Biol Blood Marrow Transplant 25 (2019) 1075–1084

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Flt3L Treatment of Bone Marrow Donors Increases Graft Plasmacytoid Dendritic Cell Content and Improves Allogeneic Transplantation Outcomes

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Article history:
Received 21 July 2018
Accepted 26 November 2018

Key Words:
Plasmacytoid dendritic cells
Flt3L
Allogeneic transplantation
Graft-versus-host disease
Graft-versus-leukemia

A B S T R A C T

A higher number of donor plasmacytoid dendritic cells (pDCs) is associated with increased survival and reduced graft-versus-host disease (GVHD) in human recipients of unrelated donor bone marrow (BM) grafts, but not granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood grafts. We show that in murine models, donor BM pDCs are associated with increased survival and decreased GVHD compared with G-CSF-mobilized pDCs. To increase the content of pDCs in BM grafts, we studied the effect of FMS-like tyrosine kinase 3 ligand (Flt3L) treatment of murine BM donors on transplantation outcomes. Flt3L treatment (300 μg/kg/day) resulted in a schedule-dependent increase in the content of pDCs in the BM. Mice treated on days -4 and -1 had a >5-fold increase in pDC content without significant changes in numbers of HSCs, T cells, B cells, and natural killer cells in the BM graft. In an MHC-mismatched murine transplant model, recipients of Flt3L-treated T cell-depleted (TCD) BM (TCD F-BM) and cytokine-untreated T cells had increased survival and decreased GVHD scores with fewer Th1 and Th17 polarized T cells post-transplantation compared with recipients of equivalent numbers of untreated donor TCD BM and T cells. Gene array analyses of pDCs from Flt3L-treated human and murine donors showed up-regulation of adaptive immune pathways and immunoregulatory checkpoints compared with pDCs from untreated BM donors. Transplantation of TCD F-BM plus T cells resulted in no loss of the graft-versus-leukemia (GVL) effect compared with grafts from untreated donors in 2 murine GVL models. Thus, Flt3L treatment of BM donors is a novel method for increasing the pDC content in allografts, improving survival, and decreasing GVHD without diminishing the GVL effect.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is curative for patients with hematologic malignances and bone marrow (BM) failure disorders [1]. Hematopoietic stem cell (HSC) grafts are typically obtained from aspiration of BM or from apheresis of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood [2,3]. The major complications of allo-HSCT include graft rejection, disease relapse, and graft-versus-host disease (GVHD) [4,5]. These adverse effects are initiated and regulated by both the content of donor immune cells in the graft and residual antigen-presenting cells in the recipient [6]. Results of the BMTCTN 0201 trial showed that an increased content of donor plasmacytoid dendritic cells (pDCs) increased survival and decreased GVHD in BM allografts, but not in G-CSF-mobilized allografts [7]. It has been reported that dendritic cell reconstitution post-transplantation is predictive of outcomes including incidences of GVHD, relapse, and death [8]. We have observed that transplantation of purified BM pDCs increased survival and decreased GVHD without affecting the graft-versus-leukemia (GVL) effect in allogeneic murine transplantation models compared with G-CSF-mobilized pDCs (Hassan, 2018, submitted). Because the pDC content of the BM is variable among allogeneic donors, methods for increasing the pDC content in BM are attractive strategies to enhance survival and the GVL effect while limiting GVHD.

Financial disclosure: See Acknowledgments on page 1083.

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https://doi.org/10.1016/j.bbmt.2018.11.029
1083-8791/

Plasmacytoid dendritic cells can be identified as Lin^-(CD3, CD14, CD16, CD19, CD20) $HLADR^+CD123^+CD11c^-CD33^-$ in humans and $PDCA1^+CD11c^+B220^+Lin^-$(CD3, CD11b, CD19, IgM, CD49b, Ter119) in mice [9–11]. As the primary source of type 1 interferon in both humans and mice, pDCs play significant roles in both innate and adaptive immunity [12]. Recipient pDCs are depleted after irradiation, allowing for examination of the effect of donor pDCs on post-transplantation GVHD and GVL [13]. Donor pDCs have been shown to have graft-facilitating functions, including enhancement of donor cell engraftment and survival post-transplantation [14]. The immunologic status—inflammatory or immunosuppressive—of donor pDCs that interact with donor T cells is paramount to their ability to limit GVHD [15]. We have shown that pDCs facilitate immunity through early post-transplantation IL-12 secretion, which enhances engraftment, the GVL effect, and late IFNγ response pathways that decrease GVHD via the induction of indoleamine 2,3-dioxygenase (IDO) production and increased numbers of Tregs [16–18].

FMS-like tyrosine kinase 3 ligand (Flt3L) is necessary for pDC differentiation, and Flt3L treatment can be used to increase the pDC content in vitro [19–22]. Although the use of CDX-301, a recombinant Flt3L, in the mobilization of HSCs has been studied, the effect of in vivo Flt3L administration alone on the content and immunologic activity of pDCs in BM remains to be determined [23,24]. We hypothesized that Flt3L treatment of BM donors would increase the pDC content of the allograft, and that transplanting Flt3L-stimulated BM grafts (F-BM) would enhance survival while limiting GVHD. We tested this hypothesis in an MHC-mismatched C57BL/6→B10.BR transplantation model in which donors were treated with PBS or 2 injections of 300 μg/kg of Flt3L. Here we report that treatment of donors with Flt3L increased the pDC content of BM grafts, increased survival, and decreased GVHD in allogeneic transplant recipients compared with BM grafts from PBS-treated donors. In addition, we observed decreased Th1 and Th17 polarization in T cells recovered from T cell-depleted (TCD) F-BM plus T cell transplant recipients on day +3. Using FACS-purified pDCs, we show that Flt3L treatment of donors led to up-regulation of adaptive immune pathways and immunoregulation checkpoints in donor pDCs without a reduction in the GVL effect. Thus, Flt3L treatment is a novel method that increases pDC content in donor grafts, increases survival, and decreases GVHD in allogeneic transplantation.

METHODS

Mice

C57BL/6 (H-$2K^b$) and B10.BR (H-$2K^k$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male donor and recipient mice were aged 8 to 10 weeks and 10 to 12 weeks, respectively. National Institutes of Health animal care guidelines were followed, and the study was approved by Emory University's Institutional Animal Care and Use Committee.

Flt3L Treatment

C57BL/6 mice were treated with various schedules of daily s.c. injections of PBS or 300 μg/kg of recombinant human Flt3L (CDX-301), generously donated by CellDex Therapeutics (Hampton, NJ).

G-CSF Treatment

C57BL/6 mice were treated with 5 consecutive days of s.c. injections of PBS or 300 μg/kg of recombinant G-CSF (Sandoz, Princeton, NJ).

