BENDAMUSTINE CONDITIONING REDUCES GVHD WHILE MAINTAINING GVL IN MURINE MHC-MISMATCHED HEMATOPOIETIC CELL TRANSPLANTATION

By

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ABSTRACT

Graft-versus-host disease (GvHD) remains a significant obstacle to the success of hematopoietic stem cell transplantation due to challenges in uncoupling GvHD from the highly beneficial graft-versus-leukemia (GvL), both of which are mediated by donor T-cells. Our laboratory has previously shown that pre-transplant conditioning with bendamustine (BEN) and total body irradiation (TBI) significantly reduces GvHD when compared to cyclophosphamide (CY)+TBI in a major histocompatibility complex (MHC) mismatched murine bone marrow transplant model. Despite reduced GvHD, conditioning with BEN+TBI retains GvL effects in mice with A20-luciferase. To determine which cells are responsible for this GvL effect, CD4+ and CD8+ T-cells and NK cells were depleted. We see the greatest deficit in anti-tumor effect when CD8+ T-cells are depleted, followed by CD4+ T-cells, with depletion of NK cells showing little change to GvL effects. This indicates that CD8+ T-cells primarily contribute to the GvL effect seen in this model. As GvHD and GvL are mediated by donor T-cells, whose effector functions are informed by dendritic cells (DCs), we characterized DCs that were conditioned with BEN+TBI compared to CY+TBI to understand the mechanism of BEN. We hypothesized that BEN inhibits STAT3 signaling causing a compensatory increase in Flt3 and Akt1 expression to increase signaling through this alternative pathway. Contrary to our hypothesis, the qRT-PCR results showed a significant decrease in Akt1 transcripts in both mice and humans as the concentration of BEN increased.

INTRODUCTION

Hematopoietic cell transplantation (HCT) is clinically indicated as a treatment for many diseases, from leukemias and lymphomas to anemias.[1,2] However, graft-versus-host disease (GvHD) remains a significant impediment for the success of HCT. While GvHD prophylaxis has been shown to be effective in suppressing GvHD, 30-70% of HCT recipients will develop GvHD.[3] Furthermore, prophylaxis has been shown to also suppress the graft-versus-leukemia effect (GvL), increasing the risk of malignancy relapse.[4,5] Therefore, there is a need for a novel treatment that can suppress GvHD while maintaining GvL effects. While cyclophosphamide (CY) + total body irradiation (TBI) is the current clinical standard of care as HCT conditioning for acute lymphoblastic leukemia, it is associated with severe GvHD.[6,7] Bendamustine has been included in chemotherapy based conditioning regimens for HCT but had not previously been studied in combination with TBI.[8] We have previously shown that pre-transplant conditioning with BEN+TBI significantly reduces GvHD morbidity and mortality when compared CY+TBI in a major histocompatibility complex (MHC) mismatched murine bone marrow transplant (BMT) model.[9] In this study, we use a tumor challenge model to evaluate the effect of BEN conditioning on GvL in the murine BMT model. We found that despite low GvHD, BEN conditioning preserves GvL effects in mice with A20-luciferase (A20-luc), a BALB/c B-cell lymphoblastic leukemia. Furthermore, we demonstrate that the GvL effects induced in this model and preserved by BEN conditioning are T-cell mediated.

GvHD and GvL effects are mediated by donor T-cells, whose effector functions are informed by antigen-presenting cells, particularly dendritic cells (DCs).[10-12] Therefore, in order to understand the biological mechanisms of BEN in this model, we characterized DCs that were conditioned with either BEN + TBI compared to CY + TBI. DCs treated with BEN were shown

to induce less allogeneic T-cell proliferation, had a significant increase in type 1 conventional DCs (cDC1) which have been shown to suppress GvHD,[13] and had a greater expression of Flt3, a receptor that contributes to the expansion of cDC1s. [14,15]

Fms-like tyrosine kinase 3 (Flt3) is a receptor tyrosine kinase that is expressed on hematopoietic cells and mediates survival, proliferation, and differentiation.[15] When bound to its ligand, Flt3 ligand (Flt3L), homodimerization and autophosphorylation occur which initiate several signal transduction cascades including Ras/MEK/Erk, STAT, and PI3K/Akt (**Figure 1**).[15-17] Flt3 has been shown to induce expansion of DCs, but cDC1s show increased expansion compared to other DC subsets.[15, 18] cDC1s have been reported to suppress GvHD through inducing tolerance of T-cells and have also been shown to cross present exogenous antigens, such as tumor antigens, to CD8+ T-cells, resulting in superior anti-tumor responses.[15, 18, 19] Through these mechanisms, cDC1s are able to uncouple GvHD and GvL.

Flt3 receptor may signal through multiple signaling molecules (**Figure 1**), including signal transducer and activator of transcription 3 (STAT3), to regulate DC survival and proliferation.[20] BEN has been reported to directly bind to and inhibit canonical STAT3 signaling,[21] and RNA sequencing of DCs found an increase in Akt1 transcripts, another downstream signaling molecule for Flt3 receptor, in BEN- compared to CY-treated DCs.[14] We therefore hypothesized that BEN inhibits STAT3 signaling, causing a compensatory increased in Flt3 expression and Akt1 expression to allow for increased signaling through this alternative Flt3 signaling pathway. To test this, murine bone marrow-derived DCs (BMDCs) and human monocytic-DCs (moDCs) were generated following exposure to BEN and qRT-PCR was used to measure the expression of Akt1 transcripts. Contrary to our hypothesis, we show a significant

decrease in Akt1 transcripts in both murine and human cells as the concentration of BEN increased.

