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Optimizing graft source for allogeneic hematopoietic stem cell transplantation

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Abstract

Optimizing graft source for allogeneic hematopoietic stem cell transplantation

By Mojibade Hassan

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative for patients diagnosed with blood cell malignancies and genetic blood disorders. Hematopoietic stem cells (HSC) are obtained either from bone marrow (BM) aspiration or are collected by apheresis post daily administration of the mobilizing agent, granulocyte-colony stimulating factor (G-CSF). Clinical outcomes for survival and acute graft-versus-host disease (GvHD) amongst recipients of bone marrow versus G-CSF-mobilized peripheral blood (G-mobilized) grafts are similar, but higher incidence and greater severity of chronic GvHD is seen in recipients of G-mobilized grafts. A critical, unmet need in the field of HSCT is understanding how graft source selection results in the highest rates of survival without inducing disease relapse, graft rejection, or acute or chronic GvHD. Because the immune cell content of stem cell grafts has been correlated with development of GvHD, modulating immune cell content is a possible method to improve the success of stem cell transplants. The graft content of donor plasmacytoid dendritic cells (pDC) has been associated with improved transplant outcomes including decreased acute GvHD in both humans and murine models. The manner in which pDC are thought to prevent GvHD will be discussed herein.

Results of the clinical study, BMTCTN0201, revealed that BM grafts that contained higher than median numbers of pDC resulted in decreased incidence of chronic graft-versus-host disease (GvHD) in allogeneic hematopoietic stem cell transplant recipients. This association was not seen in recipients of G-mobilized grafts. We hypothesized that observed differences in incidence and severity of chronic GvHD amongst recipients of BM and G-mobilized grafts are due to immune-regulatory properties of pDC. Performing murine transplants with fluorescent-activated cell sorting (FACS)-purified BM or G-mobilized pDC, combined with HSC and T cells, we show that donor BM pDC limit GvHD and improve survival compared with G-mobilized pDC. BM pDC expressed higher levels of CCR9 and secreted more IL-12 than G-mobilized pDC. Using CCR9 knockout or IL-12 knockout donor mice as the source of pDC caused severe GvHD in transplant recipients and greatly reduced survival. Additionally, gene expression analysis showed BM pDC downregulated pathways regulating T cell polarization and co-stimulation compared with G-mobilized pDC. In contrast, G-mobilized pDC had greater antigen-presenting abilities and induced higher levels of inflammatory cytokines, ultimately decreasing the content of regulatory T cell in transplant recipients. In graft-versus-leukemia (GvL) models, survival, and tumor burden were similar comparing recipients of donor BM to recipients of G-mobilized pDC. Thus, BM pDC were able to limit GvHD without diminishing GvL. These data provided mechanistic insights on differences in how donor pDC regulate T cell allo-reactivity and chronic GvHD in BM versus G-mobilized allo-transplants.

Although donor BM pDC increase survival and decrease GvHD compared to G-CSF-mobilized grafts, not all BM grafts have higher than median numbers of pDC. Thus, we developed a method to increase pDC content in BM grafts. We studied the effects of FMS-like tyrosine kinase 3 ligand (Flt3L) treatment of murine BM donors on graft pDC content and on transplant outcomes. Flt3L treatment (300μg/kg/day) caused an increase in the content of pDC in the bone marrow of donor mice in a dose dependent manner. A

treatment schedule of 300μg/kg/day on days -4 and -1 was chosen because we observed greater than 5-fold increase in pDC content without significant increase in content of hematopoietic stem cells, T cells, B cells, and natural killer cells in the bone marrow graft. Additionally, utilizing an MHC mismatched murine transplant model, recipients of Flt3L-treated BM (F-BM) and T cells had increased survival and decreased GvHD scores. Furthermore, recipients of F-BM grafts had less Th1 and Th17 polarization in donor T cells at early time-points post-transplant compared with recipients of BM and T cells. Recipients of FACS-isolated pDC from F-BM reduced GvHD and improved survival compared to recipients of equivalent numbers of untreated marrow pDC. Furthermore, upregulation of adaptive immune pathways and immunoregulatory checkpoints was observed in gene array analyses of purified F-BM pDC compared with pDC from untreated BM donors. Finally, recipients of F-BM grafts had similar tumor burden and prolonged survival in two murine GvL models. Therefore, we show that bone marrow pDC result in improved HSCT outcomes and Flt3L treatment of marrow donors is a novel method to increase the content of pDC in allografts, increase survival, and decrease GvHD without diminishing GvL effect. These insights will further the understanding of the impact of graft source on transplant outcomes and has the potential to inform clinical practice in this field.

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Chapter 1:

Introduction

1-1-0 Hematopoietic Stem Cell Transplantation

1-1-1 Overview of Hematopoietic Stem Cell Transplantation

Malignancies of the hematological system account for 9% of the cancers diagnosed in the United States. These malignancies include leukemia, lymphoma, and multiple myeloma(1). While chemotherapy and radiation are treatments for all hematological malignancies, hematopoietic stem cell transplantation (HSCT) is the only curative therapy for many patients with relapsed or refractory malignancy (2). In addition, HSCT is curative for patients that have been diagnosed with hematological disorders such as sickle cell disease and thalassemias (3, 4). HSCT was first performed in 1956 to treat a leukemia patient. The patient underwent total body irradiation and received a bone marrow graft containing hematopoietic stem cells from his identical twin brother (5).

This method is traditionally referred to as bone marrow (BM) transplantation where hematopoietic stem cells and other immune cells are obtained from the liquid center of the pelvic bones, or in extreme cases the sternum (6). This type of hematopoietic stem cell retrieval is reserved for healthy donors exclusively and donors are generally put under general anesthesia for the procedure. The major risk of this procedure to the donor is localized pain at the site of needle insertion (7). Since that the time of the first bone marrow transplant other methods of obtaining hematopoietic stem cells have been developed. Treating hematopoietic stem cell donors with cell mobilizing agents such as granulocyte-colony stimulation factor (G-CSF), plerixifor, or FMS-like tyrosine kinase 3 ligand and performing apheresis to enrich for hematopoietic stem cells are additional methods used to obtain HSC (8). G-CSF mobilized (G-mobilized) peripheral blood grafts are the most common form of HSCT in the United States (9). Due to the effect of G-CSF on all bones in the body, generalized bone pain is the major risk of this procedure for the donor (7).

As there are two methods used to obtain hematopoietic stem cells, there are also two forms of HSCT. Autologous HSCT describes a transplant in which the patient (recipient) is the source of the hematopoietic stem cell graft. Allogeneic HSCT describes a transplant where another person (donor) is the source of hematopoietic stem cell graft. Allogeneic stem cell donors are matched to recipients that share identical genes for the human leukocyte antigen (HLA). HLA is the gene that encodes for the major histocompatibility complex (MHC) gene that regulates the immune system (10). While matching between the donor and recipient at all six major HLA sites is highly desired, unfortunately not every patient has an available HLA-matched donor. An HLA-matched sibling donor is preferred (11). In 2012, of the 18,000 Americans needing an allogeneic bone marrow transplant, 12,500 were unable to find a suitable donor in their family (12), therefore these patients needed a graft from an unrelated volunteer donor, reducing the chance of a perfect match and increasing the possibility of adverse outcomes (13). At this time about 25,000 HSCT are performed each year (1) and with improvement of transplant outcomes, this number will continue to grow. Although disease relapse and graft rejection are possible outcomes following HSCT, the major complication of allogeneic HSCT is graft-versus-host disease (GvHD) and the risk of this outcome significantly increases when grafts are transplanted from HLA-mismatched, unrelated donors (14, 15). The use of immunosuppressive therapies can help combat the effects of GvHD but the use of these drugs have their own side effects such as an increased risk of disease relapse and infection(16). Thus, strategies to prevent graft-versus-host disease (GvHD) are of utmost importance in the field of hematopoietic stem cell transplantation.

1-1-2 Hematopoietic Stem Cell Transplantation Immunology

In order to perform an HSCT, the transplant recipient is “conditioned” with chemotherapy and irradiation to kill cancer cells and eliminate the host immune system that might lead to rejection of donor bone marrow cells (17). Evaluation and preparation of the donor prior to harvesting the bone marrow graft includes multiple clinical visits to assess their health, with the marrow graft typically collected as an out-patient surgical procedure. Peripheral blood donors are treated with the mobilizing agent, most commonly G-CSF, for 5 consecutive days. On day 5, the donor undergoes blood apheresis to enrich the graft collection with hematopoietic stem cells (1, 7, 8, 15). In the case of a bone marrow harvest or a G-CSF mobilized apheresis, the donor graft contains hematopoietic stem cells along with other immune cells such as T cells, B cell, natural killer cell (NK cells) and dendritic cells (18). The hematopoietic stem cell graft is transfused into the recipient after conditioning. Donor immune cells in the BM or G-mobilized graft facilitate donor hematopoietic stem cell engraftment by helping to eliminate residual host-type immune cells and reconstitute a functional donor-derived immune system in the transplant recipient (19).

Additionally, in allogeneic HSCT, expansion and activation of healthy donor immune cells results in graft-versus-tumor (GvT) response, potentially eliminating any remaining malignant cells in the recipient. The major cell type that is implicated in the GvT process are T cells (20). However, donor T cells can also cause GvHD if large numbers are present in the graft. Alternatively, rejection of the graft by residual host immune cells can occur if there are too few donor T cells. Therefore, defining an optimal concentration of donor T cells in the allogeneic HSCT is necessary for ideal outcomes (21). Reduction of GvHD incidence can be achieved by ex vivo depletion of T cells (22) or *in vivo* anti-T cell antibody therapy (23), but T cell depletion leads to increased frequency of graft rejection and relapse. For peripheral blood grafts specifically, and depending on the center, T cell concentration in the collected graft is measured, and the number of T cells in the graft can be reduced using T cell depletion techniques if it is higher than what is believed to

be an optimal number (24). However, current clinical practices of optimizing donor T cell content in bone marrow and peripheral blood stem cell transplantation have not resulted in significant improvement in clinical outcomes to date. Hence, understanding of the role of immune cell subsets in the graft and how they function post HSCT is necessary for improving outcomes.

Full understanding regarding the success of hematopoietic stem cell transplantation in some patients while other transplant recipients may reject the graft, suffer from severe GvHD or have disease relapse is lacking in the field of HSCT. Mechanistic differences contributing to a successful transplant as opposed to a failed transplant are poorly understood. The ratio, activation status, and source (BM vs. G-mobilized) of immune cells in hematopoietic stem cell grafts has been shown to be associated with various transplant outcomes including graft rejection, disease relapse, GvHD, and treatment related mortality (19). Greater understanding of the complex interactions among the major immune cell types within an HSCT graft and how these cells interact with recipient immune cells and tissues will be of great benefit to the field. Complications of GvHD have limited the availability of allogeneic hematopoietic stem cell transplants to patients that have life threatening diseases such as hematological malignancies (25). Insights into the immunological properties of donor hematopoietic cells have the potential to transform clinical practice, reduce adverse complications following HSCT, and ultimately allow the expanded the use of HSCT to more patients due to safer outcomes.

1-1-3 Complications of Hematopoietic Stem Cell Transplantation

As mentioned above, complications of HSCT include graft rejection, disease relapse, and most commonly GvHD. Successful engraftment following HSCT is defined as the recipient hematopoietic system being compromised of donor immune cells. Successful engraftment occurs as a result of irradiation and/or chemotherapy that injures or kills the hematopoietic cells including both malignant and nonmalignant cells of the recipient. The donor graft immune cells also aid in engraftment by removing any residual immune cells or cancer cells. Conversely, graft rejection is defined by the recipient hematopoietic system compromised of mainly recipient cells. Although engraftment is an endpoint measured to assess the success of transplant outcomes, there is no consensus on percentage or types of cells that should be measured (26)Graft rejection is the result of failed conditioning or less than optimal donor immune cell response to the recipient hematopoietic system (27). In this instance, pancytopenia of the blood and marrow aplasia result due to the damage of the hematopoietic system of the recipient from the chemotherapy and irradiation, and failure of donor cells to engraft. Disease relapse is another possible result of graft rejection, whereby the donor immune cells are unable to mount an efficient immune response to residual cancer cells. Thus, patients that have graft rejection or have disease relapse must undergo a second HSCT to ensure full HSC engraftment and restore normal hematopoiesis and immune function (28, 29). On the contrary, when donor immune cells engraft too robustly, GvHD ensues.

GvHD is a donor cell immune response where target organs in the recipient of an allogeneic hematopoietic stem cell transplant are attacked by donor derived allo-reactive immune cells resulting in inflammation, injury, and damage to these organs. GvHD target organs include the skin, intestines and liver (30). Acute GvHD is characterized by three steps. First, the chemotherapy regimen introduced to prepare the recipient for the bone marrow transplant creates a pro-inflammatory environment. Subsequently, this inflamed environment promotes T cell

proliferation and activation. Finally, these activated T cells attack target organs in the recipient and secrete inflammatory cytokines that enhance the pro-inflammatory environment and result in GvHD (31). Of note, this form of GvHD is reversible. Chronic GvHD is a result of prolonged injury to the GvHD target organs that results in permanent damage to these organs. Chronic GvHD that leads to fibrosis and sclerosis may result in irreversible organ damage (32).

In summary, the content of immune cells in an allogeneic stem cell graft has a complex relationship with post-transplant outcomes. Enhancing mechanistic knowledge of how donor allo-HSCT graft composition and the immune function of these cells impacts transplant outcomes has great potential to increase the success rate of HSCT while reducing adverse effects such as graft rejection, GvHD, and relapse. Hitherto, it has been shown that donor T cell content is important for engraftment, GvHD, and GvT activity. Recently, donor dendritic cell content has been studied given that T cell activation is in part due to antigen presentation by these cells (33, 34). Herein, the role of pDC in bone marrow transplant is discussed and evaluated, with suggestions of strategies to fill the critical gap in knowledge in transplant immunology through the use of murine HSCT models.

1-1-4 Regulation of Post-Transplant Immunity

The different possible clinical outcomes of allogeneic HSCT including graft rejection, disease relapse, and GvHD are mainly the result of differences in the function of donor T cells as a result of their post-transplant activation, proliferation, and pharmacological and immune-mediated suppression (14, 27). Four different types of T cells are known to play a role in post-transplant immunity and include T helper cells 1, 2, and 17 along with regulatory T cells (35). Regulatory T cells actively suppress T cell activation and autoimmunity (36). There are two types of regulatory T cells: natural and induced. 5-10% of all T cells in the body are non-antigen specific, natural regulatory T cells. These cells are $CD25^{+}$. Due to their lack of antigen specificity, these cells are not appealing candidates for cell based therapy (25). Contrarily, induced regulatory T cells do have antigen specificity. Generation of induced regulatory T cells occurs in peripheral organs and tissues. While the exact mechanism of suppression is unknown, regulatory T cells do secrete suppressive cytokines such as IL-10 and TGFβ (37).

The enhancement of regulatory T cells in donor grafts has been shown to prevent GvHD in murine allogeneic hematopoietic stem cell transplant models (38). Additionally, the use of regulatory T cells has been demonstrated to enhance engraftment in mice (39). Expansion of regulatory T cells can be achieved ex vivo by culturing donor regulatory T cells with recipient cells and IL-2. Furthermore, their ability to prevent GvHD can be sustained following a single administration (40, 41).

Interestingly, recipient regulatory T cells also play a role in inducing tolerance and preventing rejection of the donor allogeneic hematopoietic stem cell graft by host immune cells. In a murine bone marrow transplant model utilizing post-transplant adoptive transfer of regulatory T cells, recipient-type regulatory T cells were shown to be the dominant regulatory T cell population that could enhanced engraftment and reduce GvHD in transplant recipients (25). Of note, recipient-

type regulatory T cells were effective when given post-transplant in conjunction with original donor MHC-matched transplant, but not an MHC-mismatched transplant. While regulatory T cells were found within the first two weeks post-transplant, their regulatory functions were not evident until more than 5 weeks post-transplant when FoxP3 expression was higher (42).

1-2-0 Hematopoietic Stem Cell Mobilizing Agents

1-2-1 Overview of Granulocyte-Colony Stimulating Factor

Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein that is secreted by many tissues and cells including endothelium, macrophages, and immune cells. Analogs are filgrastim and lenograstim. G-CSF stimulates bone marrow to produce granulocytes and stem cells. Additionally, the bone marrow is stimulated to release these newly produced cells into the peripheral blood. The advantages of using G-CSF for HSC mobilization and collection by apheresis include less initial risk to the donor (no anesthesia or surgical procedures), ease of graft acquisition (apheresis versus bone marrow harvest), less time to reconstitution of the hematopoietic system in the transplant recipient ultimately decreasing risk of opportunistic infection due to immunosuppression, although there is no difference in graft-versus-leukemia effect (43, 44). Although survival up to seven years post-transplant is equal between recipients of BM and G-CSF mobilized peripheral blood (G-mobilized) grafts, recipients of G-mobilized grafts have more treatment related morbidity (45).

