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Alina Ulezko Antonova April 9, 2019

Bone Marrow Grafts from Donors Treated with Flt3L Have More Plasmacytoid Dendritic Cells and Lead to Improved Transplant Outcomes

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Abstract

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Hematological malignancies can be cured with hematopoietic stem cell transplantation (HSCT). The source of hematopoietic stem cells (HSC) from adult donors is either bone marrow (BM) or granulocyte-colony stimulating factor mobilized peripheral blood (G-PB). A randomized, multi-center clinical trial (BMT CTN 0201) showed better overall survival (OS) and less graft-versus-host disease (GvHD) among recipients of grafts containing higher numbers of plasmacytoid dendritic cells (pDC). This was only true for recipients of BM grafts, but not recipients of G-PB grafts. Thus, BM pDC may be more effective than G-PB pDC in regulating post-transplant GvHD. Unfortunately, not all BM grafts have optimal numbers of pDC. Therefore, a method to increase pDC numbers in all BM grafts is highly significant and may offer a novel approach to prevent chronic GvHD (cGvHD). Because FMS-like receptor tyrosine kinase 3 ligand (Flt3L) up-regulates pDC proliferation and maintains pDC homeostasis, we hypothesized that Flt3L could be used as a method to increase pDC content in BM grafts. We show that s.c. administration of 300 μg/kg of Flt3L on days -4 and -1 to mice increases >3 fold the content of pDC in BM grafts without affecting the content of HSCs. Using an allogeneic transplant model (C57BL/6→B10.BR), we report that transplantation of 5 million T-cell depleted Flt3L-stimulated BM (TCD F-BM) cells plus 4 million allogeneic donor T cells resulted in improved overall survival and decreased GvHD as compared to control TCD BM plus T cells. Donor-derived T cells in recipients of F-BM were marked by decreased Th1 and Th17 polarization. Comparative analysis of mRNA from pDC sorted from human and murine donors treated with Flt3L indicated up-regulation of immunoregulatory checkpoints and adaptive immune pathways, as well as genes involved in intrathymic thymocyte selection as compared to pDC from control untreated marrow. Moreover, Flt3L-treated donors had increased intra-thymic pDC content, and allogeneic transplantation of sorted pDC from eGFP+ mice revealed increased homing of donor-derived pDC to the recipient thymus. In conclusion, we report that donor BM graft treatment with Flt3L may improve HSCT outcomes by increasing pDC content and their GvHD-regulating activity, possibly by pDC involvement in the elimination of auto-reactive T cells that would otherwise initiate GvHD.

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I. Introduction

1. Hematopoietic Stem Cell Transplantation

1.1. Overview of HSCT

Hematopoietic stem cell transplantation (HSCT) is the transfusion of hematopoietic stem cells (HSC) to reconstitute marrow function in patients with damaged or defective bone marrow (Armitage, 1994). HSCT was first performed mice as a method of protection against otherwise lethal total-body irradiation (TBI) (Lorenz et al, 1951). In 1959, Thomas et al. reported that an end-stage leukemia patient had a three-month remission after infusion of his identical twin's marrow following TBI (Thomas et al, 1959). Currently, in the United States alone, around 20,000 transplants are performed each year (Pasquini and Zhu, 2014). Moreover, HSCT is the main treatment for non-malignant bone marrow disorders, hemoglobinopathies and relapsed and refractory hematological malignancies, and it has also been used to treat immune dysfunctions in response to HIV, which recently gained attention because a patient achieved complete remission following HSCT from a $CCR5^{\Delta 32/\Delta 32}$ donor (Gupta et al, 2019).

There are two types of HSCT. The first one, allogeneic HSCT, involves transplanting grafts from a donor to a recipient; when the donor is an identical twin, the transplant is considered syngeneic. Alternatively, when the graft is obtained from the patient itself and stored for administration following high-dose chemotherapy, the transplant is considered autologous (Armitage, 1994). Due to the potential morbidity and mortality related to donor immune cells that react against host (recipient) tissues, a condition called

graft-versus-host disease (GvHD), autologous or human leukocyte antigen (HLA)-matched allogeneic transplants are preferred over HLA-mismatched transplants especially in older patients because there is no risk of GvHD (Armitage, 1994).

1.2. Clinical Harvesting of Cells in HSCT: BM vs G-CSF

Hematopoietic stem cells (HSC) can come from two sources: bone marrow (BM) or granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood (G-PB). The methodology that is currently used for harvesting bone marrow is a modification of the original technique developed by Thomas and Storb (Thomas and Storb, 1970). Marrow is harvested by repeated aspirations from the posterior iliac crest and, in cases of low cell counts, from the anterior iliac crest and the sternum (Armitage, 1994). The number of BM cells that are obtained following this procedure is typically 100-300•10^6/kg of the recipient's body weight; a larger number is acquired in certain diagnosis like aplastic anemia and after intense conditioning regimens (Armitage, 1994). HSC can also be collected from peripheral blood by apheresis, but because the number of circulating stem cells in blood is low in the steady state, peripheral blood stem cell (PBSC) donation requires the use of mobilizing agents that translocate HSCs from the BM to the periphery. The standard mobilizing agent is G-CSF (Hölig, 2013), currently marketed under the brand names lenograstim and filgrastim (Hequet, 2015). The optimal number of HSCs to be harvested is 4x10^6 CD34+ cells/kg per patient; in all diseases, a minimum of 2x10^6 cells/kg should be collected to ensure rapid and durable hematopoietic engraftment (Hequet, 2015). When cells are harvested from G-PB, blood apheresis –

which consists in separating blood components in layers by centrifugation and harvesting HSCs in a specific layer that has other blood cells- is conducted on the donor's blood and the product is then directed to the recipient (Hequet et al, 2015).

In the case of major ABO incompatibility between donor and recipient, mature erythrocytes are removed from the graft to avoid a hemolytic transfusion reaction (Gale et al, 1977). Finally, depleting the graft from T cells has been shown to reduce the risk of GvHD, but it has been associated with an increased risk of graft rejection due to the lack of donor T cells that serve to eliminate the remnant recipient T cells and NK cells that can mediate rejection of the donor HSC graft (Armitage, 1994).

1.3. Misconceptions Around the Degree of Donor Affectation Following the Two Procedures

When cells are harvested from peripheral blood, donation does not require hospitalization and is thought to be less physically demanding for the donor (Hölig, 2013), although direct comparisons of pain for allogeneic HSC donors undergoing either BM or PBSC harvests suggest equivalent discomfort associated with both procedures (Pulsipher et al, 2013). As a matter of fact, results of a randomized clinical trial from the National Marrow Donor Program show that donors experience comparable levels of pain in the course of the two procedures, but that the timing of the pain is different: BM donors report mainly mild skeletal pain 2 days to 1 week following harvest, while G-PB donors experience mild-to-moderate pain during preparatory G-CSF mobilization on days 2-6 after the beginning of the regimen (Pulsipher et al, 2013). Moreover, studies show that

over time the concentration of total nucleated cells in harvested marrow for transplantation purposes has decreased, possibly due to this underuse of this medical procedure (Prokopishyn et al, 2019). Because of this misconception, over 90% of transplants in the United States are performed from G-PB (Hölig, 2013).

1.4. Complications of HSCT

The major complications of HSCT are GvHD, graft rejection, pulmonary complications and veno-occlusive disease of the liver. Of these, GvHD is the most common, with chronic GvHD occurring in 30% to 70% of patients (Lee et al, 2008) and acute GvHD in 35-50% of transplant recipients (Jacobsohn et al, 2007). The main targets of GvHD are the skin, the gastrointestinal system and the liver (Armitage, 1994). This disease occurs when immunologically competent cells from the graft attack the recipient's cells, which are less fit because the recipient has undergone a conditioning regimen, typically myeloablative radiation or high-dose chemotherapy (Armitage, 1994). GvHD occurs in two manners: acute GvHD (aGvHD), which usually takes place one to two months after HSCT. Clinically, a diagnosis of aGvHD is suspected when the recipient has dermatitis, cutaneous blisters, abdominal pain with or without diarrhea, persistent nausea and vomiting and/or hepatitis with elevation of bilirubin and/or liver enzymes (Jacobsohn et al, 2007). By contrast, chronic GvHD (cGvHD), which typically develops at least two to six months after transplantation and in some cases can appear many years after HSCT (Armitage, 1994). The pathophysiology of cGvHD may involve inflammation, cell-mediated immunity, humoral immunity, and fibrosis (Jagasia et al, 2014).

Prophylaxis preventing the development of GvHD include administration of cyclosporine with or without methotrexate, the use of corticosteroids and the removal of T cells from the graft (Stob et al, 1986; Ramsay et al, 1982; Storb et al, 1989).

1.5. Survival and GvHD after Transplant

Although recipients of G-PB have slightly better overall survival (OS) one year post-HSCT (Waller et al, 2014), more BM recipients are alive three to seven years post-HSCT (Lee et al, 2016). This is mainly because G-PB recipients develop higher cGvHD (Lee et al, 2016). Because of increased late OS and decreased GvHD, BM is thought to be a better source of HSC for allogeneic transplantation.

Despite conferring an advantage in transplant outcomes, long-term OS in BM recipients is as low as 50% seven years post-HSCT. Therefore, ways to improve bone marrow transplantation are being actively pursued. In 2014, BMT CTN 0201, a randomized multicenter clinical trial by the bone marrow transplantation clinical trials network (BMT CTN), showed that recipients of BM grafts with more abundant plasmacytoid dendritic cells (pDC) and naïve CD8 T cells survived longer, while a similar correlation was not found in recipients of G-PB. This finding suggested that donor pDCs from BM grafts but not from G-PB may play an important role in post-transplant tolerance.

2. Plasmacytoid Dendritic Cells

2.1. Overview of pDC

Plasmacytoid dendritic cells are the major producers of type I IFN in response to viral agents (Colonna et al, 2002); they reside mainly in lymphoid organs (Sozanni et al, 2010), and only represent 0.3-0.5% of the human peripheral blood (Ronnblom et al, 1983). Phenotypically, human pDCs express low levels of the integrin CD11c and positive for the B cell marker B220/CD45RA (Reizis et al, 2011), while murine pDC are positive for CD11c, B220, Ly6C, bone marrow stromal antigen 2 (BST2) and Siglec-H (Swiecki and Colonna, 2015). The FMS-like tyrosine kinase 3 (Flt3) receptor is necessary for HSC differentiation to pDC (D'Amico and Wu, 2003). Upon conversion to hematopoietic progenitor cells (HPC), HSC become Flt3+ and further subdivide into the common lymphoid or the myeloid precursors, both of which can eventually become pDCs (D'Amico and Wu, 2003). However, their genetic profile resembles more closely that of the lymphoid precursors (Matta et al, 2010). The commitment of HSC and committed hematopoietic progenitors to pDC differentiation is dependent upon the binding of Flt3 ligand (Flt3L) to the Flt3 receptor (CD135) and the activation of the transcription factor E2-2 (Cisee et al, 2008) through the activation of the signal transducer and activator of transcription 3 (STAT3 and phosphoinositide 3-kinase (PI3K)-dependent activation of the mammalian target of rapamycin (mTOR) (Laouar et al, 2003). Interestingly, a recent study has shown that Flt3L and type 1 interferon IFNα synergistically drive lymphoid progenitors to differentiate into pDC by inducing upregulation of Flt3 (Chen et al, 2013). Finally, the transcription factor Bcl11a is necessary for expression of IL-7R and

Flt3 receptor on HSC, as its absence in mice leads to severely decreased pDC in fetal livers and fetal BM in a Flt3L and IL-7-dependent manner (Wu et al, 2013).

2.1. Role of pDC in Transplant Tolerance

Plasmacytoid dendritic cells can be either immunogenic or tolerogenic, depending on the signaling, co-stimulatory or transcriptional context (Swiecki and Colonna, 2015). When they receive signaling through the toll-like-receptor (TLR) signaling cascades or other pattern recognition receptors, they act to induce immunogenicity. By contrast, when they are either unstimulated or activated by other means, and express indoleamine 2,3-dioxygenase (IDO) (Pallotta et al, 2011), ICOSL (Ito et al, 2017), PDL1 (Diana et al, 2011), granzyme B (Jahrsdorfer et al, 2010) or lymphocyte activation gene 3 -a CD4-like molecule that negatively regulates activated T cell expansion- (Matta et al, 2010), they induce tolerance, which is usually specific to allo-antigens or tumor cells. However, pDC have recently gained much attention due to their potent ability to induce the generation and proliferation of antigen-specific regulatory T cells (Cardenas et al, 2011). Moreover, an allogeneic transplant model in mice showed that enhancing precursors of pDCs in the graft resulted in increased numbers of regulatory T cells, leading to attenuated levels of both clinical and histopathological GvHD (Huang et al, 2011).

Despite the promise that pDCs offer to the field of bone marrow transplantation, there is insufficient evidence for the mechanisms underlying their transplant-regulating capabilities. One topic of interest in this regard is the expression of the chemokine receptor CCR9. There is an association between CCR9 expression on pDC and their

induction of tolerance and attenuation of GvHD. Upon binding of CCL25, to CCR9 positive pDC migrate to the small intestine, which is a target GvHD organ (Hadeiba et al, 2008). Moreover, CCR9+ pDC powerfully induce Treg function and are able to suppress antigen-specific immune responses both *in vitro* and *in vivo*. Interestingly, these immunosuppressive pDC are typically of lymphoid lineage. Although there are studies that report that lack of CCR9 expression on pDC dichotomizes this subset as "precursors of lymphoid tissue-resident classical dendritic cells (cDCs)" instead of fully differentiated pDC (Swiecki and Colonna, 2015), CCR9- pDC produce more IFNα following stimulation of TLR than CCR9+ pDC (Schlitzer et al, 2011).