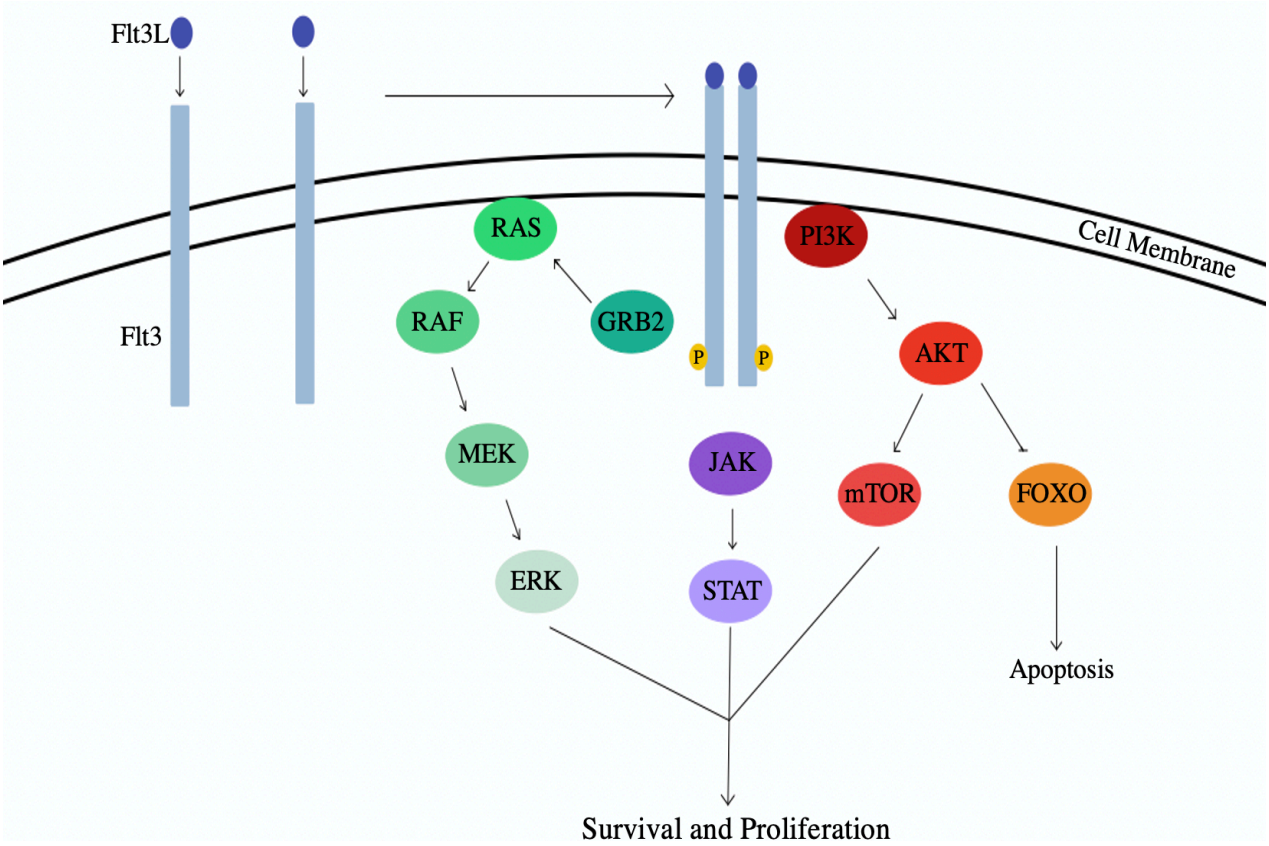


Figure 1: Flt3 signaling pathway. The Flt3 signaling pathway plays a role in the expansion of several hematopoietic lineages and DCs, particularly type 1 conventional DCs. Once the Flt3 ligand binds to the receptor, homodimerization and conformation changes occur that result in the phosphorylation of tyrosine kinase domains.[16] Activation of the receptor causes the activation of downstream signaling pathways including the Ras/MEK/Erk, STAT, and PI3K/Akt pathways.[17]

MATERIALS AND METHODS

Mice

Age matched 6-10 week old female BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in specific pathogen-free conditions and cared for following the guidelines of the University of Arizona Institutional Animal Care and Use Committee (IACUC). All experiments performed were approved and followed all guidelines set by IACUC.

BMT model

Recipient BALB/c (H-2^d) mice were given 40 mg/kg BEN intravenously (iv) or 200 mg/kg CY intraperitoneally (ip) on day -2. These drugs have been shown to be cleared by 24 hours after administration, thus had no direct effect on the donor graft.[22-24] On day -1, the mice received 400 cGy TBI using a Cesium 137 irradiator.[9] On day 0, the mice received 10^7 T-cell depleted C57BL/6 (H-2^b) bone marrow (BM) cells and 3×10^6 splenic cells (SC) iv. Survival was monitored daily and moribund mice were euthanized following IACUC approved procedures. Mice were weighed and scored every three to four days on activity, fur texture, posture, and skin integrity. Cumulative GvHD scores were calculated using scores along with percent of starting weight. After day +8, mice with a cumulative GvHD score of 8 were euthanized.

Drug preparation and administration

For *in vivo* experiments, BEN was reconstituted in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and diluted in sterile phosphate-buffered saline (Hyclone, Logan, UT) with 0.2% carboxymethylcellulose and 0.25% polysorbate 80 (Sigma-Aldrich). BEN was administered

through iv injection. CY (Sigma-Aldrich) was reconstituted in sterile water (Hyclone) and diluted in sterile saline (Fisher Scientific, Pittsburgh, PA). CY was administered through ip injection.[25] Both BEN and CY doses are at ~50% of the maximum tolerated dose for BALB/c mice.[9]

For *in vitro* experiments, BEN was reconstituted in dimethyl sulfoxide (Sigma-Aldrich) to a concentration of 75 mg/mL then diluted with complete media (CM) (RPMI-1640 with 10% FBS, 1% sodium pyruvate, 1% MEM NEAA, and 100 U/mL penicillin-streptomycin) to concentrations of 3 μM, 10 μM, 30 μM, and 100 μM for use with murine BMDCs. For human moDCs, BEN was diluted to concentrations of 25 μM, 50 μM, 75μM, and 100 μM.

Tumor cells, imaging, and *in vivo* depletions

A20-luciferase, a BALB/c B-cell lymphoblastic leukemia cell line, was provided by Dr. Xue-Zhong Yu, MD (Medical University of South Carolina) and has previously been used in murine BMT studies.[25] A20-luc was cultured in RPMI 1640 (Hyclone SH30027) with 10% FBS, MEM, sodium pyruvate (Hyclone SH30239), and 2 μL/mL Zeocin (Gibco) at 37 °C and 5% CO_2. On day 0, the cells were injected iv with the BMT.[26]

To image tumor burden, luciferin (GoldBio, LUCK) was ip injected into the mice at 0.15 mg/g. The mice were then anesthetized with isoflurane (Piramal Critical Care, 440532079) and imaged using a LagoX (Special Instruments Imaging, Tucson, AZ). Luminescence was quantified using AmiView software (Special Instruments Imaging) and presented as ln(photons/second). For depletion experiments, the mice were ip injected weekly with 200 μg of the depleting antibodies NK1.1 (BE0036), GK1.5 (BE0003-1), and 2.43 (BE0061) beginning on day +3.

Bone Marrow-Derived DCs

Bone marrow (BM) cells were collected from 6-10 week old, female BALB/c mice and red blood cells were lysed (BD Biosciences, Franklin Lakes, NJ). 3×10^6 cells were plated per well in 6-well plates at a concentration of 10^6/mL. BM was cultured at 37°C and 5% CO_2 in CM with 200 ng/mL Flt3L (Miltenyi Biotec) and BEN at concentrations of 0 μM , 3 μM, 10 μM, 30 μM, and 100 μM. After four hours, the BEN containing media was washed out and cells were cultured in CM with 200 ng/mL Flt3L. On day 6, BMDCs were collected, washed, and resuspended in PBS and RNAlater (Invitrogen, Carlsbad, CA).

Human Monocytic-DCs

Peripheral blood was collected from healthy human volunteers that were recruited as part of an institutional review board (IRB)-approved research protocol. Whole blood was diluted 1:1 with PBS, layered on top of Ficoll (GE Healthcare Life Sciences), then centrifuged. Peripheral blood mononuclear cells (PBMCs) were collected, washed with PBS, then counted. CD14+ MicroBeads (Miltenyi Biotec) were used to isolate CD14+ monocytes. CD14+ monocytes were counted and resuspended in human DC media (RPMI-1640, 10% FBS, 1% antibiotic-antimycotic solution (ThermoFisher, Waltham, MA), 500 U/mL rhIL-4 (PeproTech, Rocky Hill, NJ), 800 U/mL rhGM-CSF (PeproTech), 100 ng/mL rhFlt3L (Miltenyi Biotech)), then plated into 6-well plates at 1-1.5 $\times 10^6$ monocytes per well. BEN was added to each well at concentrations of 0 μM, 25 μM, 50 μM, 75 μM, and 100 μM. Cells were cultured at 37°C and 5% CO_2. After four hours, the BEN-containing media were washed out and cells were cultured in human DC media. On day 5, moDCs were collected, washed, and resuspended in PBS and RNAlater (Invitrogen).