The disadvantages of a G-mobilized graft include greater than 10-fold increase in T cells in the graft and increased incidence of chronic graft-versus-host disease. Use of G-CSF results in Th2 and Th17 cell polarization (46, 47). Both cell types are implicated in acute GvHD manifestation (48, 49). Additionally, dendritic cell cytokine profiles are regulated by G-CSF and Th2 inducing dendritic cells are stimulated post G-CSF administration (50, 51). G-CSF has been shown to increase the content of all dendritic cell subsets including plasmacytoid dendritic cells (pDC) (52). While the effect of G-CSF administration on donor T cells in the graft has been thoroughly studied, further characterization of how the graft source or mobilization procedure changes the functions of other immune cells, specifically pDC, in the graft would provide valuable information that will not only inform clinical practice, but also has potential to identify novel drug targets to improve allo-transplants.

1-2-2 Overview of FMS-like tyrosine kinase 3 Ligand

FMS-like tyrosine kinase 3 ligand (Flt3L) is a cytokine that promotes the differentiation and proliferation of many immune cells including T cells, B cells, NK cells, and dendritic cells, by binding its receptor Flt3 on hematopoietic progenitor cells (53-56). Additionally, Flt3L can induce mobilization of hematopoietic stem cells and terminally differentiated immune cells from the bone marrow to the peripheral blood system (57). Mutation and increased signaling by Flt3 is common in many forms of acute myeloid leukemia (58) due to the enhanced proliferation and survival conferred by Flt3L signaling in the differentiation and proliferation of many immune cells.

The use of Flt3L alone as a hematopoietic stem cell mobilizing agent was recently studied using a recombinant form of Flt3L called CDX-301 in a clinical trial. This study showed that administration of Flt3L to healthy, human volunteers resulted in mobilization of hematopoietic progenitor cells, monocytes, and dendritic cells (57). In mice, when Flt3L was combined with the CXCR4 antagonist, plerixafor, an increase in hematopoietic stem cells, NK cells, regulatory T cells and DCs was observed in the peripheral blood of donors compared to recipients of plerixafor alone. Of note, in this murine model recipients that received HSCT grafts from donors mobilized with Flt3L alone had higher overall survival than recipients of grafts from plerifaxor and Flt3L treated donors. The clinical trial mentioned above using Flt3L alone as a mobilizing agent was discontinued due to insufficient hematopoietic stem cell mobilization (57). While Flt3L may not serve as an efficient mobilizing agent for HSCT, its effects on immune cell proliferation have been well documented (59-61). The effect of Flt3L on the composition of the graft when it is administered prior to bone marrow harvest rather than its use in mobilization of peripheral blood grafts remains unknown. *In vitro* studies have shown the greatest effect of Flt3L administration to bone marrow cells is on differentiation and proliferation of plasmacytoid dendritic cells, an immune cell that has been described as a graft facilitating cell in both hematopoietic stem cell and solid organ transplantation (62).

1-3-0 Plasmacytoid Dendritic Cells and Their Role in Transplant

1-3-1 Overview of Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells are 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 (63-65). Plasmacytoid dendritic cells are derived from hematopoietic stem cells (HSC) and have morphology that is like what is seen in plasma cells, with a peri-nuclear clear zone on H&E staining consistent with an extensive endoplasmic reticulum (66). The exact development from HSC to pDC is unknown (67). Flt3+ hematopoietic stem cells become hematopoietic progenitor cells than can become lymphoid-restricted common lymphoid progenitors (CLP) or myeloid-restricted common myeloid progenitors (CMP) (61). In mice, it is known that pDC are derived from at least 2 different progenitor cells types including CLP or CMP (68). There is heterogeneity in Flt3 expression of CLP and CMP (69), but it is the Flt3+ cells that are capable of producing functioning pDC as depicted in Figure 1 (61). Commitment to the pDC lineage requires Flt3L binding Flt3, which in turn activates the transcription factor E2-2 (70). Expression of Spi-B and Bcl2A have been shown to be necessary for pDC development as well (55, 71). The importance of Flt3L for differentiation of pDC is verified by the fact that Flt3 ligand deficient mice have low numbers of pDC, but pDC number can rebound to near normal levels with Flt3 ligand injections (72). It remains to be determined if there is a difference in pDC function, migration, and activation depending on progenitor lineage. Furthermore, the requirement of Flt3 ligand for development of pDC is also necessary for human pDC (73, 74). Additionally, pDC number can be increased in bone marrow cultures with the addition of Flt3 ligand to the culture medium (73, 75, 76). The addition of Flt3L to *in vitro* bone marrow cultures greatly increases the pDC content (77).

While granulocyte-macrophage colony stimulating factor (GM-CSF) is necessary for myeloid dendritic cell generation, it is not necessary for pDC generation (78). This is validated by no

difference in pDC numbers when comparing wildtype mice with GM-CSF deficient mice (67). However, *in vitro* survival of pDC is boosted by the addition of GM-CSF to the culture medium (79). In contrast G-CSF is a powerful inducer of pDC generation and proliferation and the effects on function of pDC post G-CSF mobilization have not yet been described, neither have the effects of Flt3L induced differentiation and proliferation, but will both be thoroughly discussed herein (67, 77).

1-3-2 Plasmacytoid Dendritic Cells in Immunity

Plasmacytoid dendritic cells have been described as the primary Type I IFN producing cells (80). While pDC are thought to develop in the bone marrow, they migrate throughout the body to secondary lymphoid organs through high endothelial venules mediated by expression of CD62L (81-83). The life span of pDC has been found to be about 2 weeks (79). These cells are found fully developed with IFNα secreting capabilities in multiple tissues including the hematopoietic system, the liver, and the bone marrow (84, 85).

Activation of plasmacytoid dendritic cells most commonly occurs through stimulation of toll-like receptors (TLR) 7 and 9. Within the first 6 hours post activation of TLR7/9, the majority of the pDC gene expression is dedicated to type I IFN genes (86, 87). While antigen presentation is not the main function of pDC, in their immature state, pDC are able to uptake antigen (at a much lower level than cDC) (88). *In vitro* assays have shown that human pDC have the ability to present antigens acquired through micropinocytosis on MHC I and MHC II to the appropriate T cell (89, 90).

Issues that arise with plasmacytoid dendritic cells include their involvement in autoimmunity. Unsolicited production of IFNα by pDC has been found in patients who suffer from systemic lupus erythematous (SLE), Sjogren's syndrome, and psoriasis (91, 92). In addition to the negative impact of unnecessary IFNα production, these autoimmune pDC mature and present antigens to T cells resulting in a pathogenic autoimmune response and subsequent disease pathogenesis (93).

Most recently plasmacytoid dendritic cells have also become known as transplant facilitating cells and enhance donor HSC engraftment by creating tolerance to donor cells in immune cells of the recipient that persist following the conditioning regimen (93). The tolerogenic pDC phenotype has

been described as immature, with low levels of MHC II, CD80, and CD86 expression, and high levels of CCR9 expression. Intriguingly, $CCR9^{+}$ pDC are usually found within the intestine or thymus of mice (94, 95). In this state, pDC are unable to induce and maintain T cell proliferation (96). These cells play a role in tolerance by influencing T cell fate and activation, inducing the generation of regulatory T cells and are associated with limitation of GvHD post-transplant (25). CCR9 expression is lost when pDC are activated following triggering of TLR7/9 (94). Once pDC are activated, the number of dendritic processes increases and the cells are able to activate or inhibit T cells based on environment and signals they receive (25, 67). Due to the multiple roles of pDC, they are links between the innate and adaptive portions of the immune system (67). Additionally, it is known is that greater than median numbers of pDC in a bone marrow graft leads to improved outcomes in allogeneic HSCT recipients (97).

1-3-3 Plasmacytoid Dendritic Cells in Transplant

Immune cell modulation has shown success in allogeneic hematopoietic stem cell transplantation, resulting in innovative cell-based therapies that are being tested in clinical transplantation. One cell of interest is the pDC. Additionally, a graft facilitating cell which is $CD8^{+}/TCR^{-}$ has recently been described and shown to aid in hematopoietic engraftment and reduction in GvHD when administered to bone marrow transplant recipients (98). The major subpopulation of these facilitating cells are pDC. The ability of donor pDC to enhance engraftment and reduce GvHD is attributed to their ability to induce generation and proliferation of antigen specific regulatory T cell (25). In a murine bone marrow transplant model with an enhanced facilitating cell population, increased numbers of regulatory T cells was observed along with reductions in GvHD, clinical or histopathological (42). Secondly, facilitating cells have been shown to improve the bone marrow environment with TNFα production which in turn enhances donor hematopoietic stem cell engraftment. Direct cell contact is necessary for this process and has been validated when co-culture of facilitating cells and hematopoietic stem cells led to lower levels of TNFα production by the facilitating cells and decreased survival of the hematopoietic stem cells (99). This is in contrast to a trans-well assay, where this phenomena was not observed (25). The multifaceted effects of pDC on transplant outcomes include aiding in engraftment, stimulation of regulatory T cell polarization, and aiding in host defense (Figure 2).

The ability of a rare donor cell population to have global effects on the numbers and activity of regulatory T cells is important due to clinical and logistical issues with the maintenance of regulatory T cells' tolerogenic function following ex vivo expansion and administration to bone marrow transplant recipients. The discovery and use of facilitating cells such as pDC as part of the bone HSCT graft have the potential to expand the use of allo-HSCT to a larger number of patients with hematological disorders and autoimmune diseases who may benefit from allo-transplant but lack a HLA-matched donor.

Plasmacytoid dendritic cells in the allograft uptake alloantigen following transplantation and then migrate to peripheral lymphoid organs and lymph nodes. Once in lymph nodes, donor pDC are able to present these alloantigen and induce the generation of antigen-specific regulatory T cells (25). This is achieved by pDC production and secretion of IL-10 and TGFβ (100, 101). This induction of regulatory T cells may be achieved by direct contact between pDC and naïve T cells, increased exposure of T cells to IL-10, the reduction in IFNγ and/or the upregulation of IDO (102). These data suggest that clinical strategies to increase the content of immune-regulatory function of pDC in a donor HSCT graft could represent a potential major advancement in clinical practice of allogeneic bone marrow transplantation. The use of donor pDC as a graft-facilitating donor cell is limited by the difficulty of obtaining large enough numbers of these cells in donor marrow grafts or the ability to expand them quickly and efficiently ex vivo while retaining their beneficial immunological properties (103).

While pDC make up the majority of the facilitating cell population, the addition of purified donor pDC to an allograft may not have the same benefits as the use of a broader population of facilitating cells. Surprisingly, the removal of pDC from the facilitating cell pool does nullifies the benefits of these cells completely. Thus, pDC are essential for graft-facilitating cell function, but not sufficient. Other cells that make up the facilitating cell pool include NK FC and CD19 FSC. Therefore, the interaction of these cells may be the necessary factor to enhance allogeneic hematopoietic stem cell engraftment and reduce GvHD. It has been shown that direct contact of these facilitating cells with hematopoietic stem cells is necessary for the beneficial effects to occur (99).

Enhancement of hematopoietic stem cell engraftment by pDC is thought to be the result of induction of an immunomodulatory environment via cytokine secretion, cell surface marker

activation with its ligand, and/or regulation of the enzyme, IDO. This leads to a decrease in tryptophan, which renders T cells ineffective. The expression of IDO from macrophages and trophoblasts is induced by cytotoxic T lymphocyte antigen-4 (CTLA4) on regulatory T cells, which are generated and proliferate in the presence of pDC (25). Additionally, T cells are stimulated to produce FoxP3 in the presence of tryptophan metabolites. IDO-competent pDC can induce a tolerogenic environment as opposed to an immunogenic environment depending on the cytokine signals and stimulation that the pDC receives (104). IFNγ may play an important role in the induction of a tolerogenic environment post-transplant due to the ability of IFNγ to induce IDO synthesis by pDC. To date, IDO's tolerogenic role in transplant has been demonstrated in liver grafts, pancreatic grafts with IDO gene transfer and lung transplants with IDO gene transfection (105).

1-4-0 Murine Hematopoietic Stem Cell Transplant Models

1-4-1 Benefits of Murine Hematopoietic Stem Cell Transplant Models

Murine hematopoietic stem cell transplant models are a valuable resource and knowledge gained from them has been translated to clinical practice. Most of the scientific understanding of GvHD in humans is derived from discoveries found in mouse models (106). A recent comparison of gene expression in mice and humans post sepsis and burns showed dissimilar gene profiles (107), begging the question of whether inflammation in murine models mirrors that of man? In the field of bone marrow transplantation, many discoveries made in murine models have been confirmed by clinical practice. These include the effects of stem cell purification (108), blockade of T cell co-stimulation, the use of regulatory T cells to prevent GvHD, and the administration of post-transplant cyclophosphamide as GvHD prophylaxis (109). While murine host antigen presenting cells (APC) survive irradiation (110) and can initiate GvHD through antigen post allo-HSCT (111), relatively little is known about the influence of donor APC post-transplant. The availability of murine HSCT models in which the composition of the allo-graft can be precisely manipulated and transplantation of defined cell subsets from knock-out strains can elucidate the function of different cell types and the mechanisms by which they regulate post-transplant immunity.

To assess the successfulness of murine hematopoietic stem cell transplants clinical parameters are typically measured including tracking of weight loss/gain, behavior, skin and coat condition and behavioral signs of GvHD such as hunching of the back. Measurements of those parameters are combined to give a clinical GvHD score and can be used with results from a histopathological score to assess overall severity of GvHD (112). Additionally, post-transplant survival is the “gold-standard” in the assessment of murine GvHD studies. The murine HSCT model used for the

majority of the work discussed herein in a C57BL/6→B10.BR model in which the donors and recipients are mismatched at all of the MHC loci (Figure 3).

1-4-2 Murine Hematopoietic Stem Cell Transplant Models Limitations

One stark contrast that must be kept in mind when evaluating data that spurs from murine mouse models of transplantation is the difference in pre-transplant conditioning regimens between humans and mice. Humans are conditioned with myeloablative chemotherapy regimens and/or radiation. Whereas, mice are usually conditioned just with total body irradiation (113).

While the use of mouse models is advantageous due the amount of resources such as knockout strains and monoclonal antibodies (106), other models are used to investigate GvHD pathology including nonhuman primates (114) and rats (115). Additionally, imaging of transplanted cells and GvHD target organs to better understand immune cell homing post-transplant and causative cells and/or cytokines of GvHD can be readily done in mouse models with the use of bioluminescent imaging and MRI (116).

1-5-1 Figure Legends

Figure 1: Plasmacytoid dendritic cell lineage. Model of pDC lineage depicting development from both common lymphoid and common myeloid progenitor cells that are $Flt3^{+}$.

Figure 2: Beneficial effects of pDC in hematopoietic stem cell transplantation is multifactorial. Model depicting pDC role in various stages of post-transplant immunity including but not limited to engraftment, induction of regulatory T cells, and anti-viral host defenses.

Figure 3: C57BL/6→B10.BR murine hematopoietic stem cell transplant model. Murine transplant model used for the majority of transplants discussed here in. MHC H2 haplotype of each breed depicted in parentheses. Lightning bolt represents two separate 5.5 Gy irradiation sessions 3+ hours apart for a total of 11 Gy.

1-5-2 Figures

Figure 1.

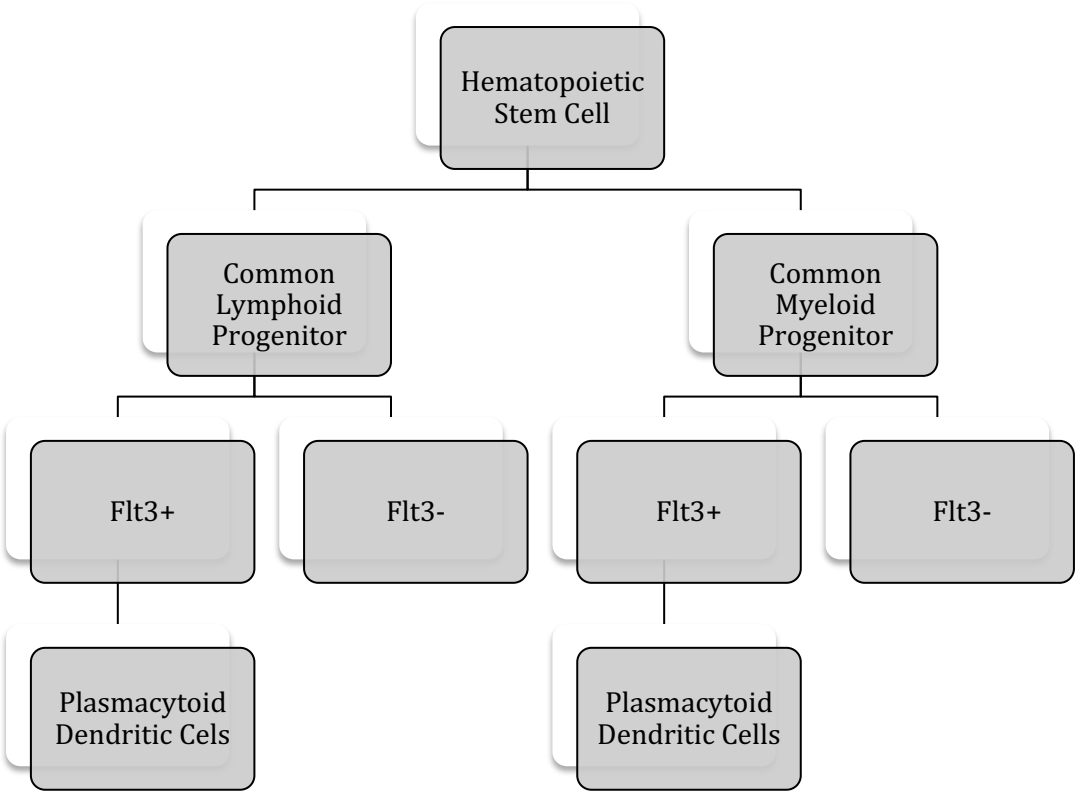


Figure 2.

