

Post-transplant bendamustine reduces GvHD while preserving GvL in experimental haploidentical bone marrow transplantation

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Summary

Advances in haploidentical bone marrow transplantation (h-BMT) have drastically broadened the treatment options for patients requiring BMT. The possibility of significantly reducing the complications resulting from graft-versus-host disease (GvHD) with the administration of post-transplant cyclophosphamide (PT-CY) has substantially improved the efficacy and applicability of T cell-replete h-BMT. However, higher frequency of disease recurrence remains a major challenge in h-BMT with PT-CY. There is a critical need to identify novel strategies to prevent GvHD while sparing the graft-versus-leukaemia (GvL) effect in h-BMT. To this end, we evaluated the impact of bendamustine (BEN), given post-transplant, on GvHD and GvL using clinically relevant murine h-BMT models. We provide results indicating that post-transplant bendamustine (PT-BEN) alleviates GvHD, significantly improving survival, while preserving engraftment and GvL effects. We further document that PT-BEN can mitigate GvHD even in the absence of Treg. Our results also indicate that PT-BEN is less myelosuppressive than PT-CY, significantly increasing the number and proportion of CD11b⁺Gr-1^{hi} cells, while decreasing lymphoid cells. *In vitro* we observed that BEN enhances the suppressive function of myeloid-derived suppressor cells (MDSCs) while impairing the proliferation of T- and B-cells. These results advocate for the consideration of PT-BEN as a new therapeutic platform for clinical implementation in h-BMT.

Keywords: bone marrow transplantation, graft-versus-host disease, graft-versus-leukaemia, bendamustine, cyclophosphamide.

Allogeneic haematopoietic cell transplantation (HCT) is potentially curative for many patients with haematological disorders and malignancies. However, the identification of suitable human leucocyte antigen (HLA)-matched donors remains a challenge, particularly for patients of minority or mixed race (Gragert *et al*, 2014; Pidala *et al*, 2013; Switzer *et al*, 2013). As every individual shares one HLA haplotype with each biological parent and with 50% of siblings, a familial haploidentical donor is immediately available in most cases, avoiding the delays associated with unrelated donor identification. Haploidentical HCT has therefore become a mainstream transplant option (Bacigalupo *et al*, 2015; Castagna *et al*, 2014; Fuchs, 2015; Handgretinger, 2014; Martelli *et al*, 2014, 2015; McCurdy *et al*, 2015; Raiola *et al*, 2014).

Graft-versus-host disease (GvHD) represents a common complication in T cell-replete haploidentical-BMT (h-BMT), but is significantly reduced by the administration of cyclophosphamide post-transplant (PT-CY) (Brodsky *et al*, 2008). Graft engineering strategies are also being evaluated with the aim of reducing GvHD, including the depletion of CD3⁺ or TCR- $\alpha\beta^+$ and CD19⁺ cells, concurrently enriching for CD34⁺ cells, TCR- $\gamma\delta^+$, NK cells and T regulatory cells (Treg) (Di Ianni *et al*, 2011a,b; Dvorak *et al*, 2013; Federmann *et al*, 2012; Locatelli *et al*, 2013; Martelli *et al*, 2014). While some of these approaches appear promising, they are restricted to centres that have graft-engineering capabilities. T-cell-replete h-BMT with PT-CY has therefore emerged as a leading h-BMT approach as it circumvents the need to manipulate stem cell grafts and can be applied at any BMT

centre(Luznik & Fuchs, 2010; Luznik *et al*, 2012). PT-CY, especially when implemented following reduced intensity conditioning (RIC), has been associated with relapse rates as high as 55%(Munchel *et al*, 2011). This has prompted the consideration of myeloablative conditioning (MAC) regimens to reduce relapse rates, but at the cost of increasing GvHD and non-relapse mortality(Bacigalupo *et al*, 2015; Ciurea *et al*, 2015; Solomon *et al*, 2015).

In this context, we investigated the feasibility of using the bifunctional mechlorethamine derivative bendamustine (BEN), an active alkylator and purine analogue, following h-BMT(Hartmann & Zimmer, 1972; Tageja & Nagi, 2010). BEN has been used effectively against lymphomas(Corazzelli *et al*, 2013; Derenzini *et al*, 2014; Kahl *et al*, 2010; Rigacci *et al*, 2012; Robinson *et al*, 2008), chronic lymphocytic leukaemia(Bergmann *et al*, 2005; Quinquenel *et al*, 2015) and, more recently, as conditioning for allogeneic HCT(Khoury *et al*, 2014). This agent has also been applied as pre-treatment for chimeric antigen receptor T-cell therapy of leukaemias, as it induces a sustained lymphodepletion earlier than other agents(Kalos *et al*, 2011). However, BEN has not been studied as a post-transplant immunomodulating agent. Based on this information, we evaluated the potential of BEN to serve as an alternative to PT-CY in mitigating GvHD following h-BMT. Our results indicate that post-transplant BEN (PT-BEN) can control GvHD following MAC and RIC h-BMT. More importantly, graft-versus-leukaemia (GvL) effects were significantly stronger in PT-BEN than PT-CY treated mice. These findings uncover a novel application for BEN and may have a major translational impact on the development of more effective clinical h-BMT approaches.

Methods

Mice

Age-matched 5–12 week-old female CAF1/J and NOD-*scid* IL2R γ ^{null} mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), CB6F1 from Charles Rivers Laboratories (Wilmington, MA, USA) and BALB/c from the National Cancer Institute (Frederick, MD, USA). 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).

Haploidentical bone marrow transplantation

Recipient CAF1/J (H-2 $^{d/a}$) mice received 600 (RIC) or 1000 (MAC) cGy total body irradiation (TBI) on day -1 using a Cesium 137 irradiator. On day 0, mice received 10⁷ CB6F1 (H-2 $^{d/b}$) bone marrow (BM) cells with or without 3x10⁷ spleen cells (SC) intravenously (i.v.). Moribund mice were euthanized following IACUC-approved procedures and survival was monitored. Mice were weighed every three to 4 d and per cent of starting weight was calculated. Mice were

Post-BMT bendamustine reduces GvHD and promotes GvL

also scored clinically on skin integrity, fur, posture and activity and cumulative GvHD scores were calculated(Cooke *et al*, 1996).

Preparation of total T-cells, CD25[−] T-cells, and T-cell-depleted BM

Total T-cells were isolated from naïve CB6F1 spleens by negative selection using mouse Pan T-Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA) with a purity of >97%. CD25⁺ T-cells were depleted from this population using CD25 Microbead Kit (Miltenyi Biotec) resulting in preparations containing less than 0.4% remaining CD25⁺FoxP3⁺ cells. T-cells were depleted from BM cells using the CD3ε MicroBead Kit (Miltenyi Biotec), with less than 0.3% CD3ε⁺ cells remaining.

Drug preparation and administration

Cyclophosphamide (CY, Sigma-Aldrich, St. Louis, MO, USA) was reconstituted in sterile water (Hyclone, Logan, UT, USA) and diluted in sterile saline (Fisher Scientific, Pittsburgh, PA, USA) for intraperitoneal (i.p.) injection. Bendamustine was reconstituted in dimethyl sulfoxide (Sigma-Aldrich) and diluted in sterile phosphate-buffered saline (Hyclone) containing 0.2% carboxymethylcellulose and 0.25% polysorbate 80 (Sigma-Aldrich) for i.v. injection.

A20 leukaemia and imaging

A20, a BALB/c B-cell lymphoblastic leukaemia cell line (American Type Culture Collection, Manassas, VA, USA), is radio-resistant and has been used previously in murine BMT studies(Chen *et al*, 2006; Zeng *et al*, 2014). A20 was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), MEM nonessential amino acids, and sodium pyruvate (Cellgro, Manassas, VA, USA) at 37°C and 5% CO₂. Luciferase-expressing A20 cells were generously provided by Dr. Xue-Zhong Yu, MD (Medical University of South Carolina, Charleston, SC, USA). At various time points, A20-Luc bearing mice were given luciferin i.p. 0.15 mg/g, anesthetized with isoflurane and imaged using a LagoX (Spectral Instruments Imaging, Tucson, AZ, USA).