Quantitative Real-Time PCR

BMDC samples harvested on day 6 were washed and resuspended in PBS and RNAlater (Invitrogen). mRNA was isolated using RNeasy Kit (Qiagen) then reverse transcribed into cDNA using iScripts reverse transcription supermix kit (Bio-Rad). qRT-PCR was performed using Sso Advanced universal probes supermix (Bio-Rad) and TaqMan probes against murine *Csf2ra, Csf2rb, Csf3r, Mycbp, STAT3, flt3, akt1, Spi1,* and *GAPDH* (Applied Biosystems). Probes were titrated to determine appropriate concentration of cDNA, as indicated in **Table 1**. Each probe was run in triplicate for each sample. qRT-PCR detection was done on a LightCycler 96 thermocycler (Roche). The $2^{-\Delta\Delta CT}$ method was used to analyze the gene expression levels.[27]

moDC samples harvested on day 5 were washed and resuspended in PBS and RNA later (Invitrogen). mRNA was isolated using RNeasy Kit (Qiagen) then reverse transcribed into cDNA using iScripts reverse transcription supermix kit (Bio-Rad). qRT-PCR was performed using Sso Advanced universal probes supermix (Bio-Rad) and TaqMan probes against human *GAPDH* and *akt1* (Applied Biosystems) Probes were titrated to determine appropriate concentrations of cDNA, as indicated in **Table 1**. Each probe was run in triplicate for each sample. qRT-PCR detection was done on a LightCycler 96 thermocycler (Roche). The $2^{-\Delta\Delta CT}$ method was used to analyze the gene expression levels.[27]

Table 1. Primers used for quantitative Real Time-Polymerase Chain Reaction

Target Gene	Taqman Assay ID	concentration of cDNA used
Mouse akt1	Mm01331626_m1	5 ng
Mouse Csf2ra	Mm00438331_g1	5 ng
Mouse Csf2rb	Mm00655745_m1	5 ng
Mouse Csf3r	Mm00438334_m1	10 ng
Mouse flt3	Mm00439016_m1	20 ng
Mouse GAPDH	Mm99999915_g1	2 ng
Mouse Spi1 (PU.1)	Mm00488140_m1	5 ng
Mouse STAT3	Mm01219775_m1	10 ng
Human akt1	Hs00178289_m1	2 ng
Human GAPDH	Hs02786624_g1	2 ng

Statistics

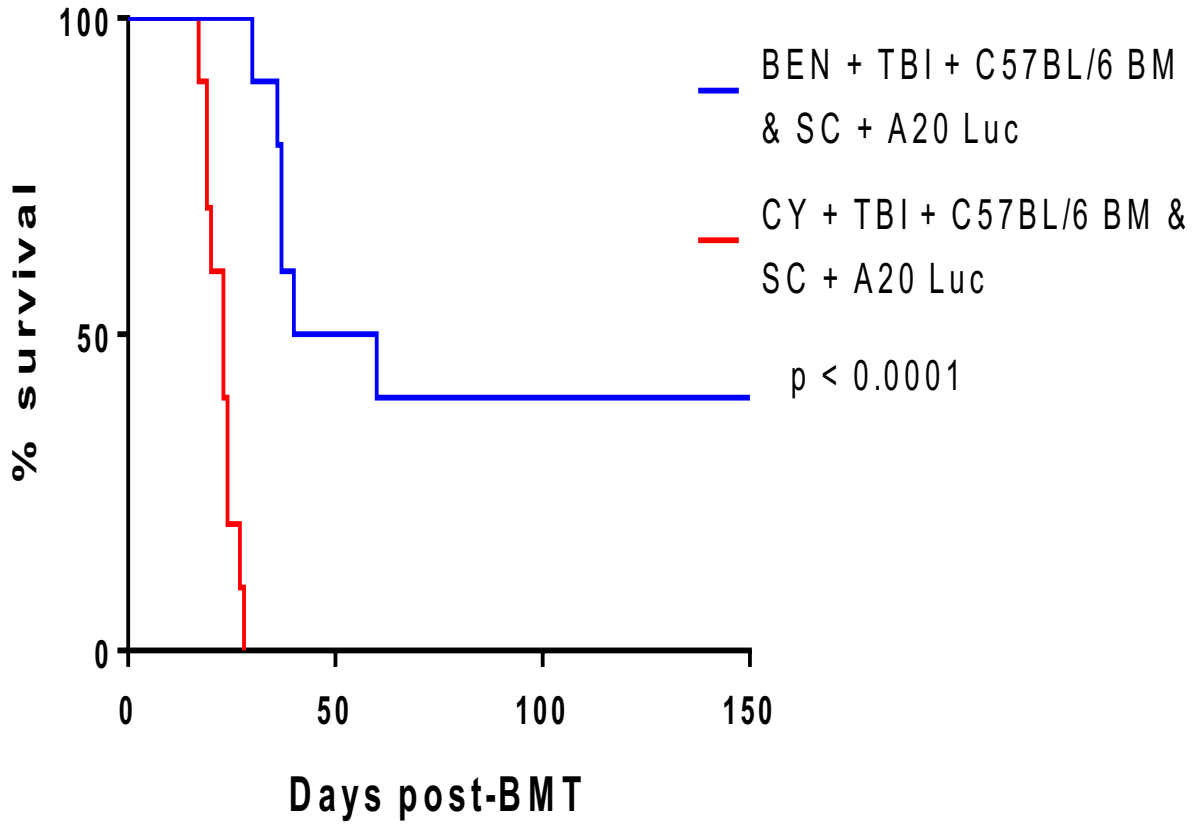
Kaplan-Meier survival curves were generated and log rank statistic was used to evaluate the differences seen between conditions. *P* values less than 0.05 were considered statistically significant. If the differences were not significant, the statistic are not indicated on graphs.

RESULTS

BEN+TBI conditioning decreases GvHD and increases survival compared to CY+TBI conditioning

Comparing BEN+TBI to CY+TBI in the MHC-mismatched murine BMT model, we are able to confirm that BEN+TBI conditioning significantly reduces GvHD morbidity and mortality. As shown in Figure 2, percent survival is significantly higher in mice that received BEN+TBI conditioning (**Figure 2A**), and GvHD scores are significantly lower (**Figure 2B**). For both conditioning regimens, comparable rates of engraftment and tissue necrosis were seen, even in syngeneic control BMTs, confirming that the differences seen can be attributed to GvHD severity.[9]