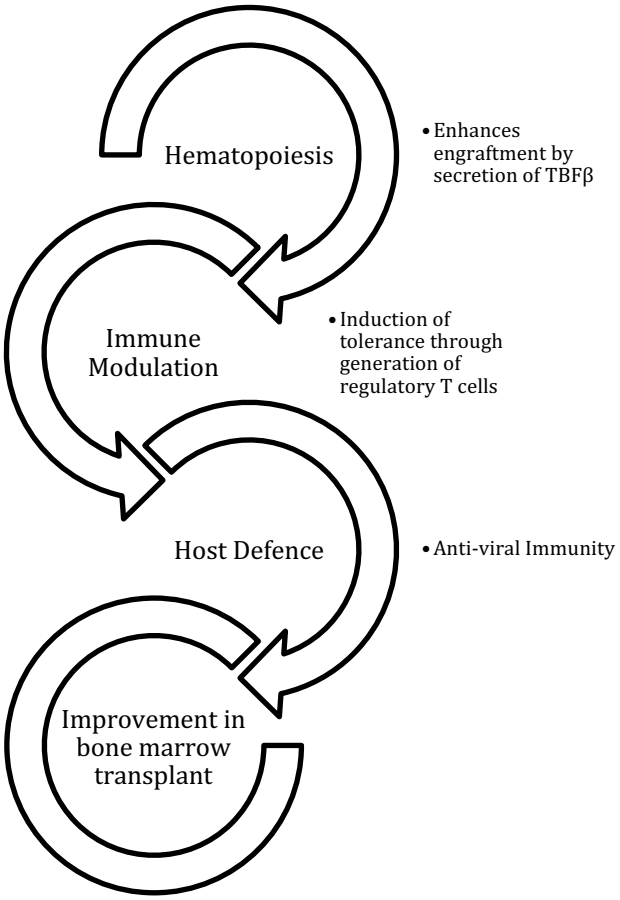
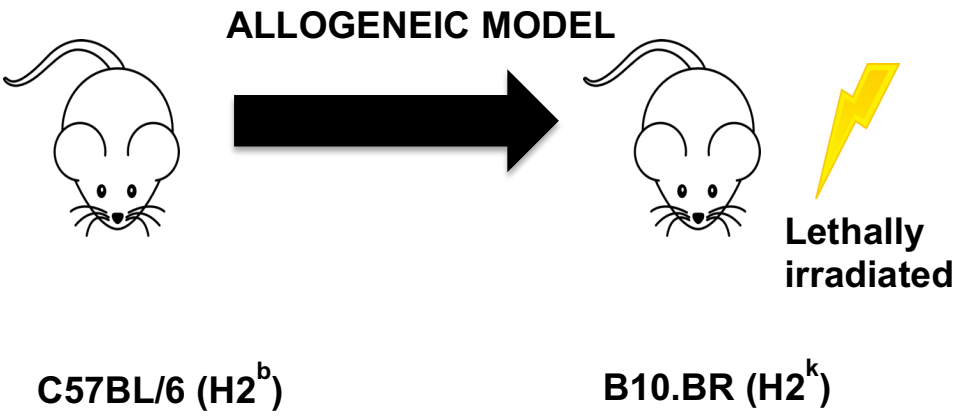

Hematopoiesis
• Enhances engraftment by secretion of TBFβ
Immune Modulation
• Induction of tolerance through generation of regulatory T cells
Host Defence
• Anti-viral Immunity
Improvement in bone marrow transplant

Figure 3.


ALLOGENEIC MODEL
Lethally irradiated
C57BL/6 (H2b)
B10.BR (H2k)

Chapter 2: Donor Bone Marrow, not Granulocyte Colony Stimulating Factor Plasmacytoid Dendritic Cell Increase Survival and Reduce Graft-versus-Host Disease in Murine Allogeneic Hematopoietic Stem Cell Transplantation

2-1-0 Abstract

Clinical data from BMTCTN0201 show bone marrow (BM) grafts containing more than median numbers of plasmacytoid dendritic cells (pDC) resulted in less chronic graft-versus-host disease (GvHD) in allo-transplant recipients, an association not seen in granulocyte-colony stimulating factor mobilized (G-mobilized) grafts. We hypothesized that the reduced incidence of chronic GvHD in recipients of BM is due to immune-regulatory properties of BM donor pDC that are lacking or downregulated in G-mobilized donor pDC. We observed increased survival and decreased GvHD in recipients of BM pDC compared with G-mobilized pDC when transplanting FACS-purified BM or G-mobilized pDC, with HSC and T cells in murine MHC mismatched allogeneic hematopoietic stem cell transplants. BM pDC expressed higher levels of CCR9 and IL-12 compared with G-mobilized pDC, and transplanting CCR9 knockout or IL-12 knockout donor pDC decreased survival and resulted in severe GvHD, supporting the role of tissue-specific homing and local cytokine synthesis in the ability of donor pDC to modulate acute GvHD. In contrast, G-mobilized pDC had greater antigen-presenting abilities and induced higher levels of inflammatory cytokines, resulting in lower numbers of regulatory T cells in transplant recipients. In graft-versus-leukemia models, survival, and tumor burden were similar comparing recipients of donor BM to G-mobilized pDC. Finally, in gene expression analysis, BM pDC had downregulated pathways regulating T cell polarization and costimulation compared to G-mobilized pDC. Mechanistic insights on the ability of BM pDC to regulate T cell allo-reactivity, reduce GvHD, and increase survival in allo-transplants is discussed herein.

2-2-0 Introduction

Complication of allogeneic hematopoietic stem cell transplantation (HSCT) are initiated and regulated by donor immune cells. These adverse outcomes include graft rejection, disease relapse, and graft-versus-host disease (GvHD) (14, 15). Clinical outcomes in allogeneic HSCT (allo-HSCT) are greatly impacted by donor graft immune cell content (18). Similar overall survival rated are observed in recipients of bone marrow (BM) or granulocyte-colony stimulating factor (G-CSF) mobilized grafts from unrelated donors at 7 years post-transplant. Interestingly, G-CSF-mobilized (G-mobilized) graft recipients had increased rates of chronic GvHD symptoms and treatment related mortality compared with BM graft recipients (45, 117). It is evident that donor T cells mediate GvHD (118), but the role of donor antigen-presenting cells in the graft, including their role T cell activation initially and proliferation has yet to be fully determined.

Patients who receive HLA-matched or single antigen mismatched allogeneic BM grafts containing greater than the median numbers of donor plasmacytoid dendritic cells (pDC) across grafts for all patients had a lower incidence of chronic GvHD and fewer deaths from acute GvHD compared to recipients of less than the median number of donor BM pDC (97). Identification of pDC by flow cytometry selects for Lin^-(CD3, CD14, CD16, CD19, CD20)$HLADR^+CD123^+CD11c^-CD33^-$ in humans and are selected by $PDCA1^+CD11c^+B220^+Lin^-$(CD3, CD11b, CD19, IgM, CD49b, Ter119) in mice (63-65). These cells are the major source of type 1 interferon in both humans and mice, supporting a significant role for pDC in both innate and adaptive immune responses (119). Furthermore, donor pDC have been described as graft-facilitating cells, leading to improved engraftment and survival post-transplant (120). The exact mechanism by which donor pDC facilitate donor HSC engraftment and regulate post-transplant immunity is yet to be defined, with hypothesized mechanisms including increased regulatory T cells post-transplant (25). Cytokines produced by donor pDC may enhance engraftment, as pDC are the major DC subset producing IL-12 in mice, while both classical DC (cDC) and pDC produce IL-12 in humans (121). Murine

pDC also produce IL-10 and TGFβ, cytokines that suppress Th1 immunity (122, 123). In mice, presentation of alloantigen by host-type cDC is thought to be the major mechanism of activating allo-reactive T cells which cause GvHD (33), while pDC-T cell interaction may also play a role in GvHD (124).

In a murine model of allogeneic HSCT, we previously showed that addition of BM pDC to donor grafts of HSC and T cells limits the incidence and severity of GvHD (35). Extrapolating from clinical transplant experience, we hypothesized that pDC isolated from BM and G-mobilized grafts have distinct immunological activities resulting in disparate abilities to regulate GvHD and affect survival in transplant recipients. To test this hypothesis, we used an MHC mismatched C57BL/6→B10.BR transplant model in which grafts contained purified populations of HSC, T cells, and donor pDC. We report herein that the addition of donor BM pDC to HSC and T cell grafts results in better survival with less GvHD than adding donor G-mobilized pDC. Using knock-out strains as the source of donor pDC we demonstrate that the protective effect of donor BM pDC on GVHD is dependent upon IFNγ-licensing of pDC that induces IL-12 and subsequently indolamine 2,3-deoxygenase (IDO) leading to generation of Treg and protection from GvHD. Thus, these murine data provide a mechanistic basis for clinical observations that recipients of G-CSF mobilized grafts experience more morbidity and mortality from GvHD than recipients of BM grafts.

2-3-0 Materials and Methods

Mice

C57BL/6 (H-2K^{b}), B10.BR (H-2K^{k}), and IL-12p40KO (B6.129S1-Il2b^{tm1jm}/J), IL-10KO (B6.129P2-Il10^{tm1Cgn}/J), IFNγR KO (B6.129s7-Ifngr1^{tm1Agt}/J), and IDO KO (B6.129-Ido1^{tm1Alm}/J) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Male donor and recipient mice were 8-10 weeks and 10-12 weeks, respectively. National Institutes of Health animal care guidelines were used and approved by Emory University Institutional Animal Care and Use Committee.

G-CSF Treatment

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

Donor Cell Preparation

Donor mice were euthanized and femora and tibias of PBS-treated C57BL/6 mice were flushed with 2% FBS PBS. Spleens of G-CSF-treated mice were flushed with 2% FBS PBS. Anti-mouse (CD3 BD 553064, CD11b BD 553311, CD19 eBioscience 12-0913-83, IgM BD 553409, Nk1.1 BD 557391, Ter119 BD 553673) PE, CD11c FITC BD 553801 or APC-CY7 BD 561241, B220 PERCP-CY5.5 Biolgend 103236 and PDCA1 ef450 eBioscience 48-3172-80 were used for pDC analysis and purchased from BD Bioscience (San Jose, CA), Biolegend (San Diego, CA), and eBioscience (San Diego, CA). HSC were stained with (CD3, CD11b, CD19, IgM, CD49b, Ter119) PE, B220 PERCP-CY5.5, CD117 APC BD 553356, Sca-1 PE-CY7 BD 558162. T cell purification was performed by incubating with biotinylated B220 BD 553086, CD49b Biolegend 108904, Gr-1 Biolegend 108404, Ter119 Biolegend 116204 antibodies, then with anti-biotin microbeads, and negative selection with a LS Miltenyi MACS column (Gladbach, Germany).

Flow Cytometry

Stimulation of pDC for cytokine profile analysis was done using 50μM of CpG (ODN 1585, Invivogen, San Diego, CA) or 10ng IFNγ (Peprotech, Rocky Hill, NJ) on whole bone marrow or splenocytes in complete RPMI 1640 supplemented with 10% FBS, 100 U/mL of penicillin, 100μg/mL of streptomycin, and 50μM of 2-mercaptoethonaol, nonessential amino acids, HEPES, and sodium pyruvate (complete media) each in 10cm wells for 9 hours at 37°C. BD golgiplug was added at hour 3. Intracellular analysis of pDC was done using BD Bioscience cytofix/cytoperm kit and anti-mouse IDO PercP eFluor 710 eBioscience 46-9473-80, IFNα FITC PBI 22100-3, and IL-12 APC BD 554480 antibodies.

Identification of T cells was done using anti-mouse CD3 FITC BD 555274, CD4 PE-CF594 BD 562464, CD8 PERCP-CY5.5 Biolegend 103235, and CD25 APC-CY7 BD 557658. T cells were stimulated with BD leukocyte activation cocktail and golgiplug for 6 hours. Intracellular staining was done using the BD Bioscience cytofix/cytoperm kit and IFNγ APC BD 554413 and TNFα PE BD 554419 antibodies. Intranuclear staining was done using the eBioscience fixation kit and Tbet PE-CY7 Biolegend 644824 and FoxP3 PE BD 563101 antibodies. Data were acquired with a FACS Aria (BD, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, Oregon).

T cells were stimulated with phorbol-12-myristate-13-acetate, ionomycin, and golgiplug from BD for 6 hours. Intracellular staining was done using the BD Bioscience cytofix/cytoperm kit and IFNγ APC, IL-17 APC-Cy7, and TNFα PE antibodies. The eBioscience fixation kit and Tbet PE-CY7, GATA3 PE, RORγT APC, and FoxP3 PE antibodies was used for intranuclear staining.

Cytospin

The protocols above were used to sort pDC to > 90% purity. 1% BSA-PBS was used to moisten the cytospin filters. Cytospin wells were loaded with 10^4 cells, placed in the Cytospin 3 by Shandon

(Runcorn Chesire, England) and spun at maximum speed. Methonal was used to fix the cells and desiccated overnight. Then Giemsa stain from Electron Microscopy Sciences (Hatfield, Pennsylvania) was used to stain the cells. Permount from Fisher Chemical (Geel, Belgium) and Mounting Medium Xylene from Fisher Scientific (Pittsburg, PA) were used to mount cells. The Zeiss Axioplan 2 (Thornwood, NY) was used to image slides.

In vitro T cell Activation

MACS purified splenic T cells from C57BL/6 mice were cultured 1:1 with irradiated B10.BR splenocytes alone or in the presence of 2:1 numbers of BM or G-mobilized pDC in 96 well plates in complete media for 72 hours at 37°C for MLR. TEα cells from C57BL/6 mice were cultured alone or 2:1 with pDC with 3μM of Eα peptide in 96 well plates in complete media for 72 hours at 37°C.

CFSE Staining

T-cells were labeled with 1μM of carboxyfluorescein succinimidyl ester for proliferation assays (Life Technologies, Eugene, Oregon).

Post-transplant Serum Analysis

Recipient mice serum was collected 10 days post-transplant. Cytokine profiles were assessed by Luminex Multiplex (Austin, TX).

Transplantation

On day -1 recipients were irradiated twice at 5.5Gy at least 3 hours apart for a total of 11Gy. Mice were transplanted with 5,000 FACS sorted HSC, 50,000 FACS sorted BM derived or G-mobilized pDC, and 10^6 MACS enriched T cells were injected on day 0. GvHD monitoring used a 10-point

scoring system including aspects concerning weight, attitude/activity, skin condition, hunching, and coat condition (125).

Tumor Cell Challenge and Bioluminescent Imaging

Luciferase-transfected C1498, an acute myeloid leukemia cell line, was generously gifted by Dr. Bruce Blazar. Mice were lethally irradiated on day-2, challenged with 50,000 C1498 cells on day -1, and then transplanted on day 0. Mice were injected with 150µg/kg of D-Luciferin and imaged with IVIS spectrum (Perkin Elmer, Waltham, MA). Measurements of luminescence are given as photons/sec/cm^2 and normalized to mice that received the same transplant without $luciferase^+$ tumor.

LBRM 33-5A4 were purchased from American Type Culture Collection (ATCC) (Manassas, VA). ATCC recommendations were used for culturing of the cell line. Transfection of these cells to express luciferase was conducted in our laboratory. Mice were irradiated on day -2, challenged with 2 million LBRM cells on day -1 of transplant, and transplanted on day 0. Tumor burden was monitored by injecting mice with 150µg/kg of D-Luciferin and imaging with IVIS spectrum (Perkin Elmer, Waltham, MA). Measurements of luminescence are given as photons/sec/cm^2/sr and normalized to mice that received the same transplant without tumor challenge.

