days in the presence of various concentrations of BEN. Cells were phenotyped and the % CD4+ CD25+ FoxP3+ of all cells remaining at the end of the culture was assessed, as well as viability. BEN was washed out and cells were plated in a suppression assay at a ratio of 1 Treg to 1 T-cell (CD3/CD28 activated, CellTrace Violet-stained, from naïve C57BL/6 mice). After 3 days of co-culture, proliferation was assessed by flow cytometry. Representative flow cytometry plots are shown, with proliferation index (PI) indicated, with higher proliferation indices representing greater proliferation. The T-cells + beads plot represents activated T-cells without Tregs (a positive control for proliferation). Averages are shown with SEM. Pooled data from 3 experiments is shown, n = 3/condition.
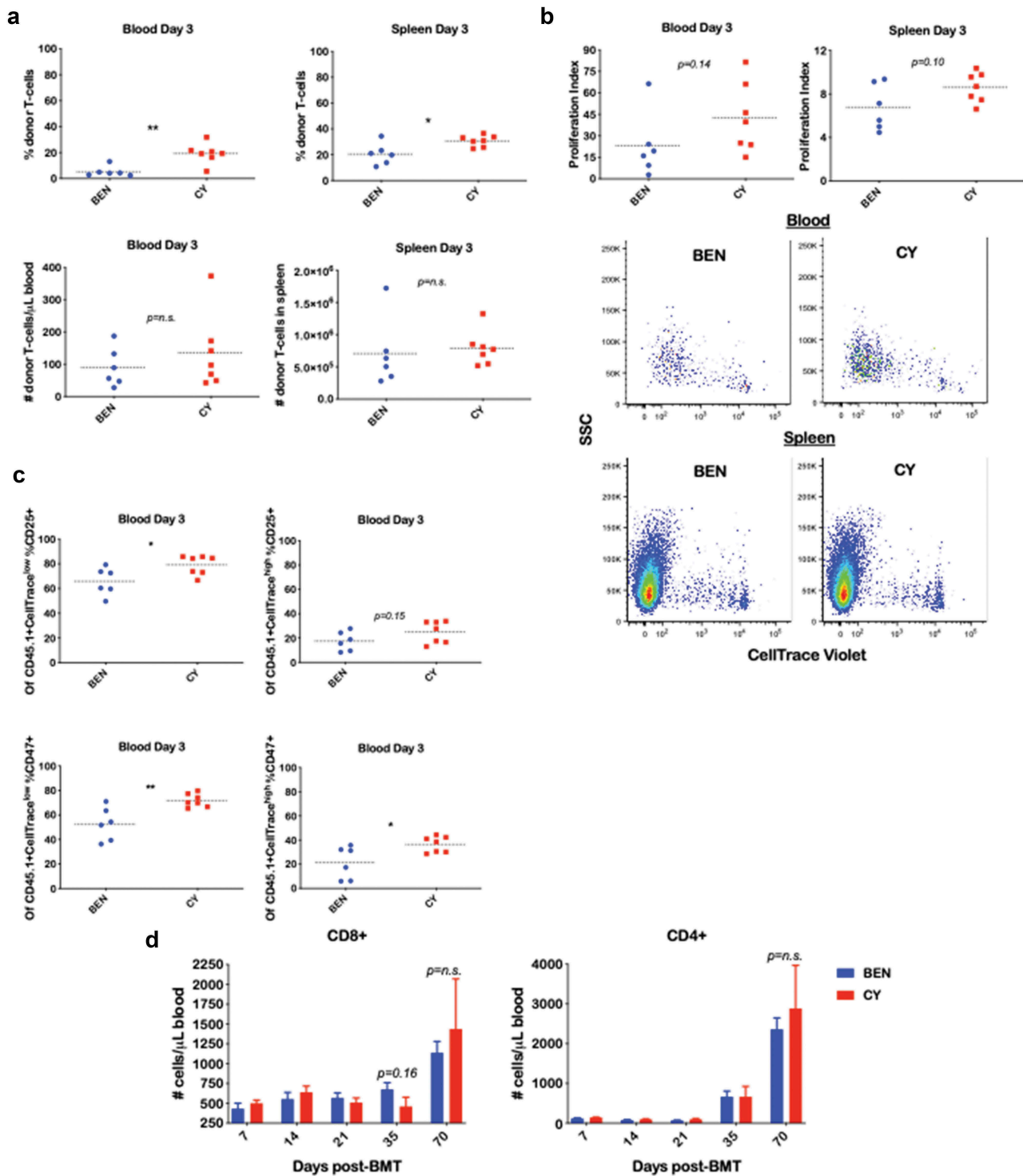
Figure 3. BEN-TBI does not result in appreciable donor T-cell phenotypic differences post-transplant when compared to CY-TBI. (a–c) BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 TCD-BM from naïve C57BL/6 mice with 3×10^6 CellTrace Violet-stained tT from naïve BoyJ mice on day 0. Blood and spleen were collected on day +3. (a) % donor T-cells (CD45.1+) was determined by flow cytometry. Using CBCs determined by HemaVet