Due to their unique circulation profile and dendritic cell phenotype and poor antigen presentation capabilities (Colonna et al, 2015), pDC have been easily studied in isolation from the rest of the dendritic cell pool. For instance, in contrast to cDC, which traffic to the lymph nodes via afferent lymphatics, pDC enter lymph nodes via high endothelial venules (HEVs) mainly through CD62L (Penna et al, 2001). To keep the pDC in the bone marrow stromal niche and for their development, pDC depend upon expression of CXCR4 (Kohara et al, 2007). These characteristics suggest strategies to mobilize pDC from the marrow and into the blood that may be attractive avenues to improve bone marrow transplantation outcomes.

3. Pharmacological strategies to modify pDC graft content and function

The randomized, multi-center clinical trial conducted by the BMT CTN and analyzed by Waller et al in 2015 showed that greater numbers of pDC in the donor

marrow grafts correlate with better transplant outcomes. Unfortunately, not all BM grafts have high numbers of pDCs. This suggests that a method to increase pDC content in BM could potentially improve transplant outcomes.

3.1. FMS-Like Tyrosine Kinase 3 Ligand

The most promising avenue for increasing pDC numbers in vivo is Flt3L, as it is widely used in *in vitro* experiments to increase pDC content and maintain pDC survival (Waskow et al, 2008). Flt3L was first cloned using a soluble form of the Flt3 receptor and a murine T cell line that expressed a surface ligand that bound to the receptor (Lyman et al, 1983).

In a recent study, healthy human volunteers received subcutaneous treatment of CDX301 human recombinant Flt3L. This treatment resulted in effective peripheral expansion of monocytes, HPCs and key subsets of myeloid DCs and pDCs, with no clear effect on regulatory T cells (Anandasabapathy et al, 2015).

Additionally, He et al combined treatment with the CXCR4 antagonist Plerixafor and Flt3L and saw an increase in HSC, NK cells, T regs and DCs in the peripheral blood when compared to treatment with Plerifaxor alone. Interestingly, recipients of Flt3L alone had higher OS than in combination with Plerifaxor (He et al, 2014).

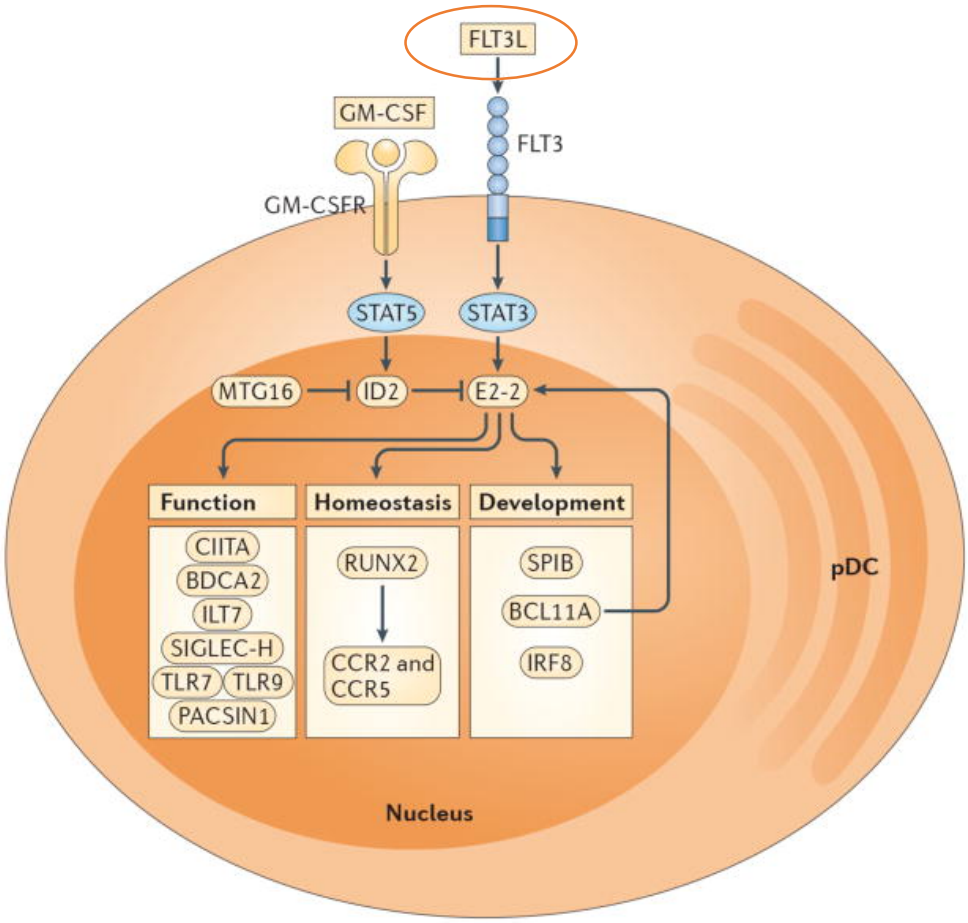


Figure 1. Plasmacytoid dendritic cells' development, homeostasis and function is dependent upon binding of Flt3L to the Flt3 receptor, which recruits the master transcription factor E2-2. Binding of GM-CSF results in inhibition of E2-2 and therefore pDC arrest. Adapted from Colonna et al, 2015.

The potential role of Flt3L treatment in reducing donor graft alloreactivity and preventing GvHD is also supported by in solid organ transplant studies, such as one where pre-transplant systemic infusion of freshly isolated immature DCs from the kidneys of Flt3L-treated mice resulted in increased heart allograft survival in a syngeneic setting (Coates et al, 2004).

3.2. Effect of Flt3L on pDC

Flt3L is crucial for pDC development (Schmid et al, 2010), and for this reason it has been widely used to increase pDC numbers *in vitro* (Maraskovsky et al, 1996). As seen in Figure 1, Flt3L is essential for pDC generation, development and function. This suggests that if exogenous Flt3L is present, it will bind to the Flt3 receptor and initiate a STAT3-mediated signaling cascade without significant feedback inhibition. pDC exposed to higher-than-normal levels of Flt3L, thus, may proliferate more than pDC exposed to steady levels of Flt3L, and exposure to exogenous Flt3L may affect pDC phenotype and function. We hypothesize that Flt3L-generated donor pDC will have an even greater potential to interact with donor T cells and reduce their alloreactivity, leading to a lower incidence of GvHD and improved survival post-transplant.

Flt3 is involved in the proliferation of NK cells, dendritic cells, and HSCs. Thus, over 70% of patients with Acute Myeloid Leukemia (AML) and Acute Lymphoid Leukemia (ALL) express mutations in the Flt3 gene. For this reason, many therapeutic agents for AML and ALL are Flt3 tyrosine kinase inhibitors (Gilliland, 2002). This suggests that if Flt3L is administered to patients who had either AML or CLL, Flt3L could potentially be harmful as it would bind to the mutated receptors and enhance leukemia blast proliferation, which would potentially result in relapse. Therefore, we believe that the best use of Flt3L is administration to donors before transplant.

4. Potential mechanisms for donor pDC to regulate chronic GvHD

The thymus is where T cells develop and where auto-reactive T cells are eliminated. cGvHD is a failure to eliminate auto-reactive T cells and B cells. Therefore, donor-derived pDC may home to the thymus and attenuate the development of cGvHD by either favoring negative selection or regulating positive selection of lymphocytes, or facilitating differentiation of natural T regulatory cells (T regs) (Huang et al, 2011; Ito et al, 2007).

4.1. Possible Role of the Thymic pDC in HSCT

The thymus is a primary lymphoid organ that typically involutes after adolescence, except in processes like HIV, aging, and bone marrow transplantation (Haynes et al, 2000). Specifically, in HSCT, immune reconstitution is achieved by generating lymphocytes that undergo positive selection in the thymic cortex and negative selection in the thymic medulla. The main cell type responsible for positive thymocyte selection are cortical thymic epithelial cells (cTECs), mainly through antigen presentation. Currently, it is thought that the total contribution of dendritic cells to positive and negative selection of T cells is about 0.5% (Klein et al, 2014); moreover, there is no clear evidence indicating whether dendritic cells in the thymus are of intrathymic or extrathymic origin. Studies of MHC II expression show that DCs are capable of inducing negative but not positive selection of thymocytes *in vivo* (Brocker et al, 1997).

pDC comprise over one third of all thymic dendritic cells. Because it is known that pDC enter the thymus from the periphery after being differentiated in the bone marrow

(Hadeiba et al, 2012), it is thought that thymic and peripheral pDC differentiate from a common precursor. It has been shown that pDC could pick up peripheral antigens and transport them to the thymus (Hadeiba et al, 2012). Interestingly, CCR9 is crucial for the homing of pDC to the thymus, and also for the homing of T cells to the thymus. All dendritic cells that have been empirically found in the thymus are in the thymic medulla, and known to pick up medullary thymic epithelial cell (mTEC)-derived antigens. pDC are exceptional in that they do not pick up mTEC-derived antigens, and that after pDC pick up periphery antigens, they halt their uptake of antigens. Finally, it is widely believed that pDC do not migrate to the thymus after TLR stimulation, since they are thought to be involved in tolerance by the generation of regulatory T cells in the thymus, and the presence of activated pDC in the thymus would be contrary to their role in promoting tolerance. Moreover, one study showed that the natural Treg that are induced by pDC in the thymus produce more IL-10 and less TGF-beta as compared to Treg induced by mDC. This suggests that thymic pDC, in the presence of self-antigens in the periphery, may induce the generation of IL10-producing Tregs (Matta et al, 2010).

The pathway used and quality of mechanistic behavior of pDC in the thymus suggest that these cells can participate in thymocyte selection in a manner that is unique from the rest of the DC pool, and contribute to the attenuation of GvHD by direct cell contact with the developing thymocytes derived from the donor grafts.

II. Hypothesis

The effect of Flt3L on bone marrow grafts remains largely unknown. Using a murine model of allogeneic transplantation, we hypothesized that treatment of BM donors with Flt3L will increase BM pDC content in the marrow and allogeneic transplantation of Flt3L-treated BM grafts will lead to improved overall survival and decreased GvHD in recipients as compared to PBS treatment. If this advantage exists, we hypothesize that donor-derived pDC from the marrow of Flt3L-treated donors will show increased homing to the recipient thymus, where pDC would be necessary but not sufficient to promote thymic function.

If our hypothesis is correct, Flt3L could potentially be used as a preparatory treatment for BM donors to improve transplant outcomes, and novel insights could be gained regarding the role of pDC in the thymus of transplanted patients.

III. Materials and Methods

1. Mice

C57BL/6 (H-2K^b), e-GFP C57BL/6 (H-2K^b), and B10.BR (H-2K^k) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Both donors and recipients were males aged 8-10 weeks and 10-12 weeks, respectively. Recipients were conditioned for one week following arrival to the mouse facilities of the Winship Cancer Institute of Emory University. Ethical considerations were followed according to Emory University's Institutional Animal Care and Use Committee, and animal care guidelines were followed according to the National Institutes of Health.

2. Flt3L Treatment

Donor C57Bl/6 mice were either treated with PBS or 300 μg/kg of human recombinant Flt3L (CDX301), generously donated by CellDex Therapeutics (Hampton, NJ).

3. T Cell Depletion and Purification

Donor C57BL/6 marrow underwent T cell depletion (TCD) and donor spleens underwent T cell purification (TCP). C57BL/6 mice were euthanized with CO_2 and femurs and tibias (TCD) or spleens (TCP) were collected and flushed with 2% FBS PBS. Cell suspensions were lysed with ACK Lysis Buffer, spun down and counted using the cell counter (Beckman Coulter, Indianapolis, Indiana). For TCD, the suspensions were incubated in ice with anti-mouse Fc Block for five minutes and biotinylated anti-mouse CD3 (BD Biosciences, San Jose, CA) for 30 minutes. For TCP, splenocytes were incubated in ice with anti-mouse B220, CD49b, Gr-1, and Ter119 antibodies (BD Biosciences, San Jose, CA). Both procedures were followed by an incubation with anti-biotin microbeads (150 μL/2x10^8 cells) and negative selection via MACS LS columns (MACS Miltenyi Biotec, Gladbach, Germany).

4. HSC and pDC Purification

Donor C57BL/6 marrow was obtained using the same procedure as described for TCD and TCP. For HSC isolation, cell suspensions were incubated with anti-mouse Lin (CD3, CD11b, CD19, IgM, CD49b, Ter119) PE, B220 PerCP-Cy5.5, c-Kit APC and Sca1 PE-

Cy7. For pDC isolation, the antibodies used were Lin (CD3, CD11b, CD19, IgM, CD49b, Ter119) PE, B220 PerCP-Cy5.5, CD11c FITC, pDCA1 ef-450. When pDC were obtained from eGFP C57BL/6 for the thymic studies, CD11c APC-Cy7 was used instead of FITC in order to avoid spectral overlap with GFP. Antibodies were purchased from either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), or eBioscience (San Diego, CA). HSC were defined as Lin- B220- c-Kit+ Sca1+, while pDC were Lin- B220+ $CD11c^{high}$ and pDCA1+. The FACSAria cell sorter (BD Biosciences, San Jose, CA) was used for purification via a 70 μm nozzle.