2A



2B

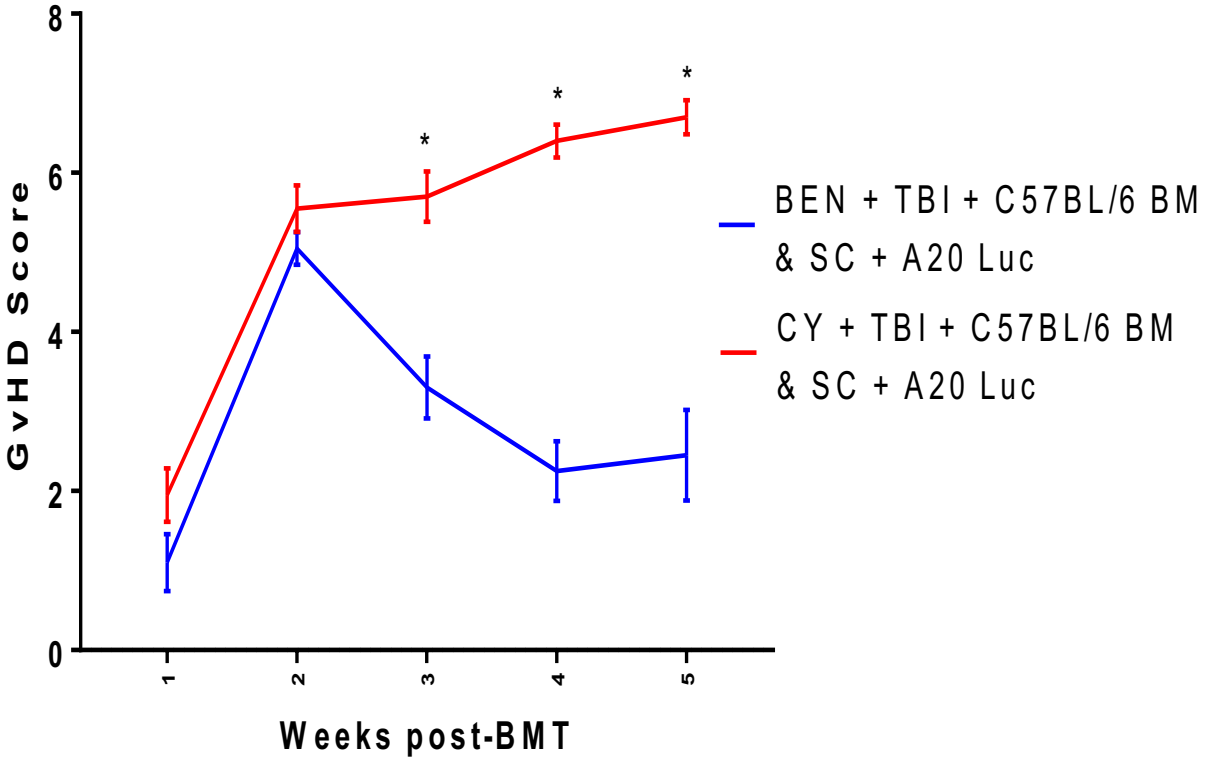


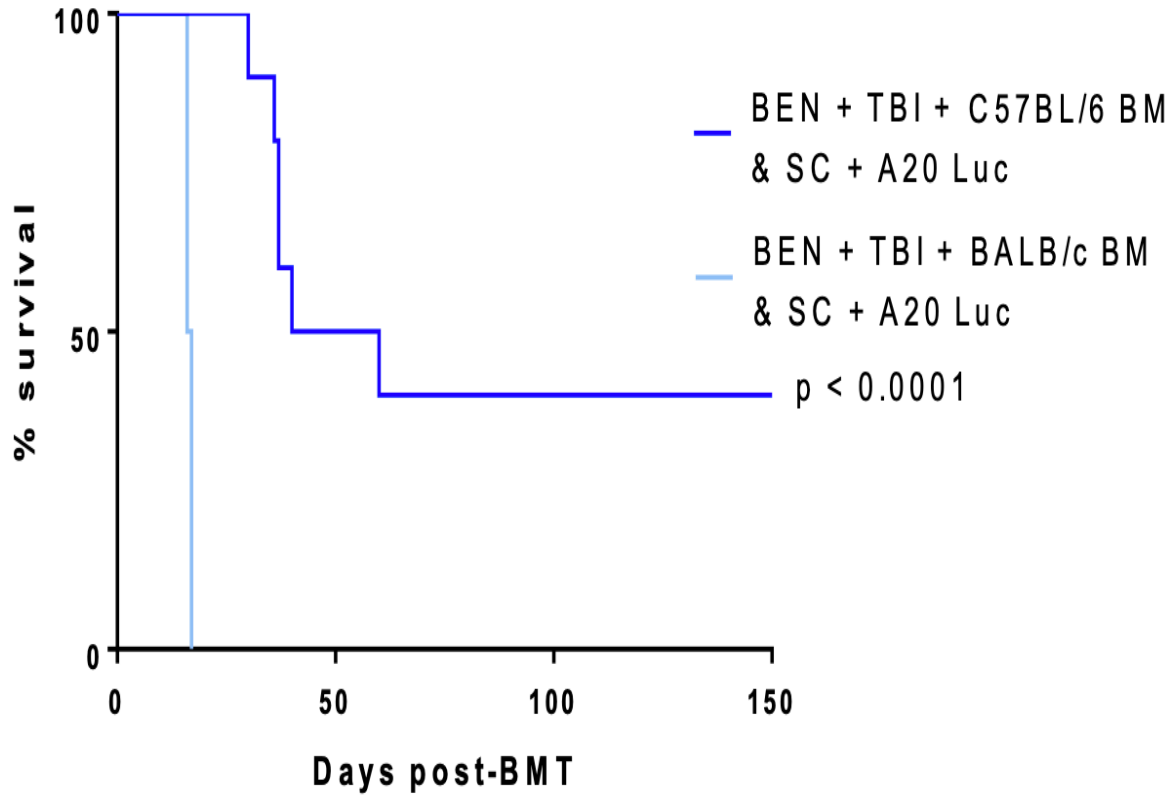
Figure 2: BEN+TBI conditioning decreases GvHD and increases survival compared to CY+TBI conditioning. BALB/c mice received BEN 40 mg/kg iv or CY 200 mg/kg ip on day -2 and 400 cGy TBI on day -1. On day 0, mice received 10^7 donor BM cells with $3*10^6$ SC from C57BL/6 mice and 10^5 A20-Luc IV. Data is pooled from 2 independent experiments (n=5 mice/group). **A**. Survival was monitored, and Kaplan-Meier curves were generated and analyzed using log rank tests to determine significance. **B.** GvHD was monitored by clinical scoring. *p <0.0001.

BEN+TBI conditioning maintains GvL effects

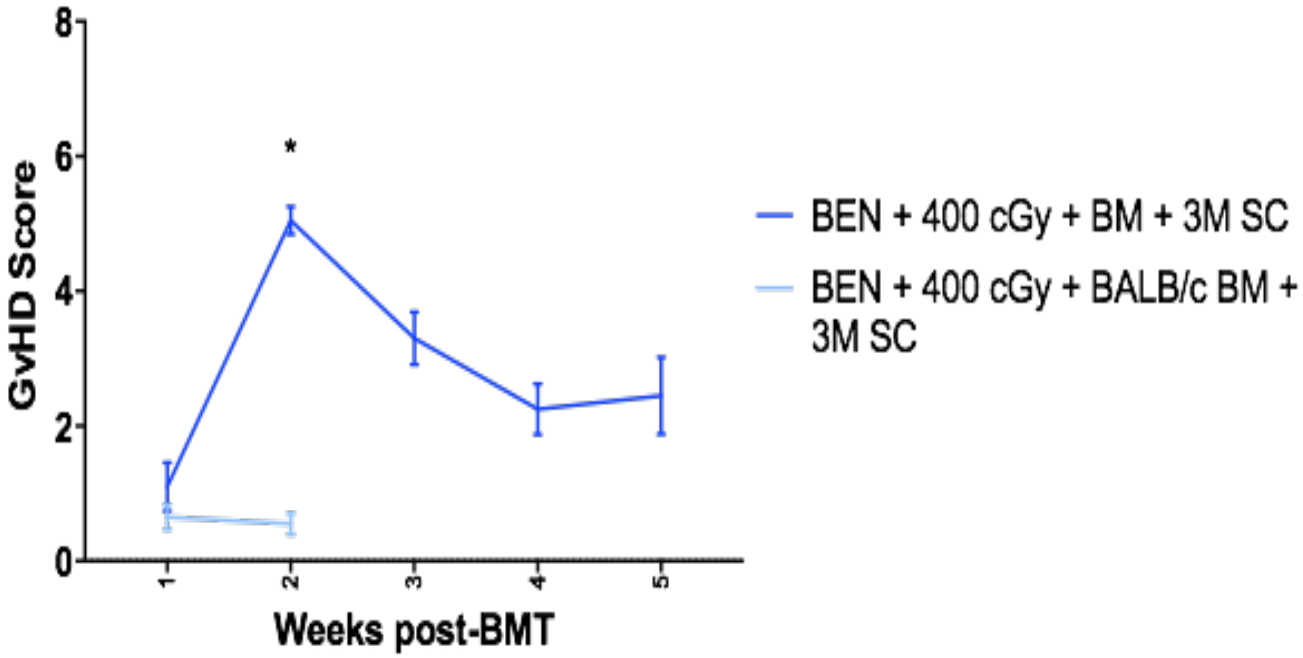
The focus of this study was to investigate the effect of BEN+TBI conditioning on GvL. Using A20-luc leukemia cells that were injected iv along with the BMT on day 0, we find that BEN+TBI conditioning prior to the MHC-mismatched transplant significantly improves survival over a syngeneic control (**Figure 3A**). Despite lower GvHD severity, mice who received the syngeneic control displayed significantly higher amounts of tumor burden than the mice who