analysis, absolute number of donor T-cells was calculated. (b) After gating on CD45.1+ cells (representing donor T-cells), CellTrace Violet dilution was analyzed using ModFit software to determine proliferation index. Representative CellTrace Violet dilution is shown. (c) Within the CD45.1+ gate, cells were stratified by CellTrace^{high} (non- proliferative) and CellTrace^{low} (proliferative) and CD25 and CD47 expression were analyzed by flow cytometry. Pooled data from 2 experiments with line at mean are shown, n = 6–7 mice/group. * $p < .05$, ** $p < .01$. (d-g) BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve C57BL/6 mice on day 0. Peripheral blood was collected on days +7, +14, +21, +35, and +70 and stained for CD8, CD4, Tbet, GATA3, RORγt, CD134, CD278, PD-1, TIM3, CTLA-4, and CD272. CBCs were determined and used to calculate absolute cell numbers. Average absolute numbers of cells per μL of blood are shown with SEM. Representative flow plots from day +7 with fluorescence minus one (FMO) controls are shown (e). (d) Pooled data from 4 experiments are shown, n = 19 mice/group. (e-g) Pooled data from 2 experiments are shown, n = 10 mice/group. ** $p < .01$.
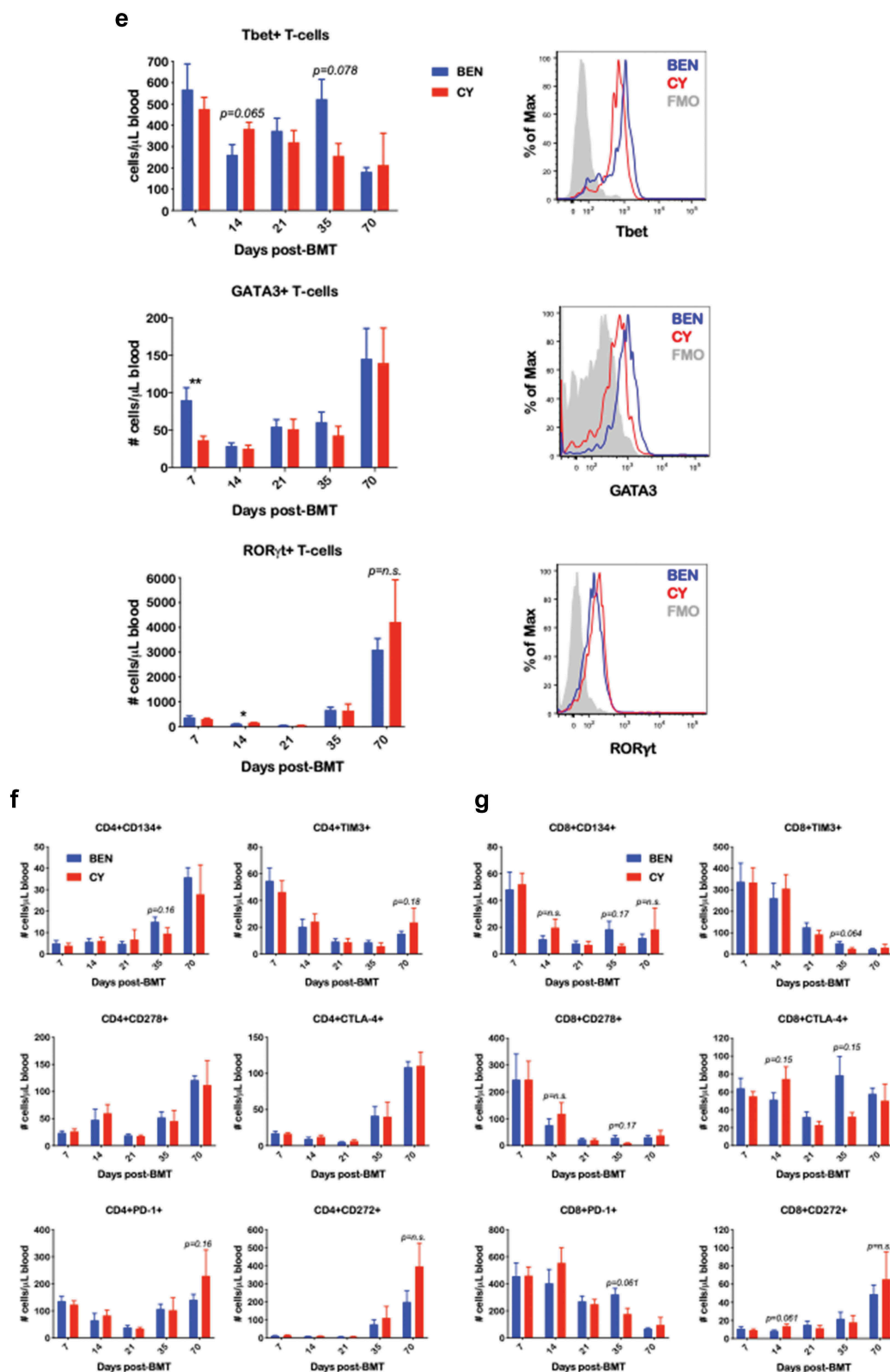
Figure 3. (Continued).