Donor Cell Preparation

Donor mice were euthanized, and femurs and tibias of donor C57BL/6 mice were flushed with 2% FBS PBS. Biotinylated anti-mouse CD3 (BD Biosciences, San Jose, CA) was used for T cell depletion. T cell purification was performed by incubation with biotinylated B220, CD49b, Gr-1, and Ter119 antibodies. TCD and purification samples were then incubated with antibiotin microbeads, followed by negative selection with MACS LS columns (Miltenyi Biotec, Gladbach, Germany).

Flow Cytometry

Anti-mouse (CD3, CD11b, CD19, IgM, CD49b, Ter119) PE or CD11b PE-CY7, CD11c FITC or APC-CY7, B220 PERCP-CY5.5, and PDCA1 ef450 were purchased from BD Biosciences, BioLegend (San Diego, CA), or eBioscience (San Diego, CA) and used for pDC analysis. Stimulation of pDCs for cytokine profile analysis was done using 50 μM CpG (ODN 1585; InvivoGen, San Diego, CA) of whole BM in complete RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM each of 2-mercaptoethanol, nonessential amino acids, HEPES, and sodium pyruvate (complete medium) in 10-cm wells for 9 hours at 37°C. BD GolgiPlug (BD Biosciences) was added at hour 3. Intracellular analysis of pDCs was done using the BD Biosciences Cytofix/Cytoperm Kit and anti-mouse IDO PerCP-Cy5.5, IFNα FITC, IL-10 PECY7, and IL-12 APC antibodies.

Splenocytes were stained using anti-mouse CD3 FITC, CD4 PE-CF594, CD8 PERCP-CY5.5, and CD25 APC-CY7. T cells were stimulated with BD Leukocyte Activation Cocktail and GolgiPlug for 6 hours. Intranuclear staining was done using the eBioscience fixation kit and Tbet PE-CY7, GATA3 PE, RORγT APC, and FoxP3 PE antibodies. Data were acquired with a FACSAria cell sorter (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, Oregon).

In Vitro T Cell Activation

MACS-purified splenic T cells from C57BL/6 mice were cultured with 2 μL of anti-CD3/28 Dynabeads (Thermo Fisher Scientific, Waltham, MA) per 10^6 T cells. A 1:2 ratio of PBS- or Flt3L-treated pDCs to T cells were incubated in 96-well plates in complete medium for 72 hours at 37°C.

Transplantation

On day -1, recipients were irradiated twice at 5.5 Gy, with fractions separated by 3 to 4 hours, for a total of 11 Gy [25]. Mice underwent transplantation with 5×10^6 TCD PBS- or Flt3L-treated BM with or without 4×10^6 T cells on day 0. GVHD monitoring used a 10-point scoring system including weight, attitude/activity, skin condition, hunching, and coat condition [26]. Histopathological GVHD grading of the small intestine was done on a scale of 0 to 4 (grade 0, no pathological findings; 1, rare apoptotic cells without crypt loss; 2, loss of individual crypts; 3, loss of contiguous crypts; 4, few or no identifiable crypts with possible mucosal ulcers). A higher grade generally includes characteristics of lower grades, but not necessarily so long as criteria of the assigned grade are met. Histopathological GVHD grading of livers was done on a scale of 0 to 1 (grade 0, no pathology; 1, evidence of lymphocytic infiltration into the portal triad and apoptotic bile duct epithelial cells) [27–29].

Cytospin

Cells were treated and sorted according to the foregoing protocols. The cytospin filter was moistened with 1% BSA-PBS. Cells were loaded into the cytospin wells and spun at maximum speed on a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher Scientific). The cells were fixed with methanol and desiccated overnight. The cells were stained overnight with Giemsa stain (Electron Microscopy Sciences, Hatfield, PA), then fixed with Permount (Fisher Chemical, Geel, Belgium) and xylene mounting medium (Thermo Fisher Scientific). The slides were imaged using an Axioplan 2 universal microscope (Carl Zeiss, Oberkochen, Germany).

Tumor Cell Challenge and Bioluminescent Imaging

An acute myelogenous leukemia cell line, luciferase-transfected C1498 (a generous gift from Dr Bruce Blazar), was used for the GVL experiments. Mice were lethally irradiated (11 Gy) on day -2 and injected with 50,000 C1498 cells on day -1. On day 0, TCD BM or BM from Flt3L-treated BM donors with or without untreated T cells were transplanted. For bioluminescent imaging, 150 μg/kg of D-luciferin was injected and mice were imaged with the IVIS Spectrum in vivo imaging system (PerkinElmer, Waltham, MA). Luminescence was measured in photons/second/cm^2/steradian and normalized to control mice (recipients of the same transplants without luciferase-positive tumors).

Gene Array Analysis

Human subjects were treated with 75 μg/kg of Flt3L (CDX-301) for 5 consecutive days and then underwent large-volume leukapheresis as part of an Institutional Review Board (IRB)-approved clinical trial (ClinicalTrials.gov identifier NCT022000380). BM samples from volunteer donors were collected as part of a separate IRB-approved clinical study of immune cells in the BM (ClinicalTrials.gov identifier NCT02485639). Gene expression of human BM and Ftl3L-mobilized peripheral blood FACS-isolated pDCs was assessed using the HumanHT-12 v4 BeadChip Kit (Illumina, San Diego, CA). Data were preprocessed, quantile-normalized, background-corrected, and log2-transformed for downstream analysis [30]. RNA-seq gene expression of murine BM and Flt3L-treated BM pDCs was assessed. cDNA was prepared using the SMART-Seq v4 Low-Input RNA Kit (Takara Bio, Susatsu, Japan). The

sequencing library was created using the NEBNext Ultra II FS DNA Kit (New England BioLabs, Ipswich, MA).

Samples were sequenced by next-generation sequencing at 2 × 151 bp in the paired ends. Fastq reads were trimmed and filtered for quality and adapter contamination with Trimmomatic (Usadel Lab, Aachen, Germany). Postfiltered reads were mapped against the Ensemble mouse GRCm38/mm10 reference genome and Gencode Release M16 gene annotation using STARaligner. Sequencing results are available at the NCBI Sequence Read Archive (accession no. SRP155387). Expression quantification was obtained using HTSeq counts, DESeq-normalized, and log2-transformed for further analysis. [31]. Differential expression analysis for both human and murine samples was performed using a modified *t* test [30]. Heatmaps were created using NOJAH (http://bbisr.shinyapps.winship.emory.edu/NOJAH/). Genes were determined to be significantly differentially expressed based on both a fold change of 1.5 and a false discovery rate cutoff of .05. Pathway analysis was performed using Cytoscape v3.6.1 and the ReactomeFI plugin [32,33].