Gene Array Analysis

Gene expression of murine BM pDC and G-mobilized pDC was assessed using purified RNA. The Takara SMART-Seq v4 low input RNA kit was used for cDNA preparation. NEBNext Ultra II FS DNA kit was used to create the sequencing library. Samples were sequenced at 2 X 151 bp in the paired ends. Trimmed Fastq reads were assessed for quality and adapter contamination with trimmomatic. Ensemble mouse GRCm38/mm10 reference genome and gencode Release M16 gene annotation were used to map post-filtered reads using STARaligner. HTSeq counts

were used for expression quantification, DESeq was used for normalization and samples were log2 transformed for further analysis (126). Moderated t-test was used for differential expression analysis of samples (127). NOJAH (http://bbisr.shinyapps.winship.emory.edu/NOJAH/) was used to generate heat maps. A fold-change of 3 and an FDR cutoff of 0.05 was used to define the significantly differentially expressed genes. Cytoscape software v3.6.1 and ReactomeFI plugin was used for pathway analysis (128, 129).

Statistics

Data were analyzed using Prism version 5 (Graphpad, San Diego, CA) for MAC and are displayed as mean+SD unless otherwise specified. Survival differences were calculated in a pairwise fashion using the log-rank test. Applicable data were compared by student-test and 1-way or 2-way ANOVA with post-hoc Bonferroni tests run. Significance was considered as a p-value of $\leq$ 0.05.

2-4-0 Results

G-CSF mobilized pDC from spleens are surrogate for G-mobilized pDC

G-mobilized splenocytes were used as a surrogate for G-mobilized blood, both in order to obtain sufficient numbers of G-mobilized pDC, and based upon previous reports that the spleen contains twice as many bone marrow-derived hematopoietic progenitors as the entire blood volume of a mouse following seven days treatment with Kit-ligand (130). To explore differences in donor pDC between BM and G-mobilized grafts, we used FACS to purify pDC from the bone marrow, spleens of untreated mice, and splenocytes of mice treated with 300μg/kg of G-CSF (Figure 1A). Post-sort purity of greater than 90% was achieved for FACS-isolated pDC populations (Figure 1B). FACS isolated G-mobilized pDC were morphologically similar to BM pDC and an equal percentage of G-mobilized pDC were able to produce IFNα compared to their BM derived counterparts (Figure 1C-D), verifying their identity as authentic pDC (119). G-CSF mobilization in mice mirrored what is commonly observed in humans, as G-CSF treatment increased the number of leukocytes in each graft source more than 2-fold, although the percentage of pDC in G-mobilized splenocytes was slightly lower, a trend reported by other researchers (50).

Addition of BM pDC to purified HSC and T cell grafts result in better transplant outcomes than adding G-mobilized pDC

We next asked the question of whether transplant outcomes would vary according to whether donor pDC were isolated from either BM or G-mobilized splenocytes. B10.BR recipient mice received 5,000 FACS-isolated HSC from unstimulated marrow, 10^6 MACS-isolated T cells from unstimulated splenocytes, and 50,000 FACS-isolated pDC from unstimulated BM or G-mobilized splenocytes from C57BL/6 donor mice. Recipients of HSC alone had a 40% survival rate, with moribund mice showing pancytopenia and only 50% donor chimerism at day 30, consistent with death from graft rejection. The addition of T cells to the HSC graft increased survival to 60%. The addition of BM pDC to the graft containing HSC and T cells increased survival to 85% (Figure

2A). Recipients of G-mobilized pDC achieved >80% donor chimerism on day 10 compared with <20% donor chimerism in recipients of BM pDC, consistent with the superior graft-facilitating activity of G-mobilized pDC reported in human HSCT (131). By day 30 post-transplant donor chimerism was ~100% for all groups that received donor T cells (Figure 2B). GvHD severity in the groups that received donor pDC was similar until 30 days post-transplant, when recipients of HSC and T cells, with G-mobilized pDC had higher average GvHD scores than recipients of BM pDC (Figure 2C). Histological analyses of the small intestine, a GvHD target organ, showed that recipients of BM pDC had limited GvHD compared to recipients of G-mobilized pDC (Figure 2D-E) (132). Recipients of G-CSF pDC developed wasting scaly skin typical of chronic GvHD (Figure 2F). Moribund mice were euthanized due to weight loss and inactivity, consistent with GvHD-related mortality.

CCR9 expression by BM pDC is necessary for their ability to increase survival

Because chemokine receptors play an important role in the migration and homing of pDC to hematolymphoid and GvHD target organs (30), we next tested levels of chemokine receptor expression on pDC from BM versus G-mobilized grafts. Based on comparisons of human pDC from BM and G-mobilized grafts (133), we focused on CCR9 expression, which facilitates pDC migration to the gut (134) and CCR7, which facilitates pDC migration to lymph nodes (135). Consistent with prior comparison of pDC from BM and G-mobilized grafts in humans (133), CCR9 and CD62L expression were significantly higher in murine BM pDC compared to G-mobilized pDC, with no significant difference in CCR7 (Figure 3A). Expression of CCR9 in G-mobilized pDC was significantly lower than untreated splenic pDC (Supplemental Figure 1). To determine the role of CCR9 expression in pDC function, we performed C57BL/6→B10.BR transplants using CCR9 KO mice as the source of pDC. There was 0% survival in mice that received CCR9 KO BM pDC, compared with 70% survival among recipients of wild-type (WT) donor BM pDC (Figure 3B). In

addition, recipients of CCR9 KO donor pDC had higher clinical GvHD scores late post-transplant, prior to their death or euthanasia (Figure 3C).

We have previously shown that BM pDC homed to and persisted in the gut and mesenteric lymph nodes for up to 21 days post-transplant (136). We next determined whether differential CCR9 expression affected pDC migration to the gut. Using luciferase$^+$ C57BL/6 mice for donor pDC, we transplanted purified HSC, T cells, and BM or G-mobilized pDC into B10.BR recipient mice. Initial migration of donor pDC to the gut was equivalent comparing recipients of BM or G-mobilized luciferase$^+$ pDC, with BLI signals still detectable in the abdomen for up to 3 weeks post-transplant, and with recipients of luc+ G-mobilized pDC also showing BLI signals coming from donor pDC in skin (Figure 3D-E).

Since gut-homing was not abrogated in pDC from G-mobilized pDC donors, which had lower CCR9 surface expression, and CCR9 expression on pDC is also a marker of their tolerogenic state (95), we tested the ability of donor pDC to limit T cell proliferation in the gut. We transplanted purified HSC, T cell, and BM, G-mobilized, or CCR9KO pDC using the C57BL/6→B10.BR model, using luciferase$^+$ donor T cells. Donor T cell proliferation in the gut was lowest among recipients of BM pDC over the course of 11 days compared with recipients of G-mobilized pDC and recipients of CCR9KO pDC (Supplemental Figure 2A-B).

Bone marrow pDC signal T cells with cytokines, while G-mobilized pDC activate T cells through antigen cross-presentation

Because recipients of CCR9 KO pDC had greater donor T cell expansion, and CCR9 expression is also a marker of tolerogenic pDC that facilitate induction of Treg, we next explored other mechanisms by which donor pDC might regulate the immunological status of donor T cells and suppress alloreactivity (95). We examined the cytokine profiles of pDC from BM and G-mobilized

grafts, focusing on expression of IL-10 and IL-12, “signal 3” cytokines produced by DC that regulate T cell polarization (137). BM or G-mobilized splenocytes were stimulated with CpG for 9 hrs. BM pDC secreted significantly more IL-10 and IL-12 than G-mobilized pDC (Figure 4A). To determine whether IL-10 production by BM pDC is responsible for their favorable effect on post-transplant immunity, C57BL/6→B10.BR transplants were performed using IL-10 KO mice as the source of donor pDC, with purified HSC and T cells. Surprisingly, the absence of IL-10 in donor pDC increased survival and decreased GvHD as compared to WT BM pDC (Supplemental Figure 3A-B), indicating that higher IL-10 production by donor BM versus G-mobilized pDC could not explain the diminished level of GvHD seen with BM pDC.

We next examined the role of IL-12 in BM pDC function using FACS-isolated donor BM pDC from IL-12 KO mice in the C57BL/6→B10.BR transplant model as described above. Absence of IL-12 in donor pDC resulted in 60% survival in allotransplant recipients, compared to 90% among recipients of WT BM pDC (Figure 4B). GvHD scores of mice receiving IL-12 KO BM were higher than those that received WT BM pDC (Figure 4C).

Finally, because pDC may regulate T cell function via cytokine or antigen cross-presentation (88, 124, 138), and G-mobilized pDC expressed significantly more CD86 and MHC II than BM pDC (Figure 4D), more MHC II than non-mobilized splenic pDC (Supplemental Figure 4) and less IL-10 and IL-12 compared with BM pDC, we next examined the ability of pDC to cross-present the TEα peptide antigen. Using purified TEα T cells and peptide, G-mobilized pDC presenting the TEα peptide were able to induce significantly more Th1 polarization of the transgenic T cells than BM pDC (Figures 4E).

G-mobilized pDC produce inflammatory cytokines post-transplant

To examine the functional interactions of pDC with donor T cells *in vivo*, we next assessed T cell cytokine profiles and expansion of specific T cell subsets using the C57BL/6→B10.BR transplant model with purified HSC, T cells, and either BM or G-mobilized pDC. Intracellular staining showed similar levels of IFNγ synthesis from $CD4^+$ T cells comparing groups on day 3 post-transplant, but more day 10 IFNγ synthesis was observed in $CD4^+$ T cells among mice that received G-mobilized pDC (Figure 5A), with no significant difference in TNFα and IL-17 production in $CD4^+$ T cells across groups (data not shown). On day 10 post-transplant significantly fewer Treg were seen in mice that received G-mobilized donor pDC compared with BM pDC (Figure 5B).

We have previously published that BM pDC respond to IFNγ *in vivo* with enhanced IDO production, and IDO produced by donor pDC induce donor Treg, thereby limiting GvHD (136). We performed a time-dependent kinetic analysis of IDO production in BM or G-mobilized pDC following *in vitro* IFNγ stimulation. BM pDC increased IDO production over 48 hours while IDO production in G-mobilized pDC transiently decreased from 12 to 24 hours, then rebounded at 48 hours (Figure 5C). Additionally, using the C57BL/6→B10.BR model, transplanting donor pDC lacking the genes encoding IFNγR or IDO increased donor T cell proliferation, consistent with the diminished ability of IFNγR or IDO KO donor pDC to limit GvHD (Supplemental Figure 5A-B).

Next, we performed a C57BL/6→B10.BR transplant and analyzed cytokine levels in the serum of recipients at 10 days post-transplant. Mice that received grafts containing G-mobilized pDC had significantly higher levels of inflammatory cytokines involved in the Th17 pathway (TGFβ, IL-1) and a non-significant trend towards higher IL-17 levels (Figure 5D) (139). Additionally, other inflammatory cytokines, TNFα and IL-31, were significantly lower in recipients of BM pDC (Figure 5E).

The source of donor pDC did not affect GvL activity of donor T cells

Finally, to determine whether the ability of BM pDC to limit GvHD affected the GvL activity of concomitantly transplanted T cells, we transplanted FACS isolated HSC, T cells, and pDC in a B10.BR→C57BL/6 murine C1498 graft-versus-leukemia transplant model. Recipient mice received 11Gy irradiation on day -2, 50,000 luciferase^{+} C1498 cells on day -1, and 5,000 FACS-isolated HSC, 10^{6} MACS-isolated T cells, and 50,000 FACS-isolated BM or G-mobilized pDC on day 0. Serial measurement of tumor burden by bioluminescence and survival showed no significant difference between groups that received pDC (Figure 6A-C). Consistent with these results, we also found no difference in survival or tumor burden with the use of donor BM versus G-mobilized pDC on the GvL effect of donor T cells in C57BL/6→B10.BR transplants with the B10.BR LBRM tumor cell line (Supplemental Figure 6A-C).

BM pDC and G-mobilized pDC have distinct gene expression profiles

To explore differences in BM and G-mobilized pDC gene expression that could confirm findings herein, we purified RNA from FACS-isolated pDC from bone marrow or spleens from mice that were treated with PBS (BM) or from spleens of mice that were treated with 300μg/kg of G-CSF (G-mobilized) for 5 consecutive days. The Takara SMART-Seq v4 low input RNA kit was used for cDNA preparation. NEBNext Ultra II FS DNA kit was used to create the sequencing library for sequencing. Sequencing results are available at the NCBI Sequence Read Archive, accession SRP155387. In an unsupervised clustering analysis, non-mobilized pDC from the spleen had a gene expression profile intermediate between splenic G-mobilized pDC and BM pDC, consistent with the effects of egress from the BM microenvironment but not G-CSF treatment on gene expression in pDC (Figure 7A). Comparing gene expression and pathway analysis between BM pDC and G-mobilized pDC using a moderated t-test and Reactome FI plugin in Cytoscape, respectively, we found that BM pDC had a distinct gene expression profile from G-mobilized pDC, significantly upregulating 22 genes, and downregulating 100 genes (Figure 7B). Of note, T cell

polarization induction and T cell co-stimulatory pathways were downregulated in BM pDC compared to G-mobilized pDC.

2-4-0 Discussion

While HSCT remains a curative treatment for relapsed blood cell malignancies, it has significant morbidity and mortality related to GvHD (140). Recipients of BM or G-mobilized grafts from unrelated donors have equal survival rates, but G-mobilized graft recipients have a higher incidence of chronic GvHD (45). Analysis of the donor cells in the BM and G-mobilized grafts in BMTCTN0201 showed that recipients of BM grafts with higher numbers of pDC had increased survival and lower treatment related mortality, while stratification of G-mobilized grafts by their content of donor pDC did not show significant survival differences (97). These clinical observations raised the question of why the activity of pDC varied by graft source. Herein, we document differences in mechanisms by which murine donor pDC regulate donor T cell activation and GvHD, depending on the tissue source of donor pDC: BM or G-mobilized grafts.

The current data confirms our previous studies, that BM donor pDC attenuate GvHD without diminishing GvL activity. The C57BL/6→B10.BR transplant model using FACS- and MACS-purified donor cells has aspects of both acute and chronic GvHD, a heterogeneity that is commonly seen in human HSCT recipients (131). We have previously described differences in chemokine receptor expression between pDC from either BM or G-mobilized human grafts (133) and BM pDC homing to the gut in a murine transplant model (136). One mechanism for the differential inhibition of GVHD but not GvL by donor pDC is homing of these cells to GvHD target tissues. Our results showed that BM and G-mobilized pDC migration to the gut is similar, thus migration to GvHD target organs alone is not the major factor influencing GvHD outcomes. Differences in gene expression between BM and G-mobilized or CCR9KO pDC did affect donor T cell proliferation in the gut, with less T cell proliferation among recipients of BM pDC, suggesting that the higher CCR9 expression in BM pDC indeed denotes tolerogenic function. GvHD is known to limit maturation of pDC and gut GvHD may eliminate donor pDC resident in gut tissue (141). Thus, the ability of BM pDC to limit T cell proliferation plays a role in the local persistence of donor

pDC in GVHD target organs. Contrary to this, the mature phenotype of donor G-CSF pDC may decrease their susceptibility to inflammation-induced elimination.

We have previously demonstrated that pDC production of IL-12 and IDO play a role in limitation of GvHD, and that IDO production from pDC *in vivo* are induced by donor T cell IFNγ, leading to Treg generation (136, 142). Remarkably, even though pDC isolated from G-mobilized grafts have a similar phenotype, we show that activated G-mobilized pDC synthesize markedly less IL-12, previously reported as a consequence of G-CSF administration (51), and have altered responsiveness to IFNγ, thus limiting their ability to regulate GvHD through T cell-pDC signaling. We now demonstrate that while both BM and G-mobilized pDC can induce Th1 polarization of donor T cells, Th1-induction by BM pDC is IL-12 mediated, while G-mobilized pDC contribute to Th1 polarization via cross-presentation of peptide antigen. Furthermore, with increased time exposure of BM pDC to IFNγ, there is a continuous increase in production of IDO, an immune regulating enzyme that catabolizes tryptophan, negatively affecting T cell growth and survival (143). This time-dependent effect is not seen in G-mobilized pDC, in which IDO expression transiently declines following IFNγ exposure.

In contrast to BM pDC, G-mobilized pDC have increased ability for antigen cross-presentation and create an immunogenic microenvironment, ultimately resulting in greater persistence of activated donor T cells and increased incidence and severity of GvHD. In accordance with other reports, we demonstrate that activation of G-mobilized pDC in transplant recipients initiates signaling pathway leading to activation of Th17 cells and increased GvHD post-transplant (46). Additionally, mice that received BM pDC have significantly more Treg 10 days post-transplant than mice that received G-mobilized pDC. In contrast to the generation of Treg by donor BM pDC, transplanting G-mobilized pDC led to higher levels of TGFβ, a cytokine that induces both Th17 and Treg polarization (139), and IL-1, a cytokine that increases Th17 differentiation while reducing

Treg differentiation through splicing of FoxP3 (144). We interpret these data to suggest that even with transiently high levels of IDO, the increased TGFβ post-transplant in recipients of G-mobilized pDC, coupled with increased IL-1 leads to an imbalance between Tregs and Th17 cell types, a common feature of chronic GvHD (145). Furthermore, BM pDC downregulate Th1, Th2, and Th17 polarization induction pathways, enhancing their ability to limit GvHD. Additionally, IL-31, associated with atopic dermatitis (146), was increased in the serum of mice that received G-mobilized pDC, and these mice had the scaly skin typical of chronic GvHD. Taken together, these data indicate that the immunological consequence of transplanting G-mobilized pDC is a pro-inflammatory state that ultimately results in more GvHD.