injected them into naïve BALB/c mice, conditioned with mye-loablative TBI, along with TCD-BM from naïve C57BL/6 mice. These T-cells did not induce GvHD and resulted in complete survival (10^6 tT MHC-mismatched; Figure 5(a)), as did the TCD-BM only control. For comparison, splenic T-cells from

age-matched C57BL/6 mice that received BEN-TBI conditioning and a syngeneic primary transplant were used as controls. Syngeneic T-cells were in their primary recipient for the same amount of time as the MHC-mismatched T-cells, but without exposure to BALB/c MHC antigens. When transplanted into
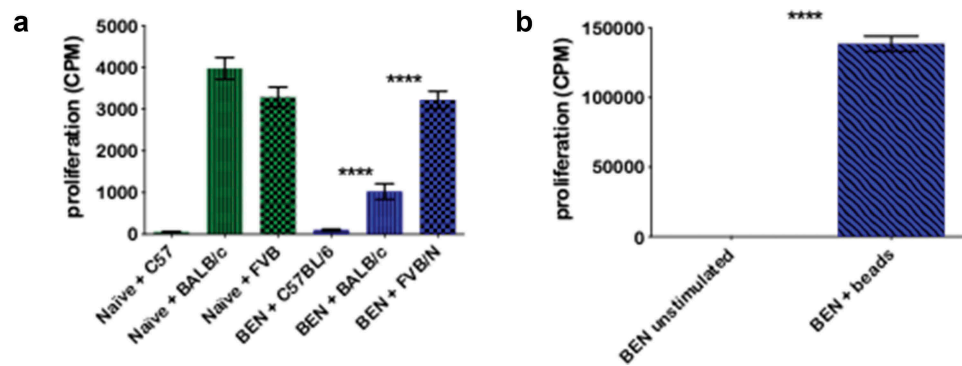
Figure 4. BEN-TBI results in T-cell tolerance to host, but not third party, MHC antigens *in vitro*. BALB/c recipient mice received 40 mg/kg BEN iv on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve C57BL/6 mice on day 0. ≥+100 days post-BMT, splenic T-cells of surviving mice were isolated and plated in an MLR with irradiated (1500 cGy) SC from naïve C57BL/6, BALB/c, and FVB/N mice. On day 3 of co-culture, 0.5 μCi tritiated-thymidine was added to each well and on day 4, cells were harvested and counts per minute (CPM), denoting proliferation, were measured using a gamma counter. T-cells from naïve C57BL/6 mice were also plated with C57BL/6, BALB/c, and FVB/N SC as a control (a). Additionally, BEN T-cells were stimulated with anti-CD3/CD28 beads and CPM were determined (b). Average CPM with SEM is shown. Pooled data from 3 experiments are shown, n = 6–12 mice/condition. **** $p < .0001$.

secondary BALB/c recipients, T-cells from syngeneic primary transplant recipients resulted in severe GvHD and complete lethality (10^6 tT syngeneic; Figure 5(a)). This indicates that when mice are conditioned with BEN-TBI and receive an MHC-mismatched BMT, the engrafted T-cells become tolerant to host MHC, resulting in suppression of GvHD. We phenotyped the tolerant T-cells from mice receiving an MHC-mismatched BMT, as well as those from syngeneic primary BMT recipients. We saw no difference in Tbet, GATA3, and FoxP3 expression (Figure 5(b)) or proportion of CD4 versus CD8 (Figure 5(c)). We saw increased expression of the co-stimulatory molecule CD278 on the CD8+ T-cells from the syngeneic primary transplant, which is consistent with increased GvHD (Figure 5(e)). However, though there are trends, we did not see any other significant or consistent differences in co-stimulatory or co-inhibitory molecules (Figure 5(d–e)). We saw no differences in CD47 or CD25 expression or effector memory, central memory, and naïve T-cells (Supplemental Figure 7). We confirmed the persistence of these tolerant T-cells in the blood following the secondary transplant. Our data indicate that the cells not only persist, but increase in number through day +70 following the secondary transplant (Figure 5(f)), while causing no discernible GvHD.

BEN-TBI preserves GvL

Having established that BEN-TBI conditioning reduces GvHD significantly and yields T-cells that are tolerant to host MHC (H-2^d) antigens, we investigated the GvL associated with this conditioning regimen. We showed that, when luciferase-expressing BALB/c (H-2^d) derived A20 leukemia cells were injected on the day of transplant, BEN-TBI conditioning followed by an MHC-mismatched BMT significantly improved survival over BEN-TBI conditioning followed by a syngeneic transplant (Figure 6(a)). This indicates that GvL effects are preserved following BEN-TBI conditioning, despite suppression of GvHD. We also showed that in an allogeneic transplant setting with leukemia, BEN-TBI conditioning significantly improves survival over CY-TBI conditioning