Flow cytometry

Cells were incubated with anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA, USA) for 15 min, then fluorochrome-conjugated antibodies for 30 min. Cells were fixed and permeabilized (Fixation/Permeabilization; eBioscience, San Diego, CA, USA) before intracellular staining. Fluorescence data were collected with an LSRFortessa cell analyser (BD Biosciences) and analysed using FlowJo 2 (Tree Star, Ashland, OR, USA). Antibodies used were anti-mouse CD4 allophycocyanin (APC) (clone RM4-5), CD25 APC-eFluor780

(clone PC61.5), CD25 APC (clone PC61.5), Gr-1 fluorescein isothiocyanate (FITC) (clone RB6-8C5), CD11b eFluor450 (clone M1/70), FoxP3 AlexaFluor700 (clone FJK-16s), FoxP3 APC (clone FJK-16s), H2kb peridinin chlorophyll (PerCP)-eFluor710 (clone AF6-88.5.3), CD8 phycoerythrin-cyanin 7 (PE-Cy7) (clone 53-6.7) (eBioscience), B220 BV510 (clone RA3-6B2) (Biolegend), H2kk PE (clone 36-7-5), and CD3ε PE-CF594 (clone 145-2C11) (BD Biosciences).

BM-derived MDSC generation, suppression assays and proliferation assays

CB6F1 BM cells were cultured for 3 d at 0.5×10^6 cells/ml with 10 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL6) (Peprotech, Rocky Hill, NJ, USA), human granulocyte colony-stimulating factor (G-CSF) (Amgen, Thousand Oaks, CA, USA) and 0–100 μ mol/l BEN in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (Hyclone)(Kurko *et al*, 2014). On day 3, cells were stained and analysed by flow cytometry or co-cultured with stimulated CB6F1 T-cells to evaluate their suppressive function. T-cells were isolated from spleens using a Pan T-cell II Isolation Kit (MiltenyiBiotec), stained with CellTrace Violet (40 μ mol/l, 5 min; Life Technologies, Carlsbad, CA, USA) and stimulated with CD3/CD28 beads (2 beads to 1 T-cell; Life Technologies). MDSCs were co-incubated with T-cells (1 MDSC to 2 T-cells) in RPMI medium with 10% FBS, MEM, sodium pyruvate and 55 μ mol/l β -mercaptoethanol (Sigma-Aldrich) for 3 d. Flow cytometry followed by Modfit (Verity Software House, Topsham, ME, USA) analysis was used to determine the proliferation index (PI) of the T-cells. % proliferation = (PI sample – PI unstimulated T-cells) / (PI stimulated T-cells – PI unstimulated T-cells) $\times 100$. Total T-cells for proliferation assays were prepared, stimulated, plated and analysed as above. On day 3 of culture, cells were stained with anti-CD4 and CD8 and analysed by flow cytometry. B-cells were isolated using a MiltenyiBiotec B-cell Isolation Kit, stained with CellTrace Violet

and cultured for 5 d with 10 μ g/ml lipopolysaccharide (LPS) (Sigma-Aldrich). Cells were plated and analysed as above.

Statistics

Kaplan–Meier curves were analysed by log-rank test to determine survival percentages and differences between groups (Kaplan & Meier, 1958; Peto & Peto, 1972). Mann–Whitney tests were used to determine cell count, weight and GvHD score differences between groups. *P* values <0.05 were considered statistically significant.

Study approval

All animal studies were approved by the IACUC at The University of Arizona.

Results

A fully haploidentical-BMT model is utilized as an experimental platform for GvHD

Most reported mouse haploidentical models use parent→F1 combinations, which are limited by a lack of rejection of parent donor cells. To more closely mimic the environment encountered in human h-BMT, we established a more clinically relevant, fully haploidentical model consisting of BALB/c \times C57BL/6 F1 (CB6F1, H-2^{d/b})→BALB/c x A/J F1 (CAF1/J, H-2^{d/a}). Besides offering the advantage of evaluating rejection and engraftment, transplantation of CB6F1→CAF1/J recipients is associated with significantly more severe GvHD when compared to that of parent→F1 (Figure S1). As T-cells constitute about 2% of murine bone marrow(Sykes, 1990), recipients receiving only h-BMT did not develop lethal GvHD, irrespective of the intensity of conditioning. Co-injecting 3×10^7 h-SC with 10^7 h-BM cells (h-BMT/SC) resulted in GvHD lethality (Fig 1). An increase in GvHD mortality relative to TBI intensity was evident in h-BMT/SC

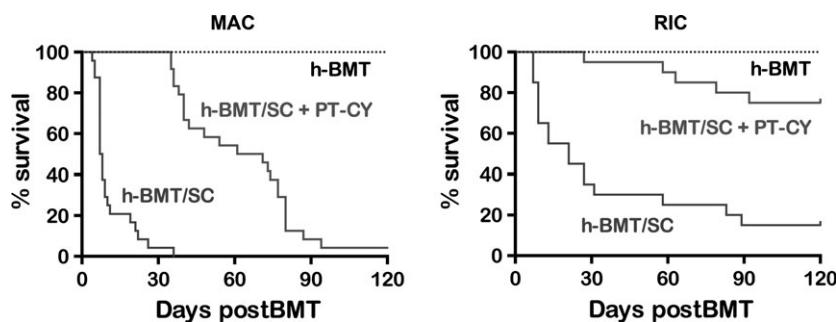


Fig 1. Survival from GvHD following MAC and RIC haploidentical BMT (h-BMT) increases with PT-CY. CAF1/J mice (H-2^{d/a}) received myeloablative conditioning (MAC) 1000 cGy or reduced intensity conditioning (RIC) 600 cGy total body irradiation (TBI) on day -1 and h-BM (10^7) with or without h-SC (3×10^7) from CB6F1 mice (H-2^{d/b}) on day 0. Post-transplant cyclophosphamide (PT-CY) 75 mg/kg was given i.p. on days +3 and +4. Pooled data from 4 experiments (MAC; $n=16$) and 6 experiments (RIC; $n=24$). Kaplan–Meier survival curves were generated and analysed using the log-rank test. MAC: *h-BMT/SC+PT-CY vs h-BMT/SC and h-BMT* $P<0.0001$. RIC: *h-BMT/SC+PT-CY vs h-BMT/SC* $P<0.0001$ and *vs h-BMT* $P<0.035$.

recipients, with mice receiving MAC dying of GvHD at a median time of 7 d *versus* 21 d for RIC (Fig 1).

PT-CY significantly prolonged survival with both conditioning regimens. However, >95% of mice eventually died of GvHD following MAC and 25% following RIC (Fig 1). Given these limitations of PT-CY, especially with MAC, we sought to evaluate novel agents that can be administered post-transplant to better control GvHD morbidity and mortality and focused our studies on PT-BEN.

Donor cell engraftment is maintained with PT-BEN administration

Before evaluating the effects of BEN on GvHD, it was essential to determine the impact of this drug on donor cell engraftment. Chimerism was evaluated following MAC or RIC. Conditioned CAF1/J recipient mice received CB6F1 h-BM with or without h-SC. All mice receiving MAC achieved full donor chimerism following h-BMT. Mice retained this engraftment with the addition of both h-SC and PT-BEN (Fig 2). Mice receiving RIC followed by h-BMT had mixed chimerism on day +7, with only half of the mice becoming fully chimeric by day +14. The addition of h-SC to the bone marrow infusion augmented donor chimerism to 60–95% by day +7 and 100% by day +14, which remained stable when assessed on day +77. Mice receiving PT-BEN exhibited 80–95% donor chimerism on day +7, increasing to complete donor chimerism in all mice by day +14 (Fig 2). These results confirm that PT-BEN maintains donor cell engraftment.