Statistical Analysis

Data were analyzed using Prism version 5 for Mac (GraphPad Software, San Diego, CA) and are displayed as mean ± SD unless specified otherwise. Survival differences were calculated in a pairwise fashion using the log-rank test. Applicable data were compared using the Student *t* test and 1-way or 2-way ANOVA. A *P* value ≤.05 was considered to indicate statistical significance.

RESULTS

Flt3L Administration Expands pDC Content in BM In Vivo

To examine whether Flt3L treatment could enhance pDC content in vivo, we tested the effect of Flt3L administration on murine BM donors owing to the known effect of Flt3L in stimulating the differentiation and expansion of pDCs [34]. We measured the content of HSCs, pDCs, T cells, B cells, and natural killer (NK) cells in BM from mice treated with different dosing schedules of PBS or Flt3L (Table 1). The increased pDC content in BM was proportionate to the number of Flt3L doses administered (Figure 1A). HSC, T cell, B cell, and NK cell contents were not significantly affected in mice treated with fewer than 4 doses of Flt3L (Figure 1B-E). A schedule of 2 doses of Flt3L (schedule C) was chosen for all subsequent experiments because the pDC content was increased by 5-fold without significant differences in the content of other immune cells that might modulate GVHD, including HSCs, T cells, B cells, and NK cells.

Flt3L Administration to BM Donors May Affect Homing and Lineage But Not Phenotype of pDCs

Because homing of donor pDCs to GVHD target organs is dependent on their chemokine receptor expression [35], we measured the expression of CCR4, CCR5, CCR7, CCR9, and CXCR4 in pDCs from untreated BM and F-BM [36]. Mice were treated with PBS or 300 μg/kg of Flt3L on days -4 and -1, after which BM was harvested. There was significantly less CCR9 and more CXCR4 expression in pDCs from F-BM compared with control pDCs (Figure 2A), suggesting that the ability of pDCs to migrate to the gut and lymph nodes could be altered by treatment with Flt3L [37,38].

Table 1
Flt3L Administration Schedule

Dosage Schedule (Days)
BM: Control
A: -7
B: -4
C: -4, -1
D: -7, -5, -3, -1
E: -7, -6, -5, -4, -3, -2, -1

BM controls were treated with PBS and experimental mice were treated with 300 μg/kg of Flt3L following this schedule.

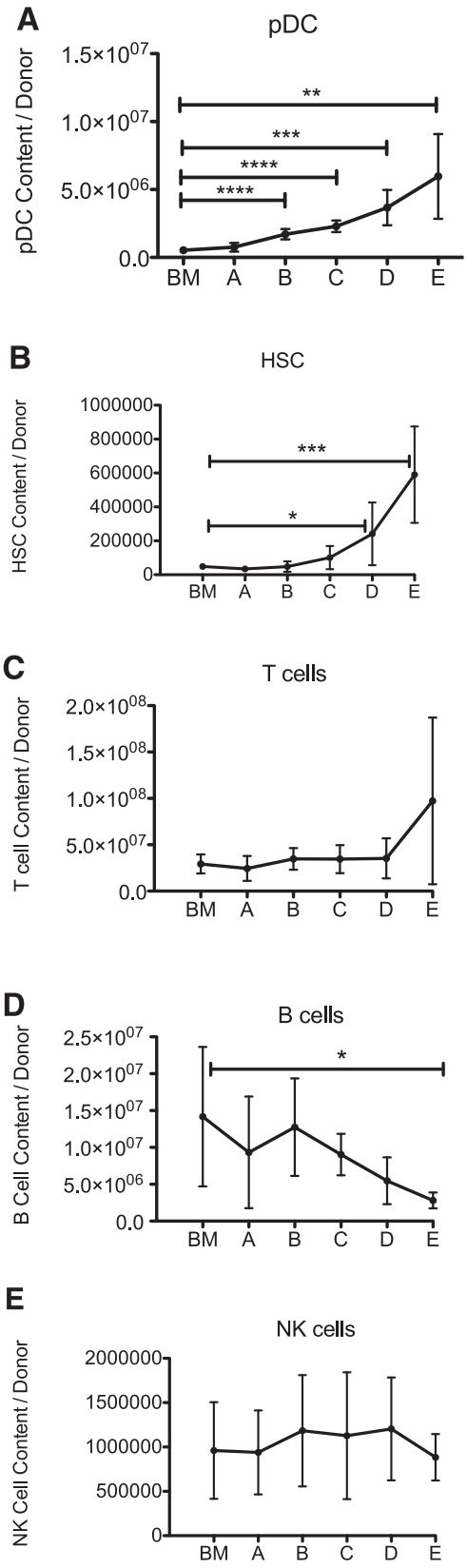

Figure 1. Flt3L administration to BM donors increased pDC content in grafts. Mice were treated with PBS or 300 μg/kg of Flt3L according to the schedule showb in Table 1. Contents of pDCs (A), HSCs (B), T cells (C), B cells (D), and NK cells (E) were measured by flow cytometry. n = 6 per group, combined data from 2 independent experiments. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

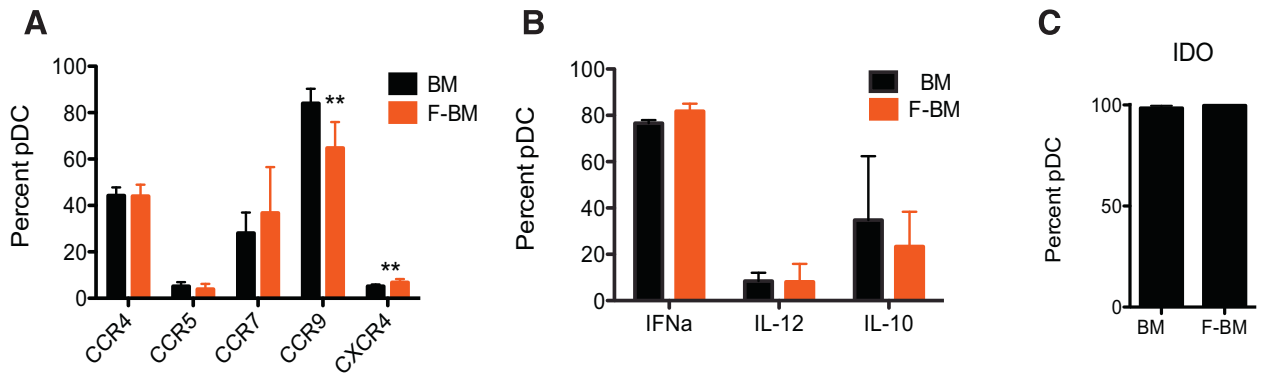

Figure 2. Phenotype of pDCs from BM and F-BM grafts are similar. Mice were treated with PBS or 300 μg/kg Flt3L on days -4 and -1. (A) Surface marker expression of chemokine receptors was measured by flow cytometry. (B) Whole BM or F-BM grafts were treated with 50 μg of CpG for 9 hours at 37°C. (C) Intracellular staining for cytokine and IDO expression was measured by flow cytometry. n = 3 to 6 per group, from 2 independent experiments. $^{**}P < .01$.