(Figure 6(b)). Figure 6(a,b) includes deaths from tumor, GvHD, and combinations of the two. When we censor deaths that occurred with no visible tumor (i.e. deaths due solely to GvHD), BEN-TBI still had significantly improved survival over CY-TBI, indicating that the difference in survival was not solely attributable to a difference in GvHD lethality (Figure 6(c)). We additionally evaluated graft-versus-host disease free, relapse-free survival (GRFS). BEN-TBI conditioning had a significantly higher percentage of mice without incidence (Figure 6(d)), indicating a superior BMT outcome with BEN-TBI conditioning, when accounting for both tumor and GvHD. Of note, in a syngeneic transplant setting, there was no significant difference between BEN-TBI and CY-TBI survival (Supplemental Figure 8), indicating that the difference in GvL observed in our MHC-mismatched BMT setting is likely due to favorable immune modulatory effects of BEN-TBI. We further confirmed superior GvL with BEN-TBI conditioning by imaging mice serially and quantifying luminescence as photons/second, indicative of their A20-luc burden. BEN-TBI conditioned mice that received a syngeneic transplant consistently demonstrated significantly more photons/second than BEN-TBI or CY-TBI conditioned mice receiving an MHC-mismatched transplant. Following day +14, CY-TBI mice showed on average significantly more luminescence than BEN-TBI, indicating a greater tumor burden (Figure 6(e)). This is also evident visually through bioluminescent imaging (Figure 6(f)). These data indicate that BEN-TBI conditioning preserves a GvL effect superior to that seen with a syngeneic transplant or with CY-TBI conditioning.