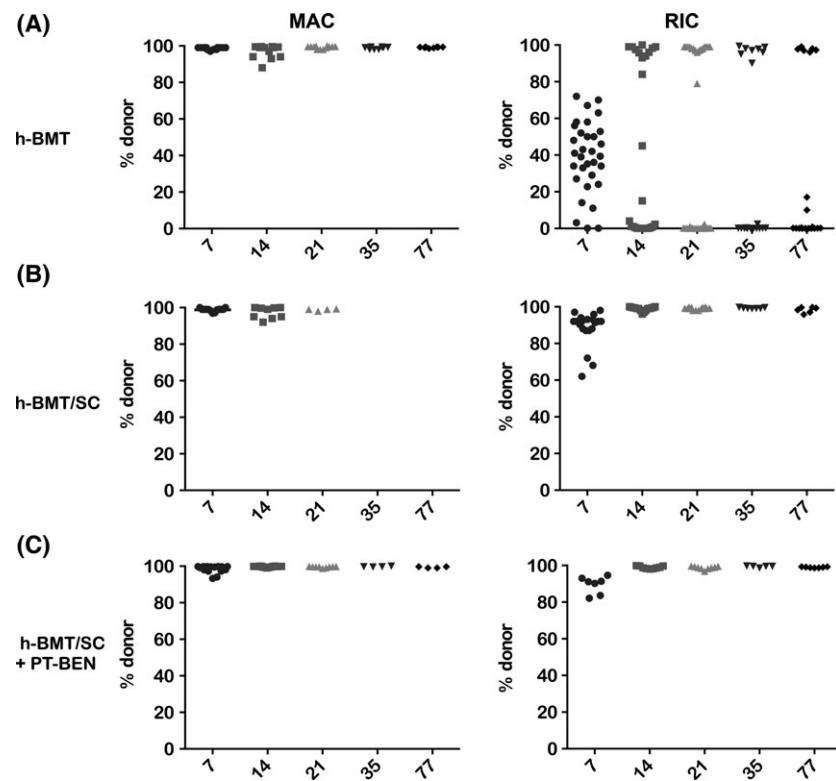


Fig 2. PT-BEN preserves engraftment following MAC and RIC haploidentical-BMT (h-BMT). CAF1/J mice ($H-2^{d/a}$) received myeloablative conditioning (MAC) or reduced intensity conditioning (RIC) total body irradiation (TBI), (A) h-BMT from CB6F1 mice ($H-2^{d/b}$), (B) h-BMT/SC, (C) h-BMT/SC and post-transplant bendamustine (PT-BEN; 30 mg/kg day +3 or 15 mg/kg days +3 and +4 i.v.). Percentage donor chimerism determined by flow cytometry is shown. Peripheral blood cells were gated on live cells from FSC/SSC and then gated on $H2 k^{b+}$ cells. MAC n=16 mice (pooled data from 4 experiments), RIC n=16 (PT-BEN) or 32 (h-BMT and h-BMT/SC) mice/group (pooled data from at least 4 experiments).

PT-BEN is as efficient as PT-CY at mitigating GvHD

Experiments were first undertaken to evaluate the most effective dose and timing of PT-BEN. We found that a single dose of 30 mg/kg on day +3 was most effective at reducing GvHD (Figure S2). Based on our determination of the maximum tolerated doses (MTDs) of BEN and CY in CAF1/J (Figure S3), we chose a comparable CY dose of 150 mg/kg, remaining within the range (66–200 mg/kg) shown by others to effectively mitigate GvHD (Ganguly *et al*, 2014; Ross *et al*, 2013). Our data confirmed that 150 mg/kg PT-CY on day +3 or 75 mg/kg on days +3 and +4 were equally effective at reducing GvHD (data not shown).

To compare the efficacy of PT-BEN to PT-CY, mice received MAC or RIC TBI, followed by h-BMT/SC and PT-BEN or PT-CY. Compared to animals receiving h-BMT/SC alone, survival was significantly improved or prolonged by both PT-BEN (31% MAC and 88% RIC) and PT-CY (0% MAC and 63% RIC) with no significant difference between the treatment groups (Fig 3A). This indicates that PT-BEN is endowed with GvHD protective properties equivalent to those of PT-CY in our model. Mice receiving PT-BEN or PT-CY exhibited comparable weight loss and early GvHD scores (Fig 3B,C). However, signs of clinical GvHD progressively improved in PT-BEN treated mice, leading to GvHD scores significantly lower than those of PT-CY treated mice (Fig 3C). As expected, mice from all groups receiving MAC had more prominent weight loss and corresponding increases in GvHD scores when compared to mice receiving RIC

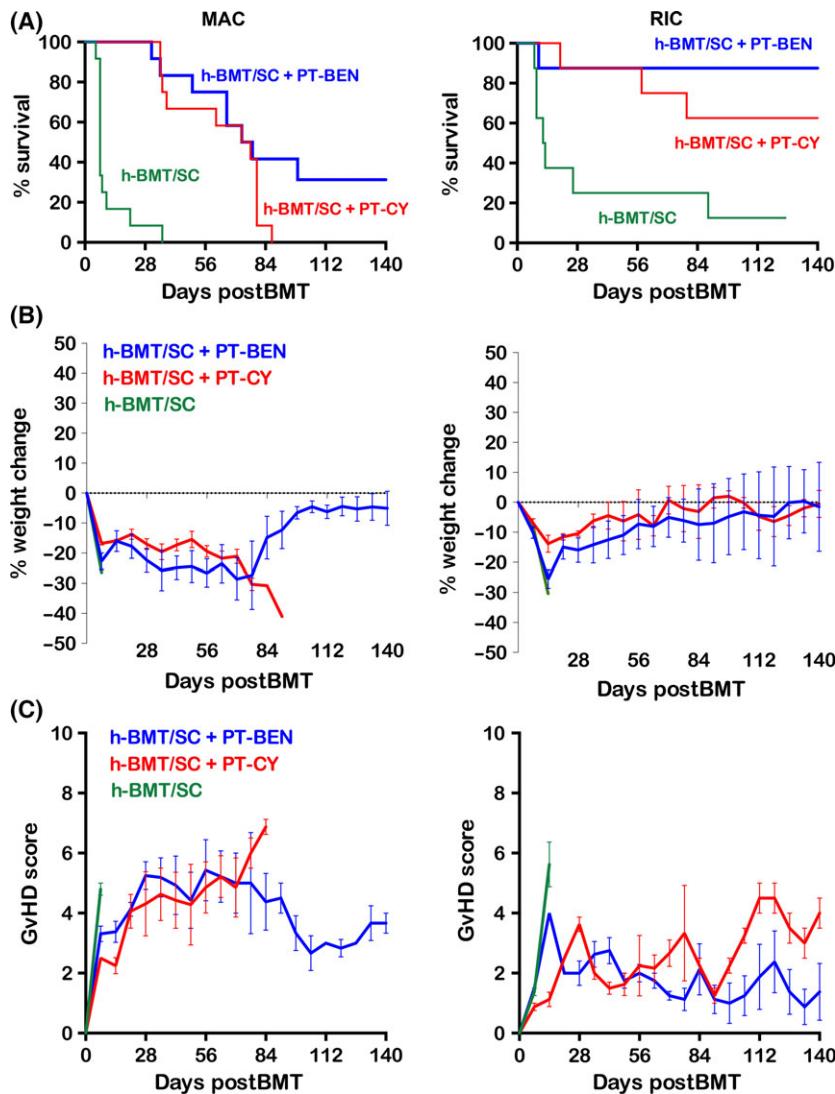


Fig 3. PT-BEN is as efficient as PT-CY at mitigating GvHD in h-BMT. CAF1/J mice received myeloablative conditioning (MAC) or reduced intensity conditioning (RIC) total body irradiation (TBI) on day -1 and h-BM (10^7) with h-SC (3×10^7) from CB6F1 mice on day 0. Post-transplant bendamustine (PT-BEN) 30 mg/kg was given on day +3 and post-transplant cyclophosphamide (PT-CY) 75 mg/kg was given on days +3 and +4. Kaplan-Meier survival curves were generated and analysed using the log-rank test. (A) Overall survival is shown. $n=12-16$ mice/group, pooled data from at least 3 experiments. MAC: h-BMT/SC vs h-BMT/SC+PT-BEN and vs h-BMT/SC+PT-CY $P<0.0001$, h-BMT/SC+PT-BEN vs h-BMT/SC+PT-CY $P=ns$. RIC: h-BMT/SC vs h-BMT/SC+PT-BEN $P<0.004$ and vs h-BMT/SC+PT-CY $P=0.017$. h-BMT/SC+PT-BEN vs h-BMT/SC+PT-CY $P=ns$. (B) Mean per cent weight change with SEM based on pre-transplant weight. (C) Mean clinical GvHD scores (based on weight, skin integrity, fur, activity and posture) with SEM. Shown are representative data from 4 experiments. $n=4$ (RIC) or 8 (MAC) mice/group.