5. pDC Characterization

To study differences in homing, the marrow of either PBS-treated or Flt3L-treated C57BL/6 mice was obtained and incubated with pDC-specific anti-mouse antibodies (described above) and their chemokine receptors CCR9 PE-Cy7, CCR5 APC, CCR7 FITC, CCR4 PE-Cy7, and CXCR4 APC. Maturity status of pDC was assessed using MHC II FITC, PDL-1 PE-Texas Red and CD62L APC. All antibodies were purchased from either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), or eBioscience (San Diego, CA).

Cytokine analysis of pDC consisted in stimulating whole BM from C57BL/6 mice with 50 μM CpG (ODN 1585; InvivoGen, San Diego, CA) 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 aminoacids, HEPES, and sodium pyruvate (complete medium) in 10-cm wells for 9 hours at 37 °C. At hour 3, BD GolgiPlug (BD

Biosciences) was added. Finally, intracellular staining was performed using the Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA) and anti-mouse IL-10 PE-Cy7, IL-12 APC, IDO PerCP-Cy5.5 and IFNα FITC, purchased from either BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), or eBioscience (San Diego, CA).

6. Bone Marrow Transplantation

Bone marrow from C57BL/6-derived tibias and femurs was subjected to TCD, and T cells were obtained via TCP from the spleens of C57BL/6 mice as described above. For GvHD studies, recipient B10.BR mice above 22.0g were irradiated on day -1 relative to bone marrow harvest, at a total dose of 11Gy at two time points 3 hours apart at 5.5 Gy each using a Cesium irradiator. On day 0, mice were transplanted with $5x10^6$ TCD-BM cells from either PBS-treated or Flt3L-treated donors with or without $4x10^6$ T cells from untreated mice. The rationale for controlling for T cells was that clinical marrow transplantation is often contaminated by blood-derived T cells; thus, TCD served as a control for GvHD. Following transplantation, mice were housed in sterile cages with antibiotic water containing neomycin sulfate (1.1 mg/mL) and polymyxin sulfate (1000 U/mL) during the first 30 days, and in auto-water cages after that period. Mice were monitored for weight loss daily during the first 10 days, and then for clinical GvHD every other day. GvHD monitoring was based on a 10-point scale that included weight, attitude/activity, skin condition, hunching, and coat condition. Histopathological GvHD grading of the small intestine was done using a scale from 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). Histopathological GvHD grading of the liver 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) (reference NIH guidelines on GvHD).

7. Cytospin

Murine pDC were obtained from the marrow of C57BL/6 mice and sorted according to the strategy described above. 10^5 cells were suspended in 100 µL of cold 1% BSA-PBS and the filter was wetted with an aliquot of the original suspension. Cell suspensions were loaded onto the Cytospin wells, and the samples were spun at maximum speed on a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher Scientific). The cells were fixed with 100 µL of methanol and dried in a dissecation chamber overnight. Slides were stained overnight with Hematoxylin and Eosin (Electron Microscopy Sciences, Hatfield, PA) and then fixed with Permount (Fisher Chemical, Geel, Belgium) and mounted with xylene mounting medium (Thermo Fisher Scientific). A Zeiss Axioplan 2 microscope was used to image the slides (Carl Zeiss, Oberkochen, Germany).

8. Tumor Cell Challenge and Bioluminescent Imaging

C1498, an acute myelogenous leukemia cell line transfected with luciferase, was generously donated by Dr. Brace Blazar. On day -2, C57BL/6 mice were irradiated at 11 Gy in two separate rounds, each 5.5 Gy. On day -1, they were injected with 500,000 C1498 cells. Then, on day 0, mice were transplanted with 5 million B10.BR-derived TCD-BM

from mice treated with either PBS of Flt3L plus or minus 1 million T cells from untreated mice. Another model called LBRM, an acute lymphoblastic leukemia tumor cell line, was used using the same protocol as with C1498.

Bioluminescent imaging was performed using the IVIS Spectrum in vivo imaging system (PerkinElmer, Walham, MA). Mice were injected i.p. with 150 μg/kg of D-luciferin and placed in supine with the ventral surface facing upward in the imager. Luminescence was calculated as the average radiance across all Regions of Interest in the animals, determined manually by the experimenter, and measured in photons/second/cm^2/steradian and normalized to transplanted mice that were not transfused with the cell line.

9. Gene Array Analysis

Two types of human samples were obtained. First, large-volume leukapheresis was performed on human subjects treated s.c. with 75 μg/kg/day of human recombinant Flt3L (CDX-301) for 5 consecutive days, as part of the Institutional Review Board (IRB)-approved clinical trial NCT022000380; these samples are referred to in this thesis as hF-PB, for they are from peripheral blood collected from Flt3L-treated human subjects. Second, marrow samples were obtained from volunteer donors as part of the IRB-approved clinical trial NCT02485639; these samples will be referred to as hBM, for they were obtained from the marrow of human untreated subjects. pDC were FACS-isolated from hF-PB and hBM samples, and their gene expression was assessed using the HumanHT-12 v4 BeadChip Kit (Illumina, San Diego, CA). All data were processed,

quantile-normalized, background-corrected, and log2-transformed for subsequent analysis.

Two types of murine samples were obtained: murine marrow from PBS-treated mice (BM) and from Flt3L-treated mice (F-BM). Then, FACS-isolated pDC were analyzed for RNA-seq gene expression. Specifically, cDNA was prepared using SMART-Seq v4 Low-Input RNA Kit (Takara Bio, Susatsu, Japan). The sequencing library was created using NEBNext Ultra II FS DNA Kit (New England BioLabs, Ipswich, MA). These samples were sequenced by Next Generation Sequencing (NGS) at 2x151 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. Differential expression analysis for both human and murine samples was performed using a modified t test. 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.

10. Thymic Collection and Confocal Microcopy Analysis of Recipient Thymi

Donor thymi were collected by making a continuous incision into the chest cavity perpendicular to the direction of the ribs. The thymus had dimensions of ~2mmx2mmx1mm and was white in appearance. Once excised, it was ground using a 70 μm cell strainer and 2% FBS PBS. The resulting cell suspension was stained with the pDC-specific markers using the protocol described above and analyzed via flow cytometry. Of note, CD11c expression in thymic pDC was higher than in pDC typically characterized in the bone marrow for the experiments conducted in this thesis.

Confocal analysis was performed in order to detect GFP+ pDC in the recipient thymus. An allogeneic transplant was performed using FACS-purified pDC from eGFP C57BL/6 mice, and FACS-purified and MACS-isolated T cells from WT C57BL/6 mice into B10.BR recipients. On day 7 post-transplantation, the thymus was collected in OCT embedded cryomolds and frozen sections were processed and sectioned by the Pathology Core Laboratory at Winship Cancer Institute of Emory University. Slides were stained with anti-pDCA1-Alexa Fluor 568 and DAPI. Images were visualized and analyzed using the Leica XP8 microscope.

IV. Results

Flt3L treatment increases BM pDC in vivo

To understand whether Flt3L alone is capable of expanding pDC in the marrow of donors, we administered s.c. injections of Flt3L to C57BL/6 mice and harvested their marrow for flow cytometry analysis. Control mice (BM group) were treated with PBS, while experimental mice (groups A-E) were treated with increasing dosages of Flt3L

according to the schedules depicted in Table 1, with the days being relative to harvest day, which we considered day 0. Although the effect of Flt3L on pDC expansion seemed insignificant in the case of a sole administration seven days prior to harvest, a single administration four days before harvest was sufficient to increase pDC content three-fold in the marrow, and up to five-fold upon daily administration (Figure 2A).

While Flt3L did not significantly affect T cells, B cells or NK cells (Figure 2 C-E), the highest doses increased HSC content (Figure 2B), a phenomenon that has been previously described (Mueller et al, 2002). We chose schedule C (Table 1C) for all subsequent experiments because it expanded pDCs three-fold without significantly altering the content of other immune cells that are present in the graft, namely HSCs, T cells, B cells, and NK cells.

BM	Control
A	-7
B	-4
C	-4, -1
D	-7, -5, -3, -1
E	Daily (-7 to -1)

Table 1. Flt3L administration schedule. Human recombinant Flt3L (CDX301, 300µg/kg) was given subcutaneously to C57BL/6 mice once a day on the days indicated on Table 1. All days are relative to BM harvest, which is day 0. Control mice were treated with PBS.

Flt3L treatment alters the chemokinetic, but not the cytokinetic, profile of pDC

Once we established that Flt3L alters marrow pDC quantity, we examined its effect on pDC phenotype because we hypothesized that the expanded pDC would migrate to

GvHD target organs and attenuate the disease. Using flow cytometry, we analyzed marrow-derived pDC from Flt3L-treated or PBS-treated mice for the expression of the chemokine homing receptors CCR4, CCR5, CCR7, CCR9 AND CXCR4. We found that F-BM pDC had increased expression of CXCR4 (Figure 3A), which may be important in either orchestrating their proliferation in the marrow microenvironment by keeping them attached to the stromal environment or in homing to the lymph nodes. Interestingly, F-BM pDC had reduced CCR9 expression (Figure 3A). This finding was potentially significant because, as previously mentioned in this thesis, CCR9+ pDC are believed to be highly tolerogenic and ameliorative in the context of GvHD. Moreover, CCR9 is responsible for the homing of pDC to the gut -a target GvHD organ- and the thymus. Downregulation of this receptor may indicate reduced homing or, alternatively, a CCR9-independent strategy to access these sites.

Next, we phenotyped donor pDC activated *in vitro* with CpG for expression of cytokines relevant to their ability to regulate T cell activation and polarization. We studied IFNα, IL-12 and IL-10 as indications of anti-viral responses, Th1 and Treg polarization, respectively. There were no significant differences in cytokine profiles between F-BM pDC and BM pDC (Figure 3B). IDO production was similar and close to 100% across the two groups (Figure 3C), indicating pDC ability to confer Tregs their immunosuppressive function (Lippens et al, 2016).

Recipients of F-BM grafts had increased survival and less GvHD

After analyzing the modifications in graft content upon Flt3L treatment in donors, we performed an MHC-mismatched transplant to assess the effect of a Flt3L-stimulated marrow graft on post-transplant survival and GvHD in allogeneic recipients. B10.BR mice were transplanted with 5 million TCD BM cells with the addition of 4 million spleen-derived T cells. All mice that received TCD bone marrow with no additional T cells had 100% survival regardless of donor treatment (Figure 4A) and GvHD scores that were between 0 and 0.5 (data not shown), as assessed on the clinical GvHD scoring system described previously (Cooke et al, 1996). Among recipients of T cells, mice that received allogeneic TCD F-BM + T cells had significantly higher OS (Figure 4A) and lower incidence of GvHD (Figure 4C) that mice that received the same grafts from PBS-treated donors. All groups had ~100% donor hematopoietic engraftment 30 days post-transplantation in the peripheral blood derived from the ocular vein (Figure 4B).

Histological analysis of the liver and small intestine showed evidence of GVHD pathology in recipients of control TCD BM, without the addition of donor T cells (Figure 4 D-F). Using National Institutes of Health criteria for histological diagnosis of GVHD (Shulman et al, 2006; Jacobsohn et al, 2007; Snover, 1984), 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 4D-F), 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.

The benefit of F-BM may be attributable to pDCs in the graft

In order to isolate the benefits of F-BM pDC from that of the whole TCD F-BM graft, we transplanted 5,000 HSC and 1 million T cells from PBS-treated donors and 50,000 pDC from either PBS-treated or Flt3L-treated mice in an allogeneic BMT model (C57BL/6 → B10.BR). Recipients of HSC alone had the worst survival (Figures 5A and 5C) due to poor engraftment (data not shown), and recipients of pDC from both groups had better survival than mice that received HSC or HSC + T cells only (Figure 5A). Moreover, recipients of F-BM pDC had better OS than recipients of BM pDC (Figure 5A), although this difference was not statistically significant. Recipients of F-BM had a lower incidence of GvHD than recipients of BM pDC (Figure 5B). Interestingly, when we replicated this transplant with 2 million T cells –a higher dose that is semilethal—, recipients of F-BM pDC had a significant advantage in survival compared to recipients of HSC and T cells only (Figure 5C). This suggested a trend towards improved survival of recipients of F-BM pDC over recipients of BM pDC, as well as a slightly lower incidence of GvHD compared to recipients of BM pDC up to day 27 post-transplant (Figure 5D). Thus, the results of these two transplant experiments suggest that that F-BM pDC are superior to BM pDC in their ability to increase survival and regulate post-transplant GvHD.

The genetic profile of purified pDC from Flt3L-treated donors differs from that of control donors

Due to the observed benefits of isolated pDC from F-BM as compared to pDC

from BM in their ability to improve transplant outcomes, we next explored the mechanism by which Flt3L modifies pDC function. We FACS-isolated pDC from the marrow of Flt3L-treated and PBS-treated C57BL/6 mice (Figure 6A) and analyzed RNA from these cells using Next Generation Sequencing (NGS).

Genetic expression was compared in a pairwise fashion, specifically pair-end (2x151), meaning that each gene had two reads 151 bp long. The log fold change (logFC) was expressed according to the following formula:

Equation 1.

$$logFC = log_2(\frac{Expression\ of\ gene\ in\ BM\ pDC}{Expression\ of\ gene\ in\ F-BM\ pDC})$$

According to Equation 1, if the amount of gene-specific RNA in BM pDC is greater than that in F-BM pDC, then the ratio (FC) will be a value greater than 1. A base-two log of an integer greater than 1 is another positive integer, which will result in a positive logFC. In contrast, if the amount of gene-specific RNA in BM pDC is less than that in F-BM pDC, FC will have a numerical value between 0 and 1, and it will have a negative exponent if expressed in scientific notation. Because a negative exponent of the base can be rearranged as a factor that multiplies the whole logarithm, in the latter case the value of logFC will be negative.