received the MHC-mismatched transplant, regardless of the conditioning regimen, indicating that death of syngeneic controls are due to tumor (**Figure 3B**). On day +14, the mice receiving syngeneic controls show increased tumor burden over either group indicating an absence of GvL (**Figure 3C**). On days +14 and +21, mice that received CY+TBI conditioning showed higher tumor burden than the mice that had been conditioned with BEN+TBI, indicating that BEN+TBI conditioning preserves GvL. On day +21, mice that do not show tumor are emaciated due to GvHD. By day +28, all mice that had received CY+TBI died due to GvHD or tumor. Mice that received BEN+TBI conditioning survived through the end of the experiment.

3A



3B


GvHD Score
8
6
4
2
0
*
1
2
3
4
5
Weeks post-BMT
BEN + 400 cGy + BM + 3M SC
BEN + 400 cGy + BALB/c BM + 3M SC

3C

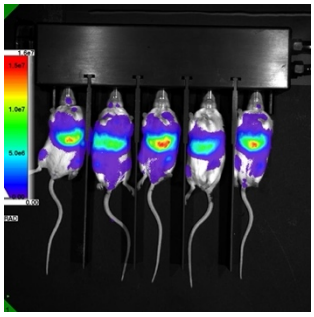
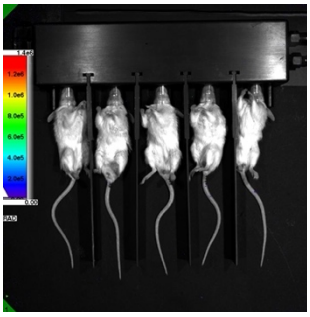
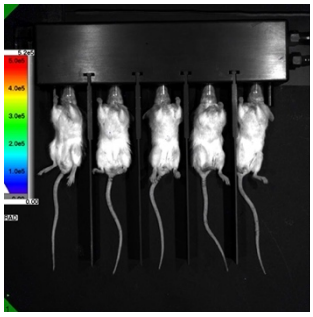
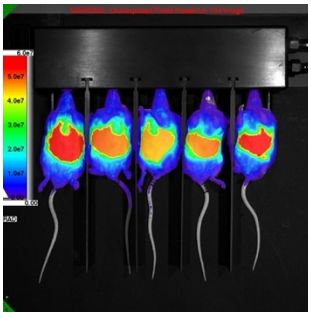
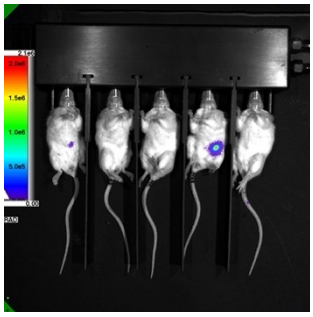
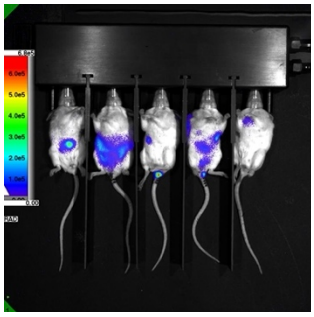
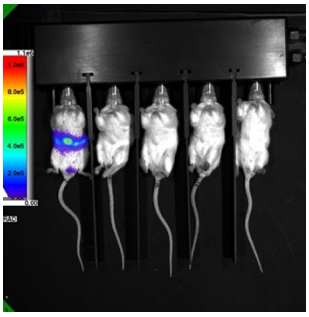
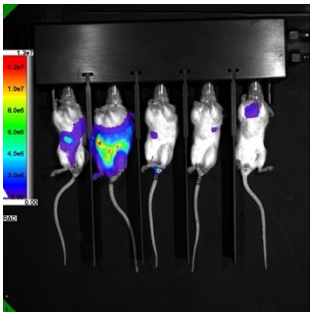
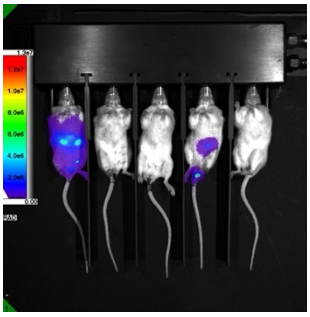
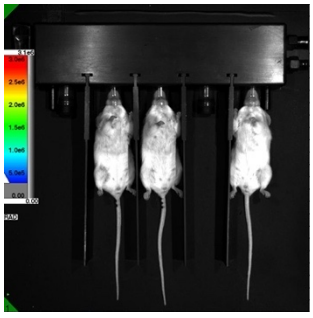
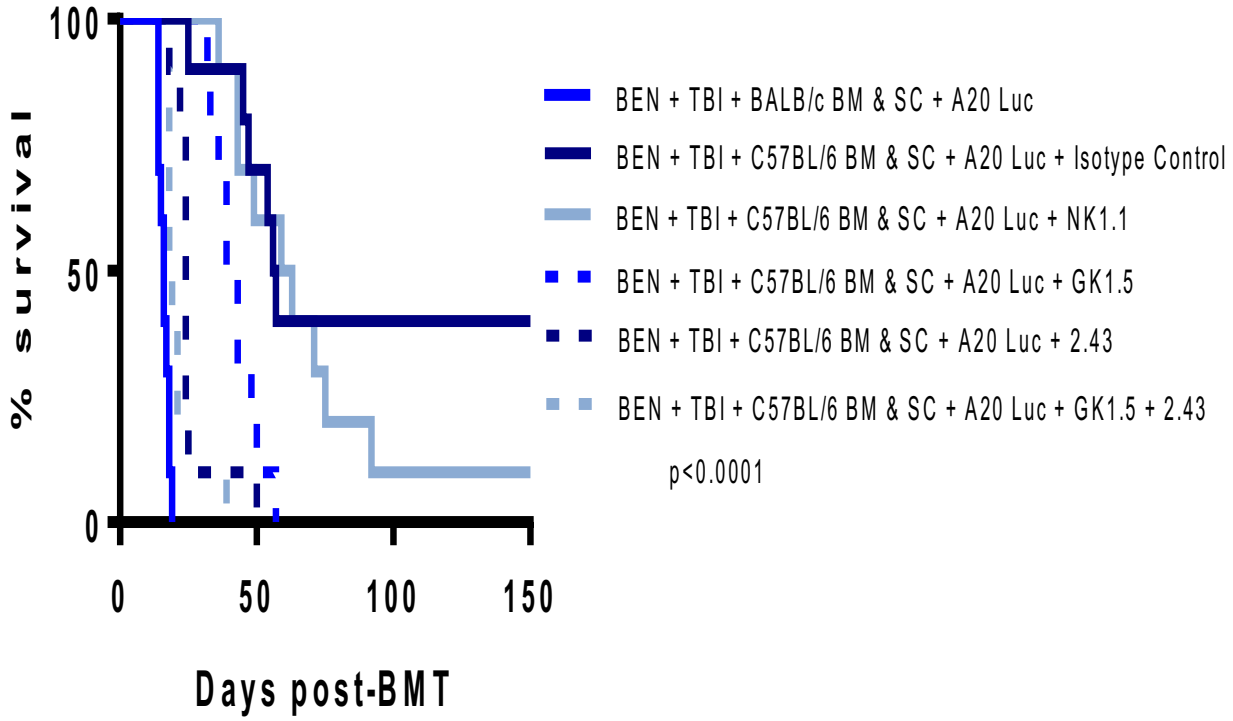
Days post BMT	BEN+TBI + BALB/c BM + SC + A20-Luc	BEN+TBI + C57BL/6 BM + SC + A20-Luc	CY+TBI + C57BL/6 BM + SC + A20-Luc
7			
14			
21			
28			
144			