Although our proposed mechanism of BM pDC's role in both activating donor Th1 polarized donor T cells and limiting GvHD may appear paradoxical, we demonstrate that these processes occur sequentially due to bi-directional interactions and signaling between donor pDC and T cells. We also show that induction of Th1 cells is cytokine, not antigen presentation mediated. This coupled with downregulation of genes involved with Th1 induction in cognate T cells may help limit the expansion of Th1-polarized T cells that cause GvHD in recipients of BM pDC. IFNγ has been reported to promote a pro-inflammatory environment which aids in engraftment and contributes (147). Alternatively, IFNγ can also induce IDO expression, suppressing inflammation (148-152). Our data are consistent with a model in which initial pro-inflammatory activity of BM pDC is mediated through IL-12 secretion that induces Th1 cells that facilitate donor HSC engraftment, reconstitution of the hematopoietic system, and GvL activity (136, 142). The data also suggest that the inflammatory milieu generated by BM pDC induces donor Treg (via feedback from IFNγ-producing T cells) that suppress inflammatory responses and limit GvHD (Figure 8). These data highlight significant differences in post-transplant immune reconstitution between BM versus G-mobilized grafts and provide insights into the quality and function of pDC from different sources and the mechanisms by which pDC affect post-transplant outcomes.

The present study is not without limitations. In order to isolate the effect of G-CSF mobilization on pDC, we transplanted highly purified immune cell populations, and the current results do not preclude contributions of other cell populations in the graft (e.g. NK cells) from regulating post-transplant immunity and GvHD in a manner that is affected by the graft source (153). In order to facilitate collection of adequate numbers of FACS-isolated pDC following G-CSF mobilization we used spleen as the source of pDC rather than blood. In addition, it is possible that the beneficial effects of donor BM pDC in the graft could be recapitulated by exogenous administration of Th1 inducing cytokines, Th1 cytokines, and/or Treg inducing cytokines. Thus, in the future, developing methods to transiently induce Th1 polarization along with IFNγ response pathways and targeting the pathways that increase BM pDC content of donor grafts, offer a novel approach to limit GvHD without diminishing GvL activity, and improve transplant outcomes with less morbidity and mortality.

In summary, the present data uses established murine model systems of allogeneic HSCT to confirm the clinical benefit of increased number of donor BM pDC in allotransplant and the decreased levels of chronic GvHD seen in recipients of BM versus G-mobilized grafts (45, 97). While GvHD was reduced and survival improved in murine transplants comparing recipients of BM pDC versus G-mobilized pDC, there was no difference in GvL activity or survival, consistent with clinical data from BMTCTN0201 showing equivalent rates of relapse between recipients of BM versus G-PB grafts (45). In conclusion, these data suggest important functional differences in immune cells based upon graft source that should continue to be explored in clinical trials.

2-5-0 Figure Legends

Figure 1. G-CSF mobilization in mice. Mice were treated with PBS or 300μg/kg of G-CSF for 5 consecutive days and PBS treated bones and splenocytes or G-CSF treated splenocytes were examined by flow cytometry. (A) Representative dot plots of gating strategies for selection of pDC based upon surface markers as follows: Lineage$^-$ (CD3, CD11b, CD19, IgM, CD49b, Ter119) B220$^+$ CD11c$^+$ PDCA1$^+$. (B) Representative dot plots of post sort purity. (C) FACS purified pDC from bones and spleens of PBS treated mice and spleens of G-mobilized spleens were imaged following cytospin. (D) Whole BM or whole splenocytes were stimulated with CpG for 9 hrs. at 37°C. Percentage of pDC from BM and G-mobilized grafts positive for IFNα production by intracellular cytokine staining. n=3 per group. Statistical comparison using unpaired T test.

Figure 2. Bone marrow pDC increase survival and reduce GvHD more than G-mobilized pDC. (A) C57BL/6→B10.BR transplant recipients received 5,000 FACS isolated HSC, 1 million MACS isolated T cells, and 50,000 FACS isolated pDC as indicated. Survival of recipients. (B) Recipient chimerism 10 and 30 days post-transplant. (C) GvHD scores of Figure 2B transplant. * Indicates significance at that point between bone marrow and G-mobilized pDC recipients. (D) Representative histopathological samples of small intestine (600X) on day 15 post-transplant. (E) Histopathological scores of GvHD associated pathology. Histopathological grades of GvHD associated pathology. Graded 0-4. Score of 0: no pathology, 1: Apoptotic cells, 2: Crypt loss, 3: Contiguous crypt loss. (F) Picture of mice with magnification of tails depicting the scaly skin associated with GvHD pathology in recipients of G-mobilized grafts. n=28 per group. Mantel-Cox, two-way ANOVA with post-hoc Bonferroni and linear regression were run. *P < .05, **P < .01, ***P < .001.

Figure 3. CCR9 is important for BM pDC function. Mice were treated with PBS or 300μg/kg G-CSF for 5 consecutive days. (A) Expression of CCR4, CCR7, CCR9, CXCR3, and CD62L on pDC was examined by flow cytometry. (B) C57BL/6→B10.BR transplant recipients received 5,000 HSC, 1 million T cells, 50,000 wild-type or CCR9 KO BM pDC. Survival curve. (C) GvHD scores of recipients in Figure 3B. *Indicates significance at that time-point. n =12 per group. Two-way ANOVA with post-hoc Bonferroni, Mantel-Cox, and linear regression were run. *P < .05. (D) Murine recipients of a C57BL/6→B10.BR transplant received 5,000 HSC, 1 million T cells, 50,000 luciferase^{+} BM or G-mobilized pDC. Serial bioluminescent imaging of recipient mice with (E) quantification of bioluminescence. n=4 mice per group.

Figure 4. IL-12 secretion is key to BM pDC function. Mice were treated 5 consecutive days with PBS or 300μg/kg G-CSF. Cytokine production was assessed by flow cytometry. (A) Quantification of IL-10 and IL-12 production. (B) C57BL/6→B10.BR transplant recipients received 5,000 HSC, 1 million T cells, 50,000 wild-type BM pDC or IL-12 KO BM pDC. (B) Survival of recipients by treatment group. (C) GvHD scores of mice in Figure 4B. n=20 per group. (D) Expression of CD86 and MHC II on pDC. n=5 per group. (E) BM or G-mobilized pDC were incubated with T cells alone (control), T cells in a MLR (MLR), or TEα T cells and peptide (Peptide) for 72 hours. Tbet expression was assessed by flow cytometry. *I indicated significance of G-mobilized compared to all other groups. n=3 per group. Two-way ANOVA with post-hoc Bonferroni correction, Mantel-Cox, and linear regression were run. *P < .05, **P< .01.

Figure 5. Donor G-mobilized pDC induce inflammatory cytokines post-transplant. Mice were treated 5 consecutive days with either PBS or 300μg/kg G-CSF. Transplant recipients

received 5,000 FACS isolated HSC, 1 million MACS isolated T cells, 50,000 FACS isolated pDC as indicated in the legend. Intracellular, intranuclear, and serum analyses were conducted post-transplant and analyzed by flow cytometry and Luminex Multiplex, respectively. (A) IFNγ production by T cells 3 and 10 days post-transplant. (B) FoxP3 expression in T cells 3 and 10 days post-transplant. (C) IDO production by BM or G-mobilized pDC after IFNγ stimulation at 12, 24, and 48hrs. (D) IL-17, TGFβ, and IL-1 in serum of mice 10 days post-transplant. (E) TNFα and IL-31 in the serum of mice 10 days post-transplant. n=3-4. Two-way ANOVA with post-hoc Bonferroni were. *P < .05.

Figure 6. BM pDC limit GvHD without limiting GvL. Mice were treated 5 consecutive days with PBS or 300μg/kg G-CSF. B10.BR→C57BL/6 transplant recipients received 5,000, 1 million T cells, 50,000 BM or G-mobilized pDC. Recipient mice received 50,000 luciferase^{+} C1498 cells. (A) Survival of recipients. (B) Quantification of tumor burden by (C) serial bioluminescent imaging of recipient mice. n=20 per group.

Figure 7. BM pDC and G-mobilized pDC have distinct gene expression profiles, increasing BM pDC ability to limit GvHD. Mice were treated 5 consecutive days with either PBS or 300μg/kg G-CSF. BM pDC and G-mobilized pDC were sorted by FACS. The Takara SMART-Seq v4 low input RNA kit was used for cDNA preparation. NEBNext Ultra II FS DNA kit was used to create the sequencing library. (A) Heatmap depicting the significantly differentially expressed genes in the murine samples using z-score scaling, 1-pearson correlation distance, and ward.D clustering. Mapped as BM vs. G-CSF. The grey gene cluster represents genes

upregulated in BM samples, and the black gene cluster represents genes downregulated in BM samples. (B) Volcano plot of gene upregulation and downregulation (BM vs. G-mobilized).

Figure 8. Bone marrow pDC limit GvHD through donor pDC function. Model of pDC effect on donor T cells signaling cascades post-transplant.

2-6-0 Figures

Figure 1.

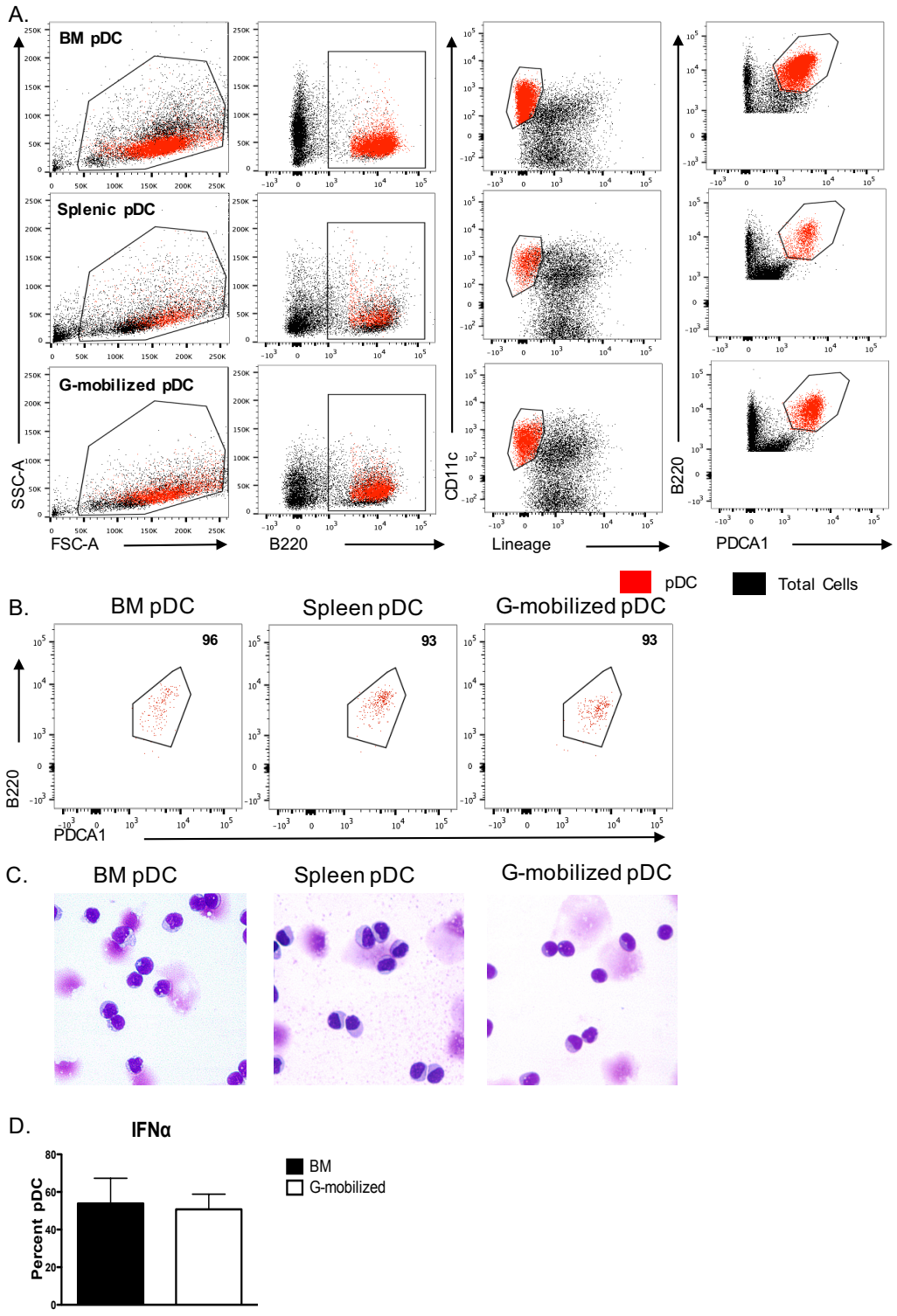


Figure 2.

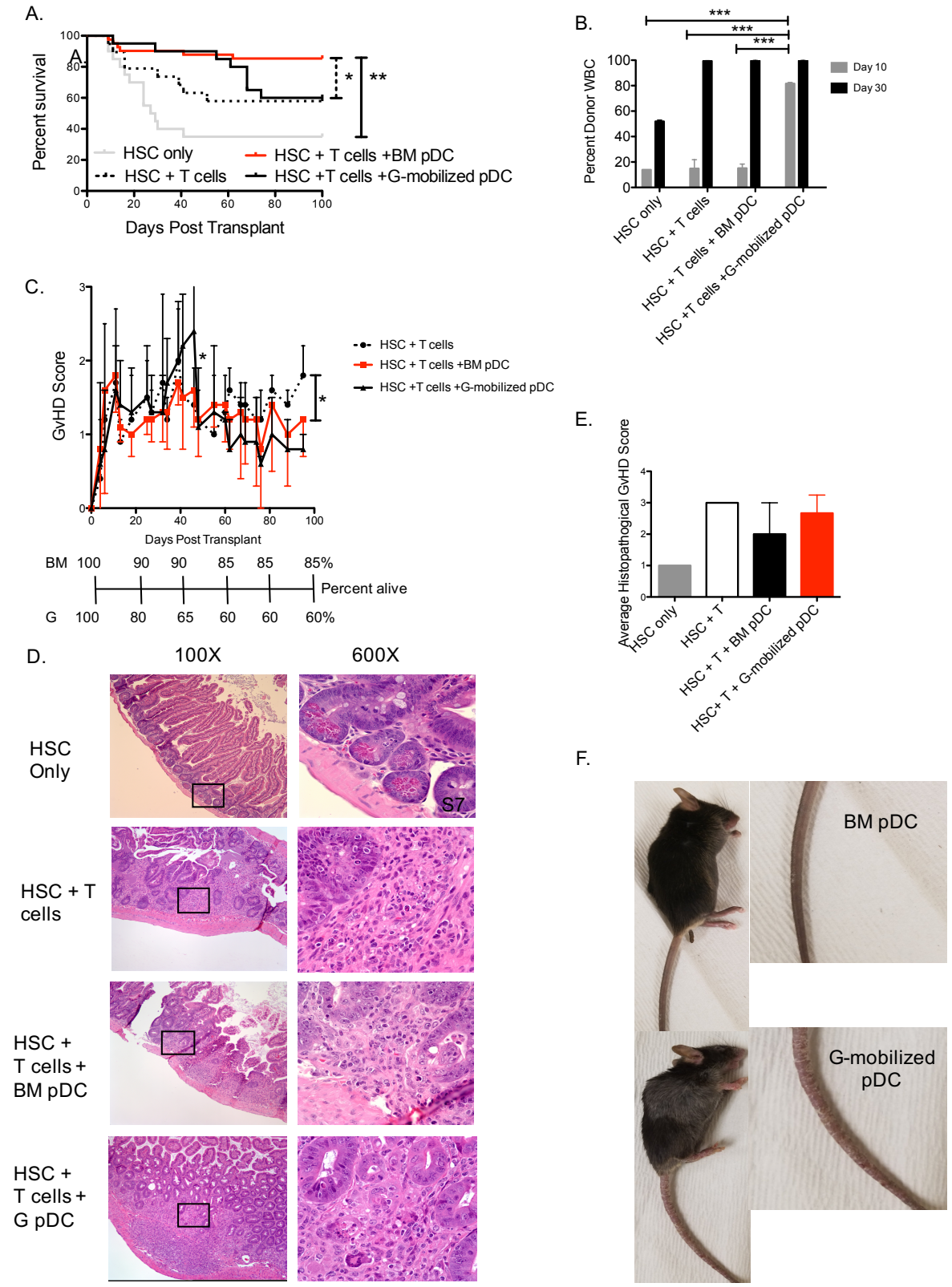

A.
Percent survival
Days Post Transplant
HSC only
HSC + T cells
HSC + T cells +BM pDC
HSC +T cells +G-mobilized pDC
*
**
B.
Percent Donor WBC
Day 10
Day 30
***
***
***
HSC only
HSC + T cells
HSC + T cells + BM pDC
HSC +T cells +G-mobilized pDC
C.
GvHD Score
Days Post Transplant
HSC + T cells
HSC + T cells +BM pDC
HSC +T cells +G-mobilized pDC
BM 100 90 90 85 85 85%
G 100 80 65 60 60 60%
Percent alive
E.
Average Histopathogical GvHD Score
HSC only
HSC + T
HSC + T + BM pDC
HSC+ T + G-mobilized pDC
D.
100X
600X
HSC Only
HSC + T cells
HSC + T cells + BM pDC
HSC + T cells + G pDC
S7
F.
BM pDC
G-mobilized pDC

Figure 3.