GvL with BEN-TBI is T-cell dependent

We lastly sought to further characterize the effector cells responsible for the GvL effect observed with BEN-TBI conditioning by depleting natural killer (**NK**), CD4+, or CD8+ cells. Control mice received an isotype antibody injection. Depleting antibodies were injected ip weekly beginning on day +3 following transplant. We confirmed that depletion of target lymphocyte subsets in the blood endured the entire interval between
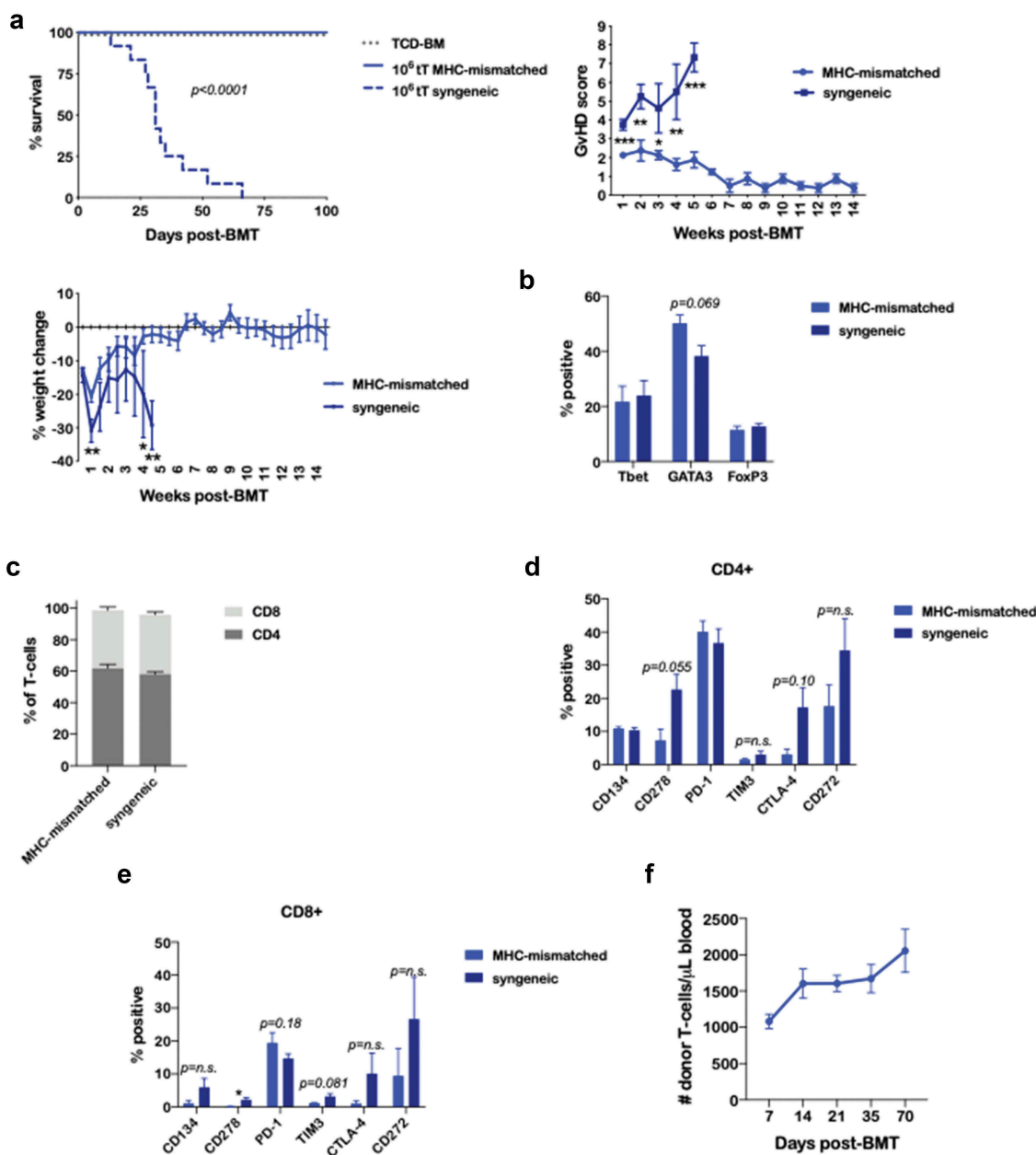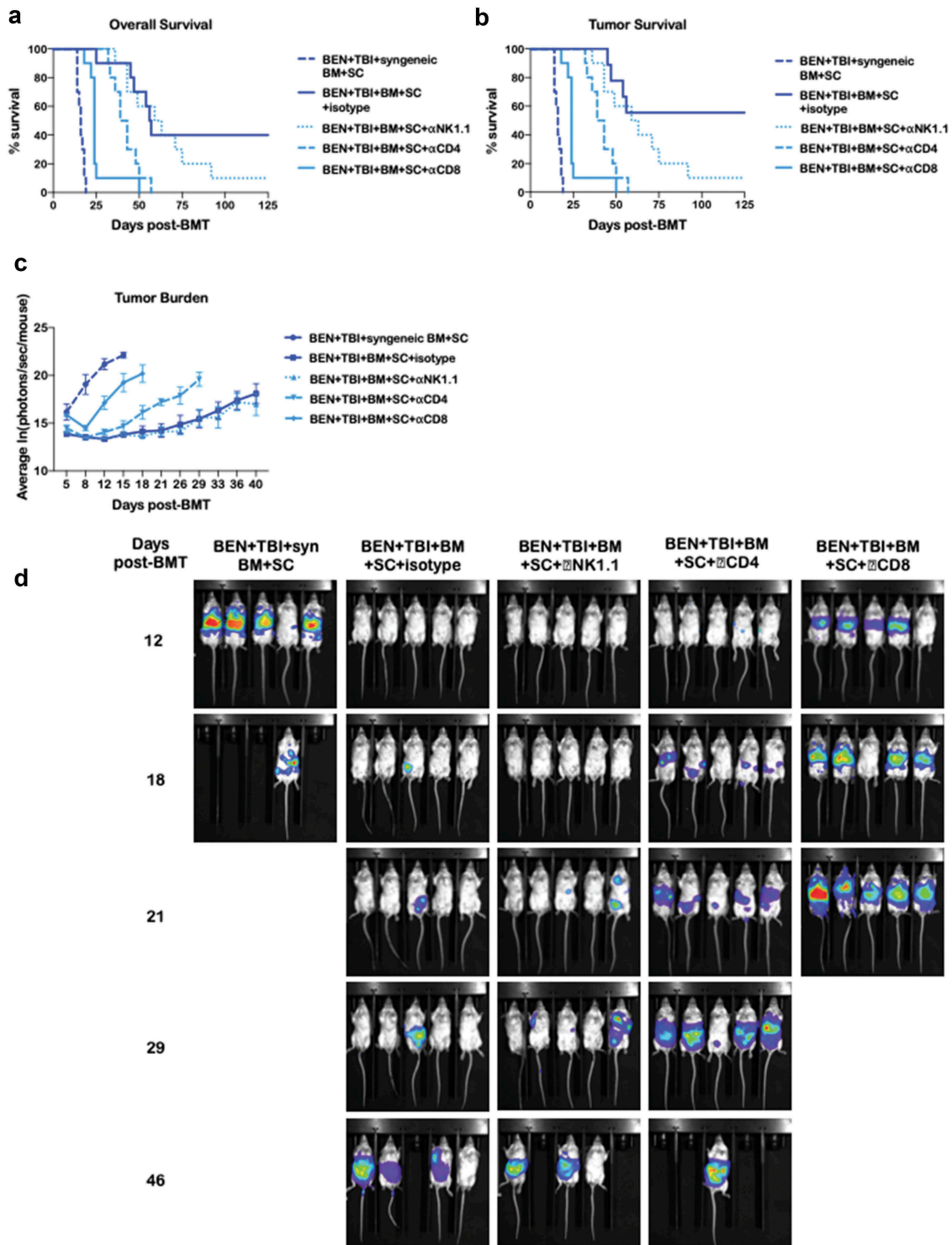
Figure 5. BEN-TBI results in T-cell tolerance to host MHC antigens *in vivo*. BALB/c recipient mice received 40 mg/kg BEN iv on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve BoyJ mice on day 0. ≥+100 days post-BMT, splenic T-cells of surviving mice were isolated and given as donor T-cells in a secondary transplant. Secondary recipients received 850 cGy TBI on day −1 and 10^7 TCD-BM from naïve C57BL/6 mice on day 0 with or without T-cells from surviving BEN-TBI conditioned mice (called MHC-mismatched). As a control, secondary recipients received T-cells from C57BL/6 mice that had previously received BEN-TBI conditioning and a syngeneic BMT (called syngeneic). (a) Survival is shown. MHC-mismatched vs. syngeneic $p < .0001$. Pooled data from 3 experiments are shown, n = 12 mice/group. Average GvHD score and % weight change from starting weight are shown with SEM. Representative data from 3 experiments are shown, n = 5 mice/group. * $p < .05$, ** $p < .01$, *** $p < .001$. (b–e) On day 0, prior to infusion in the secondary transplant, isolated T-cells from MHC-mismatched and syngeneic primary transplants were phenotyped. Average % expression with SEM is shown. Pooled data from 3 experiments is shown, n = 3/condition. * $p < .05$. (f) Following the secondary transplant, peripheral blood was collected on days +7, +14, +21, +35, and +70 and stained for CD45.1, denoting T-cells from the primary transplant. CBCs were determined and used to calculate absolute number of donor T-cells per µL of blood. Average number of donor T-cells with SEM is shown. Pooled data from 2 experiments are shown, n = 8 mice/group.