Figure 3: BEN+TBI conditioning maintains GvL effects. BALB/c mice received BEN 40 mg/kg iv or CY 200 mg/kg ip on day -2 and 400 cGy TBI on day -1. On day 0, mice received 10^7 donor BM cells with $3*10^6$ SC from C57BL/6 mice and 10^5 A20-Luc IV. **A.** Survival was monitored, and Kaplan-Meier curves were generated and analyzed using log rank tests to determine significance. **B.** GvHD was monitor by clinical scoring. $*p < 0.000001$. **C.** Imaging showing tumors in mice receiving BEN versus mice receiving CY along with syngeneic controls. Images are representative of two experiments, n = 5 mice per group.

BEN+TBI preserves T-cell dependent GvL effects

Anti-cancer responses are mediated by cytotoxic lymphocytes such as natural killer (NK) cells and, with the help of CD4+ T helper cells, cytotoxic CD8+ T-cells.[28] To better understand the how BEN+TBI conditioning preserves GvL, we depleted NK cells, CD4+ T-cells, and CD8+ T-cells. Depleting antibodies NK 1.1, GK 1.5, and 2.43, respectively, were ip injected weekly beginning on day +3. Control mice received an ip isotype antibody injection at the same time. Depletion of NK cells did not result in significant changes to survival or tumor burden (**Figure 4A)**. Depletion of CD4+ T-cells caused a significant decrease in survival, but depletion of CD8+ T-cells resulted in a further decrease of survival. Bioluminescent imaging shows no difference between NK cells depleted mice and isotype control mice, as well as significantly higher tumor burden in CD4+ and CD8+ depleted mice (**Figure 4B**). Furthermore, depletion of CD8+ T-cells also resulted in the greatest loss of GvL. This data indicates that while NK cells may not play a significant role in GvL seen after BEN+TBI conditioning, both CD4+ and CD8+ T-cells do play a significant role.

4A


100
50
0
% survival
0
50
100
150
Days post-BMT
BEN + TBI + BALB/c BM & SC + A20 Luc
BEN + TBI + C57BL/6 BM & SC + A20 Luc + Isotype Control
BEN + TBI + C57BL/6 BM & SC + A20 Luc + NK1.1
BEN + TBI + C57BL/6 BM & SC + A20 Luc + GK1.5
BEN + TBI + C57BL/6 BM & SC + A20 Luc + 2.43
BEN + TBI + C57BL/6 BM & SC + A20 Luc + GK1.5 + 2.43
p<0.0001

4B

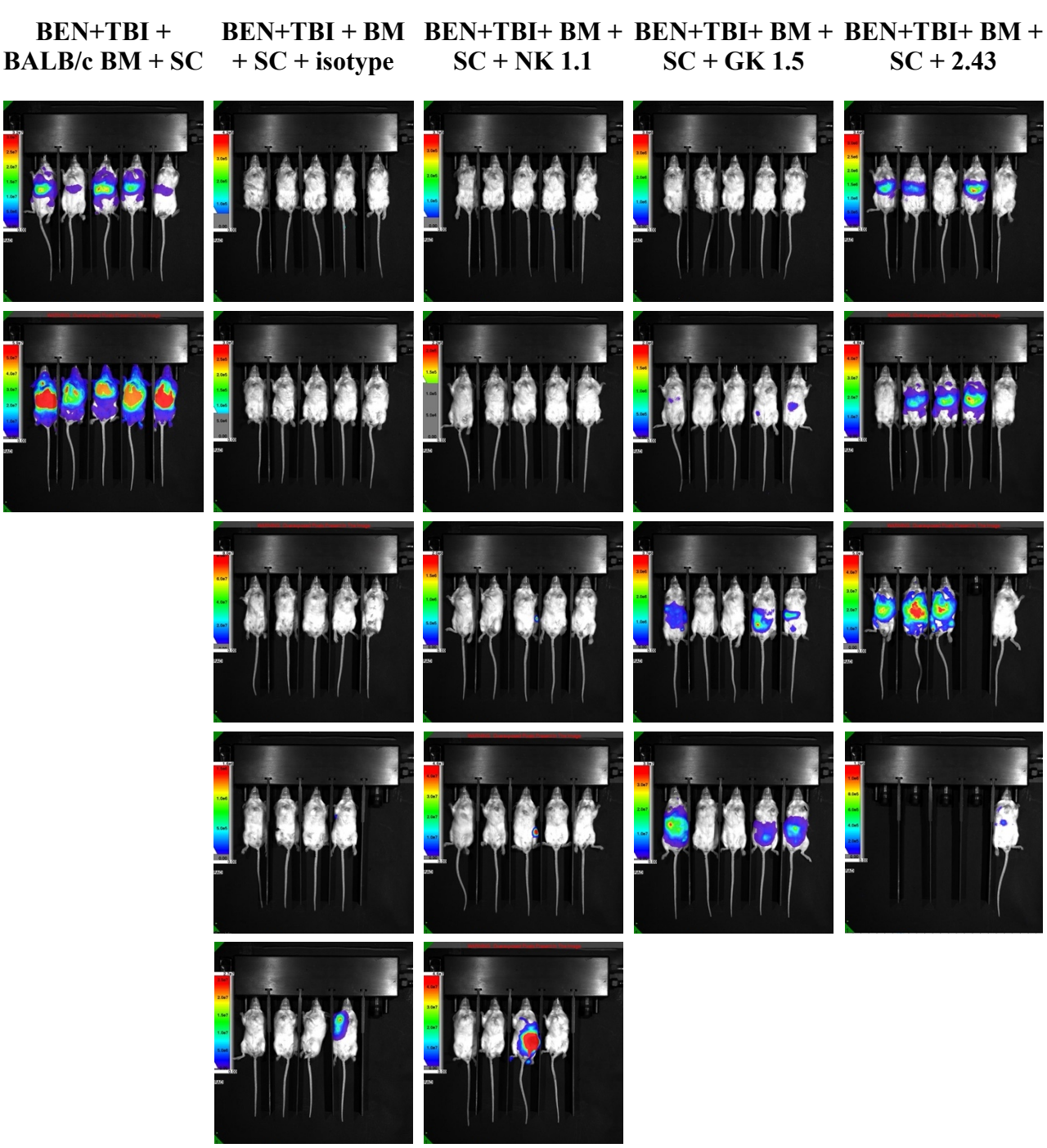

Days post BMT
BEN+TBI + BALB/c BM + SC
BEN+TBI + BM + SC + isotype
BEN+TBI+ BM + SC + NK 1.1
BEN+TBI+ BM + SC + GK 1.5
BEN+TBI+ BM + SC + 2.43
7
14
21
28
54

Figure 4: BEN+TBI preserves T-cell dependent GvL effects. BALB/c mice received BEN 40 mg/kg IV on day -2 and 400 cGy TBI on day -1. On day 0, mice received 10^7 donor BM cells with $3*10^6$ SC from C57BL/6 mice and 10^5 A20-Luc iv. Weekly, starting at day +3, mice received 200 μg/mouse of NK1.1, GK1.5, 2.43 or isotype control ip. **A.** Mouse survival was monitored, and a Kaplan-Meier curve was generated and analyzed with a log rank test to determine significance. **B.** Imaging showing tumors in mice receiving depleting antibodies as well as syngeneic and isotype controls. Images are representative of two experiments, n = 5 mice per group.