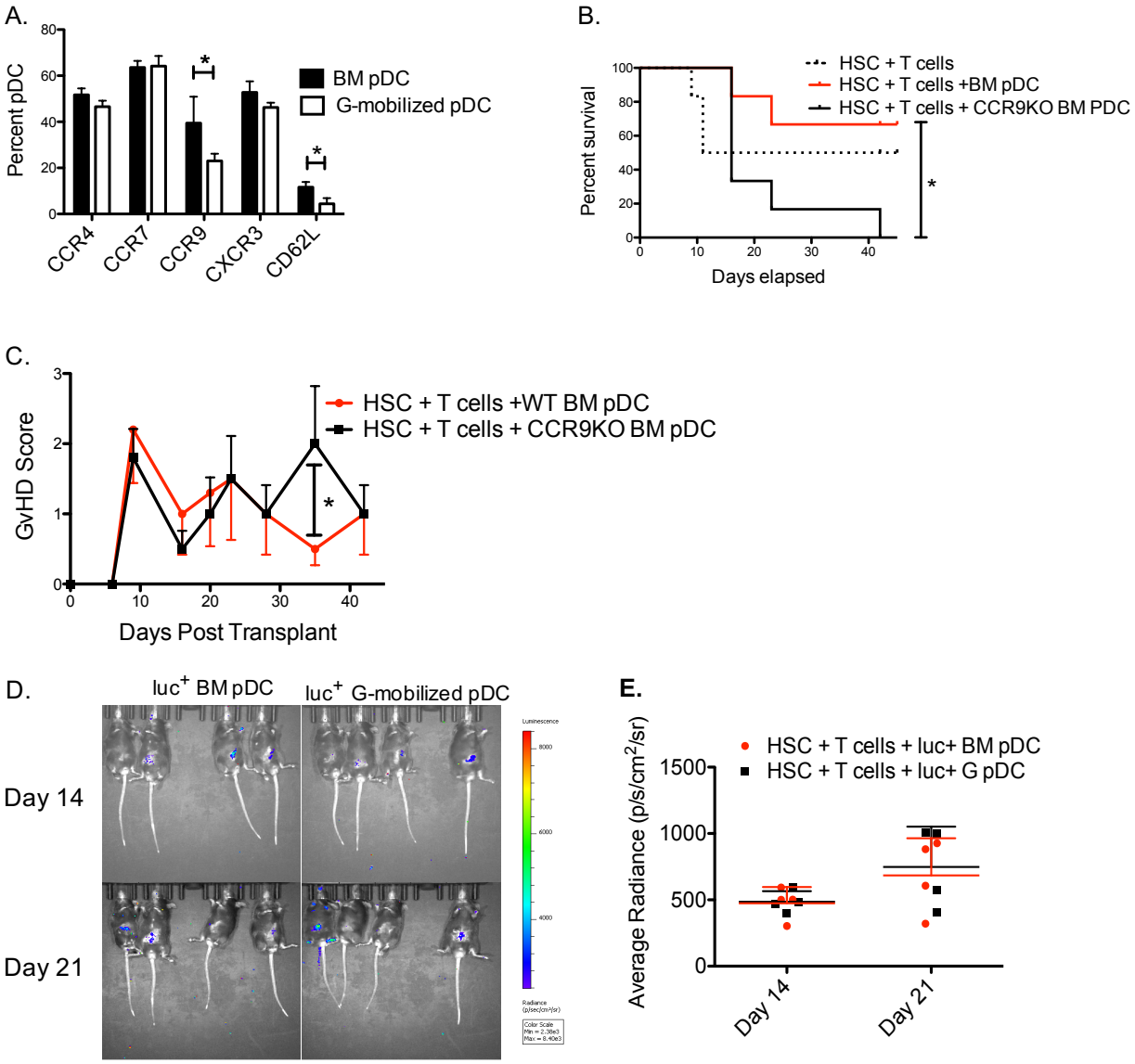

A.
Percent pDC
BM pDC
G-mobilized pDC
CCR4
CCR7
CCR9
CXCR3
CD62L
B.
Percent survival
HSC + T cells
HSC + T cells +BM pDC
HSC + T cells + CCR9KO BM PDC
Days elapsed
C.
GvHD Score
HSC + T cells +WT BM pDC
HSC + T cells + CCR9KO BM pDC
Days Post Transplant
D.
luc+ BM pDC
luc+ G-mobilized pDC
Day 14
Day 21
E.
Average Radiance (p/s/cm2/sr)
HSC + T cells + luc+ BM pDC
HSC + T cells + luc+ G pDC
Day 14
Day 21

Figure 4.

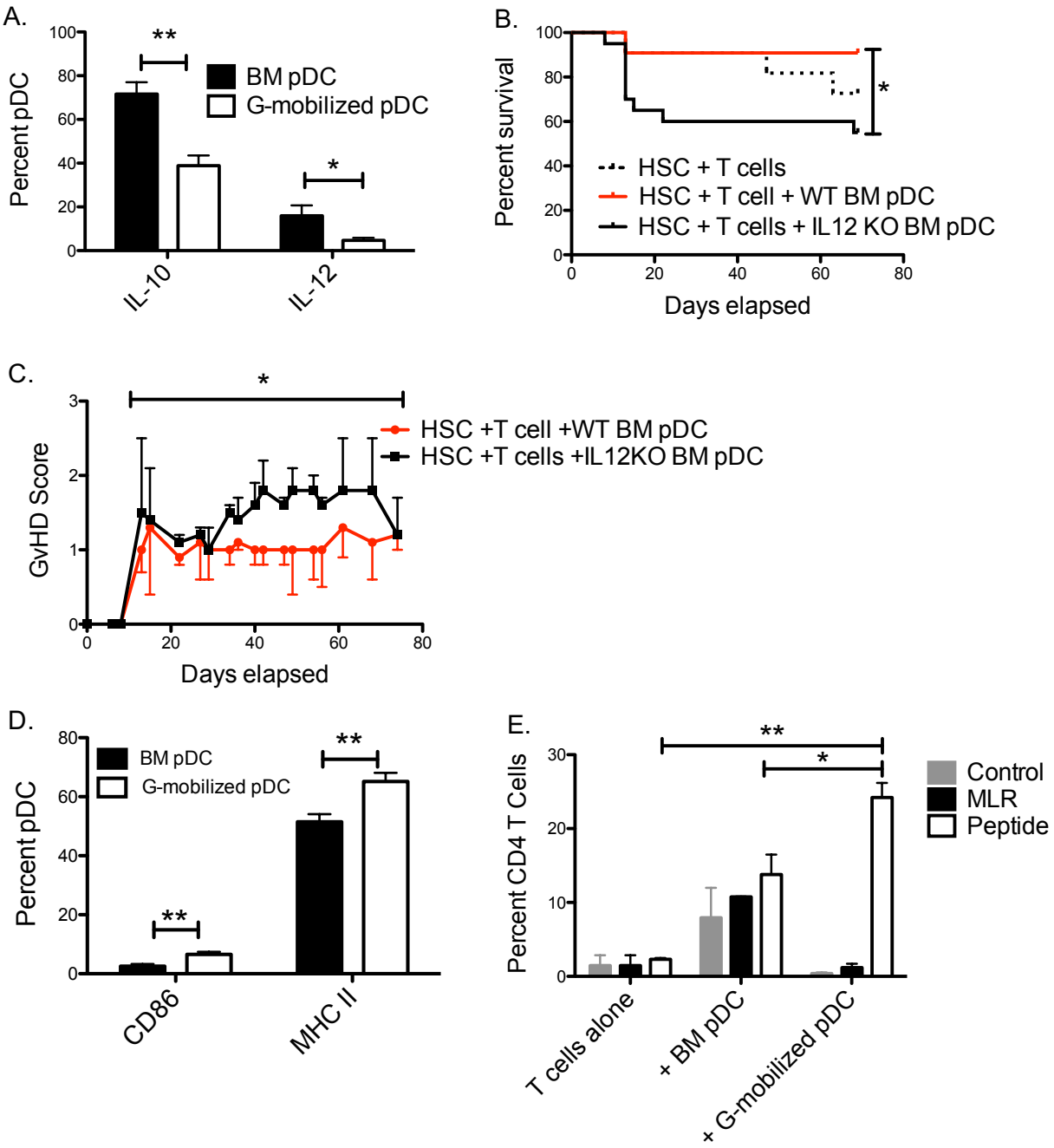

A.
Percent pDC
BM pDC
G-mobilized pDC
IL-10
IL-12
B.
Percent survival
HSC + T cells
HSC + T cell + WT BM pDC
HSC + T cells + IL12 KO BM pDC
Days elapsed
C.
GvHD Score
HSC +T cell +WT BM pDC
HSC +T cells +IL12KO BM pDC
Days elapsed
D.
Percent pDC
BM pDC
G-mobilized pDC
CD86
MHC II
E.
Percent CD4 T Cells
Control
MLR
Peptide
T cells alone
+ BM pDC
+ G-mobilized pDC

Figure 5.

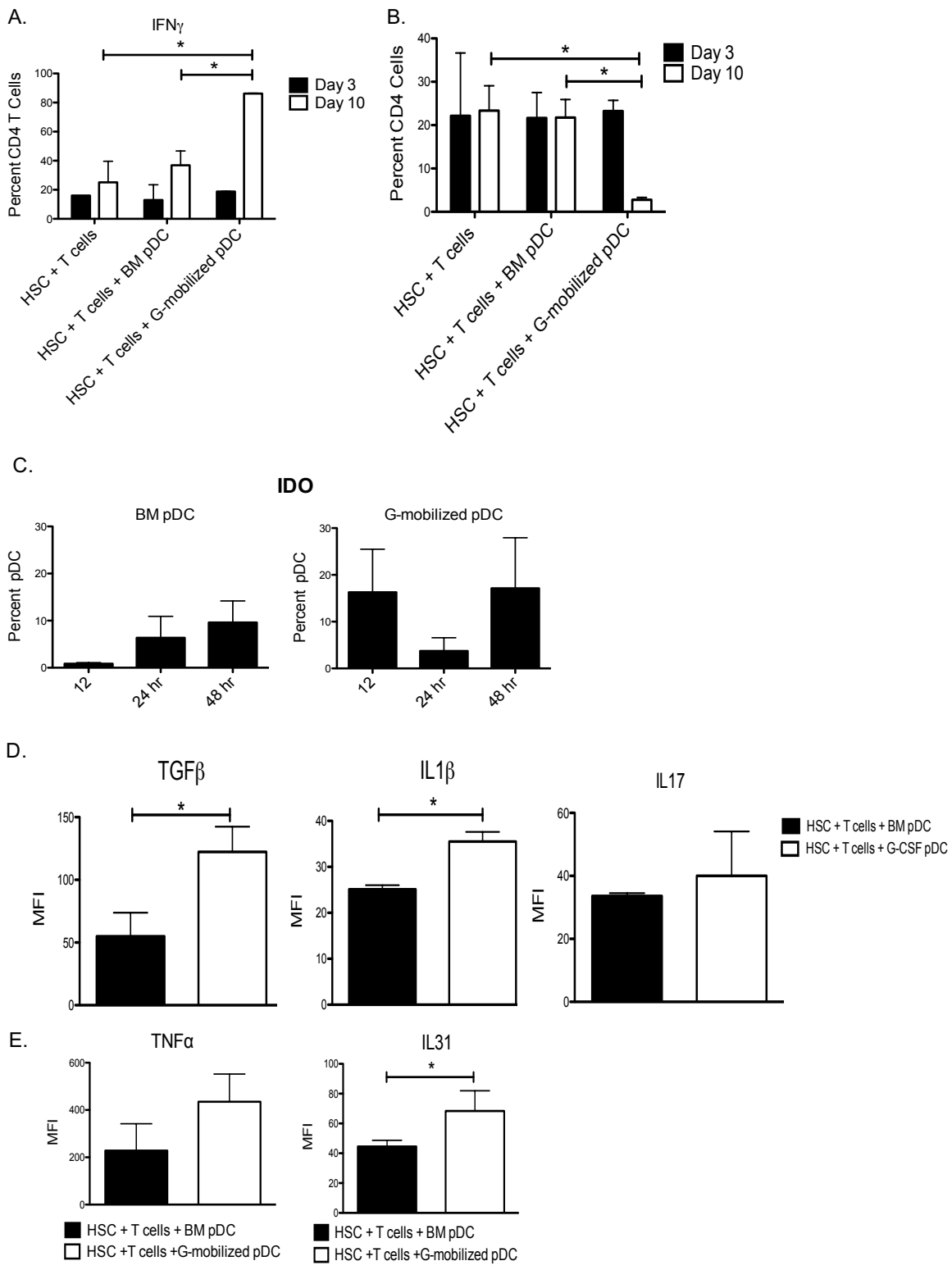

A.
IFNγ
Percent CD4 T Cells
Day 3
Day 10
HSC + T cells
HSC + T cells + BM pDC
HSC + T cells + G-mobilized pDC
B.
Percent CD4 Cells
Day 3
Day 10
HSC + T cells
HSC + T cells + BM pDC
HSC + T cells + G-mobilized pDC
C.
IDO
BM pDC
G-mobilized pDC
Percent pDC
12
24 hr
48 hr
D.
TGFβ
IL1β
IL17
MFI
HSC + T cells + BM pDC
HSC + T cells + G-CSF pDC
E.
TNFα
IL31
MFI
HSC + T cells + BM pDC
HSC +T cells +G-mobilized pDC

Figure 6.

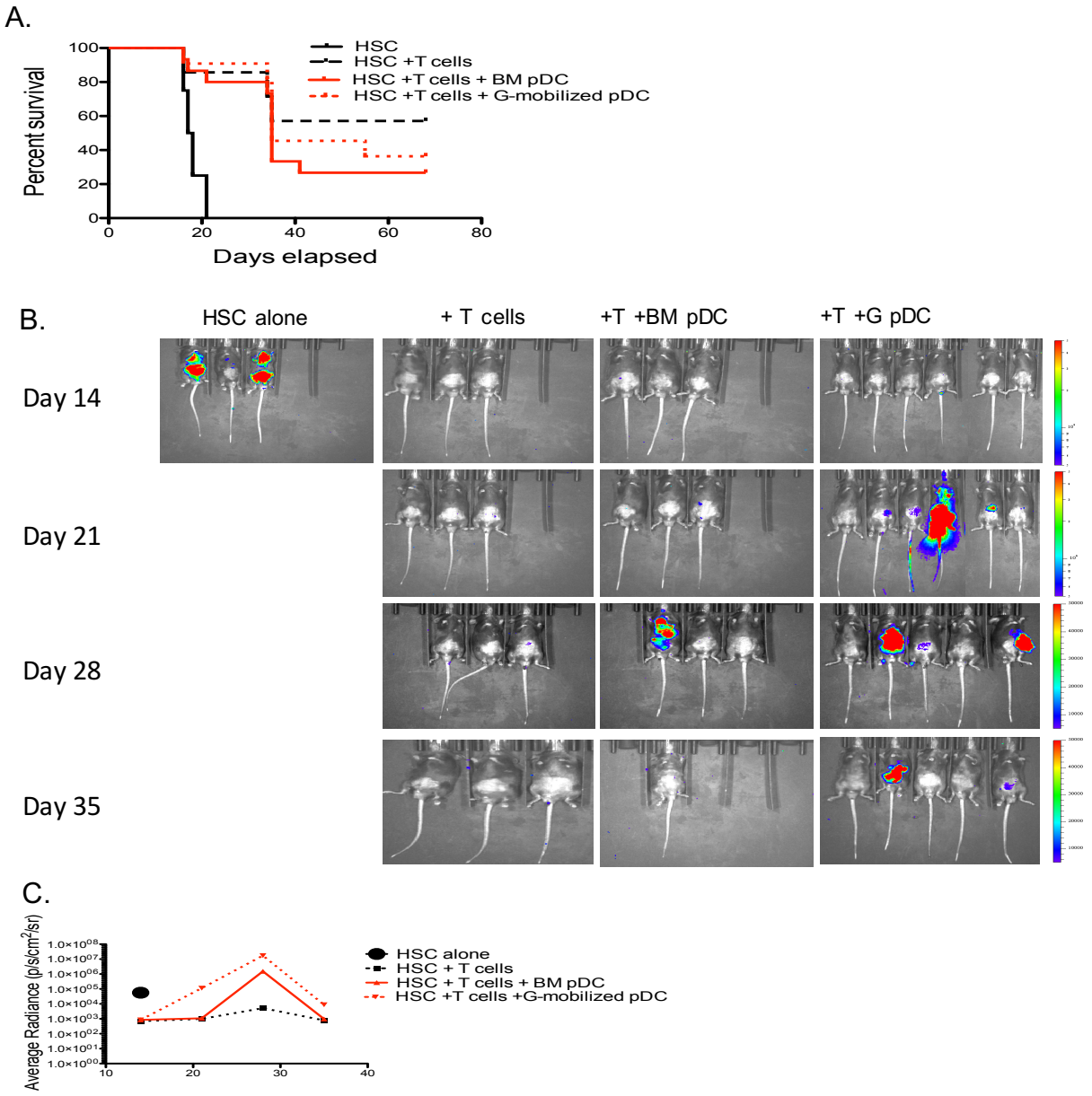

A.
Percent survival
Days elapsed
HSC
HSC +T cells
HSC +T cells + BM pDC
HSC +T cells + G-mobilized pDC
B.
HSC alone
+ T cells
+T +BM pDC
+T +G pDC
Day 14
Day 21
Day 28
Day 35
C.
Average Radiance (p/s/cm²/sr)
HSC alone
HSC + T cells
HSC + T cells + BM pDC
HSC +T cells +G-mobilized pDC