To better understand the effect of pDCs on T cell activation and polarization, we next examined the cytokine profiles and IDO production of pDCs from BM and F-BM grafts. Flt3L treatment did not result in any substantive changes in the levels of IFNα, IL-12, IL-10, and IDO in FBM pDCs compared with pDCs from untreated BM (Figure 2B and C).

FBM Recipients Had Increased Survival and Less GVHD Compared with Untreated BM Recipients

We next performed transplantations using untreated BM or F-BM with the addition of donor T cells from untreated mice to assess the effect of Flt3L treatment of donor BM on post-transplantation survival and GVHD. Lethally irradiated (11 Gy) B10.BR mice underwent transplantation with 5 million TCD BM cells from C57BL/6 donor mice treated with PBS or 300 μg/kg of Flt3L on days -4 and -1 in combination with 4 million T cells from PBS-treated C57BL/6 donor mice. On average, TCD BM grafts consisting of 5 million nucleated cells from PBS-treated donors contained ~50,000 pDCs and TCD F-BM contained ~250,000 pDCs. Mice that received allogeneic TCD F-BM with the addition of donor T cells had significantly longer survival compared with recipients of TCD BM plus T cells (Figure 3A). Donor hematopoietic engraftment in all groups was nearly 100% (Figure 3B). Recipients of TCD F-BM plus T cells had significantly lower GVHD than recipients of control TCD BM plus T cells (Figure 3C). Histological analysis of the liver and small intestine showed scant evidence of GVHD pathology in recipients of control TCD BM, without the addition of donor T cells. Using National Institutes of Health criteria for histological diagnosis of GVHD [27–29], and euthanizing mice on day +30 post-transplantation, GVHD histopathology was not significantly different comparing scoring sections of small intestine or liver between recipients of TCD BM plus T cells and TCD F-BM plus T cells (Figure 3D and E), although the small numbers of mice studies sacrificed at this time point and possible differences in sampling of tissue limit the statistical power of this comparison.

To isolate the role of pDCs from F-BM in this transplantation model, we transplanted B10.BR recipients with purified 5000 HSCs, 10^6 T cells, and 50,000 FACS-isolated pDCs from BM of untreated or Flt3L-treated C57BL/6 BM. Recipients of F-BM pDCs had 90% survival and lower GVHD scores compared with recipients of BM pDCs, in which survival was 80% (P not significant; Supplementary Figure 1A and B).

Flt3L-Treated Donors Have Different Gene Expression Profiles Than BM Donors

After observing a trend toward increased survival in the group that received an equal number of FACS isolated FBM pDCs compared with untreated BM pDCs in a model system using purified HSCs, T cells, and pDC transplants, we hypothesized that quantitative effects of Flt3L treatment on the increased number of pDCs in donor BM is not the sole determinant of improved transplantation outcomes following Flt3L treatment. Thus, to explore qualitative effects of Flt3L treatment on pDCs, we analyzed gene expression profiles of pDCs from F-BM versus BM from untreated donor mice. pDCs were isolated by FACS from the BM of mice treated with PBS or 300 μg/kg Flt3L on days -4 and -1. The isolated cells had >95% purity and uniform pDC morphology when imaged following cytospin (Figure 4A). RNA was obtained from these purified pDCs and sequenced using next-generation sequencing.

Pairwise analyses were conducted on individual genes and immunologic pathways using a P value cutoff of <.05 (Supplementary Tables 1 and 2). F-BM pDCs have different gene expression profiles than pDCs from untreated BM grafts (Figure 4B and C). Immunologic pathways that showed significant differences ($P < .05$) include the adaptive immune pathways and immune checkpoint pathways. Up-regulated genes in the adaptive immune pathway include *APRIL, Hmox1*, and *Etv5*, all of which may affect the ability of donor pDCs to regulate T cell polarization and activation [39]. Immune checkpoint pathway genes *Bcl2, Cyclin D3, TIM-3*, and *ACK1* are up-regulated in pDCs from F-BM grafts, suggesting an increased ability for immune cell regulation [40]. Finally, pDCs from F-BM grafts up-regulated *Tox1* and *Prss16*, genes that regulate T cell selection in the thymus (Table 2) [41,42].

To better interpret the translational relevance of these murine studies, gene expression profiles were compared between FACS-isolated human pDCs from untreated BM donors and pDCs isolated from apheresis products of Flt3L-treated sibling donors. Because we did not have access to the BM from healthy human volunteers treated with Flt3L, we used grafts from sibling stem cell donors that underwent mobilization with 5 daily injections of 75 μg/kg of CDX-301 (recombinant Flt3L) as a single mobilization agent from an IRB-approved clinical study (ClinicalTrials.gov identifier NCT022000380) that assessed the efficacy of Flt3L in mobilizing stem cells, and compared gene expression to pDCs isolated from untreated BM acquired on a separate clinical study (ClinicalTrials.gov identifier NCT02485639). RNA from pDC samples were sequenced by Illumina chromatin immunoprecipitation,