(Fig 3B,C). Together, our data demonstrate that, at these doses, PT-BEN is as effective as PT-CY in reducing GvHD morbidity and mortality in both MAC and RIC murine h-BMT settings.

To further investigate GvHD morbidity, animals receiving h-BMT or h-BMT/SC followed by PT-BEN or PT-CY were euthanized on days +21, +35, or +49 for histological evaluation of colonic and hepatic GvHD. As the majority of mice receiving h-BMT/SC die prior to day +21, we were not able to include them as untreated positive controls. Colons from mice receiving h-BMT without h-SC showed little evidence of GvHD early, with some colonic abnormalities at later time points, probably due to the presence of T-cells in the BM inoculum. In contrast, mice receiving h-BMT/SC with PT-BEN or PT-CY demonstrated mild to moderate subacute multifocal to coalescing colitis with moderate crypt hyperplasia, mixed lymphocytic and neutrophilic infiltrates and, in some cases, mucosal ulceration consistent with GvHD. Histological colon GvHD scores (Burich *et al*, 2001) of mice

receiving PT-BEN were comparable to those receiving PT-CY (Fig 4A,B). Similarly, mice receiving h-BMT alone showed no significant pathology in their livers, while those receiving h-BMT/SC with PT-BEN or PT-CY demonstrated similar degrees of mild multifocal chronic active cholangitis indicative of liver GvHD (Fig 4C).

The protective effects of PT-BEN against GvHD are not dependent on donor CD25⁺ T-cells

Additional experiments were performed to assess the role of donor Treg in PT-BEN-mediated protection from GvHD. Recipient mice received T-cell-depleted (TCD) h-BMT on day 0 with 6×10^6 purified splenic T-cells (total T) or CD25⁺ depleted T-cells (CD25⁻ T). The GvHD observed with total T was not severe enough to allow significant survival advantage with PT-BEN (data not shown). When CD25⁺ T-cells were eliminated from the haploididentical T-cell inoculum, GvHD lethality and morbidity significantly

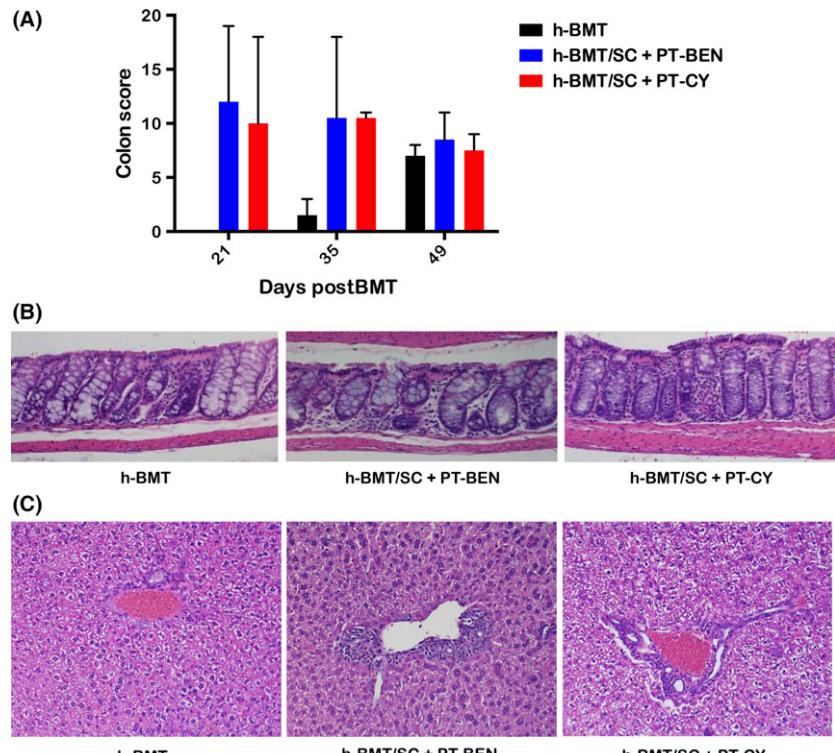


Fig 4. PT-BEN and PT-CY show comparable levels of colonic and hepatic GvHD. CAF1/J mice received reduced intensity conditioning total body irradiation on day -1 and h-BM (10^7) with h-SC (3×10^7) from CB6F1 mice on day 0. Post-transplant bendamustine (PT-BEN) 30 mg/kg and post-transplant cyclophosphamide (PT-CY) 75 mg/kg were given on days +3 and +4. (A) Proximal and distal colons were collected and blindly scored for abnormalities. $n=4$ mice/group. Total colon scores (sum of proximal and distal scores) from days +21, +35 and +49 are shown. (B) Representative images of haematoxylin and eosin staining of distal colons collected on day +21 are shown at $50\times$ magnification. (C) Representative images of haematoxylin and eosin staining of livers collected on day +21 are shown at $50\times$ magnification.

increased (Fig 5). Infusion of 6×10^6 CD25⁻ T-cells on day 0 led to the death of all recipients, with most occurring before day +10, substantiating the importance of CD25⁺ Treg in suppressing GvHD in this h-BMT model. Treatment of mice receiving 6×10^6 CD25⁻ T-cells with PT-BEN significantly improved survival, from 0% to 50%, at +140 d. Moreover, these surviving mice regained their weight and showed minimal signs of GvHD at day +140 (Fig 5). These data clearly demonstrate that PT-BEN can suppress GvHD in the absence of donor Treg.

PT-BEN is less myelosuppressive than PT-CY

To further explore the potential benefits of PT-BEN over PT-CY, mouse peripheral blood counts were analysed serially post-transplant. PT-BEN was less myelosuppressive than PT-CY with a day +7 average white blood cell (WBC) count of $4.5 \times 10^9/l$ compared to $0.32 \times 10^9/l$ following MAC ($P<0.001$) and $5.7 \times 10^9/l$ compared to $0.48 \times 10^9/l$ following RIC ($P<0.00001$) (Fig 6A). PT-BEN maintained a higher WBC count than PT-CY up to day +12 and by day +14 counts had similarly recovered in both groups (Figure S4A). PT-BEN was relatively more lymphodepleting than myelosuppressive when compared to PT-CY with a myeloid to lymphoid ratio of 14.6 compared to 0.48 following MAC and 17.6 compared to 0.55 following RIC on day +7 (Fig 6A). When analysed daily, PT-BEN treated mice had a higher myeloid to lymphoid ratio up to day +13. By day +14, the ratios were comparable (Figure S4B). PT-BEN

treated mice, in contrast to PT-CY treated mice, never became neutropenic (defined as <100 neutrophils $\times 10^9/l$ blood) (von Vietinghoff & Ley, 2008; Zuluaga *et al*, 2006) when analysed from days +4 to 14 (Figure S4C). When compared to PT-CY, PT-BEN treated mice exhibited higher haemoglobin, platelet and red blood cell counts up to day +14 following MAC (Figure S5A). There was no clear pattern following RIC (Figure S5B).