Murine BMDCs exposed to BEN exhibit a concentration-dependent decrease in mRNA transcript levels of Akt1.

DCs are professional antigen presenting cells (APCs) that present antigen (Ag) to Ag-specific T-cells which induces T-cell activation and proliferation.[12] We have previously shown that DCs treated with BEN induced less allogeneic T-cell proliferation, had a significant increase in type 1 conventional DCs (cDC1) which have been shown to suppress GvHD, and had a greater expression of Flt3 than DCs treated with CY.[14] STAT3 has been shown to be indispensable for Flt3L induced expansion of DCs, with STAT3 deficient mice showing a severe deficit in DCs after administration of Flt3L.[29] As BEN has been shown to inhibit STAT3 signaling,[21] we hypothesized that BEN inhibits STAT3 signaling, causing a compensatory increased in Flt3 expression and Akt1 expression to allow for increased signaling through this alternative Flt3 signaling pathway (**Figure 1**). To investigate this, quantitative real-time polymerase chain reaction (qRT-PCR) was run using TaqMan probes against murine *Csf2ra, Csf2rb, Csf3r, Mycbp, STAT3, flt3, akt1, Spi1,* and *GAPDH* on BMDCs that were exposed to BEN in concentrations of: 0 μM, 3 μM, 10 μM, 30 μM, and 100 μM for four hours.

For *Csf2ra, Csf2rb, Csf3r, STAT3, flt3,* and *Spi1* there was no apparent trend, nor was there significance of the data. For *Mycbp,* there was no data collected for any samples. For *akt1*, there

was a concentration-dependent decrease in mRNA transcript levels; as the concentration of BEN increased the transcript levels significantly decreased (**Figure 5**).

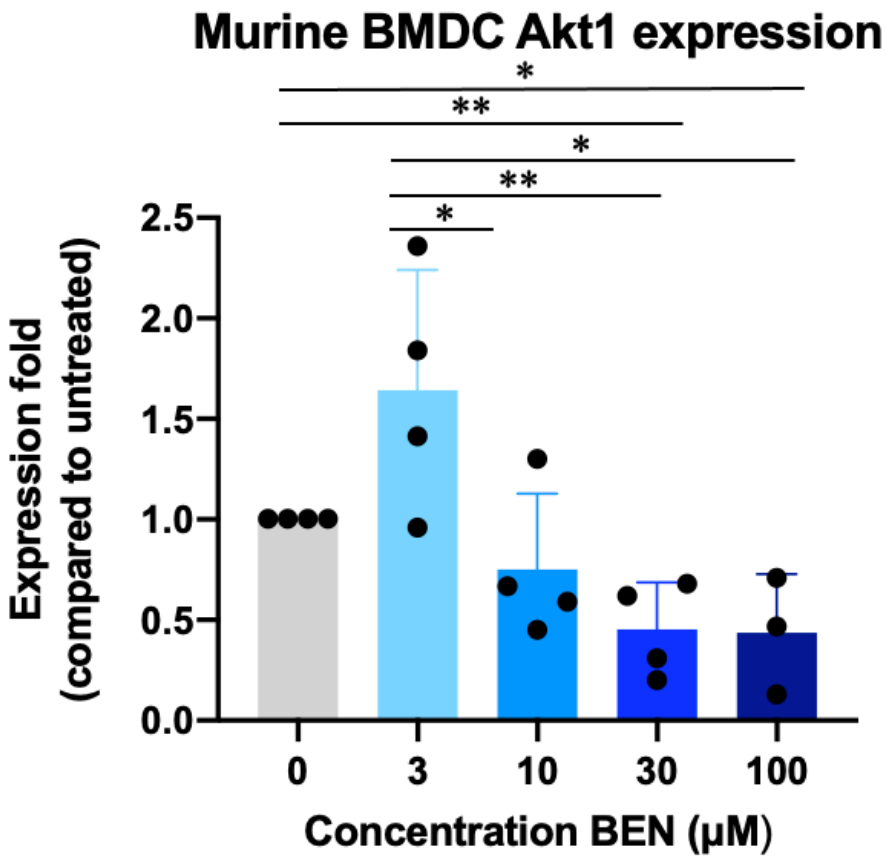


Figure 5: Murine BMDCs exposed to BEN exhibit a concentration-dependent decrease in mRNA transcript levels of Akt1. Data was normalized to BMDCs exposed to 0 µM BEN using the $2^{-\Delta\Delta CT}$ method and shown as expression fold change with SEM. Pooled data from 2 experiments are shown, n=4. Unpaired t test was used to determine significance. *p < 0.05, **p < 0.01.

Human moDCs exposed to BEN exhibit a concentration-dependent decrease in mRNA transcript levels of Akt1.

To better understand the mechanism of BEN, we also characterized moDCs that were exposed to BEN at concentrations of: 0 µM, 25 µM, 50 µM, 75 µM, and 100 µM for four hours.

qRT-PCR was run using TaqMan probes against human *Akt1* and *GAPDH*. Similar to murine BMDCs, there was a concentration-dependent decrease in Akt1 mRNA transcript levels; as the concentration of BEN exposure increased the transcript levels of Akt1 significantly decreased (**Figure 6**).