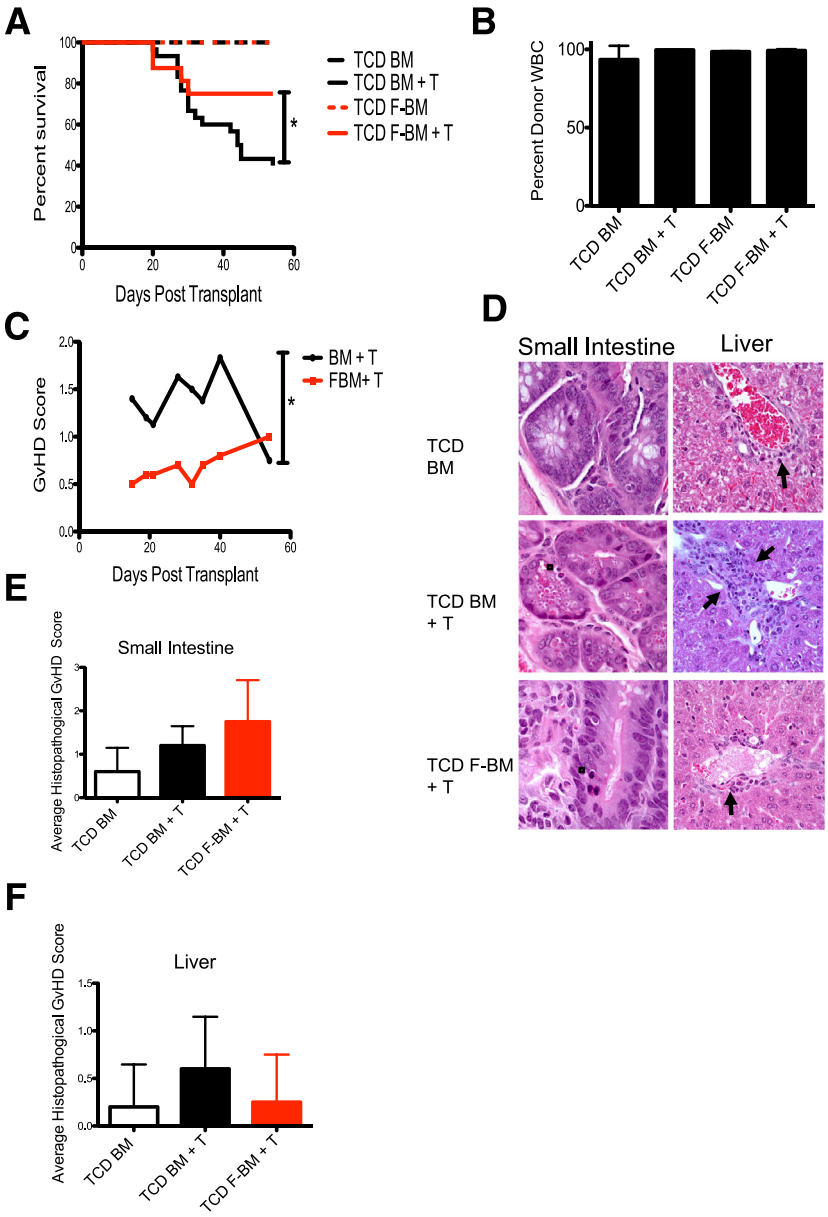

Figure 3. Grafts from Flt3L-treated donors increased survival and decreased GVHD. C57BL/6 donor mice were treated with PBS or 300 μg/kg Flt3L on days -4 and -1. B10.BR recipient mice underwent transplantation with 5 million TCD BM or F-BM cells with or without the addition of 4 million T cells. (A) Survival of murine transplant recipients. Recipient groups included TCD BM, TCD F-BM, TCD BM plus 4 million T cells, and TCD FBM plus 4 million T cells. $^*P < .05$, Kaplan-Meier survival analysis. (B) Chimerism of recipients at 30 days post-transplantation by group. (C) Clinical GVHD scores of mice that received T cells. n = 30 per group, from 2 independent experiments. $^*P < .05$, 2-way ANOVA. (D) Representative histopathological samples of small intestine and liver at day +30 from each treatment group photographed at 600× magnification. Black squares denote apoptotic cells in the small intestine crypts. Histopathological grades of GVHD-associated pathology are 0, no pathology; 1, apoptotic cells; 2, crypt loss; 3, contiguous crypt loss. Liver pathology included lymphocytic infiltration to the portal triad and rare apoptotic bile duct epithelial cells (black arrows), graded as 0, no pathology, or 1, portal triad lymphocytic infiltration and apoptotic bile duct epithelial cells. (E and F) Average histopathological grade of GVHD-associated pathology in the small intestine (E) and liver (F). n = 4 to 6 per group, from 2 independent experiments.

and differential gene expression along with immune pathway analysis was conducted (Supplementary Tables 3 and 4). Similar to the murine gene array analysis, *KLRF1* and *SLAMF6* in the adaptive immune pathway and *BCL2* and *BIRC3* in the immune checkpoint pathway were up-regulated in pDCs from the Flt3L-treated donors compared with pDCs from untreated BM volunteers (Figure 4D and E, Table 3). In addition, Toll-like receptor (TLR) genes, including those in the TLR4 pathway (*APP, MAP2K6, CD36, ACTG1*, and *IRF7*), were down-regulated in Flt3L-treated donor pDCs, indicating a decreased ability to stimulate innate immune pathways (Table 4) [43].

F-BM Reduced T Cell Polarization Post-Transplantation

Based on the results of the gene array analyses of pDCs from Flt3L-treated mice and humans, we next studied the ability of pDCs to interact with and influence donor T cell activation and immune polarization post-transplantation in a nonparacrine cytokine fashion [44,45]. We examined the potential of pDCs to stimulate or inhibit alloactivation of T cells by measuring surface expression of MHC II, CD86, and PDL1 on pDCs. pDCs from Flt3L-treated donors expressed less MHC II and CD86 and expressed more PDL1 (Figure 5A). To determine whether changes in surface expression of costimulatory and