PT-BEN increases MDSC frequency

Flow cytometric analysis was used to further examine peripheral blood cell populations. Treg, phenotypically defined as CD4⁺CD25⁺FoxP3⁺ cells, and myeloid-derived suppressor cells (MDSCs), defined as suppressive CD11b⁺Gr-1⁺ cells, are two main immune cell populations widely described for their immunosuppressive properties, which play an essential role in the control of GvHD (Colonna *et al*, 2011; Highfill *et al*, 2010). As the lymphoid counts were extremely low on day +7, it was not feasible to perform an analysis of potential differences in the percentage of Treg, T-cells or B-cells between the PT-BEN and PT-CY groups. FoxP3⁺ cells comprised 0.1–0.2% of peripheral blood cells in PT-BEN and PT-CY groups following MAC or RIC on day +14, with a higher absolute number in PT-CY treated mice. On days +35 and +77, there was a two-fold increase in FoxP3⁺ cells in the peripheral blood of PT-CY relative to PT-BEN treated groups (Fig 6B). The absolute lymphoid subset numbers between PT-BEN and PT-CY were not significantly or consistently different,

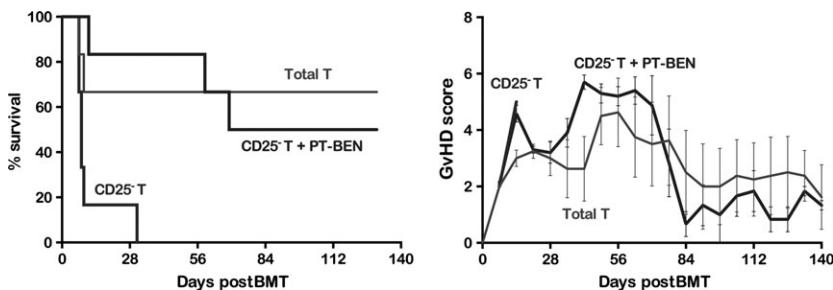


Fig 5. PT-BEN is protective against GvHD even in the absence of donor CD25⁺ T-cells. Recipient CAF1/J mice received reduced intensity conditioning and T-cell depleted h-BM (10^7) with 6×10^6 total T-cells or 6×10^6 CD25-depleted T-cells from CB6F1 mice on day 0. Post-transplant bendamustine (PT-BEN) 30 mg/kg was given on day +3. Shown are survival data and mean clinical graft-versus-host disease scores with SEM. Representative data from 2 experiments, n=6 mice/group. Kaplan–Meier survival curves were generated and analysed using the log-rank test. CD25[−] T vs CD25[−] T+PT-BEN $P<0.002$, total T vs CD25[−] T+PT-BEN $P=ns$, total T vs CD25[−] T $P<0.03$.

except that CD4⁺ cells were higher with PT-CY following MAC (Fig 6C,D). In contrast, the number of CD11b⁺Gr-1^{hi} cells was significantly higher in recipients of PT-BEN when compared to PT-CY on day +7 following MAC and days +7 and +14 following RIC (Fig 6E). Importantly, Gr-1^{hi} cells isolated from the blood and spleen of PT-BEN treated mice strongly suppressed T-cell proliferation (Figure S6), confirming their identity as MDSCs.

BEN enhances MDSC suppressive function and impairs T- and B-cell proliferation in vitro

PT-BEN is lymphodepleting, relatively spares the myeloid compartment and significantly increases MDSCs. We therefore further investigated the differential effects of BEN on these subsets *in vitro*. MDSCs were generated from naïve BM in the presence of increasing concentrations of BEN. After 3 d in culture, >90% of cells were CD11b⁺Gr-1⁺ (Figure S7), with no significant differences between groups. MDSCs generated with higher concentrations of BEN were significantly more suppressive than those generated with lower concentrations (Fig 7A).

Importantly, T-cells from naïve mice stimulated by anti-TCR/CD28 antibodies in the presence of BEN concentrations identical to those used in MDSC generation were less proliferative than those cultured in the absence of BEN. Both CD4⁺ and CD8⁺ T-cell proliferation were reduced by BEN, with more significant suppression of CD4⁺ proliferation. Suppression of proliferation could not be attributed to loss of T-cell viability (Fig 7B). When B-cells were cultured with the same concentrations, they became even less proliferative with a significant decrease in viability at 100 μ mol/l (Fig 7C).

PT-BEN mitigates GvHD but maintains GvL in the A20 leukaemia model

As BEN significantly suppresses GvHD, it was essential to determine its effects on GvL, specifically assessing whether anti-tumoural activity was maintained with PT-BEN

treatment. First, we determined the possible direct effect of BEN on A20 *in vivo*. This was an important step to discriminate between the possible immunomodulatory effects of BEN and its direct anti-cancer activity. BEN 30 mg/kg per d was administered 5 and 6 d after i.v. A20 inoculation of immunocompetent BALB/c and immunodeficient NOD-*scid* IL2R γ ^{null} mice (lacking mature T-cells, B-cells and functional NK cells). In immunocompetent tumour-bearing BALB/c mice, BEN had a minimal anti-A20 effect, increasing the median survival by only 4 d compared to no treatment (Fig 8A). Similarly, in NOD-*scid* IL2R γ ^{null} mice with A20 leukaemia, BEN only prolonged survival by 3 d (Fig 8B). By comparison, CY demonstrated a more potent direct anti-tumour activity against A20 (CY vs BEN $P<0.002$ in BALB/c and $P<0.006$ in NOD-*scid* IL2R γ ^{null}). The total dose of BEN 60 mg/kg given to these mice represents 85% of the MTD, compared to only 34% of the MTD for CY (150 mg/kg). This result confirms that A20 are more sensitive to CY than BEN. Therefore, PT-BEN could not be compared to PT-CY when tumour was given before transplant, as it would be impossible to distinguish the GvL effects from the direct anti-tumour activity.

Experiments were undertaken to determine the impact of PT-BEN on the survival of leukaemia-bearing mice undergoing h-BMT. Given that anti-A20 leukaemia effects of BEN are minimal, an increase in survival of leukaemia-bearing mice would be attributable primarily to GvL activity. A20 tumour-bearing mice received RIC and h-BMT or h-BMT/SC + PT-BEN. PT-BEN significantly increased survival from leukaemia compared to untreated mice (Fig 8C).

The preservation of significant GvL activity by PT-BEN was confirmed using luciferase-expressing A20 injected i.v. 1 d after chemotherapy (thereby eliminating any possible direct anti-leukaemia activity). All mice receiving h-BMT/SC died of GvHD without evidence of leukaemia. The survival of h-BMT/SC mice treated with PT-BEN was significantly better than that of animals treated with PT-CY. Importantly, the survival of mice receiving h-BMT/SC + PT-CY was not significantly different than that of mice receiving h-BMT