injections (data not shown). There was no difference in survival between the isotype control group and the NK cell depleted group. When CD4+ cells were depleted, survival was significantly decreased. Depletion of CD8+ cells resulted in the greatest loss of GvL with significantly decreased survival even in comparison to the CD4+ depleted mice (Figure 7(a)). These data indicate NK cells do not play a significant role in the GvL effect seen following BEN-TBI conditioning, while CD4+ cells, and even more so, CD8+ cells do play a significant role. When tumor-free deaths (deaths due solely to GvHD) were censored, the same differences were observed (Figure 7(b)). Bioluminescent imaging confirmed these results, with no
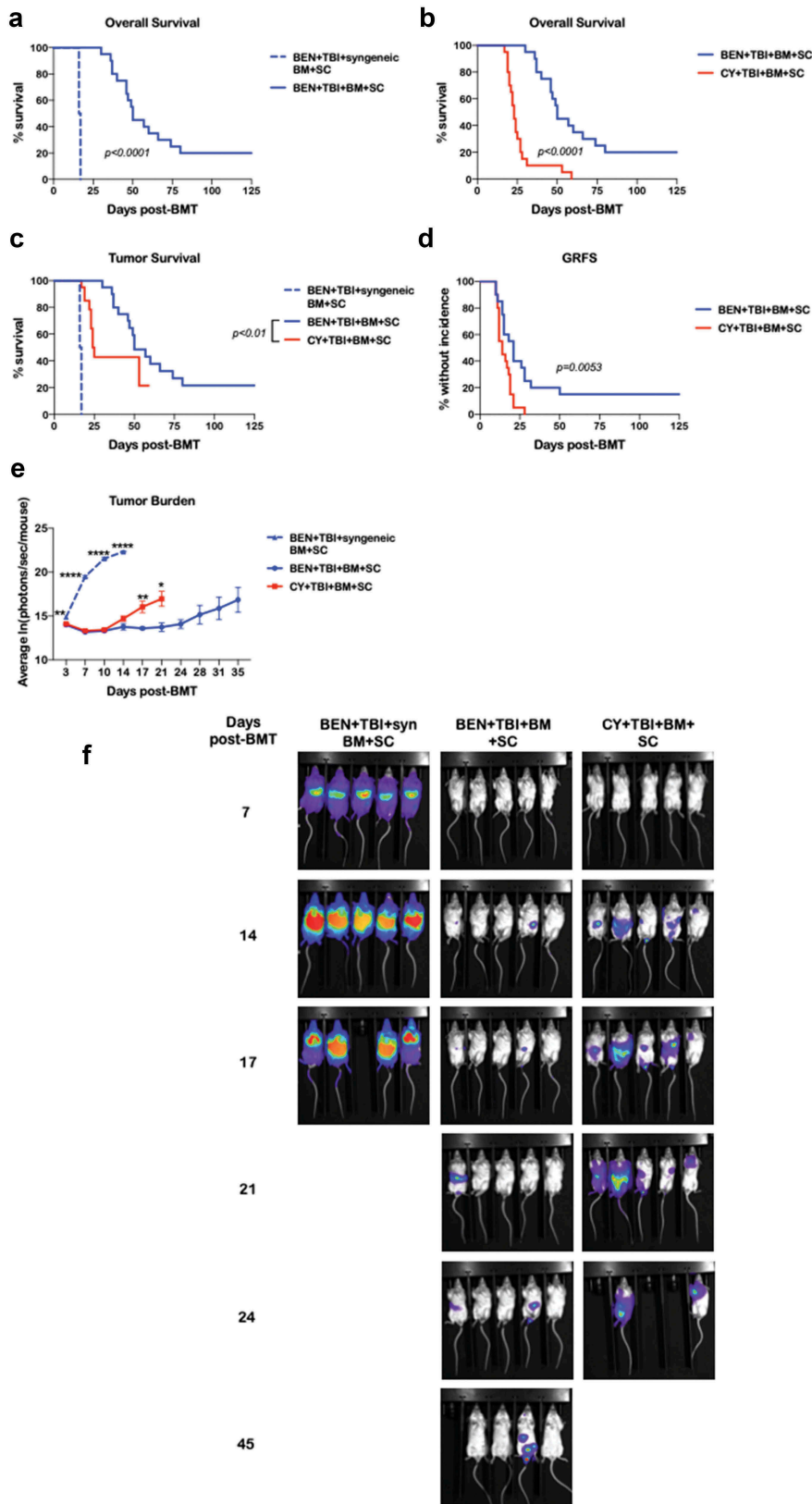
Figure 6. BEN-TBI preserves GvL. BALB/c recipient mice received 40 mg/kg BEN iv or 200 mg/kg CY ip on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve C57BL/6 mice on day 0 with 0.1×10^6 A20-luc tumor cells. (a) As a control, BEN-TBI conditioned mice received syngeneic BM and SC with A20-luc. Survival of BEN-TBI conditioned mice receiving an MHC-mismatched BMT and syngeneic BMT are shown, $p < .0001$. (b) Survival of BEN-TBI and CY-TBI conditioned mice receiving an MHC-mismatched BMT with A20-luc cells are shown, $p < .0001$. (c) Survival of BEN-TBI and CY-TBI conditioned mice receiving MHC-mismatched BMT with A20-luc are shown with deaths occurring without visible tumor censored. Only deaths with measurable tumor are included. BEN vs. CY $p < .01$. (d) Graft-versus-host disease free, relapse free survival (GRFS) is shown. A GvHD score ≥6 or visible tumor following day +8, as well as death, were considered incidents. % without incidence is shown. Pooled data from 4 experiments are shown, n = 10–20 mice/group. (e) Following BMT and A20-luc infusion, mice were imaged and luminescence was measured twice a week. Average ln(photons/sec/mouse) with SEM is shown. Representative data from 4 experiments is shown, n = 5 mice/group. * $p < .05$, ** $p < .01$. (f) Luminescent imaging representative of 4 experiments is shown, n = 5 mice/group.