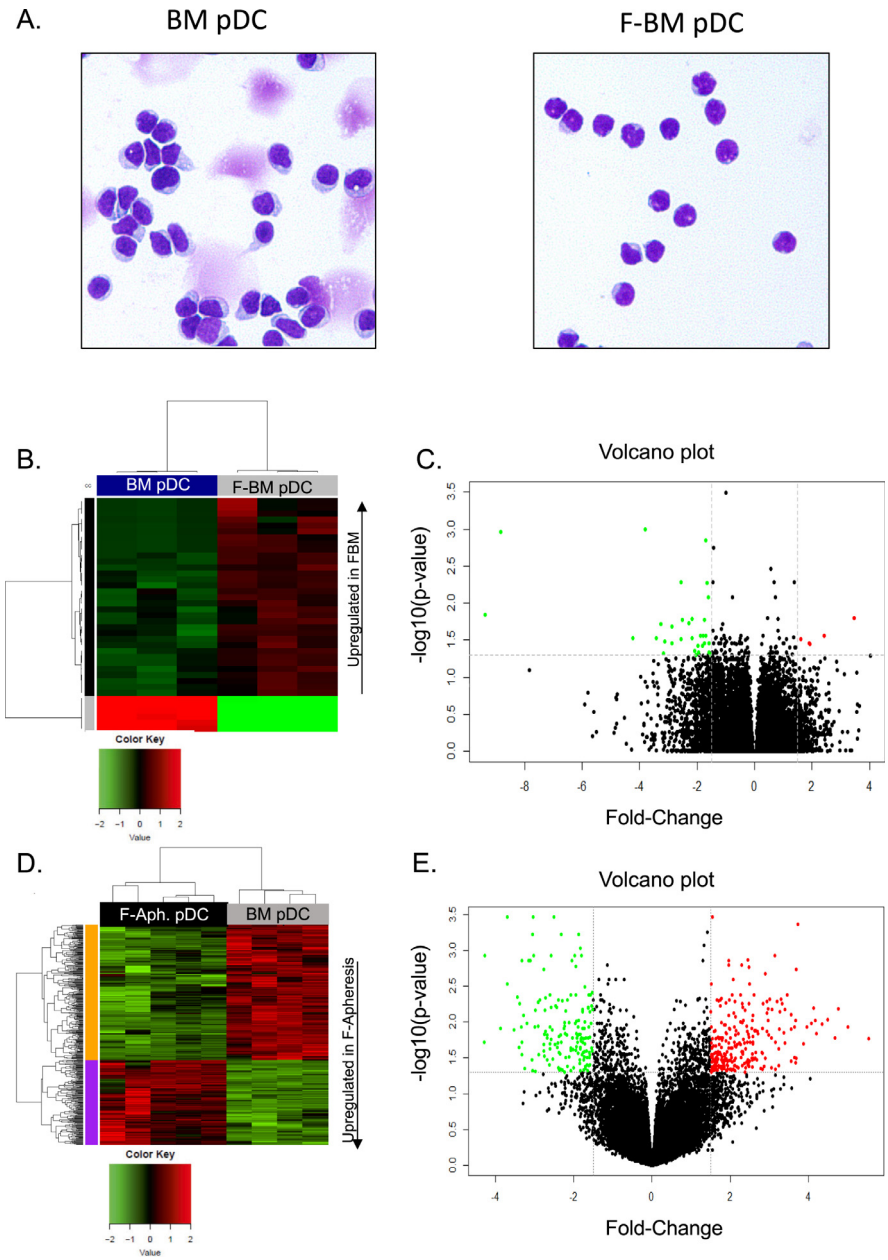

Figure 4. F-BM pDCs have a different gene expression profile then BM pDCs. Mice were treated with PBS and 300 μg/kg of Flt3L on days -4 and -1. pDCs were isolated by FACS. (A) Slides of pDCs from both groups were prepared by cytospin. RNA was sequenced by next-generation sequencing. (C) Heatmap depicting the significantly differentially expressed genes in the murine samples using *z*-score scaling, 1-Pearson correlation distance, and ward.D clustering. (D) Volcano plot of gene up-regulation and down-regulation (BM versus F-BM). n = 3 per group from 1 experiment. (E) Healthy human donors were untreated or treated with 75 μg/kg of CDX-301 (recombinant Flt3L) for 5 consecutive days. Untreated donors underwent BM harvest, and Flt3L-treated donors underwent leukapheresis on day +6. pDCs were isolated by FACS. RNA sequencing was done with the HumanHT-12 v4 BeadChip Kit (Illumina). The heatmap depicts the significantly differentially expressed genes in the human samples using *z*-score scaling, Euclidean distance, and complete clustering. (F) Volcano plot depicting up-regulation and down-regulation of genes (BM versus F-Apheresis). n = 4 to 5 per group from 1 experiment.

coinhibitory molecules affects T cell polarization, we performed a C57BL/6→B10.BR transplantation with 5 million TCD BM or F-BM cells and 4 million untreated T cells. On day 3 post-transplantation, donor T cells were analyzed for T cell transcription factor content by flow cytometry. Mice that received TCD F-BM plus 4 million T cells had donor T cells with significantly lower levels of Tbet and RORγT expression on day 3 post-transplantation, consistent with decreased Th1 and Th17 polarization, respectively (Figure 5B).

To better determine the role of pDCs in the pattern of transcription factor expression in T cells post-transplantation, we performed a C57BL/6→B10.BR transplantation using purified HSCs, purified T cells, and purified populations of BM or F-BM pDCs, and again measured T cell transcription factor expression on day 3. The same trend toward lower transcription factor expression in T cells post-transplantation was observed in this experiment as well along with lower production of cytokines (Supplementary Figure 2A and B). The effect of FBM pDCs on T cells does not appear to limit T cell proliferation, because T cell proliferation was equivalent comparing the addition of F-BM pDCs versus untreated BM pDCs to T cells in a mixed-lymphocyte reaction (Supplementary Figure 3A and B).

Table 2
Genes Up-Regulated in F-BM pDCs

Gene	Pathway	LogFC	-Log10 (P Value)
Bcl2	Immune checkpoint	-1.66	2.27
Cyclin D3	Immune checkpoint	-1.60	2.08
Etv5	Adaptive immunity	-2.30	1.73
APRIL	Adaptive immunity	-3.25	1.71
TIM-3	Immune checkpoint	-1.79	1.56
Tox1	T cell selection	-4.24	1.53
Prss16	T cell selection	-3.12	1.48
Hmox1	Adaptive immunity	-1.73	1.46
ACK1	Immune checkpoint	-1.97	1.43

Shown are up-regulated genes in F-BM versus BM in adaptive immune, immune checkpoint, and thymic-induced T cell selection pathways that are shown in Figure 4B, plotted as logFC by −log10 (*P* value).

Table 3
Genes Up-Regulated in F-Apheresis pDCs

Gene	Pathway	LogFC	-Log10 (*P* value)
BCL2	Immune checkpoint	-2.34	1.35
KLRF1	Adaptive immunity	-3.70	2.53
SLAMF6	Adaptive immunity	-2.74	2.16
BIRC3	Immune checkpoint	-2.37	1.58

Shown are up-regulated genes in F-apheresis pDCs versus BM in adaptive immune and immune checkpoint pathways that are shown in Figure 4D, plotted as logFC by −log10 (*P* value).

Table 4
Genes Down-Regulated in F-Apheresis pDCs

Genes	LogFC	-Log10 (*P* Value)
APP	3.67	2.02
MAPK6	1.85	1.47
CD36	3.14	2.93
ACTG1	1.91	1.32
IRF7	3.24	1.39

Shown are down-regulated genes in Toll-like receptor cascades including the TLR4 pathway that are shown in Figure 4D, plotted as logFC by −log10 (*P* value).

GVL Activity Was Not Diminished in F-BM Recipients

To determine whether Flt3L treatment of BM donors affected the GVL activity of the allogeneic transplant, we compared the growth of leukemia cells in recipients of F-BM compared with recipients of untreated BM using bioluminescent imaging of luciferase-positive leukemia cells and survival analyses of leukemia-bearing transplant recipients. C57BL/6 mice were irradiated (11 Gy) on day -2, inoculated with 50,000 syngeneic luciferase-positive C1498 tumor cells on day -1, and then transplanted with 5 million TCD BM or F-BM cells plus 1 million T cells from MHC-mismatched B10.BR donors on day 0. Tumor burden was measured serially by bioluminescence. There was no significant difference in tumor burden between recipients of untreated TCD BM plus 1 million T cells and recipients of TCD F-BM plus 1 million T cells (Figure 6 A-C).