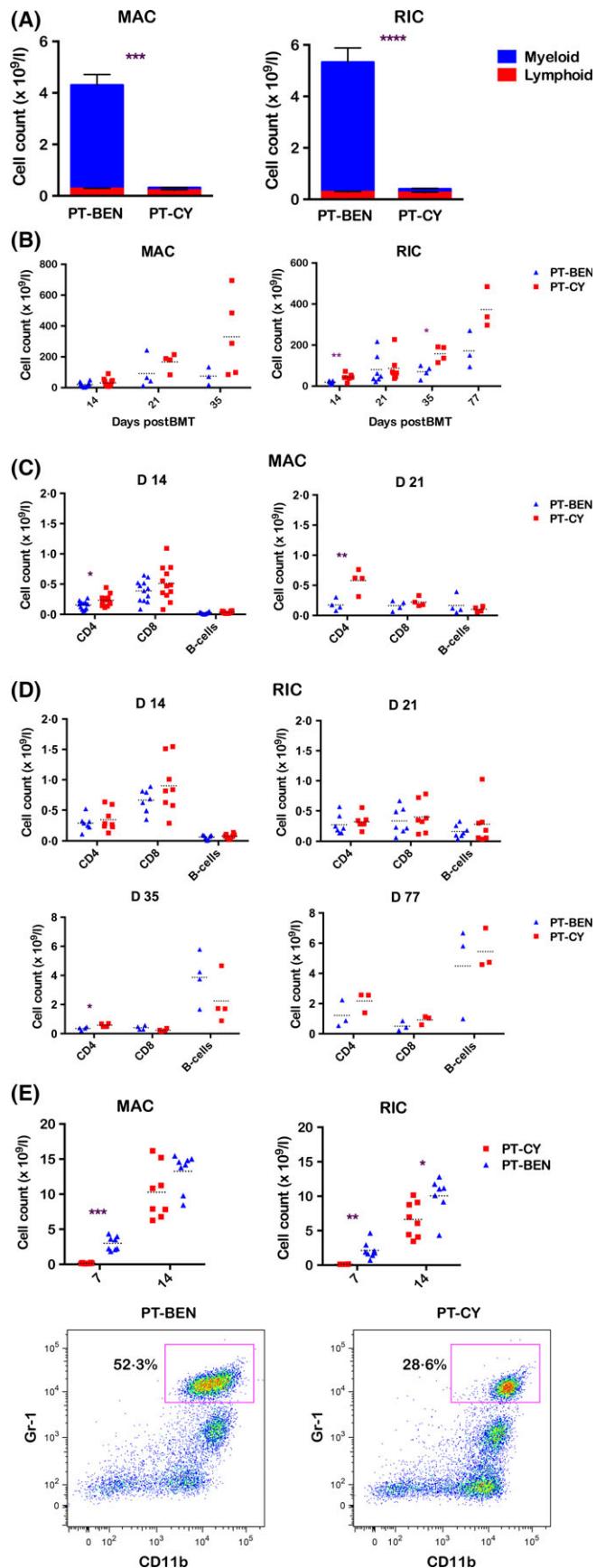


Fig 6. PT-BEN is less myelosuppressive than PT-CY following MAC and RIC h-BMT. CAF1/J mice received myeloablative conditioning (MAC) or reduced intensity conditioning (RIC) total body irradiation (TBI) on day -1 and h-BMT/SC from CB6F1 mice on day 0. Post-transplant bendamustine (PT-BEN) 30 mg/kg was given on day +3 and post-transplant cyclophosphamide (PT-CY) 75 mg/kg was given on days +3 and +4. Peripheral blood was collected on the indicated days by tail tipping. (A) Day +7 white blood cell (WBC) counts broken down into myeloid/lymphoid differentials are shown with SEM. Blood was analysed using a HemaVet blood counter and broken down into neutrophils, monocytes, eosinophils, basophils (myeloid) and lymphocytes (lymphoid). MAC n=8 mice/group, RIC n=14 mice/group (pooled data from 2–3 experiments). (B) Absolute numbers of donor CD4⁺FoxP3⁺ Treg are shown. n=4–8 (pooled data from 2–3 experiments). (C, D) Absolute numbers of donor CD4, CD8 and B-cells in peripheral blood are shown. Cells were stained with anti-H2 k^b, CD4, CD8 and B220 and analysed by flow cytometry. Cells were gated on live cells from FSC/SSC, H2 k^{b+} cells and then on CD4⁺, CD8⁺ or B220⁺ and absolute cell number was calculated based on total WBC count. (E) Absolute number of donor CD11b⁺Gr-1^{hi} cells is shown. Day +7 and +14 blood was stained with anti-H2 k^b, Gr-1 and CD11b and analysed by flow cytometry. Cells were gated on live cells from FSC/SSC, H2 k^{b+} cells and then on CD11b⁺Gr-1^{hi}. Gating strategy and representative flow from RIC day +14 are shown. Absolute cell number was calculated using the WBC count and differences were analysed using Mann–Whitney test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

alone, which did not develop clinically significant GvHD or GvL (Fig 8D). Similarly, in another experiment, mice received RIC, TCD-h-BMT or h-BMT/SC with and without PT-BEN or CY on day +3 and A20-Luc cells i.v. on day +4. Mice were imaged serially. PT-BEN treated mice demonstrated no evidence of A20-Luc leukaemia as late as day +70, while those receiving PT-CY showed onset of leukaemia as early as day +22 with all mice dying of leukaemia by day +48. All untreated h-BMT/SC mice died of GvHD by day +45. This is shown in the images and confirmed by quantification of the photons/s (Fig 9A,B). These results indicate that although PT-CY efficiently reduces mortality related to GvHD, it concomitantly reduces GvL activity in this model, while PT-BEN does not. The effectiveness of PT-BEN at preserving GvL was confirmed in the MAC and RIC models, using two different doses of SC to induce GvHD with different degrees of severity (Figure S8). This highlights the potential therapeutic benefit of BEN over CY in this setting.

Discussion

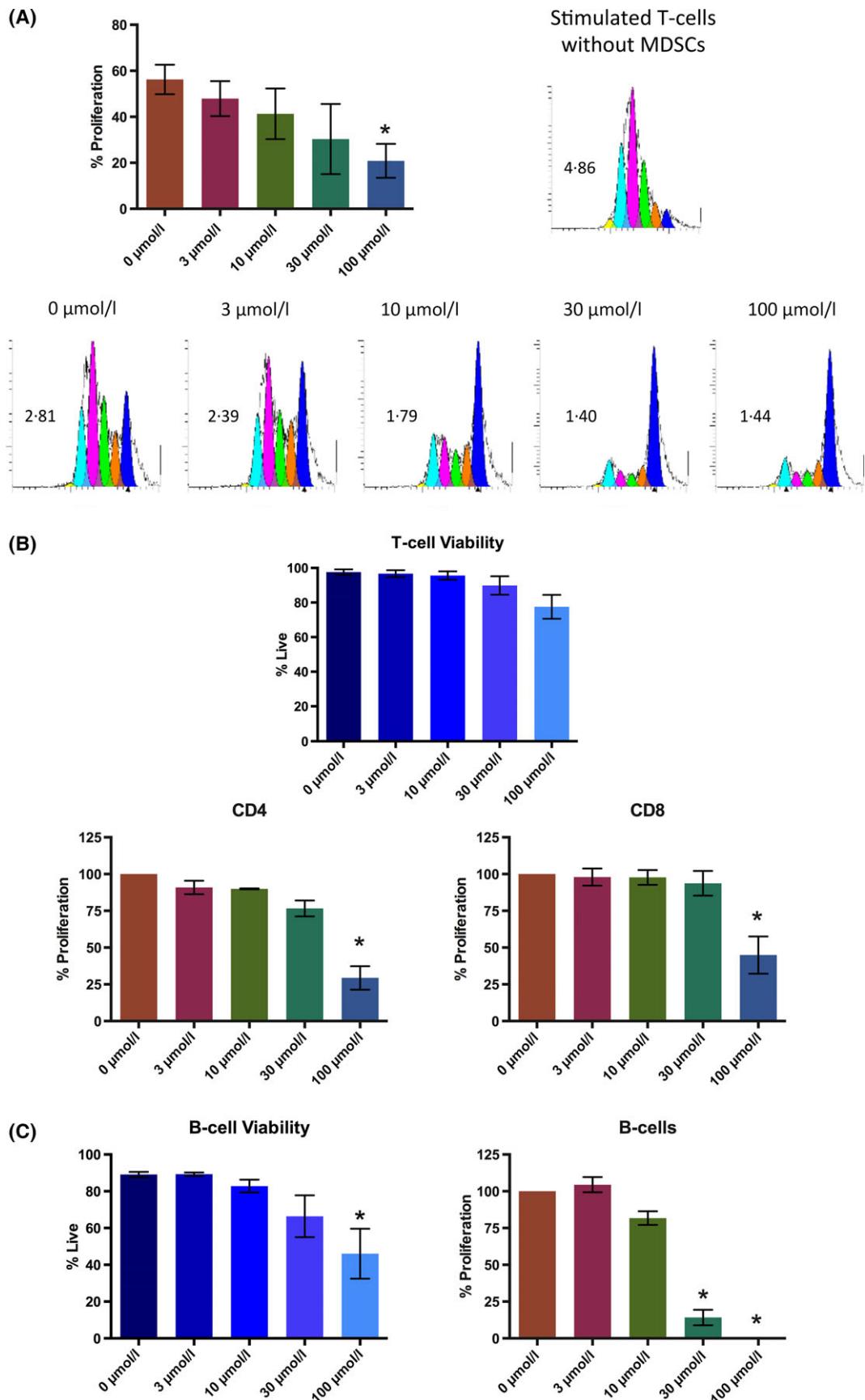
Recently, the application of haploidentical HCT has significantly expanded. The integration of PT-CY has emerged as the most widely applied h-BMT approach, partly because of its simplicity. However, high relapse rates observed in the setting of RIC have dampened enthusiasm for this strategy, particularly for patients with acute leukaemias (Ciurea *et al*, 2015). In an effort to identify an alternative agent that can readily be used to overcome the deleterious impact of GvHD while maintaining GvL, we evaluated PT-BEN. While BEN is noted for potent immunosuppressive and lymphodepleting activities, its ability to prevent or suppress GvHD when given post-transplant has not been published previously in human or murine BMT models. BEN has, however, been incorporated as a conditioning agent prior to matched allogeneic BMT and donor lymphocyte infusion (Khouri *et al*, 2014; Sala *et al*, 2014).