Figure 7

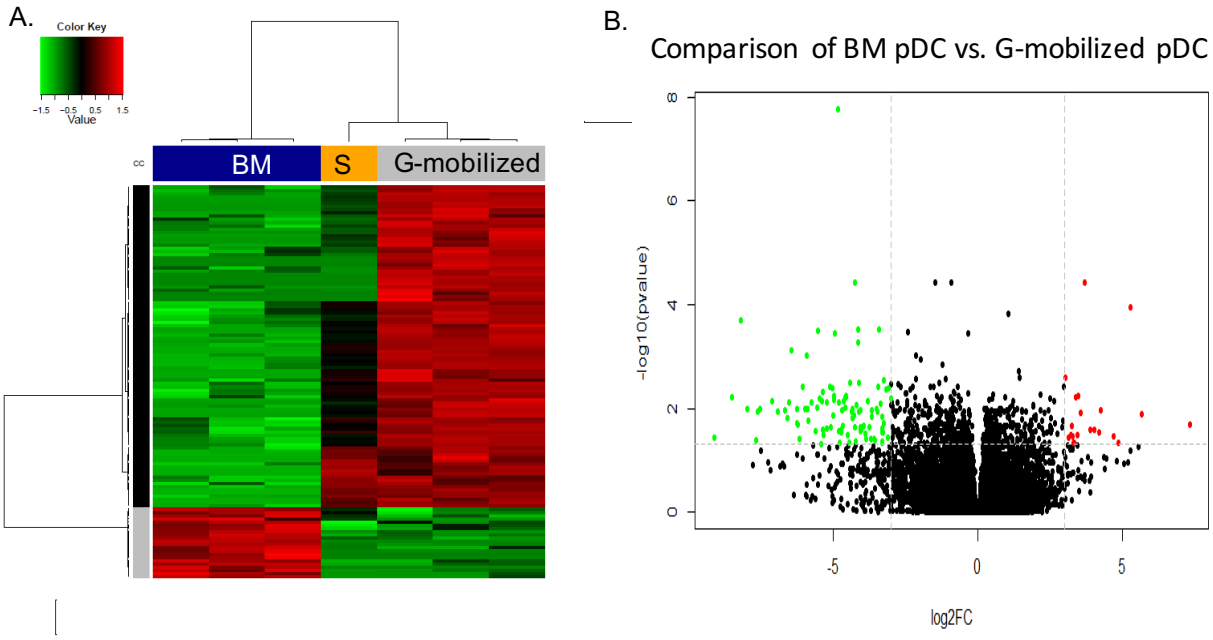
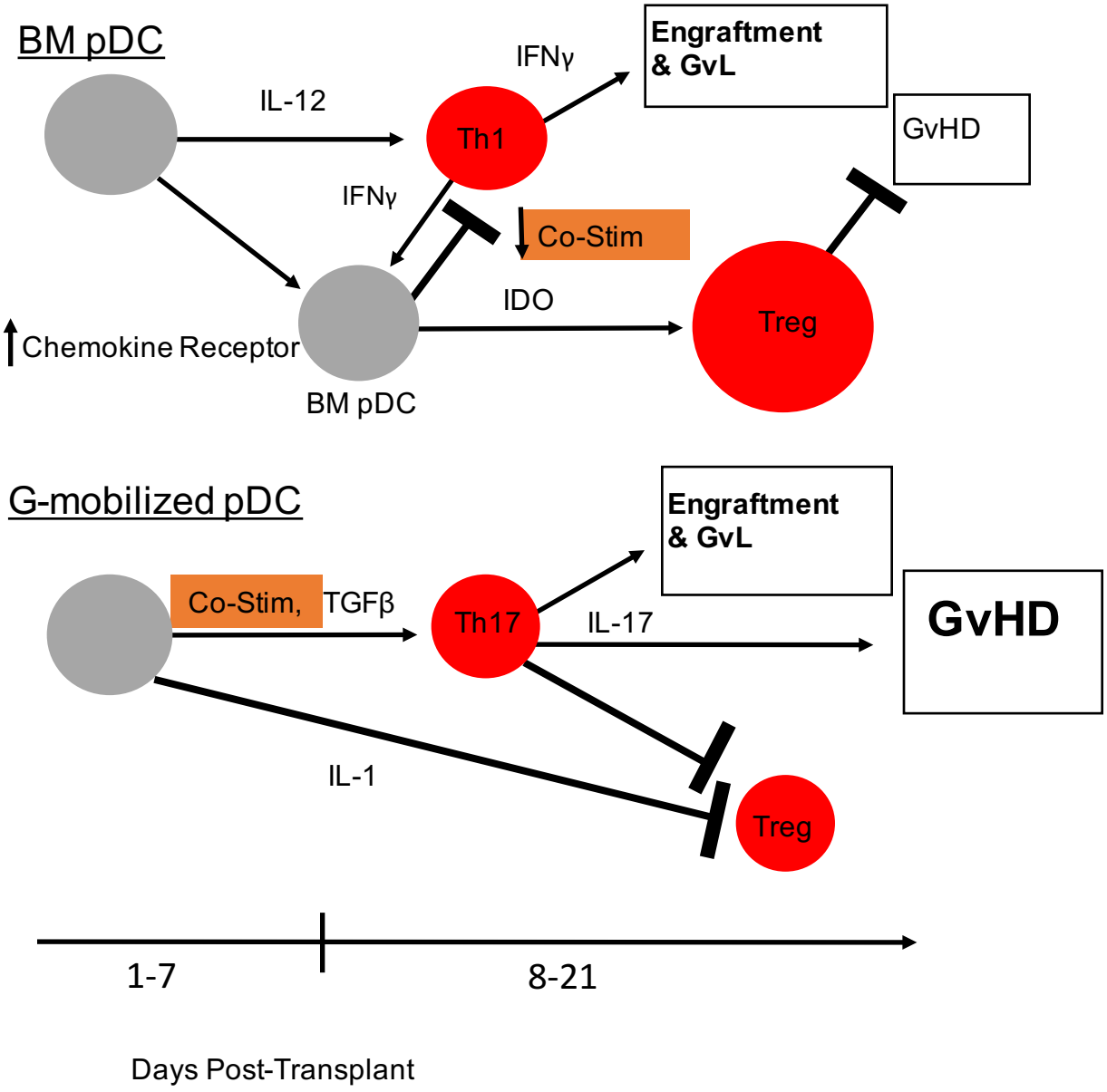

A.
Color Key
Value
BM
S
G-mobilized
B.
Comparison of BM pDC vs. G-mobilized pDC
-log10(pvalue)
log2FC

Figure 8.


BM pDC
IL-12
IFNγ
Th1
Engraftment & GvL
GvHD
IFNγ
Co-Stim
IDO
Treg
Chemokine Receptor
BM pDC
G-mobilized pDC
Co-Stim, TGFβ
Th17
Engraftment & GvL
IL-17
GvHD
IL-1
Treg
1-7
8-21
Days Post-Transplant

2-7-0 Supplementary Figure Legends

Supplemental Figure 1. G-CSF mobilization alters character of pDC. Mice were treated with PBS or 300μg/kg G-CSF for 5 consecutive days. Expression of CCR9 and CD62L on pDC was examined by flow cytometry. ****P < .0001.

Supplemental Figure 2. Lack of CCR9 increases T cell proliferation in the gut. Murine recipients of a C57BL/6→B10.BR transplant received 5,000 FACS sorted HSC, 1 million MACS sorted luciferase^{+} T cells, 50,000 FACS sorted BM, G-mobilized, or CCR9 KO pDC as indicated in the legend. (A) Serial bioluminescent imaging of recipient mice with (B) quantification of T cell proliferation.

Supplemental Figure 3. Absence of IL-10 increases survival. C57BL/6→B10.BR murine transplant recipients received 5,000 FACS isolated HSC, 1 million MACS isolated T cells, 50,000 FACS isolated WT or IL-10KO BM pDC. (A) Survival curve of murine transplants. (B) GvHD scores of mice from figure S2A. Results are from 2 independent experiments.

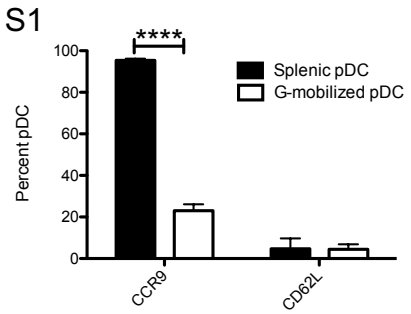
Supplemental Figure 4. G-CSF mobilization increases MHC II expression on pDC. Mice were treated with PBS or 300μg/kg G-CSF for 5 consecutive days. Expression of CD86 and MHC II on pDC was examined by flow cytometry.

Supplemental Figure 5. Absence of IDO production or IFNγR in BM pDC increase T cell proliferation. (A) C57BL/6→B10.BR murine transplant recipients received 2,500 FACS isolated HSC, 250,000 MACS isolated luciferase^{+} T cells, 50,000 FACS isolated WT, IDO KO or IFNγR KO pDC. Bioluminescent imaging of recipient mice 4 days post-transplant (B) T cell luminescence quantification. n=4 per group. *P < .05.

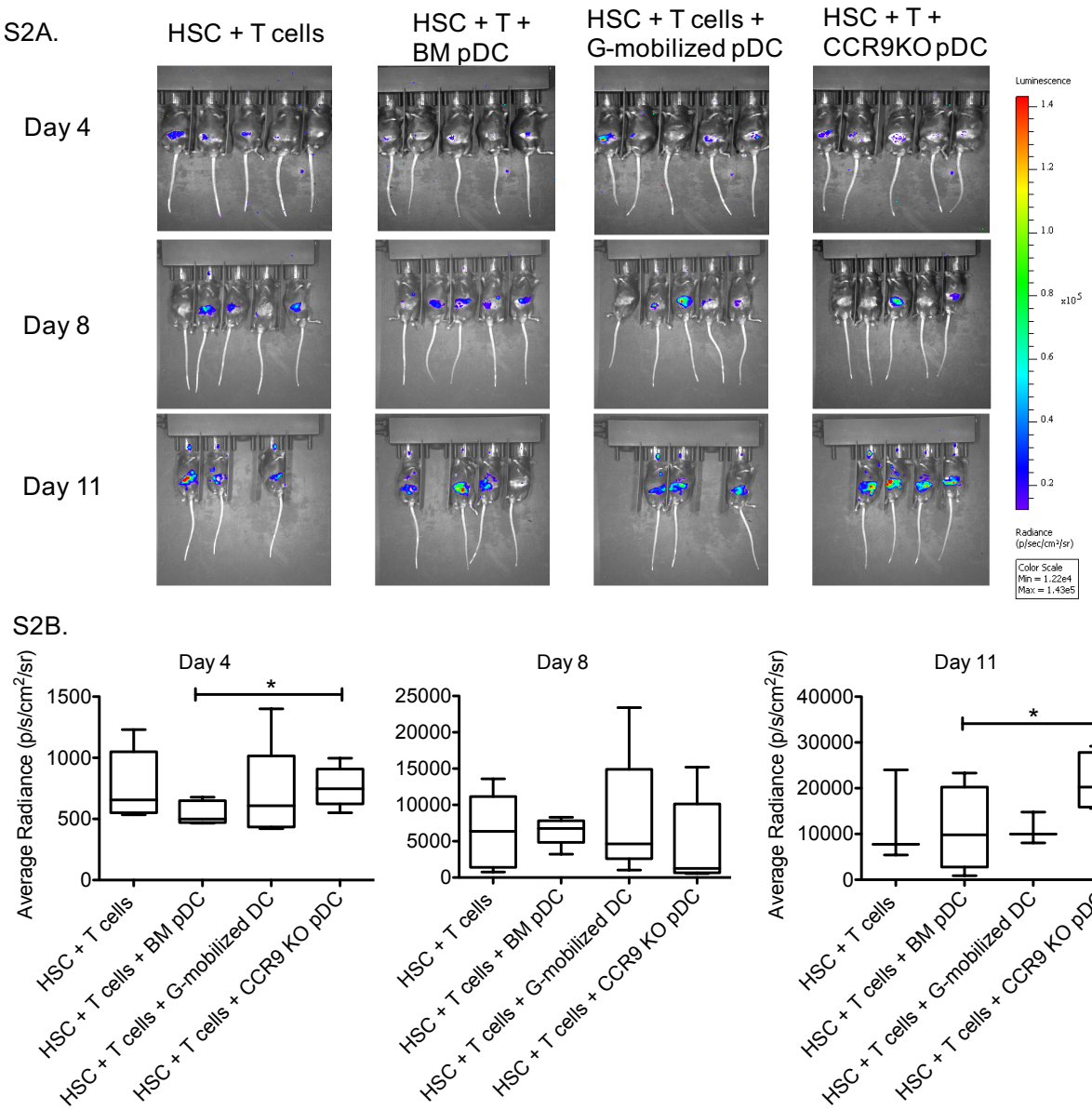
Supplemental Figure 6. BM pDC limit GvHD with equivalent GvL activity of G-PB pDC. C57BL/6→B10.BR murine transplant recipients received 5,000 FACS isolated HSC, 1 million MACS isolated T cells, 50,000 FACS isolated BM or G-mobilized pDC. Recipient mice received 50,000 luciferase^{+} LBRM cells. (A) Survival curve of murine transplant recipients. (B) Serial bioluminescent imaging of recipient mice. (C) quantification of tumor burden. Results from 2 independent experiments.

2-8-0 Supplementary Figures

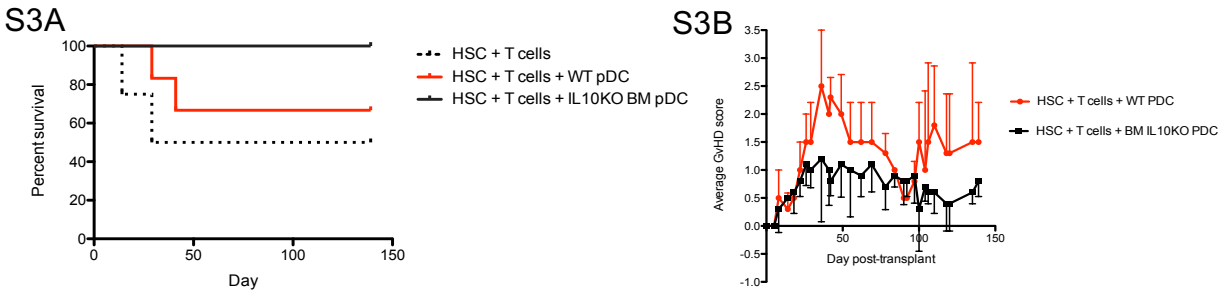
Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.