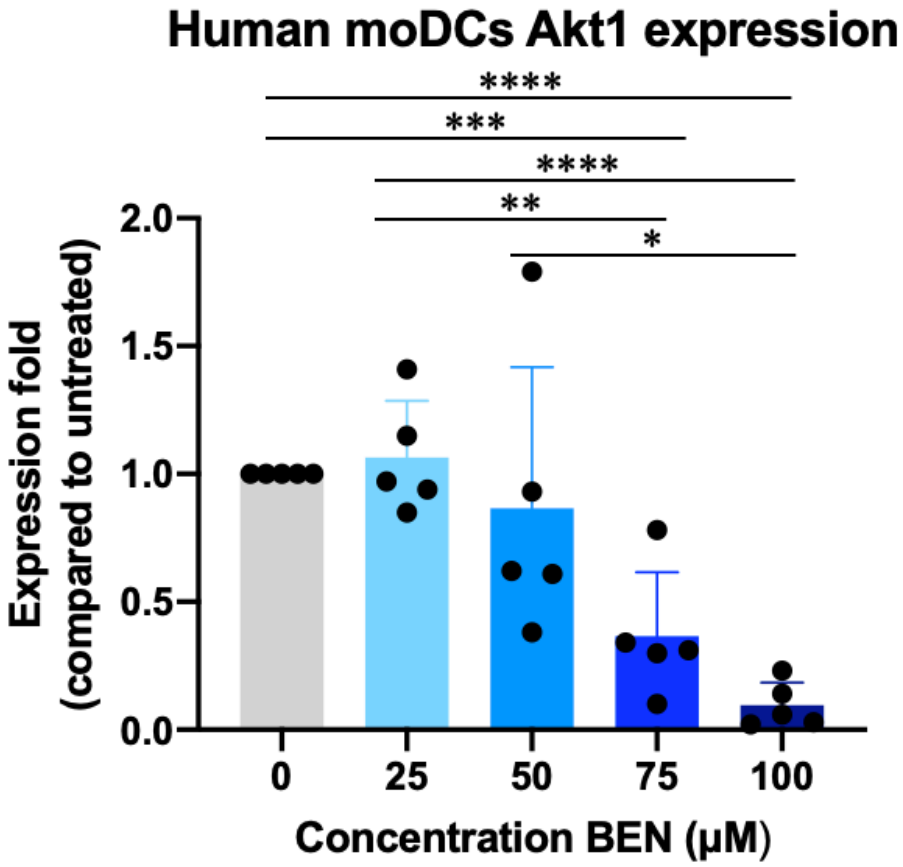


Figure 6: Human moDCs exposed to BEN exhibit a concentration-dependent decrease in mRNA transcript levels of Akt1. Data was normalized to moDCs exposed to 0 μM BEN using the $2^{-\Delta\Delta CT}$ method and shown as expression fold change with SEM. Pooled data from 2 experiments are shown, n=5. Unpaired t test was used to determine significance. *p < 0.05, **p < 0.01, ***p <0.0005, ****p < 0.0001.

DISCUSSION

As GvHD remains a significant impediment to the success of HCT,[31] a need for novel strategies to prevent or reduce GvHD arises. We have previously shown that substituting BEN for the current standard of care, CY, combined with TBI decreases the severity of GvHD, thus also improving GvHD survival in a murine MHC-mismatched BMT model.[9] In this study, we confirm this finding and also show that pre-transplant conditioning with BEN+TBI reduces GvHD without compromising GvL. To better understand how BEN mediates GvHD and promotes GvL, we depleted NK cells, CD4+ T-cells, and CD8+ T-cells to determine which cells are responsible for GvL. We show that the greatest deficit in anti-tumor effect occurs when CD8+ T-cells were depleted, followed by CD4+ T-cells, with depletion of NK cells showing no change to GvL effects. Therefore, CD8+ T-cells primarily contribute to GvL effects seen in this model, followed closely by CD4+ T-cells. NK cells play little to no role in GvL effects seen post BEN+TBI conditioning in this model. CD8+ T-cells mediate the killing of tumor cells, but CD4+ T-cells are required to induce a robust CD8+ response.[28, 32] CD4+ T-cells are also required for maturation of DCs, which in turn interact with CD8+ T-cells, enhancing anti-tumor responses.[32] Therefore, while CD8+ T-cell depletion results in a loss of anti-tumor responses, without CD4+ T-cells the anti-tumor response is greatly dampened.

As we utilized a complete MHC-mismatched model, the GvHD observed was much more severe than would be seen in an MHC-matched HCT.[33, 34] Therefore, treatments that significantly decrease GvHD in a complete MHC-mismatched model show potential for use in clinical setting. On another note, due to the severity of GvHD seen in this MHC-mismatched model, many of the mice that received CY+TBI die within the first 5 weeks. This is a limitation to the study as any mice that live beyond 5 weeks (i.e. the healthier mice in the group) causes the data to skew

toward those mice. This skewing is not seen in BEN+TBI conditioned mice, as their average survival is much longer.

We still lack a complete understand of the mechanisms of how BEN+TBI conditioning suppresses GvHD while maintaining GvL. In a previous publication, we have shown that BEN increases the proliferation of type 1 conventional DCs (cDC1s), which have been shown to suppress GvHD.[28] Furthermore, we have shown the BEN also increases expression of Flt3 and induces less allogeneic T-cell proliferation.[14] Flt3 is a receptor tyrosine kinase that upon binding of Flt3L controls survival and expansion of early hematopoietic cells.[15] Administration of Flt3L has also been shown to expand and mobilize DCs.[35] As Flt3 signaling plays a role in the expansion of cDC1s and DCs play an essential role in educating T-cells,[12, 30] increased Flt3 signaling induced cDC1 expansion is one possible explanation for the reduction of GvHD following BEN conditioning. To investigate this possible explanation, we looked toward Flt3, which when activated can signal though many signaling pathways including Ras/MEK/Erk, STAT, and PI3K/akt1.[17]

As BEN has been reported to directly bind to and inhibit STAT[21] and RNA sequencing data has shown greater Akt1 transcripts in BEN treated DCs compared to CY[14], we hypothesized that the expression of Flt3 and Akt1 are upregulated following conditioning with BEN to allow for an increase in survival and proliferative signaling that accounts for the expansion of cDC1s with BEN- treated DCs. Contrary to our hypothesis, the qRT-PCR data shows a significant decrease in the expression fold of Akt1 transcripts in both murine BMDCs and human moDCs as the concentration of BEN exposure increases compared to non-exposed DCs. As qRT-PCR can only measure transcript levels, the results reported only indicate that the DCs were not actively producing higher levels of Akt1 when they were harvested. It is possible that Akt1 transcript

expression increased earlier on during culturing. To test this, qRT-PCR could be performed at earlier time points to see if transcript levels increase earlier than 5-6 days. Furthermore, a Western blot can be performed to measure Akt1 protein levels within the cells at the time of harvesting. This would provide data that more directly pertains to protein expression, rather than transcript levels, and may allow for a better understanding of the biological mechanism of BEN.

These results suggest that there may be an alternative explanation for the increase in the expression of Flt3 on DCs. Flt3 may be upregulated with increasing concentrations of BEN to compensate for the loss of the STAT3 and Akt1 signaling pathways. Through increased recycling of the Flt3 receptor, DCs may be able to receive enough signaling to account for the expansion of cDC1s in BEN conditioned samples. Another possible explanation for the increase in Flt3 is that Flt3 may signal through an alternative pathway that does not require STAT3 or Akt1, such as through signaling through GRB2. This could be tested using qRT-PCR to measure transcript levels or using Western blots to measure protein levels with probes against *GRB2, RAS, RAF, MEK,* and/or *ERK.* To get a more accurate picture, both qRT-PCR and Western blots can be run at multiple points during culturing as well.

There are many other factors that could be contributing to suppression of GvHD that have not been investigated in this study. For example, various cytokines have been shown to play a role in GvHD pathophysiology. Tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) are the cytokines that are critical in promoting GvHD, but other cytokines also promote GvHD, including: IL-1, IL-2, IL-12 and IL-21. On the other hand, IL-10, IL-12, and transforming growth factor beta (TGF-β) have been shown to have protective properties in preventing GvHD.[36, 37] As blocking pro-GvHD cytokines has been shown to suppress GvHD,[38] it may be possible

that BEN suppresses GvHD by modulating the production of some of these various cytokines and would be interesting to investigate further.

In summary, we have confirmed our earlier finding that BEN+TBI conditioning reduces GvHD when compared to CY+TBI and also show that pre-transplant conditioning with BEN+TBI not only reduces GvHD but does so without compromising GvL. As pre-transplant BEN+TBI conditioning induces a superior GvL effect with less GvHD compared to CY+TBI pre-transplant conditioning, this suggests that using BEN instead of CY may be a safer and more effective conditioning regimen. We also show that BEN+TBI conditioning induces a T-cell mediated GvL response. Lastly, we demonstrated that BEN exposure of DCs leads to a concentration dependent decrease in Akt1 transcript levels. As our studies have shown that BEN may be a safer and more effective alternative to CY, further investigation is necessary to fully understand the mechanisms of bendamustine.

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