Using clinically relevant fully haploidentical F1 → F1 models, we provide the first evidence that PT-BEN represents an efficient and feasible alternative to PT-CY. Mice receiving h-BMT/SC followed by PT-BEN developed sustained donor chimerism. PT-BEN used at a comparable dose, based on deter-

mined MTDs in recipient CAF1/J mice, protected against GvHD as effectively as PT-CY. Additionally, we determined that PT-BEN significantly improved the survival of leukaemia-bearing mice receiving RIC and h-BMT/SC compared to those receiving h-BMT alone. This anti-leukaemic effect was mediated primarily through GvL, as even a two-fold increase in the dose of BEN demonstrated minimal direct cytotoxic activity against A20 leukaemia in non-transplanted BALB/c and NOD-*scid* IL2R γ ^{null} mice. Furthermore, when A20-Luc was given after chemotherapy to avoid the inequity of direct cytotoxic effects of BEN and CY, mice receiving PT-BEN demonstrated a significant delay in leukaemia development compared to mice receiving PT-CY, where both GvHD and GvL were significantly suppressed. Taken together, these data demonstrate that PT-BEN effectively controls GvHD and has significant advantages over PT-CY in preserving GvL activity.

Treg play an essential role in the maintenance of immunological self-tolerance (Sakaguchi *et al*, 2008). Extensive evidence has shown the balance between effector T-cells and Treg is critical for the control of GvHD (Ermann *et al*, 2005; Nguyen *et al*, 2008; Taylor *et al*, 2004; Zeiser *et al*, 2008). Consistent with previous studies in various allogeneic mismatched murine BMT models, our results indicate that in RIC h-BMT, removal of CD25 $^{+}$ T-cells (which include CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$ Treg) from the T-cell infusion significantly increases GvHD lethality (Zeiser *et al*, 2006). It is believed that the predominant effect of PT-CY depletes alloreactive T-cells early post-HCT (Luznik *et al*, 2002, 2001, 2008). However, the same group recently reported that CD4 $^{+}$ FoxP3 $^{+}$ Treg were essential for GvHD protection by PT-CY in major histocompatibility complex (MHC)-matched and MHC-mismatched models, with T-cell allografts containing both CD4 $^{+}$ and CD8 $^{+}$ cells (Ganguly *et al*, 2014). PT-BEN is also an effective lymphodepleting agent, probably partially exerting its effects by eliminating alloreactive T-cells. In our haploidentical model, PT-BEN significantly increased survival even when allografts were depleted of Treg (7–9% of the total T-cell population). As reported in other models (Ganguly *et al*, 2014), we observed that PT-CY led to an increase in Tregs at later time points, while PT-BEN did not. The mechanism of action of PT-BEN therefore appears to differ from that of PT-CY.

Fig 7. BEN treatment *in vitro* enhances MDSC function and decreases the proliferation of CD4 $^{+}$ and CD8 $^{+}$ T-cells and B-cells. (A) Myeloid-derived suppressor cells (MDSCs) were generated using bone marrow (BM) cells from naïve CB6F1 mice. BM cells were cultured for 3 d with 10 ng/ml murine IL6, murine GM-CSF and human G-CSF and various concentrations of bendamustine (BEN; 0–100 μ mol/l). MDSCs were then co-cultured for 3 d with CellTrace Violet stained, stimulated total T-cells from naïve CB6F1 spleens at a ratio of 1 MDSC to 2 T-cells. Average % T-cell proliferation (with SEM) after 3 d of co-culture is shown. Pooled data from 5 experiments and representative ModFit analysis with proliferation index are shown. This includes T-cells without MDSCs as a negative control for suppression. Significance is shown as compared to 0 μ mol/l BEN, using Mann–Whitney tests. (B) T-cells isolated from the spleens of naïve CB6F1 mice were CellTrace stained, stimulated with CD3/CD28 beads and cultured for 3 d in the presence of various concentrations of BEN. On day 3, viability was determined and T-cells were stained with anti-CD4 and CD8 for proliferation analysis by flow cytometry. Cells were gated on live cells, CD4 $^{+}$ or CD8 $^{+}$ and then CellTrace fluorescence was analysed. Shown are pooled data from 4 experiments. (C) B-cells isolated from the spleens of naïve CB6F1 mice were CellTrace stained, stimulated with 10 μ g/ml lipopolysaccharide and cultured for 5 d in the presence of various concentrations of BEN. Shown are pooled data from 4 experiments. Viability and average percent proliferation as compared to the culture with 0 μ mol/l BEN are shown, using Mann–Whitney tests. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.