In addition, using the LBRM tumor line in C57BL/6→B10.BR transplant recipients, recipients of TCD F-BM plus 4 million T cells had significantly longer survival compared with recipients of TCD BM plus 4 million T cells (Supplementary Figure 4A-C).

DISCUSSION

GVHD remains the most significant complication following HSCT [46]. Although recipients of BM or G-CSF-mobilized grafts from unrelated donors have equal survival up to 7 years post-transplantation, the incidence of chronic GVHD is higher in G-CSF-mobilized graft recipients [47,48]. Furthermore, the BMTCTN 0201 study demonstrated that recipients of BM grafts

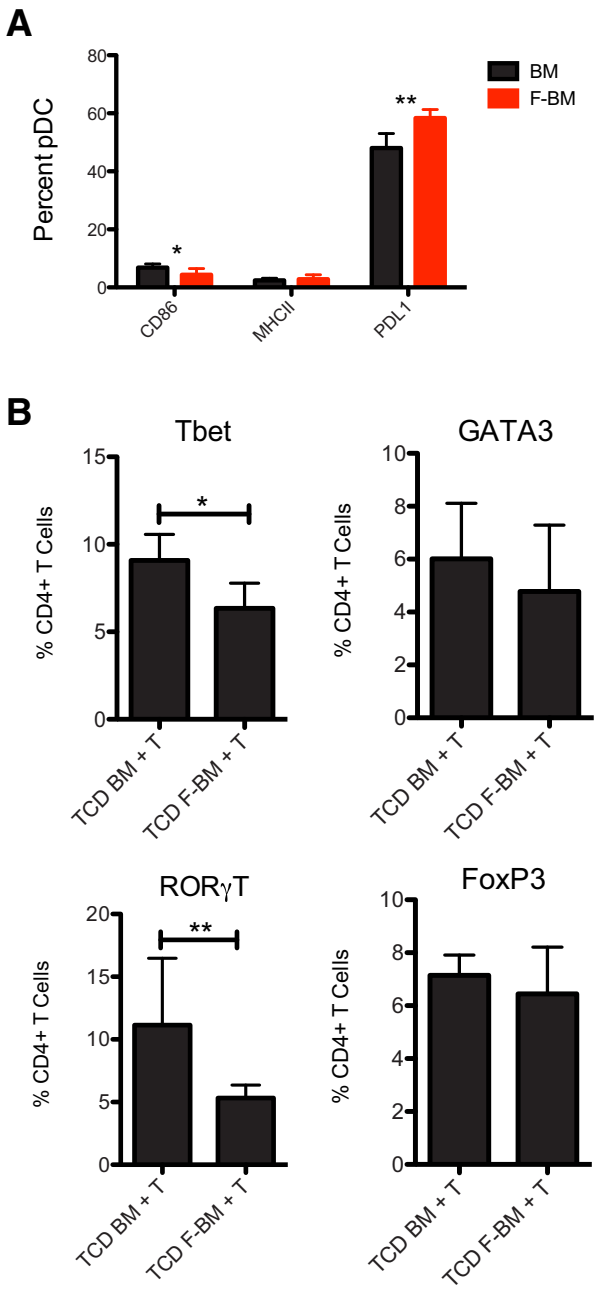

Figure 5. Flt3L treatment of BM donors decreased the expression of T helper cell transcription factors in recipients. Donor mice were treated with PBS or 300 μg/kg of Flt3L on days -4 and -1. (A) CD86, MHC II, and PDL1 surface expression was measured by flow cytometry. (B) In C57BL/6→B10.BR transplantation, mice received 5 million TCD BM or F-BM cells plus 4 million T cells. Intranuclear staining of transcription factors Tbet, GATA3, RoRγT, and FoxP3 in T cells was assessed by flow cytometry at 3 days post-transplantation. n = 6 per group, combined data from 2 independent experiments. $^{*}P < .05$; $^{**}P < .01$.

containing higher numbers of pDC had increased survival and lower treatment-related mortality owing to less GVHD [7]. The results of this study also showed a widely varying pDC content of the BM grafts among volunteer BM donors, raising the question of how to increase the content of immunoregulatory donor pDCs in all BM allografts. Here we report that administration of Flt3L to BM donors increased the pDC content of grafts, and that graft recipients of F-BM or purified donor pDCs from F-BM had increased survival and less GVHD after allogeneic transplantation.

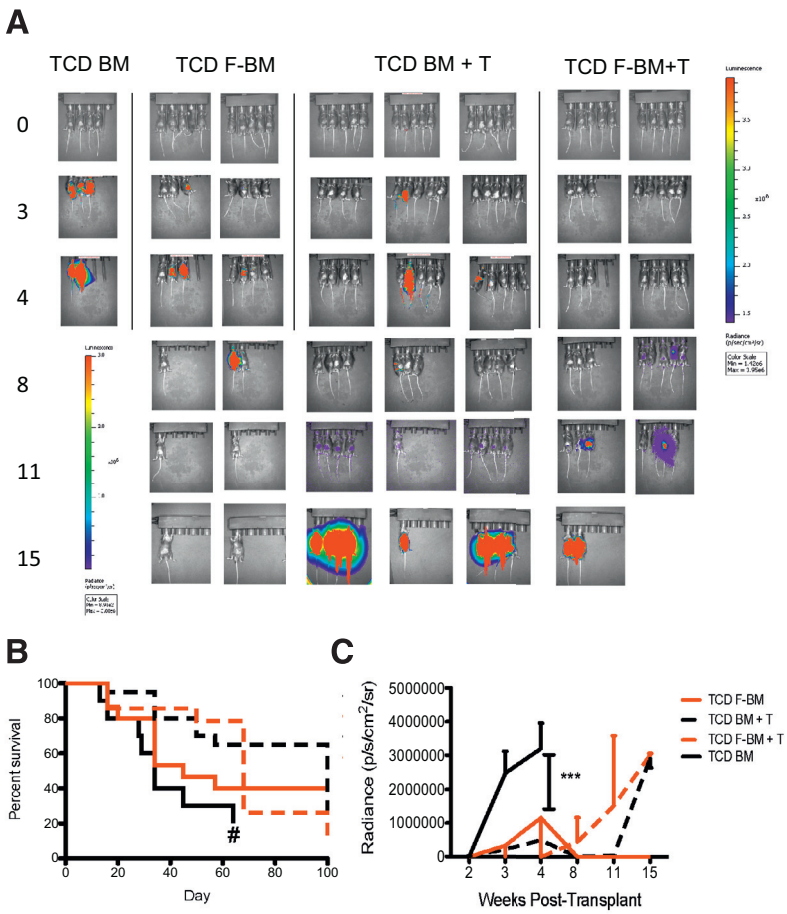

Figure 6. There was no loss of the GVL effect in recipients of F-BM. B10.BR→ C57BL/6 murine transplant recipients received 5 million TCD BM cells and 1 million T cells. Recipient mice received 50,000 luciferase-positive C1498 cells. (A) Serial bioluminescent imaging of recipient mice. (B) Survival curve of recipient mice. #Censored subjects. (C) Quantification of tumor burden. n = 10 to 15 per group from 2 independent experiments. $^{***}P < .01$.