LogFCs of each individual gene were compared side by side. Because there were three to four samples per group, it was possible to determine statistical significance

using a t-test. P value was expressed as NegLog10Pvalue. Thus, significance was achieved at a NegLog10Pvalue less than ~3.39.

The genes that were upregulated in F-BM pDC were ACK1, whose protein product is involved in antigen uptake by endocytosis during MHC II loading (Watts et al, 2010), and Hmox1, an enzyme involved in heme catabolism (Figures 6B and 6C, Table 2). Interestingly, two genes involved in T cell selection in the thymus were also up-regulated: PRSS16 and Tox1 (Figures 6B and 6C, Table 2). PRSS16 encodes the thymus-specific serine protease and has been reported to be exclusively expressed on cTECs and is thought to participate in positive selection of T cells, while Tox 1 encodes the thymocyte selection associated high mobility group box protein, which is involved in the commitment of double-positive thymocytes to the CD4 T cell lineage. Neither of these gene products have been found to be expressed on pDC or any other type of DC. Adaptive immune pathway genes APRIL and Etv5 (Figures 6B and 6C, Table 2) could be involved in the ability of pDCs to affect T cell polarization and activation status (McKenna et al, 2005). Finally, TIM3, cyclin D3 and Bcl2 (Figures 6B and 6C, Table 2) are involved in immune checkpoint pathways; for instance, cyclin D3 is necessary for cell proliferation in germinal centers (Cato et al, 2011).

ACK1	Antigen uptake	-1.97	1.43
Hmox1	Adaptive Immunity	-1.73	1.46
PRSS16	T cell selection	-3.12	1.48
Tox1	T cell selection	-4.24	1.53
TIM-3	Immune Checkpoint	-1.79	1.56
APRIL	Adaptive Immunity	-3.25	1.71
Etv5	Adaptive Immunity	-2.30	1.73
Cyclin D3	Immune Checkpoint	-1.60	2.08

Bcl2	Immune Checkpoint	-1.66	2.27

Table 2. Genes up-regulated in murine F-BM. Genes are displayed in order of statistical significance, as indicated by a smaller NegLog10pvalue. F-BM pDC have increased expression of genes involved in antigen uptake processes, adaptive immunity, immune checkpoint and T cell selection processes.

After understanding the possible mechanistic differences that may account for the ability of F-BM pDC to modulate post-transplant outcomes, we decided to explore the translational potential of our findings by examining the effect of Flt3L on human pDC. FACS-isolated pDC were obtained from the peripheral blood of CDX301-treated human subjects (75 μg/kg) participating in an IRB-approved clinical trial that was evaluating the efficacy of single agent Flt3L as a stem cell mobilizer (ClinicalTrials.gov identifier NCT02485639). Control samples were obtained from the marrow of healthy volunteers participating in a different IRB-approved clinical study (ClinicalTrials.gov identifier NCT022000380). RNA obtained from FACS-isolated pDC was sequenced by Illumina chromatin immunoprecipitation. Mathematical analysis of gene expression revealed that, like in the murine findings, SLAMF6 and KLRF1 (implicated in the adaptive immune pathways) and BCL2 and BIRC3 (that act as immune checkpoints) were up-regulated in pDC from the peripheral blood of Flt3L-treated donors compared to pDC from the marrow of volunteers (Figures 6D and 6E, Table 3). Interestingly, the most significant genes that were down-regulated in Flt3L-treated donor pDC (APP, MAP2K6, CD36, ACTG1, and IRF7) belonged to the TLR 4 pathway ((Figures 6D and 6E, Table 4). Because the ligand for TLR4 is LPS, an antigen relevant in GvHD, these findings indicate that pDC from Flt3L-treated donors may be less responsive upon stimulation of this innate immune pathway (Takeda et al, 2005).

BCL2	Immune Checkpoint	-2.34	1.35
BIRC3	Immune Checkpoint	-2.37	1.58
SLAMF6	Adaptive Immunity	-2.74	2.16
KLRF1	Adaptive Immunity	-3.70	2.53

Table 3. Genes up-regulated in pDC from apheresis of Flt3L-treated human donors. Genes are displayed in order of statistical significance, as indicated by a smaller NegLog10pvalue. Isolated pDC from blood apheresis of Flt3L-treated subjects have increased expression of genes involved in immune checkpoint and adaptive immune pathways compared to pDC from the marrow of healthy volunteers, as indicated by a negative logFC value.

ACTG1	1.91	1.32
IRF7	3.24	1.39
MAPK6	1.85	1.47
APP	3.67	2.02
CD36	3.14	2.93

Table 4. Genes down-regulated in pDC from apheresis of Flt3L-treated human donors. Genes are displayed in order of statistical significance, as indicated by a smaller NegLog10pvalue. pDC from Flt3L-treated donors have significantly decreased expression of genes involved in the TLR4 innate immune pathway.

F-BM pDC are phenotypically immature and have reduced T cell polarization abilities

Because we determined that donor pDC have different expression profiles upon Flt3L treatment, we decided to examine the effects of donor BM pDC on T cells upon transplantation. Donor F-BM pDC have a more immature phenotype than BM pDC, as indicated by higher expression of the co-inhibitory molecule PD-L1 and lower expression of the co-stimulatory molecule CD86 (Figure 7A). To assess the ability of donor F-BM pDC to induce CD4 T cell polarization, we performed an allogeneic transplant (C57BL/6 → B10.BR) with 5 million MACS-TCD BM or F-BM cells with 4 million MACS-purified splenic T cells from PBS-treated mice. We then collected splenocytes on day 3 post-

transplant and performed intranuclear T cell staining for the transcription factors Tbet, GATA3, RORyT and FoxP3 to measure the abundance of Th1, Th2, Th17 and regulatory T cells (Tregs), respectively. We found that recipients of TCD F-BM + T had reduced Th1 and Th17 polarization, as evidenced by less Tbet+ and RORyT+ CD4 T cells in the spleen (Figure 7B).

In order to isolate the effects of Flt3L treatment on marrow pDC versus the whole graft, we performed a transplant in which we FACS-purified 5,000 HSC and 50,000 BM pDC or F-BM pDC and MACS-isolated 1 million T cells. We collected the recipient spleens at day 3 post-transplant and examined CD4 T cell polarization via the same transcription factors as outlined above. In line with our hypothesis, we found that mice that received F-BM pDC had less Th2 T cell polarization as evidenced by a decreased percentage of GATA3+ CD4 T cells, and a trend towards decreased Th1 and Th17 polarization (lower Tbet+ and RORγT+ CD4 T cells). Additionally, recipients of both BM pDC and F-BM pDC showed decreased levels of Treg polarization, consistent with lower levels of CD25+FoxP3+ CD4 T cells compared to recipients of HSC and T cells only (Figure 8A). This observation may be indicative of the relative reduction of alloactivtion of donor T cells that would otherwise lead to induction of Tregs in transplant recipients without donor pDC.

We then sought to examine the signaling patterns of these recipient-derived splenic T cells by looking at IFNγ, TNFα and IL-17. We saw a sharp decrease in IFNγ production by CD4 T cells in recipients of F-BM pDC compared to recipients of BM pDC or no pDC at all (Figure 8B), consistent with the slightly reduced Th1 polarization

phenomenon seen in the previous experiment (Figure 7B). Taken together, these data indicate that F-BM pDC are phenotypically different from BM pDC and induce a tolerant immunogenic environment independent of Treg polarization.

Finally, we examined the ability of F-BM pDC to induce T cell proliferation by co-co-culturing BM and F-BM pDC with T cells in a Mixed Lymphocyte Reaction (MLR) and accessing T cell proliferation via CFSE analysis 48 h post-culture. There was no difference in T cell proliferation with the addition of either BM or F-BM pDC (Figure 8C and 8D), providing further evidence for the ability of F-BM pDC to modify T cell phenotype without altering total T cell counts. If the benefit of Flt3L relied solely on the quantitative amplification of pDC without altering pDC phenotype, addition of pDC would increase T cell proliferation on a cell-per-cell basis. Thus, this suggests that Flt3L alters the quality, not only the quantity, of donor pDC.

Tumor-bearing recipients of Flt3L-treated marrow show enhanced graft-versus-leukemia effect

Because the GvHD-attenuating effect of donor Flt3L treatment has the risk of causing immunosuppression associated with diminished graft-versus-leukemia (GvL) and increased risk for relapse, we sought to examine transplant outcomes in tumor-bearing mice. Recipient C57BL/6 mice were irradiated at 11 Gy on day -2 relative to transplant and injected with 500,000 C1498 luciferase+ cells on day -1, an acute myelogenous leukemia cell line that luminesces in the presence of luciferin. Mice were transplanted with 5 million TCD marrow from either PBS-treated or Flt3L-treated B10.

BR mice and enriched with 1 million T cells. The T cell dose was reduced from 4 million to 1 million to separate GvL from GvHD, as the latter would not occur with such a low amount of T cells. Although there were no significant differences in survival (Figure 9B), recipients of TCD F-BM had decreased tumor burden as compared to recipients of TCD BM (Figure 9A and 9C). Interestingly, although this experiment was done twice, two mice that had received TCD F-BM were alive tumor-free past the endpoint of the experiment, but had to be sacrificed to comply with IACUC policies. This suggests that TCD F-BM, even in the absence of T cells, has the potential to enhance post-transplant GvL.

We then proceeded to confirm these findings using LBRM, a T cell lymphoma cell line. Using the same transplant protocol as described above for C1498 but with 4 million T cells instead of 1 million, we performed a C57BL/6→B10.BR allogeneic transplant. Mice that received TCD F-BM plus T cells survived significantly longer (Figure 10B), but eventually died. Although histological samples of these recipients could not be obtained due to the coordinated and unexpected death of the animals, we suspect that the death was due to cGvHD. A possible mechanism for this phenomenon would be that in the case of a semilethal dose of T cells, graft-derived T cells participate in the onset and progression and cGvHD, rendering ineffective any capabilities of contributing to GvL. This can be supported by evidence of significantly elevated levels of tumor burden at the time of death of the animals (Figures 10A and 10C).

Combination of Flt3L and G-CSF mobilization results in worse transplant outcomes than G-CSF alone

Because over 90% of transplants performed in the United States use G-CSF-mobilized blood as the stem cell source, we hypothesized that the combination of G-CSF and Flt3L would lead to improved transplant outcomes. We reasoned that Flt3L, despite having poor stem cell mobilization abilities (Figure 11A), would increase pDC content in the marrow and G-CSF would mobilize them into the periphery. We performed an allogeneic transplant (C57BL/6 → B10.BR) with 5 million TCD splenocytes from donors treated with either G-CSF alone (G) on days -5 to -1 (350μg/kg) or in combination with Flt3L (FG) (300 μg/kg) on days -4 and -1, with overlapping schedules on days -4 and -1, with or without 4 million T cells from PBS-treated mice. Interestingly, we found that survival was worse for recipients of donor splenocytes from the cytokine combination, both in mice that did not receive T cells and mice that did (Figure 11C). When we studied the pDC mobilization capabilities of G-CSF versus Flt3L, we found that G-CSF was superior to Flt3L (Figure 11B). This suggested that the combined donor treatment resulted in more pDC being mobilized due to G-CSF than Flt3L. Because we know that G-CSF treatment alters pDC phenotype (Hassan et al, BBMT, in revision), we believe that the combined treatment results in an aggravating effect due to an excess of G-CSF pDC present in the graft.

Flt3L increases donor-derived thymic pDC that home to the recipient thymus post-transplant

To gain mechanistic insight into the ability of F-BM pDC to attenuate post-transplant GvHD, we examined the thymus as it is the site of elimination of autoreactive T cells. First, we treated C57BL/6 donors with Flt3L (350 µg/kg on days -4 and -1) and we collected their thymi for flow cytometric analysis. We found that mice treated with Flt3L had a ~3 fold increase in total pDC content (Figure 11A), a factor that is very similar to the relative increase in pDC content in the marrow of mice undergoing the same treatment schedule (Figure 12A). Thus, because there could be a systemic increase in pDC that is not specific to the thymus, we proceeded to examine the content of pDC in the recipient thymus following transplantation. We preformed an allogeneic transplant in a C57BL/6 to B10.BR background with FACS-isolated marrow-derived HSC, MACS-isolated splenic-derived T cells from WT donors and FACS-isolated marrow-derived pDC from eGFP+ donors. B10.BR recipients were irradiated at 11 Gy on day -1 and received 5,000 HSC, 1 million T cells and 50,000 eGFP+ pDC from mice that were either treated with PBS or Flt3L according to the usual dosing schedule. On day 7 post-transplant, we extracted the recipient thymus and preserved it as cryofrozen sections. We then stained it with anti-pDCA1 and DAPI. Preliminary data shows that recipients of F-BM pDC have increased thymic cellularity, pDCA1 expression and GFP+pDCA1+ cells (Figure 12B), suggesting that F-BM pDC home to the thymus more frequently than BM pDC and participate in the negative selection of T cells.

V. Figure legends

Figure 2. Flt3L administration to BM donors increased pDC content in grafts. Mice were treated with PBS or 300 mg/kg of Flt3L according to the schedule shown 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 3. The chemokinetic profile of pDCs from BM and F-BM grafts may be different, but the cytokinetic production is similar. Mice were treated with PBS or 300 mg/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 mg 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.