Figure 7. GvL with BEN-TBI is T-cell dependent. BALB/c recipient mice received 40 mg/kg BEN iv on day −2, 400 cGy TBI on day −1, and 10^7 BM with 3×10^6 SC from naïve C57BL/6 mice on day 0 with 0.1×10^6 A20-luc tumor cells. Mice received syngeneic BM and SC as a control. All mice received weekly ip antibody injections of 200 μg beginning on day +3 (isotype, NK1.1, CD4, or CD8). (a) Survival is shown. Pooled data from 2 experiments is shown, n = 10 mice/group. Syngeneic vs. all other conditions $p < .0001$; isotype vs. NK1.1 $p = n.s.$, vs. CD4 $p = .0068$, vs. CD8 $p < .0001$. (b) Deaths without measurable tumor are censored and survival is shown. Syngeneic vs. all other conditions $p < .0001$; isotype vs. NK1.1 $p = n.s.$, vs. CD4 $p = .0011$, vs. CD8 $p < .0001$. (c) Following BMT and A20-luc infusion, mice were imaged and luminescence was measured twice weekly. Average ln(photons/sec/mouse) with SEM is shown. Syngeneic vs. all other conditions $p < .05$ at all time points; isotype vs. NK1.1 $p = n.s.$ at all time points, vs. CD4 $p < .05$ day +12, +21, +26, +29, vs. CD8 $p < .05$ at all time points. (d) Representative luminescent imaging of 2 experiments is shown, n = 5 mice/group.

differences between isotype control and NK cell depletion, and significantly increased photons/second in the CD4+ and CD8 + depletion groups (Figure 7(c–d)).

Discussion

GvHD remains a serious, often fatal impediment to the success of HCT. There are many strategies used to prevent GvHD, such as graft modification and post-transplant immunosuppression. Another, less-studied strategy to prevent GvHD is modification of the conditioning regimen. Pretransplant conditioning regimens have been shown to damage host tissues, creating an inflammatory environment, leading to activation of antigen presenting cells and induction of GvHD. CY-TBI is a myeloablative conditioning regimen most commonly used clinically for ALL, though it is associated with severe tissue damage and GvHD.[11,17,18] We have previously published that substituting BEN for CY, combined with TBI, leads to reduced mortality and morbidity from GvHD in a murine MHC-mismatched BMT model.[19] Here, we confirm our previous findings that BEN-TBI reduces GvHD compared to CY-TBI and further show that although we could not ascertain a specific tolerant phenotype, the engrafted donor T-cells following BEN-TBI conditioning are indeed tolerant to host MHC without compromising GvL.