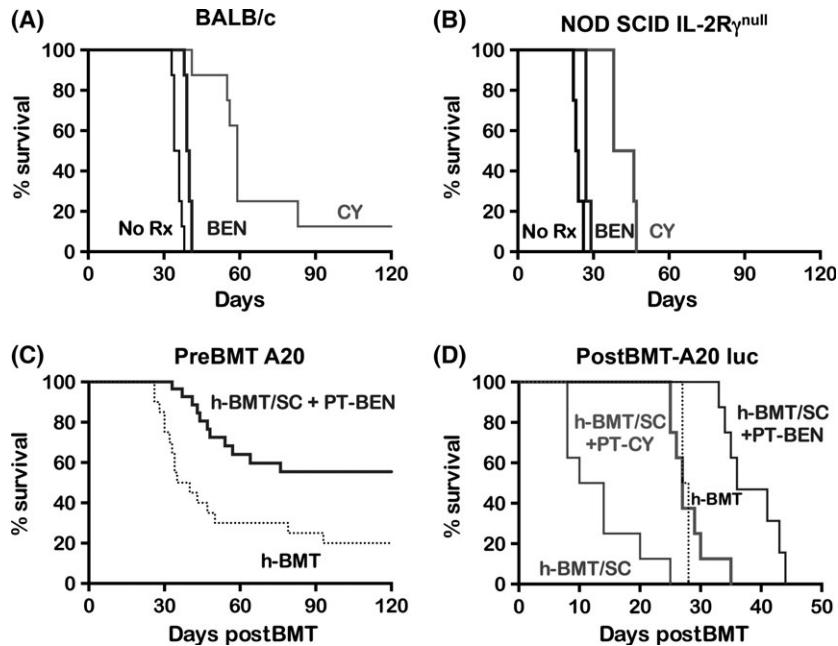


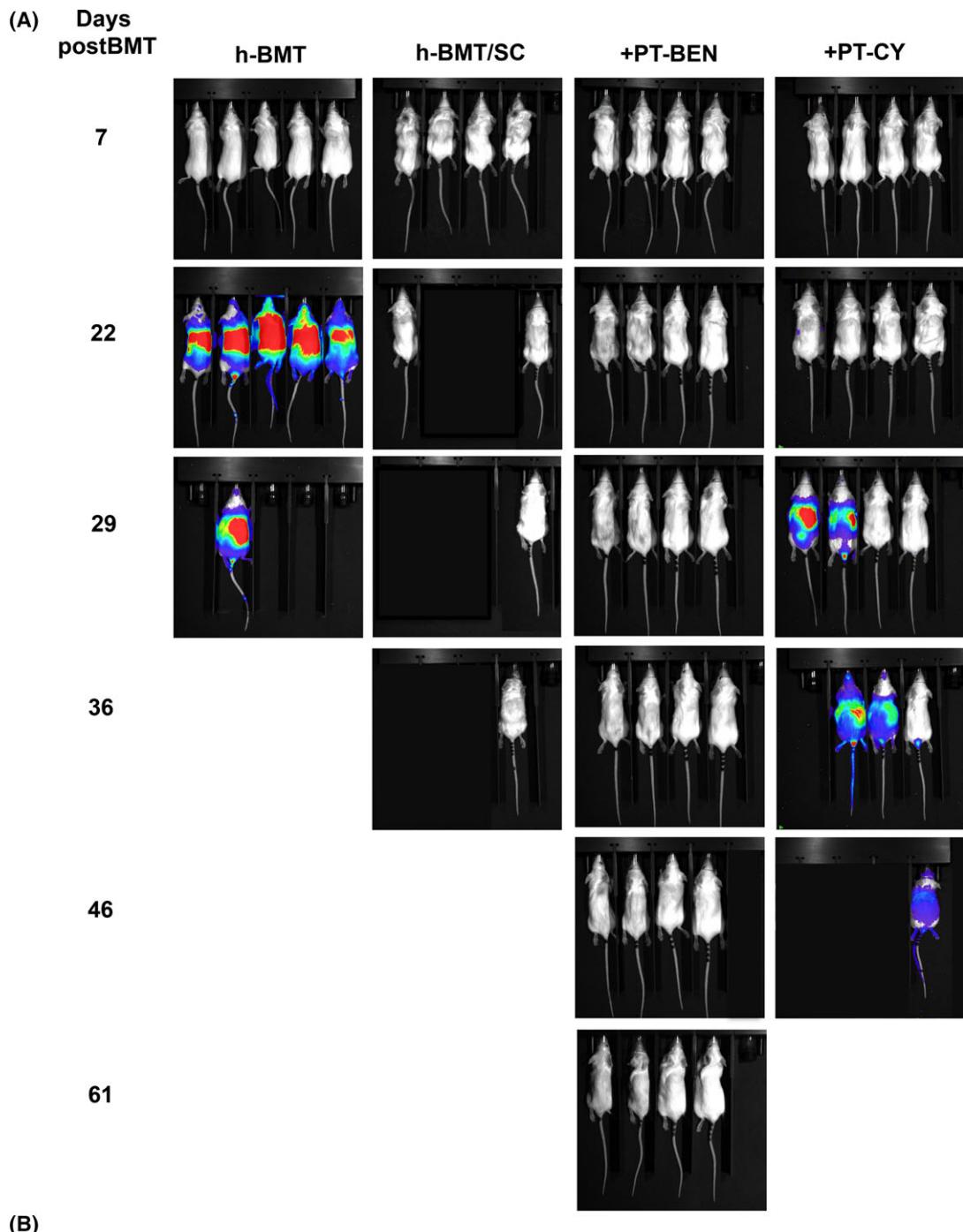
Fig 8. BEN has minimal direct anti-tumour activity against A20 leukaemia and preserves GvL when given post-h-BMT. A20 leukaemia-bearing (A) BALB/c and (B) NOD-*scid* IL2R γ ^{null} were treated with bendamustine (BEN) 30 mg/kg or cyclophosphamide (CY) 75 mg/kg 5 and 6 d post-injection of 10⁶ A20 cells. CY vs BEN $P<0.002$ in BALB/c and $P<0.006$ in NOD-*scid* IL2R γ ^{null}, n=8 (BALB/c) or 4 (NOD-*scid*) mice/group. (C) CAF1/J mice received 10⁶ A20 tumor cells i.v. on day -4, RIC TBI on day -1 and h-BM (10⁷) or h-BMT with h-SC (3 \times 10⁷) from CB6F1 mice on day 0. PT-BEN 30 mg/kg as a single dose or divided over 2 d was given starting on day +3. Pooled data from 5 experiments, n=20–34 mice/group. Kaplan-Meier survival curves were generated and analysed using the log-rank test. h-BMT vs h-BMT/SC+PT-BEN $P<0.002$. (D) CAF1/J mice received RIC TBI on day -1 and h-BMT or h-BMT/SC from CB6F1 mice on day 0. PT-BEN (33 mg/kg) or CY (150 mg/kg) was given on day +3 and 1 \times 10⁵ A20-Luc cells were injected i.v. on day +4. h-BMT/SC+PT-BEN vs h-BMT $P<0.0004$ and vs h-BMT/SC+PT-CY $P<0.0005$, h-BMT/SC+PT-CY vs h-BMT $P=ns$. Pooled data from 2 experiments, n=8 mice/group.

As PT-BEN is more lymphodepleting (including Treg) than myelosuppressive relative to PT-CY, and results in higher numbers of suppressive CD11b⁺Gr-1^{hi} cells in the blood, it is conceivable that MDSCs contribute to its mechanism of GvHD suppression. Additionally, the presence of BEN during MDSC generation *in vitro* increased their suppressive activity. The same concentrations of BEN markedly decreased the proliferation of CD4⁺ and CD8⁺ T-cells and B-cells *in vitro*. These data suggest that BEN improves MDSC function while reducing T- and B-cell function. It is noteworthy that CD4⁺ T-cells were more affected by BEN than CD8⁺ T-cells, suggesting the relative sparing of CD8⁺ T-cells could contribute to GvL activity. In addition, BEN exhibited more pronounced effects on the proliferation of B-cells than T-cells. B-cells play a pivotal role in the development of chronic GvHD (Schultz *et al*, 1995; Srinivasan *et al*, 2012; Young *et al*, 2012). It is plausible that the pronounced, long-lasting depletion of B-cells by BEN may contribute to its effective long-term control of GvHD in our model. The role

of PT-BEN on the interplay of these and other immune cells related to GvHD and GvL requires further elucidation.

In summary, we have documented that PT-BEN is an effective agent in suppressing GvHD while maintaining GvL in clinically relevant RIC and MAC experimental haploid-identical BMT models. With PT-BEN, preservation of GvL was in striking contrast to the abrogation seen with PT-CY. Additionally, BEN is less myelosuppressive than CY and may decrease the duration of severe neutropenia, significantly reducing the risk of infections (Khouri *et al*, 2014). BEN used alongside fludarabine and rituximab as conditioning in allogeneic transplantation was well-tolerated and effective (Khouri *et al*, 2014). Its safety profile was recently confirmed in a randomized trial of BEN plus rituximab versus R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) with fewer haematological and other toxicities and infections (Becker *et al*, 2015). BEN has not been noted to have the significant cardiac and pulmonary toxicity (Becker *et al*, 2015) or augmentation of TBI-induced lung damage (Sampath *et al*,

Fig 9. PT-BEN preserves GvL when given post-h-BMT. (A) CAF1/J recipient mice received reduced intensity conditioning on day -1 and T-cell depleted h-BMT or h-BMT/SC from CB6F1 mice on day 0. Post-transplant bendamustine (PT-BEN) or post-transplant cyclophosphamide (PT-CY) was given day +3 and 0.5 \times 10⁶ A20-Luc cells on day +4. Mice were injected with luciferin and imaged serially. At times, cages were imaged concurrently, so images are cropped to only show mice from the indicated group. (B) Quantification of (A) is shown in ln(photon/s/mouse). Group average with SEM is shown (left) until any mouse from the group died and analysed using Mann-Whitney tests. Individual mouse values are also shown (right). Images were analysed using AmiView. n=4–5 mice/group.



2005; Shi-Xia *et al*, 2010) that is often seen following CY conditioning (Ayash *et al*, 1992; Braverman *et al*, 1991; Cazin *et al*, 1986; Malik *et al*, 1996; Mills & Roberts, 1979). BEN, however, may be associated with hyperbilirubinaemia, rash and diarrhoea, which may be confused with onset of GvHD, and its safety post-h-BMT remains to be determined in humans (Becker *et al*, 2015). Regardless of these potential limitations, PT-BEN deserves further investigation as a potentially effective post-transplant approach following T cell-replete h-BMT. Given that PT-CY on day +3 and 4 is the standard of care in T-replete h-BMT, a proposed phase I trial at our institution will gradually de-escalate PT-CY while escalating BEN to a dose of 100 mg/m², starting with the day +4 dose.

Author contributions

J.S. and E.H. designed and performed experiments, analysed and reviewed data, and co-wrote the manuscript. Y.Z. and N.L. contributed to experimental design, data interpretation and discussion and revised the manuscript. E.K. designed the project, supervised and advised on the implementation and conduction of experiments, reviewed and interpreted data, and wrote the manuscript. The authors have declared that no conflict of interest exists.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. GvHD is more severe in an F1 into F1 model than a parent into F1 model.

Fig S2. Determination of dose and schedule of PT-BEN.

Fig S3. Determination of maximum tolerated doses of BEN and CY in CAF1/J mice.

Fig S4. PT-BEN treated mice exhibited higher WBC and neutrophil counts and myeloid to lymphoid ratios.

Fig S5. PT-BEN treated mice exhibited higher hemoglobin, platelet and red blood cell counts.

Fig S6. Gr-1hi cells from PT-BEN treated mice suppressed T-cell proliferation.

Fig S7. Generation of MDSCs from BM in the presence of BEN.

Fig S8. PT-BEN preserves GvL in the setting of varying levels of GvHD.

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