Figure 4. Grafts from Flt3L-treated donors increased survival and decreased GVHD. C57BL/6 donor mice were treated with PBS or 300 mg/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 F-BM 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 600X 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.

Figure 5. F-BM pDC increased survival and decreased GvHD. Mice were treated with PBS or 300μg/kg of Flt3L on days -4 and -1. C57BL/6→B10.BR murine transplant recipients received 5,000 FACS isolated HSC, 1 million (A, B) or 2 million (C, D) MACS isolated T cells, 50,000 FACS isolated BM or F-BM pDC. (A) Survival curve of murine transplants. (B) GvHD scores of mice from figure 4A. n=10 per group. *P < .05. (C) Survival curve of murine recipients that were infused with 2 million T cells. (B) GvHD scores of mice from figure 4C. n=5 per group. *P < .05.

Figure 6. F-BM pDCs have a different gene expression profile then BM pDCs. Mice were treated with PBS and 300 mg/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. (B) Heatmap depicting the significantly differentially expressed genes in the murine samples using z-score scaling, 1-Pearson correlation distance, and ward.D clustering. (C) Volcano plot of gene up-regulation and down-regulation (BM versus F-BM). n = 3 per group from 1 experiment. (D) Healthy human donors were either untreated or treated with 75 mg/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. (E) Volcano plot depicting up-regulation and down-regulation of genes (BM versus F-Apheresis). n = 4 to 5 per group from 1 experiment.

Figure 7. Flt3L treatment of BM donors decreased the expression of T helper cell transcription factors in recipients. Donor mice were treated with PBS or 300 mg/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.

Figure 8. Transplantation of isolated F-BM pDC decreased T helper cell transcription

factors and function in donor T cells, and these differences are not due to a decrease in T cell proliferation as shown by MLR. Mice were treated with PBS or 300μg/kg of Flt3L on days -4 and -1. C57BL/6→B10.BR murine transplant recipients received 5,000 FACS isolated HSC, 1 million MACS isolated T cells, 50,000 FACS isolated BM or F-BM pDC. (A) Intranuclear staining of transcription factors Tbet, GATA3, RoRγT, and FoxP3 and (B) intracellular staining of IFNγ, TNFα, and IL-17 in donor CD3+CD4+ T cells was assessed by flow cytometry 3 days post-transplant. n=4 per group, in one experiment. *P < .05. (C) 50,000 FACS isolated C57BL/6 pDC from BM or F-BM grafts were added to an MLR containing 1 million C57BL/6 responder T cells and 1 million irradiated B10.BR stimulator cells. Proliferation was assessed by measuring CFSE dilution by flow cytometry. Histogram of CFSE fluorescence, showing T cells that underwent proliferation represented by the peak to the left. (D) Quantification of total proliferation as the fraction of T cells with lower levels of CFSE fluorescence compared with total population of T cells. n=3 per group. *P < .05.

Figure 9. No loss of GvL effect in recipients of F-BM in a C1498 model. B10.BR→ C57BL/6 murine transplant recipients received 5 million T cell depleted bone marrow cells and 1 million T cells. Recipient mice received 50,000 luciferase+ C1498 cells. (A) Serial bioluminescent imaging of recipient mice. (B) Survival curve of murine transplant recipients. (C) Quantification of tumor burden. n=10-15 per group, from 2 independent experiments. *P < .05.

Figure 10. No loss of GvL effect in recipients of F-BM in an LBRM model. C57BL/6→ B10.BR murine transplant recipients received 5 million T cell depleted bone marrow cells and 4 million T cells. Recipient mice received 50,000 luciferase+ LBRM cells. (A) Serial bioluminescent imaging of recipient mice. (B) Survival curve of murine transplant, (C) Tumor burden quantification following serial bioluminescent imaging of recipient mice.

Figure 11. Donor Flt3L treatment does not mobilize HSC and pDC to the same extent as treatment with G-CSF. Mice were treated with PBS, 300μg/kg of Flt3L on days -4 and -1, or 300μg/kg of G-CSF for 5 consecutive days. Splenocytes were harvested from each treatment group and (A) HSC and (B) pDC were enumerated. n=3 per group. **P < .01. (C) Survival curve of a C57BL/6→B10.BR transplant receiving either 5 million TCD splenocytes from G-CSF-treated donors or Flt3L and G-CSF-treated donors plus or minus 4 million MACS-purified T cells from PBS-treated mice.

Figure 12. Donors treated with Flt3L have increased thymic pDC content and donor-derived pDC from Flt3L-treated donors home more to the recipient thymus. (A) C57BL/6 mice were treated with Flt3L following schedule C and their thymi were collected and stained for pDC. Total pDC content was analyzed via flow cytometry and quantified using AccuCheck Counting Beads. (B) An allogeneic transplant was performed in a C57BL/6 to B10.BR background with FACS-isolated marrow-derived HSC, MACS-isolated splenic-derived T cells from WT donors, and FACS-isolated

marrow-derived pDC from eGFP+ donors. B10.BR recipients were irradiated at 11 Gy on day -1 and received 5,000 HSC, 1 million T cells, and 50,000 eGFP+ pDC from mice that were either treated with PBS or Flt3L according to the usual dosing schedule. On day 7 post-transplant, we extracted the recipient thymus and preserved it as cryofrozen sections. pDCA1-Alexa Fluor 568 and DAPI were visualized using Leica Xp8 and overlaid with GFP to differentiate endemic pDC from donor-derived pDC.

VI. Figures

Figure 2.

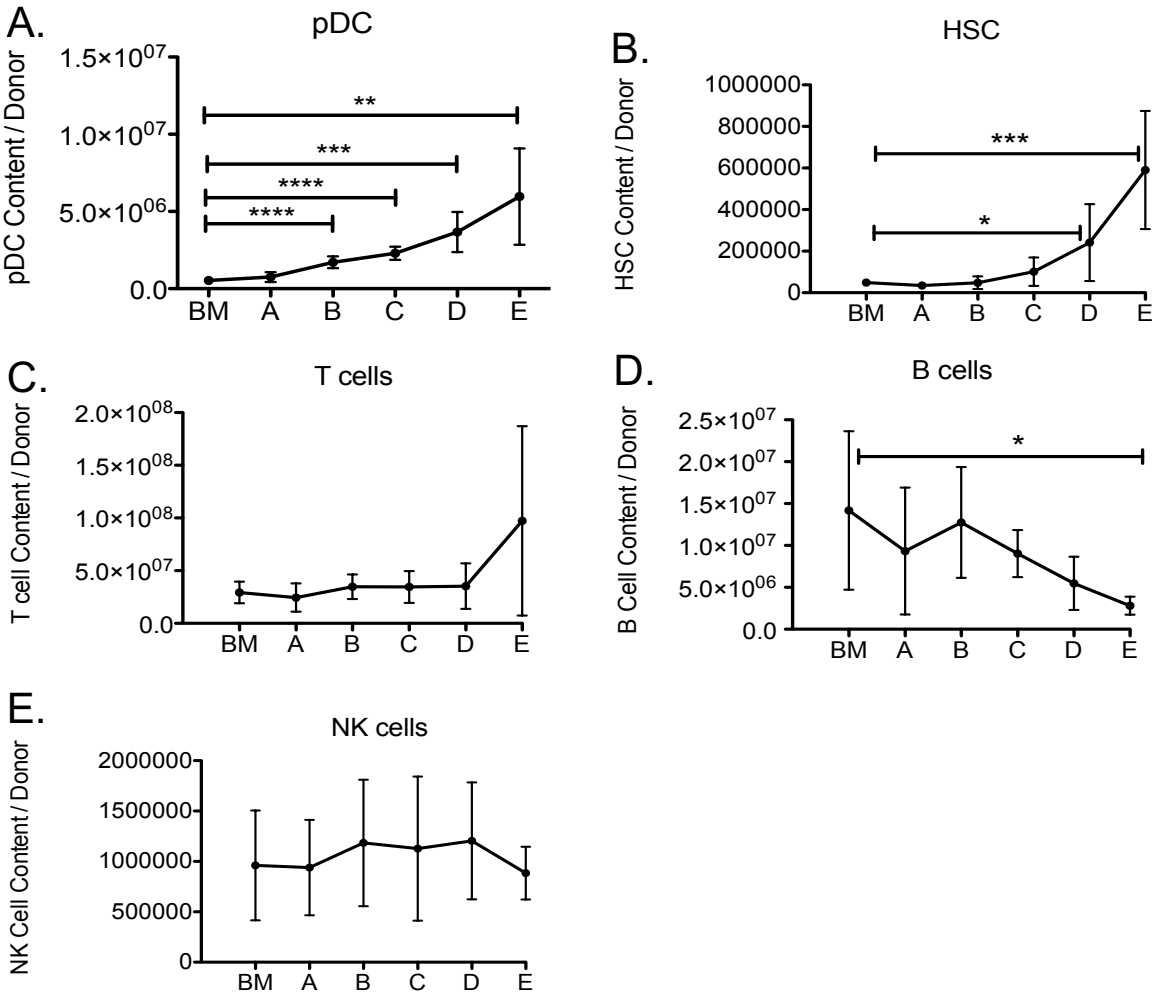
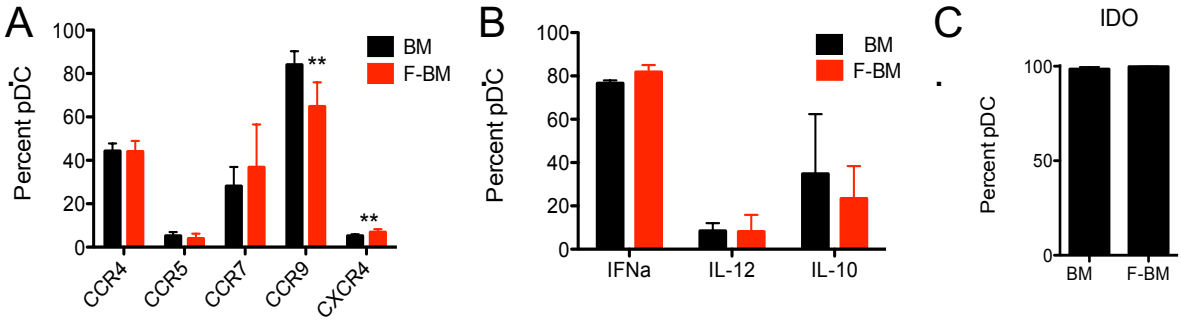


Figure 3.



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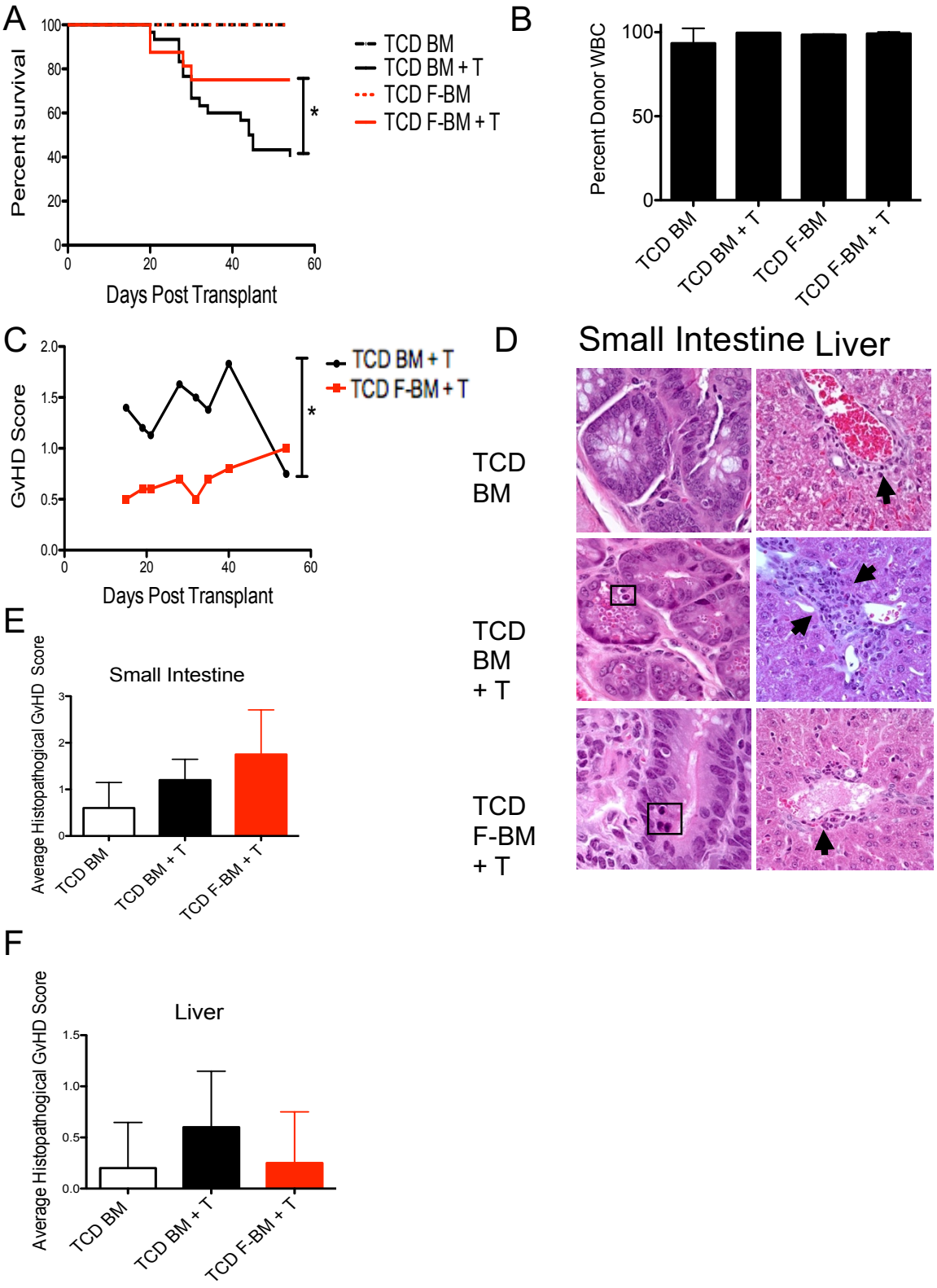

A
Percent survival
100
80
60
40
20
0
0
20
40
60
Days Post Transplant
*
TCD BM
TCD BM + T
TCD F-BM
TCD F-BM + T
B
Percent Donor WBC
100
50
0
TCD BM
TCD BM + T
TCD F-BM
TCD F-BM + T
C
GvHD Score
2.0
1.5
1.0
0.5
0.0
0
20
40
60
Days Post Transplant
*
TCD BM + T
TCD F-BM + T
D
Small Intestine
Liver
TCD BM
TCD BM + T
TCD F-BM + T
E
Small Intestine
Average Histopathogical GvHD Score
3
2
1
0
TCD BM
TCD BM + T
TCD F-BM + T
F
Liver
Average Histopathogical GvHD Score
1.5
1.0
0.5
0.0
TCD BM
TCD BM + T
TCD F-BM + T

Figure 5.