We show that the donor-derived T-cells taken from BEN-TBI conditioned mice are tolerant, specifically to BALB/c antigens, but remain functional otherwise, capable of responding to other foreign antigens and retaining the capacity to be activated by anti-CD3/CD28 beads (Figure 4). Antigen-specific T-cell anergic states have been shown to be a mechanism of peripheral tolerance in transplant.[28,59,60] There are many ways in which tolerance can be achieved following HCT, including through expression of co-inhibitory molecules.[28,60,61] For this reason, we evaluated the expression of various co-inhibitory and, conversely, co-stimulatory molecules on T-cells in the peripheral blood. We saw that when BEN-TBI DCs were co-cultured with T-cells overnight, the T-cells expressed more PD-1 (Supplemental Figure 6c), a co-inhibitory molecule that has been shown to suppress GvHD.[25,27,28,51,62] Though this could be impacting GvHD, the difference between the groups is modest ($p = .047$). Additionally, when evaluated prior to infusion in the secondary transplant, BEN-TBI T-cells expressed lower levels of the co-stimulatory molecule CD278 (Figure 5(e)). This also may have contributed to these cells causing less GvHD.[29,63] We also demonstrated that BEN-TBI increased GATA3 expression, a marker of Th2 cells, in the first week following transplant (Figure 3(e)). Though these cells have typically been considered innocuous in terms of GvHD,[20,48] other studies have found the role of Th2 cells in GvHD to be more pleiotropic, with these cells sometimes causing GvHD.[21,64,65] Of note, we saw decreased CD47 expression early post-transplant (Figure 3(c)), which could indicate that alloreactive T-cells were more effectively eliminated following BEN-TBI than CY-TBI.[47] Despite these observed differences, we did not find a clear, durable phenotypic difference that fully explained the significant tolerance achieved. One possible explanation for this is that the differences lie in markers

we did not evaluate. Though we attempted to evaluate a variety of markers associated with increases and decreases in GvHD, it was by no means an exhaustive list. Another limitation of this methodology is that while the majority of BEN-TBI conditioned mice survive past day +70, the majority of CY-TBI conditioned mice die prior to day +35, meaning later time points are skewed toward CY-TBI mice with increased survival.

Additionally, the reduction of GvHD may simply be explained by the effect of BEN-TBI on other immune cell subsets. Though we do not have a well-defined picture of the mechanism by which BEN is reducing GvHD, we have clearly shown in previous publications that BEN leads to an increase in the number of MDSCs.[2,19] MDSCs have been shown to induce T-cell tolerance, potentially explaining the tolerance to host antigens observed in our study.[66,67] As the ratio of MDSC to T-cell is higher in BEN-TBI mice than CY-TBI mice,[19] the large number of MDSCs may allow BEN-TBI conditioned mice to achieve tolerance, while CY-TBI mice do not have enough MDSCs to overcome the alloreactive T-cell response. The higher numbers of MDSCs in BEN-TBI mice shown previously is also congruent with the data in this manuscript demonstrating a lower percentage of donor T-cells in the spleens and blood of BEN-TBI conditioned mice on day 3 (Figure 3(a)). Though Tregs have been shown to be essential for GvHD control in other settings,[22,44-46] our data do not support a vital role of Tregs in our BEN-TBI model (Figure 2(c)). Our data also indicate that though we have shown that BEN can effectively increase the suppressive function of MDSCs through *in vitro* exposure,[2] the same is not true for Tregs (Figure 2(d)).

Importantly, there are many other factors that can impact GvHD and peripheral tolerance that have not been addressed in this study. For example, T-cells in target tissues may express differing levels of co-inhibitory and co-stimulatory molecules that were not apparent in the blood.[68] Cytokine levels can also both promote and prevent GvHD. We observed a transient decrease in TNFα in BEN-TBI circulating donor T-cells, which is consistent with decreased GvHD,[69] but many cytokines remain unexplored in this model. IL-10, for example, has been shown to mitigate GvHD,[54,70,71] while IL1-β and IL-6 have been shown to enhance GvHD.[56,72] Interestingly, in a purely *in vitro* study, BEN has been shown to increase IL-10 production by a B-cell cancer line.[73] This makes the cytokine profile following BEN administration an interesting candidate for further investigation of the mechanism by which BEN is reducing GvHD. Other possible factors include BEN resulting in differing chemokine expression, as chemokine inhibition can reduce GvHD by preventing homing of alloreactive T-cells to target tissues.[74,75] This is a particularly interesting potential explanation as we have previously shown fewer T-cells in the intestines of BEN-TBI conditioned mice than CY-TBI conditioned mice.[19]

Though blood T-cell phenotype does not appear to differ between BEN-TBI and CY-TBI conditioning, we do see significant differences in tumor control. Disease recurrence remains a major cause of mortality following HCT and efforts to reduce GvHD frequently lead to increased relapse rates.[1,76] Having confirmed that BEN-TBI conditioning significantly reduces GvHD, it was vital that we determine the impact of

BEN-TBI on GvL effects. We have previously shown that administration of BEN post-haploidentical BMT yields superior GvL effects over administration of CY.[2] Herein, we provide the first evidence that BEN-TBI conditioning, while reducing GvHD (Figure 1), also preserves GvL, yielding superior survival over CY-TBI (Figure 6). Additionally, this GvL effect is almost entirely dependent on T-cells (Figure 7), confirming that their tolerance to host antigens does not negatively impact their anti-tumor effect.

In summary, we have built upon our previous studies by confirming the reduction of GvHD following substitution of BEN for CY in the traditionally applied CY-TBI conditioning regimen. We demonstrate that BEN-TBI conditioning generates T-cells that are tolerant to host antigens, but that retain their anti-leukemia activity. BEN-TBI warrants further exploration both mechanistically and clinically, as it may provide a safer option than CY-TBI, in terms of both GvHD and relapse.

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