In previous studies, we showed that murine BM donor pDCs limit GVHD without attenuating the GVL effect [17,18]. Furthermore, we have unpublished data confirming that with transplantation of allo-grafts containing highly purified HSCs, T cells, and pDCs, recipients of BM pDCs have increased survival and decreased GVHD incidence compared with recipients of G-CSF-mobilized pDCs (Hassan 2018, submitted). Thus, data from the current study showing that Flt3L administration to BM donors increased pDC content by 5-fold spurred further characterization of the effects of Flt3L treatment on the quantity and quality of pDC in BM and HSCT outcomes. Transplanting 5 million TCD BM cells from Flt3L-treated donors with the addition of 4 million T cells from untreated donors increased survival with decreased GVHD compared with transplantation of an equal number of untreated TCD BM cells and T cells.

Interestingly, although pDCs from untreated BM and F-BM have a similar phenotype, we show that F-BM pDCs have an enhanced cell-intrinsic ability to limit GVHD and a gene expression profile that supports greater immunomodulatory capacity compared with pDCs from untreated BM in a C57BL/6→B10.BR heterogeneous transplant model with characteristics of both acute and chronic GVHD, a common aspect of HSCT recipients in a clinical setting. Of note, T cells from recipients of F-BM pDC had less Th1 (Tbet) and Th17 (RoRγT) polarization than T cells from recipients of pDCs from untreated BM (Figure 5B and Supplementary Figure 2A). Thus, the up-regulation of adaptive immune and immune checkpoint pathways in pDCs from F-BM may be responsible for their ability to regulate donor T cell immune polarization, decrease Th1 and Th17 polarization, and limit the incidence and severity of GVHD compared with pDCs from untreated BM [48–51]. This, coupled with increased expression of genes involved in positive and negative selection in the thymus, may enable F-BM pDCs to induce post-transplantation tolerance of donor stem cell-derived T cells. In addition, pDCs from Flt3L-treated human donors have down-regulated the expression of genes involved in TLR cascades and innate immune cell pathways compared with pDCs from untreated BM. Down-regulation of the TLR4 pathway also may play a role in limiting GVHD in the gut, because gut GVHD can activate the release of lipopolysaccharide from bacteria, ultimately activating TLR4 on pDCs, which can further aggravate injury in the gut and augment GVHD [52–54]. Thus, the down-regulation of genes involved in TLR cascades and other innate immune pathways in F-BM pDCs may also play a role in the attenuation of GVHD following transplantation of pDCs from F-BM.

The present study has some limitations. Although we focus on donor pDCs and the effect of Flt3L administration to BM donors on pDCs, other donor cell types may contribute to the transplantation outcomes following Flt3L treatment of BM donors. We have shown with 1 dose and 1 schedule of Flt3L administration (day -4 and day -1 with respect to BM harvest), there is a significant increase in the content and quality of pDCs. Although there was no significant change in the content

of HSCs, NK cells, T cells, and B cells in Flt3L-treated donor BM grafts, determining the effect of Flt3L administration on the cell-intrinsic qualities of these cells to interact with donor T cells and regulate immune responses may further clarify the mechanisms that result in increased survival and decreased GVHD in F-BM recipients [48]. Thus, the clinical utility of Flt3L treatment of human BM donors may be studied by characterizing the effect on immune cell content and quality within the BM graft. Among volunteers treated with Flt3L, some previous Phase I studies have demonstrated the safety of daily administration for 1 week or longer [23]. In addition, we did not test the ability of peripheral blood- mobilized Flt3L grafts to affect transplantation outcomes, because we observed that HSC and pDC mobilization was not equivalent to G-CSF mobilization (Supplementary Figure 5A and B). Furthermore, Flt3L as a single agent for stem cell mobilization is not efficient (ClinicalTrials.gov identifier NCT022000380), and Flt3L would need to be combined with other agents, such as CXCR4 antagonists for this approach to be feasible for clinical practice. In addition, we compared gene expression in different sources of Flt3L-stimulated pDCs in mice and humans, isolating F-BM pDCs from Ftl3L-treated murine BM and F-apheresis pDCs from human donor apheresis products owing to the lack of available human F-BM samples. Nevertheless, the similarities in gene expression between pDCs from murine samples and human samples, and the striking findings of improved survival with less GVHD in the murine transplant models suggest that characterization of pDCs from BM of Flt3L-treated human donors is warranted in a planned clinical study to further validate the clinical translation potential of these findings.

In conclusion, we report a novel method using Flt3L treatment of BM donors to reduce the GVHD-promoting activity of BM grafts and BM pDCs, thereby improving survival of allogeneic BM transplant recipients. Flt3L treatment increased the content and immunoregulatory capacity of pDCs in BM grafts, leading to decreased severity of GVHD in murine recipients. Notably, the reduction of GVHD activity following Flt3L treatment of BM donors was not associated with an attenuation of the GVL activity of donor T cells. Thus, our present preclinical data provide an impetus to test the clinical effect of Flt3L treatment of BM donors as a novel method to improve transplantation outcomes.

ACKNOWLEDGMENTS

Financial disclosure: This work was supported by National Institutes of Health Grant 5R01 CA188523, National Cancer Institute Grant P30 CA138292, the Biostatistics and Bioinformatics Shared Resource and Emory Integrated Genomics Core Shared Resource of the Winship Cancer Institute and the Research Pathology Shared Resource and Cancer Animal Models of the Winship Cancer Institute of Emory University. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest statement: There are no conflicts of interest to report.

Authorship statement: M.H., A.U.A, S.H., S.D., J.K., H.M., M.Y., and E.K.W. designed experiments; M.H, A.U.A, J.M.L., and S.H. performed experiments; M.H., A.U.A, M.R., A.J.P., and D.L.J analyzed results and created the figures; and M.H. and E.K.W wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.11.029.

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