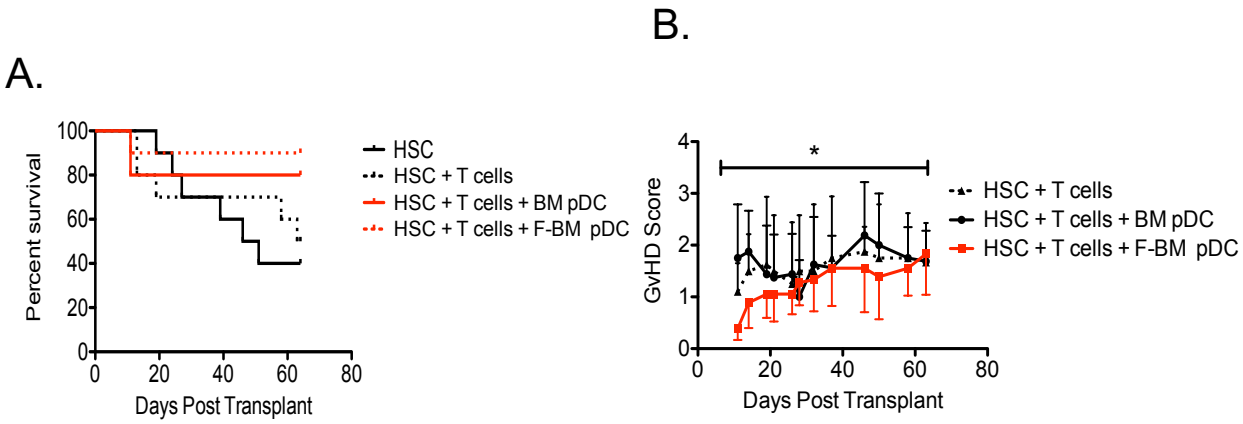
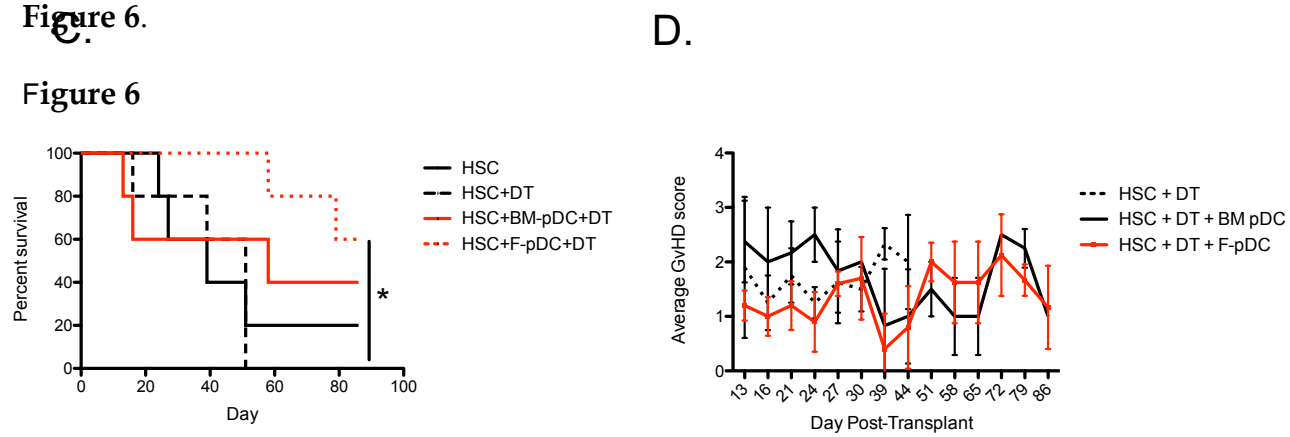


Figure 6.

Figure 6



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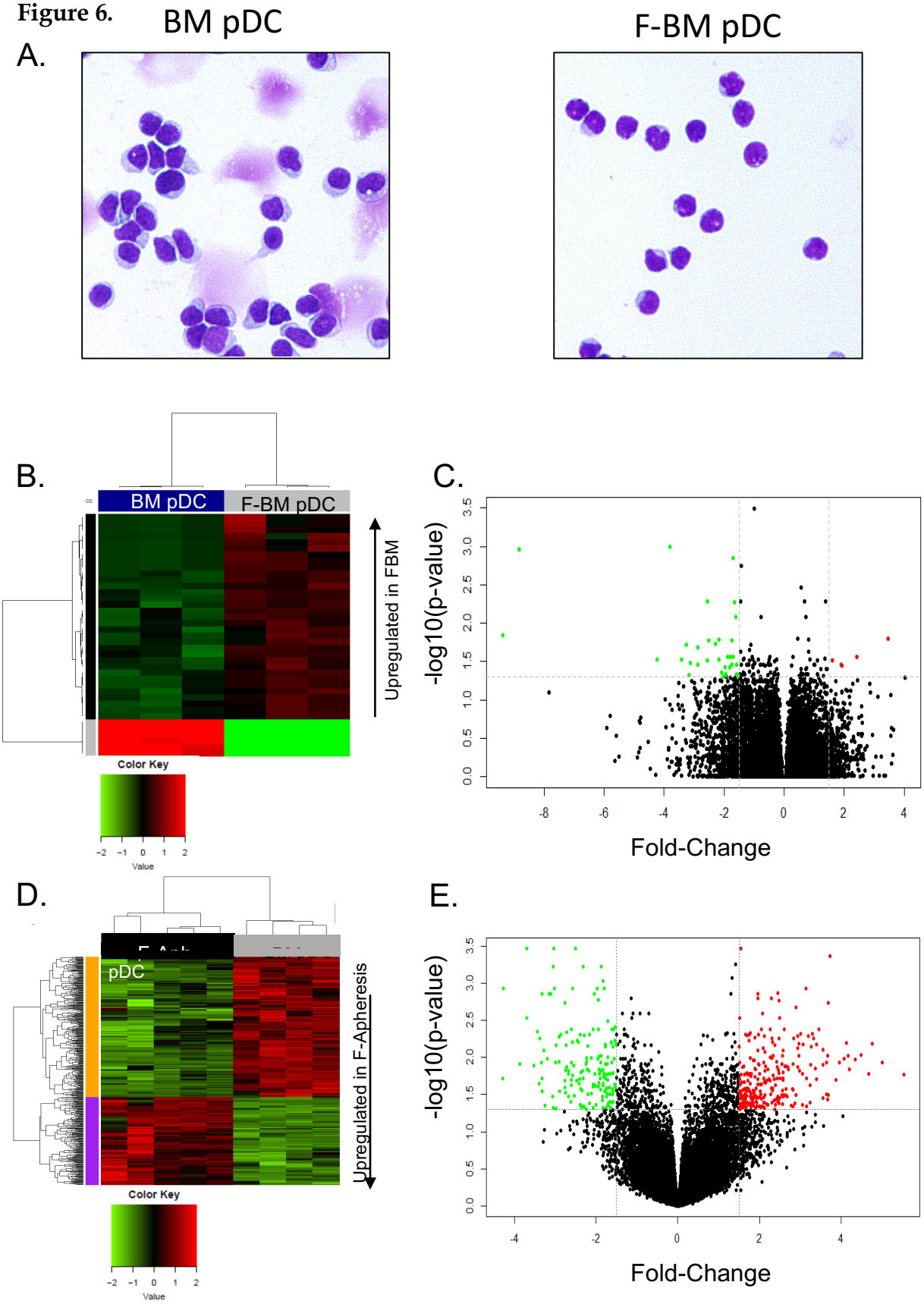

A.
BM pDC
F-BM pDC
B.
BM pDC
F-BM pDC
Upregulated in FBM
Color Key
Value
C.
-log10(p-value)
Fold-Change
D.
pDC
Upregulated in F-Apheresis
Color Key
Value
E.
-log10(p-value)
Fold-Change

Figure 7.

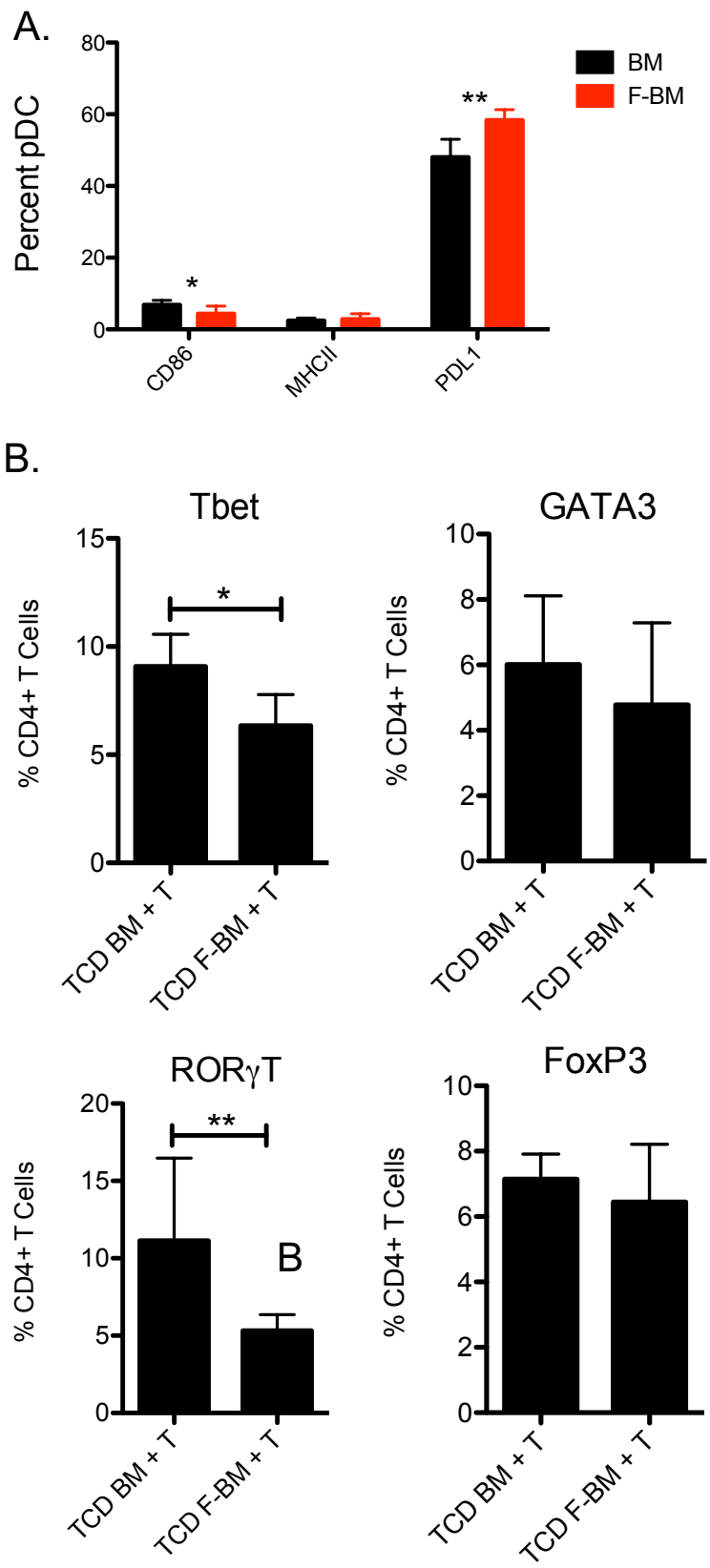

A.
Percent pDC
80
60
40
20
0
BM
F-BM
*
**
CD86
MHCII
PDL1
B.
Tbet
% CD4+ T Cells
15
10
5
0
*
TCD BM + T
TCD F-BM + T
GATA3
% CD4+ T Cells
10
8
6
4
2
0
TCD BM + T
TCD F-BM + T
RORγT
% CD4+ T Cells
20
15
10
5
0
**
B
TCD BM + T
TCD F-BM + T
FoxP3
% CD4+ T Cells
10
8
6
4
2
0
TCD BM + T
TCD F-BM + T

Figure 8.

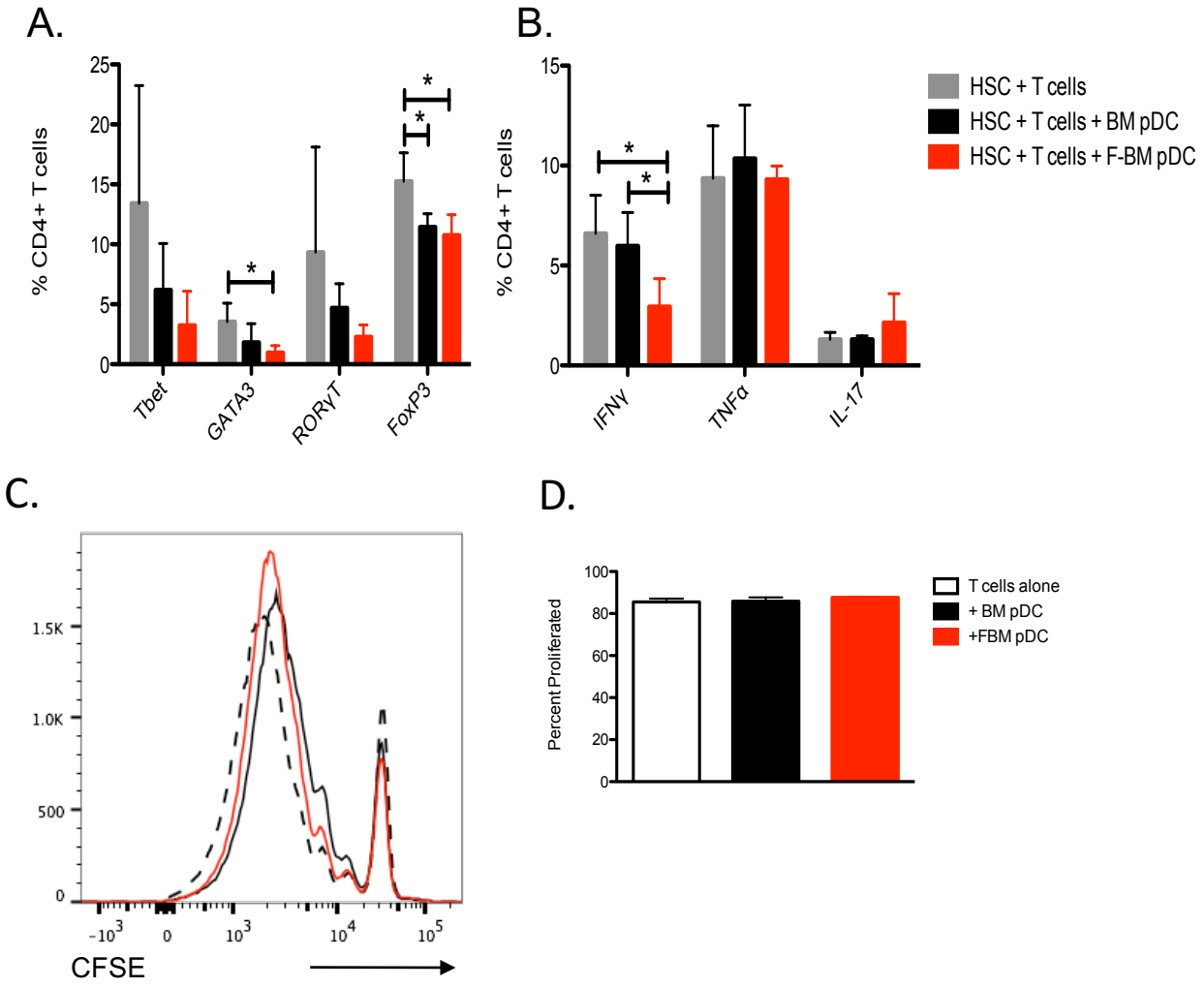

A.
% CD4+ T cells
25
20
15
10
5
0
*
*
*
Tbet
GATA3
RORγT
FoxP3
B.
% CD4+ T cells
15
10
5
0
*
*
IFNγ
TNFα
IL-17
HSC + T cells
HSC + T cells + BM pDC
HSC + T cells + F-BM pDC
C.
1.5K
1.0K
500
0
-10^3
0
10^3
10^4
10^5
CFSE
D.
Percent Proliferated
100
80
60
40
20
0
T cells alone
+ BM pDC
+FBM pDC

Figure 9.

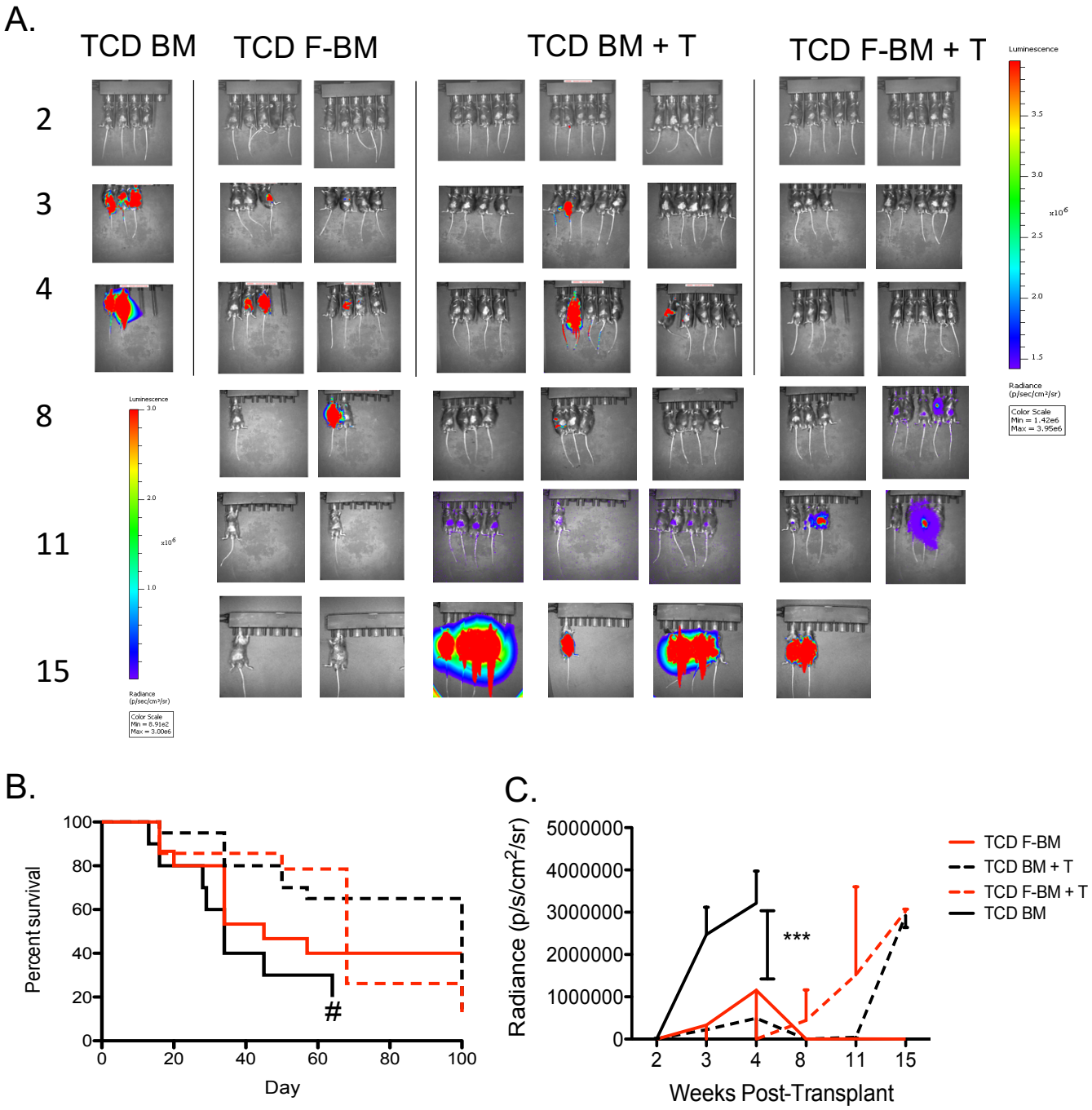

A.
TCD BM
TCD F-BM
TCD BM + T
TCD F-BM + T
2
3
4
8
11
15
Luminescence
Radiance (p/sec/cm²/sr)
B.
Percent survival
Day
#
C.
Radiance (p/s/cm²/sr)
Weeks Post-Transplant
***
TCD F-BM
TCD BM + T
TCD F-BM + T
TCD BM

Figure 10.

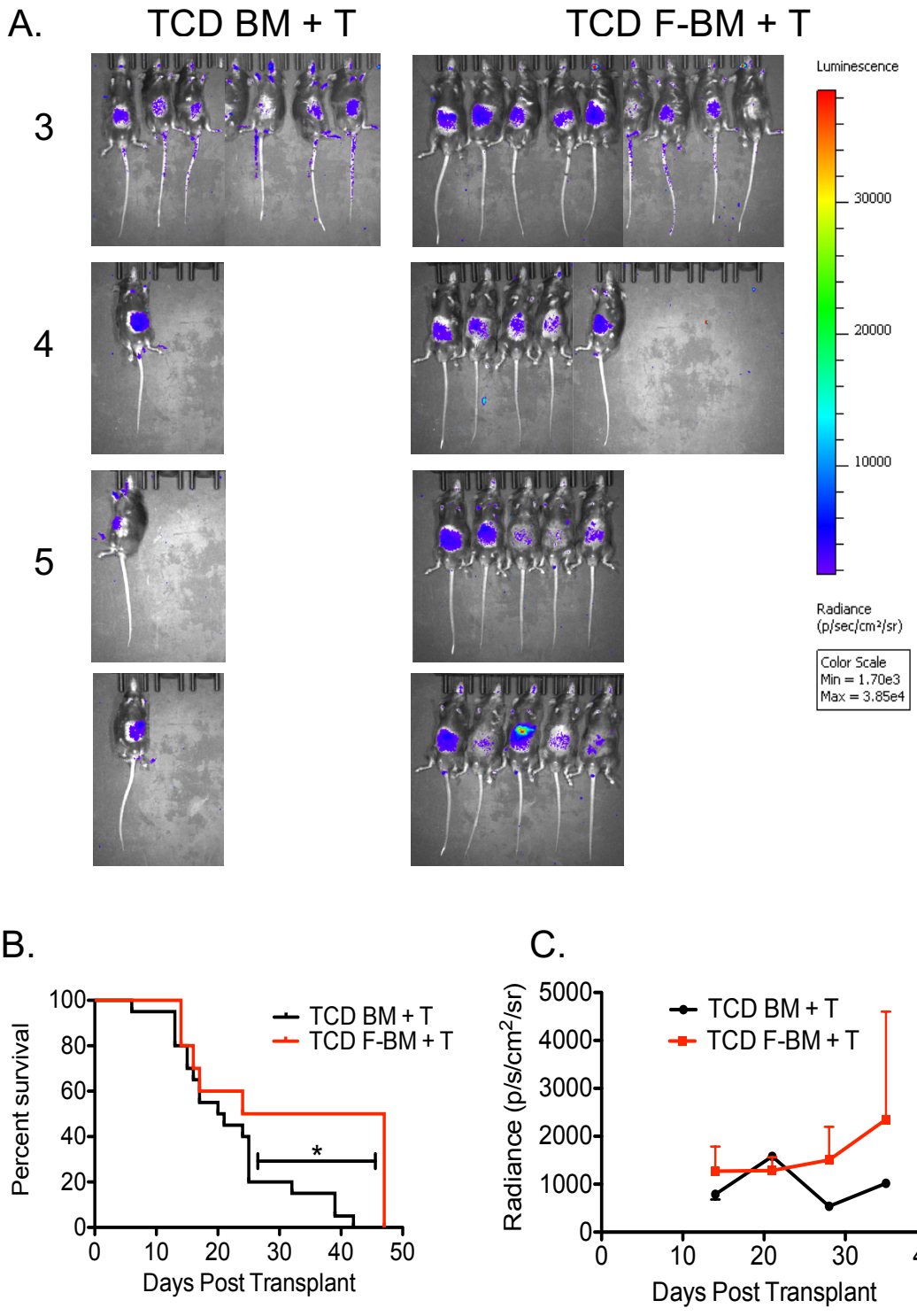

A.
TCD BM + T
TCD F-BM + T
3
4
5
Luminescence
30000
20000
10000
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.70e3
Max = 3.85e4
B.
Percent survival
100
80
60
40
20
0
0
10
20
30
40
50
Days Post Transplant
TCD BM + T
TCD F-BM + T
*
C.
Radiance (p/s/cm2/sr)
5000
4000
3000
2000
1000
0
0
10
20
30
40
Days Post Transplant
TCD BM + T
TCD F-BM + T

Figure 11.

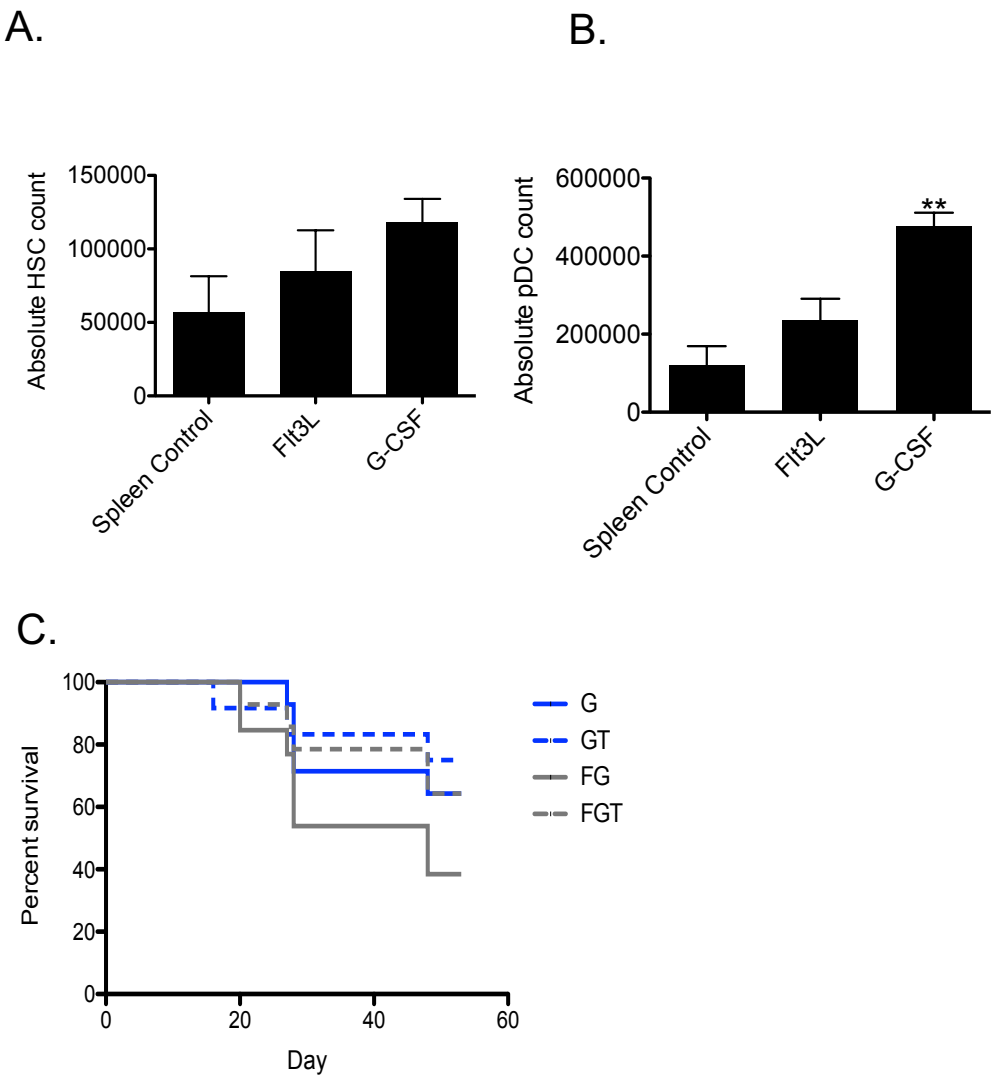
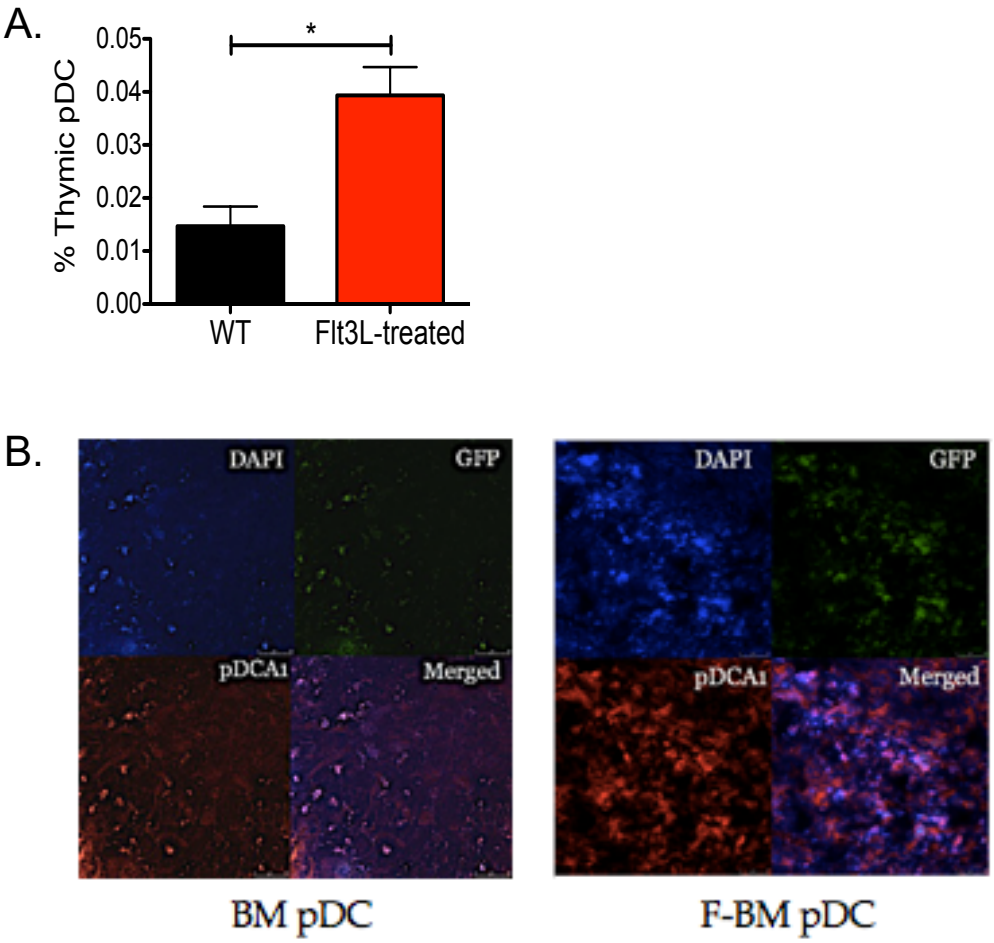

A.
Absolute HSC count
150000
100000
50000
0
Spleen Control
Flt3L
G-CSF
B.
Absolute pDC count
600000
400000
200000
0
**
Spleen Control
Flt3L
G-CSF
C.
Percent survival
100
80
60
40
20
0
0
20
40
60
Day
G
GT
FG
FGT

Figure 12.


A.
% Thymic pDC
0.05
0.04
0.03
0.02
0.01
0.00
*
WT
Flt3L-treated
B.
DAPI
GFP
pDCA1
Merged
DAPI
GFP
pDCA1
Merged
BM pDC
F-BM pDC

VII. Discussion and Future Directions

The multicenter, randomized clinical study BMT CTN 0201 from the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) found that recipients of higher numbers of pDC from BM grafts had better overall survival, less treatment-related mortality, and less cGvHD than recipients of BM grafts with lower numbers of pDC (Waller et al, 2014), while the same correlation could not be found among recipients of G-PB. Moreover, while the 2-year assessment showed similar survival, non-relapse mortality and relapse between the two groups (Waller et al, 2014), the incidence of cGvHD at 5 years post-transplant was significantly lower for BM recipients (Lee et al, 2016). Furthermore, BM recipients were more likely to be working full or part-time than recipients of G-PB (Lee et al, 2016). These data suggest that BM grafts lead to better clinical outcomes than G-PB grafts. One factor that could explain these differences is increased concentrations and altered phenotypes of pDC in BM grafts as opposed to G-PB grafts.

Using a murine model of allogeneic transplantation, we show that increasing the content of pDC in donor bone marrow with Flt3L injections prior to bone marrow harvest leads to improved transplant outcomes. Recipients of Flt3L-treated bone marrow had better OS, less GvHD –both clinical and histopathological—and retained GvL activity as compared to recipients of bone marrow from donors treated with PBS. Moreover, we demonstrate that transplantation of isolated HSC, T cells and F-BM pDC resulted in better OS and less GvHD than transplantation of the same HSC and T cell components plus pDC from control mice instead of F-BM pDC, although these differences were not

statistically significant. This indicates that F-BM contains pDC that lead to improved transplant outcomes. While data from our transplant model does not prove that the observed benefit of transplanting F-BM can be attributable to pDC in isolation, we believe that the cellular and molecular context of increased pDC concentration after Flt3L treatment of BM donors represents a novel insight into improving transplant outcomes that is highly translatable.

Upon close examination of pDC phenotype from BM and F-BM, we found that F-BM pDC express more CXCR4 and less CCR9 than BM pDC. Because CXCR4 is up-regulated when pDC proliferate and develop in the stromal niche (Kohara et al, 2007), the up-regulation of this marker supports the stimulant effect of Flt3L on pDC. In contrast, the decrease in CCR9 expression on pDC following Flt3L treatment of donors is striking. Unpublished data from our group shows that pDC from murine BM express more CCR9 than pDC from murine G-PB. Because data from BMT CTN 0201 indicated that BM pDC are more effective in regulating GvHD than G-PB pDC, our murine data suggest that increased CCR9 expression on pDC is associated with optimal transplant outcomes. Moreover, CCR9 is a chemokine receptor for the homing of pDC to the gut (Wendland et al, 2007) and the thymus (Hadeiba et al, 2012) – the former a target GvHD organ and the latter the site of elimination of autoreactive T cells during GvHD. Despite these conflicting findings, F-BM pDC may use a CCR9-independent homing strategy to localize to these organs, although its high expression (60% of pDC) may still enable them to conserve their biological function to a significant extent. Protein analysis of

intracellular cytokine production showed that there are no differences in cytokine emission between BM- and F-BM-derived pDC.

Interestingly, TCD F-BM grafts are limited in their ability to polarize Th1 and Th17 cells, and isolated F-BM pDC lead to less Th2 polarization and FoxP3+ regulatory T cells on day 3 post-transplant. This indicates that F-BM pDC may use a Th2 and/or Treg signaling-independent mechanism to immuno-modulate the initiation of the post-transplant alloimmune response. Of note, although our current findings may suggest that F-BM pDC are either anergic or exhausted as compared to BM pDC they have a more immature phenotype and have lower abilities to induce T cell polarization, we believe that this is not the case. This is explained by the retained ability of F-BM pDC to stimulate T cell proliferation to the same extent as BM pDC *in vitro* using an MLR.

Of note, 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. We demonstrate that F-BM pDC have up-regulated genes involved in the adaptive immune pathways and immune checkpoints, but have downregulated genes involved in the toll-like receptor cascades. Specifically, genes involved in the TLR4 pathway were down-regulated in F-BM pDC compared to BM pDC

as a group. The ligand for TLR4 is lipopolysaccharide, a polysaccharide present on the walls of gram-negative bacteria that often infiltrate into the bloodstream from GvHD-promoted processes (Ferrara, 1998; Raetz, 1990 and Koyama et al, 2015). This suggests that the responsiveness of F-BM pDC to TLR4 ligands induced by transplant-related inflammation and damage to the gut epithelial barrier is reduced, thus limiting indirect alloantigen presentation that could contribute to GvHD.

A compelling finding was that F-BM pDC had up-regulated genes that are involved in thymic processes. One of these genes, PRS116, codes for the protein thymus-specific serine protease, which is exclusively expressed in cTECs and serves as a marker for these cells. However, there is contradictory data both supporting and rejecting the role of this protein in intrathymic positive selection (Cheunsuk et al, 2008; Gommeaux et al, 2009). Although there is not evidence of such process yet, its up-regulation in F-BM pDC could be indicative of pDC's ability to participate in pos-transplant thymocyte selection, potentially contributing to increased generation of Tregs. A next step to determining if this hypothesis is valid would be to perform a Western Blot with anti-PRSS16 antibody comparing BM pDC and F-BM pDC in the presence of a cTEC positive control. This confirmatory study is especially important to perform because we have found that sorted F-BM pDC were found to have up-regulated genes that code for the light chain of B-cell produced antibodies, which is probably an artifact due to the inability of pDC's to produce these proteins.

Here, we report an additional piece of evidence for pDC's involvement in the thymus. We show that donor treatment with Flt3L increases pDC content in the donor

thymus. Moreover, using a eGFP-C57BL/6→B10.BR allogeneic transplant model, we report that donor-derived pDCs home to the recipient thymus by an apparent increase in GFP+ pDC in the recipient thymus via confocal microscopy. Because this could be a systemic effect since Flt3L increases pDC content in other somatic compartments such as the peripheral blood (data not shown) and the spleen, a deeper look into pDC's involvement with the thymocytes through direct cell-to-cell contact is needed, possibly using in vitro systems such as the fetal thymus organ culture (fTOC).

Taken together, these data indicate that donor treatment with Flt3L increases pDC content in the marrow, and transplantation of Flt3L-treated BM leads to improved transplant outcomes by increasing OS and decreasing cGvHD while retaining GvL in the recipients. Moreover, Flt3L increases the immunoregulatory ability of pDCs in BM grafts by decreasing post-transplant T cell polarization as compared to pDC from BM grafts that have not been treated by Flt3L. Furthermore, the pDC-intrinsic ability to improve transplant outcomes may be explained by F-BM pDC's increased migration to the recipient thymus and participation in the elimination of auto-reactive T cells. Finally, we show that the combination of Flt3L and G-CSF is not an effective strategy for clinical translation due to the effect of G-CSF on pDC phenotype (Hassan et al, BBMT, in revision), thus nullifying the benefit of Flt3L on BM pDC. Because more than 80% of transplants performed in the United States use G-PB instead of BM as the source of stem cells, Flt3L could be used in combination with CXCR4 agonists like Plerixafor, another

mobilizing agent, which has already been shown to lead to improved transplant outcomes by mobilizing HSCs more effectively than G-CSF (He et al, 2014).

VIII. Works Cited

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