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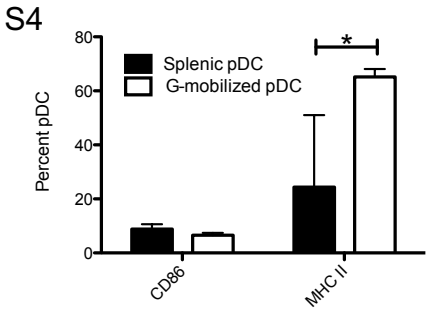

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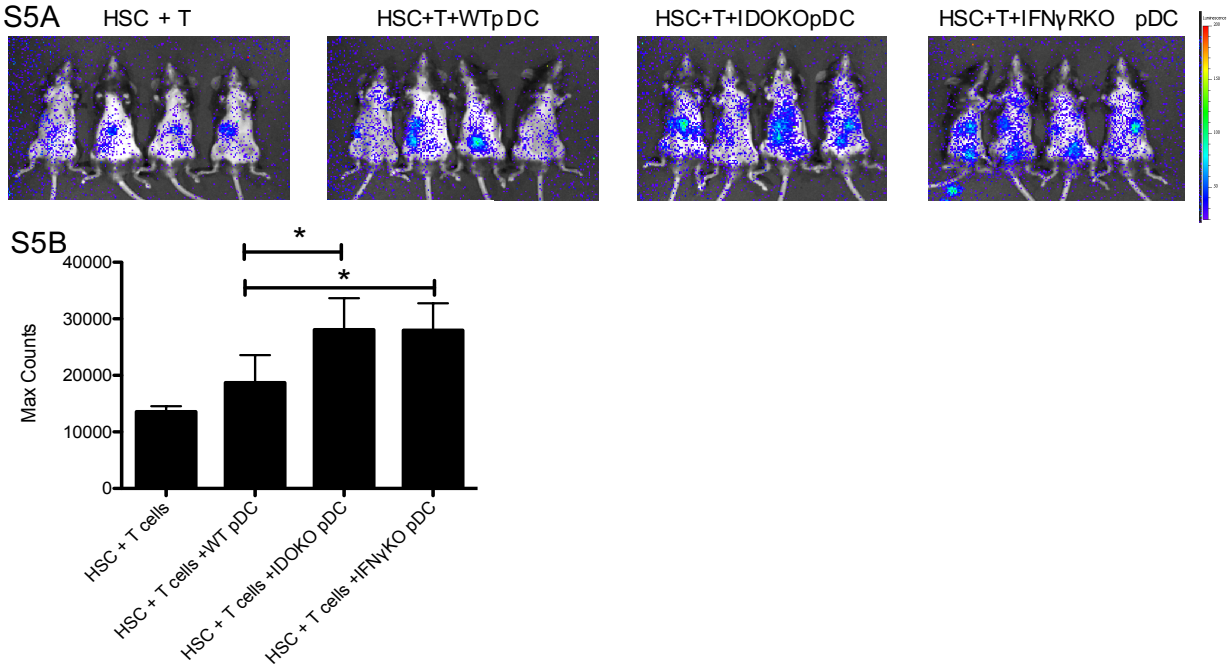
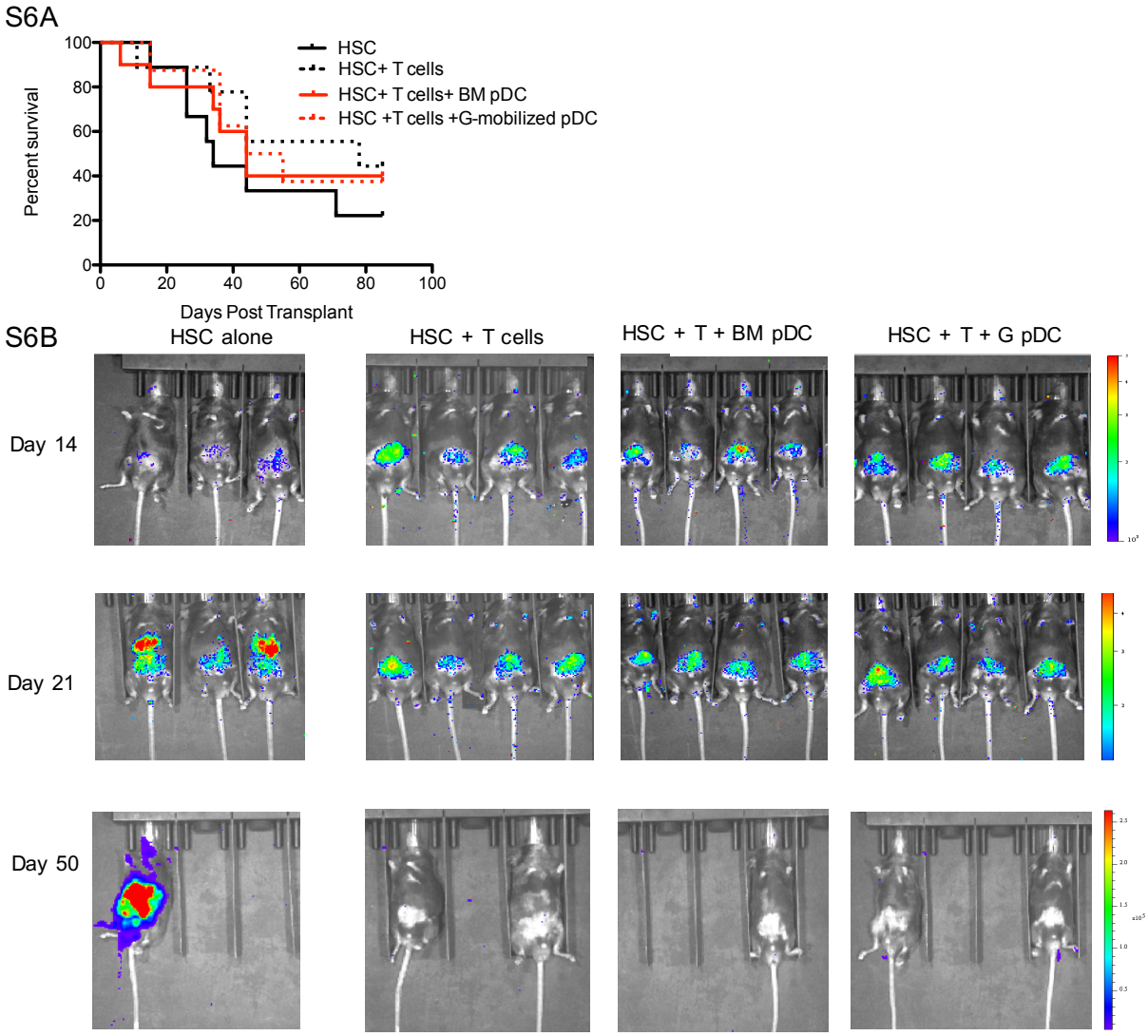


Supplemental Figure 6.



Chapter 3: FMS-like Tyrosine Kinase 3 Ligand Increases Plasmacytoid Dendritic Cell Content in Bone Marrow Grafts and Increases Survival and Decreases GvHD in Allogeneic Hematopoietic Stem Cell Transplantation

3-1-0 Abstract

Higher numbers of donor plasmacytoid dendritic cells (pDC) increased survival and reduced graft-versus-host disease (GvHD) in human recipients of unrelated donor bone marrow (BM), but not G-CSF peripheral blood grafts. In murine models, we have shown that donor BM pDC increase survival and decrease GvHD compared to G-CSF-mobilized pDC. To increase the content of pDC in BM grafts we studied the effect of FMS-like tyrosine kinase 3 ligand (Flt3L) treatment of murine BM donors on transplant outcomes. Flt3L treatment (300μg/kg/day) resulted in a schedule-dependent increase in the content of pDC in the marrow. Mice treated on days -4 and -1 had greater than 5-fold increase in pDC content without significant changes in numbers of HSC, T cells, B cells, and NK cells in the marrow graft. In an MHC mismatched murine transplant model, recipients of Flt3L-treated T cell depleted 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-transplant compared with recipients of equivalent numbers of untreated donor TCD BM and T cells. Gene array analyses of pDC from Flt3L-treated human and murine donors showed upregulation of adaptive immune pathways and immunoregulatory checkpoints compared with pDC from untreated BM donors. Transplantation of TCD F-BM plus T cells resulted in no loss of graft-versus-leukemia effect compared with grafts from untreated donors in two murine GvL models. Thus, Flt3L treatment of marrow donors is a novel method to increase the content of pDC in allografts, increase survival, and decrease GvHD without diminishing the GvL effect.

3-2-0 Introduction

Hematopoietic stem cell transplantation (HSCT) is curative for patients with hematological malignances and bone marrow failure disorders (2). HSC grafts are typically obtained from aspiration of bone marrow (BM) or from apheresis of granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood (154, 155). The major complications of allo-HSCT including graft rejection, disease relapse, and graft-versus-host disease (GvHD) (14, 15). These adverse effects are initiated and regulated by both the content of donor immune cells in the graft and residual antigen presenting cell in the recipient (18). Results of BMTCTN 0201 showed that an increased content of donor plasmacytoid dendritic cells (pDC) in BM allografts increased survival and decreased GvHD, but not in G-CSF mobilized (G-mobilized) allografts (97). It has been reported that dendritic cell reconstitution post-transplant predicts outcomes including incidence of GvHD, relapse and death (156). We have observed that transplantation of purified BM pDC increased survival and decreased GvHD without affecting graft-versus-leukemia (GvL) in allogeneic murine transplant models compared to G-mobilized pDC (Hassan, 2018, submitted). Because pDC content of the marrow is variable among allogeneic donors, methods to increase the pDC content in marrow are attractive strategies to enhance survival and GvL while limiting GvHD.

Plasmacytoid dendritic cells can be identified as Lin^-(CD3, CD14, CD16, CD19, CD20) $HLADR^+CD123^+CD11c^-$ in humans and $PDCA1^+CD11c^+B220^+Lin^-$(CD3, CD11b, CD19, IgM, CD49b, Ter119) in mice (63-65). Plasmacytoid dendritic cells play a significant role in both innate and adaptive immunity because they are the primary source of type 1 interferon in both humans and mice (157). Recipient pDC are depleted following irradiation, allowing for examination of the effect of donor pDC on post-transplant GvHD and GvL (141). Donor pDC have been shown to possess graft-facilitating functions including enhancing donor cell engraftment and survival post-transplant (120). The immunological status, inflammatory or immunosuppressive, of donor pDC that interact with donor T cells is paramount to their ability to limit GvHD (158). We have shown

that pDC facilitate immunity through early post-transplant IL-12 secretion which enhances engraftment and GvL effect and late IFNγ response pathways that decrease GvHD via induction of IDO production and increased numbers of Treg (136, 142, 152).

FMS-like tyrosine kinase 3 ligand (Flt3L) is necessary for pDC differentiation and Flt3L treatment can be used to increase pDC content *in vitro* (59-61, 159). Although the use of CDX-301 in mobilization of HSC has been studied, the effect of *in vivo* Flt3L administration alone on the content and immunological activity of pDC in bone marrow is yet to be determined (57, 160). We hypothesized that Flt3L treatment of bone marrow donors would increase the pDC content of the allograft and transplanting Flt3L-stimulated marrow grafts would enhance survival while limiting GvHD. We tested this hypothesis in an MHC mismatched C57BL/6→B10.BR transplant model in which donors were treated with PBS or two injections of 300μg/kg of Flt3L. Herein, we report that treatment of donors with Flt3L (F-BM) increased the pDC content of the graft, increased survival, and decreased GvHD in allo-transplant recipients compared to marrow grafts from PBS-treated donors. Using FACS purified pDC, we show that Flt3L treatment of donors led to upregulation of adaptive immune pathways and osmoregulation checkpoints in donor pDC. Additionally, we observed decreased Th1 and Th17 polarization in T cells recovered from F-BM transplant recipients on day 3. Therefore, Flt3L treatment is a novel method that increases pDC content in donor grafts, increases survival, and decreases GvHD in allogeneic transplantation.

3-3-0 Materials and Methods

Mice

C57BL/6 (H-2K^b) and B10.BR (H-2K^k) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Male donor and recipient mice were 8-10 weeks and 10-12 weeks, respectively. National Institutes of Health animal care guidelines were used and approved by Emory University Institutional Animal Care and Use Committee.

Flt3L Treatment of Mice

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

G-CSF Treatment

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

Donor Cell Preparation

Donor mice were euthanized and femora and tibias of donor C57BL/6 mice were flushed with 2% FBS PBS. Biotinylated anti-mouse CD3 from BD Bioscience (San Jose, CA) was used for T cell depletion. T cell purification was performed by incubating with biotinylated B220, CD49b, Gr-1, Ter119 antibodies. T cell depletion and purification samples were then incubated with anti-biotin microbeads and negative selection with a LS Miltenyi MACS column was performed (Gladbach, Germany).

Donor mice were euthanized and femora and tibias of PBS-treated C57BL/6 mice were flushed with 2% FBS PBS. Anti-mouse (CD3, CD11b, CD19, IgM, CD49b, Ter119) PE, CD11c FITC or

APC-CY7, B220 PERCP-CY5.5 and PDCA1 ef450 were used for pDC analysis and sorting. These antibodies were purchased from BD Bioscience (San Jose, CA), Biolegend (San Diego, CA), and eBioscience (San Diego, CA). HSC were stained with (CD3, CD11b, CD19, IgM, CD49b, Ter119) PE, B220 PERCP-CY5.5, CD117 APC, Sca-1 PE-CY7. T cell purification was performed by incubating with biotinylated B220, CD49b, Gr-1, Ter119 antibodies, then with anti-biotin microbeads, and negative selection with a LS Miltenyi MACS column (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 used for pDC analysis and purchased from BD Bioscience (San Jose, CA), Biolegend (San Diego, CA), and eBioscience (San Diego, CA). Stimulation of pDC for cytokine profile analysis was done using 50μM of CpG (ODN 1585, Invivogen, San Diego, CA) of whole bone marrow in complete RPMI 1640 supplemented with 10% FBS, 100 U/mL of penicillin, 100μg/mL of streptomycin, and 50μM each of 2-mercaptoethonaol, nonessential amino acids, HEPES, and sodium pyruvate (complete media) in 10cm wells for 9 hours at 37°C. BD golgiplug was added at hour 3. Intracellular analysis of pDC was done using BD Bioscience 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 FACS Aria (BD, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, Oregon).

T cells were stimulated with phorbol-12-myristate-13-acetate, ionomycin, and golgiplug from BD for 6 hours. Intracellular staining was done using the BD Bioscience cytofix/cytoperm kit and IFNγ APC, IL-17 APC-Cy7, and TNFα PE antibodies. The eBioscience fixation kit and Tbet PE-CY7, GATA3 PE, RORγT APC, and FoxP3 PE antibodies was used for intranuclear staining.

In vitro T cell Activation

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

Transplantation

On day -1 recipients were irradiated twice at 5.5 Gy, with fractions separated by 3-4 hours, for a total of 11Gy.(125) Mice were transplanted with 5 x 10^6 T cell depleted PBS or Flt3L-treated BM with or without 4 x 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 (112). Histopathological GvHD grading of small intestine was done using a 0-4 scale. 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 it not necessary as long as criteria of the assigned grade are met. Histopathological GvHD grading of liver was done using a 0-1 scale. Grade of 0: no pathology. 1: Evidence of lymphocytic infiltration into the portal triad and apoptotic bile duct epithelial cells. (132, 161, 162)

Cytospin

Cells were treated and sorted according to the protocols above. The cytospin filter was moistened with 1% BSA-PBS. Cells were loaded into the cytospin wells and spun at maximum speed on the

Cytospin 3 by Shandon (Runcorn Chesire, England). The cells were fixed with methanol and desiccated overnight. The cells were stained overnight with Giemsa stain from Electron Microscopy Sciences (Hatfield, Pennsylvania). Then fixed with Permount from Fisher Chemical (Geel, Belgium) and Mounting Medium Xylene from Fisher Scientific (Pittsburg, PA). The slides were imaged using the Zeiss Axioplan 2 (Thornwood, NY).

Tumor Cell Challenge and Bioluminescent Imaging

A generous gift by Dr. Bruce Blazar, an acute myeloid leukemia cell line, luciferase-transfected C1498, was used for graft-versus-leukemia experiments. On day -2, mice were lethally irradiated (11Gy), on day -1 injected with 50,000 C1498 cells, and on day 0 T cell depleted bone marrow or bone marrow from Flt3L treat bone marrow donors with or without untreated T cells were transplanted. For bioluminescent imaging, 150µg/kg of D-Luciferin was injected and mice were imaged with IVIS spectrum (Perkin Elmer, Waltham, MA). Luminescence was measured in photons/sec/cm^2/sr and normalized to control mice (recipients of the same transplant without $luciferase^+$ tumor).

LBRM 33-5A4 were purchased from American Type Culture Collection (ATCC) (Manassas, VA).(163) ATCC recommendations were used for culturing of the cell line. Transfection of these cells to express luciferase was conducted in our laboratory. Mice were irradiated on day -2, challenged with 50,000 LBRM cells on day -1 of transplant, and transplanted on day 0. Tumor burden was monitored by injecting mice with 150µg/kg of D-Luciferin and imaging with IVIS spectrum (Perkin Elmer, Waltham, MA). Measurements of luminescence are given as photons/sec/cm^2 and normalized to mice that received the same transplant without tumor challenge.

Gene Array Analysis

Human subjects were untreated or treated with 75μg/kg of Flt3L (CDX-301) for 5 consecutive days. Untreated donors had bone marrow harvested and Flt3L-treated donors underwent leukapheresis. Gene expression of human BM and Ftl3L mobilized peripheral blood FACS isolated pDC was assessed using Illumina HumanHT-12 v4 beadchip microarray. Data was preprocessed, quantile normalized, background-corrected and log2 transformed for downstream analysis.(127) RNASeq Gene expression of murine BM and Flt3L-treated BM pDC was assessed using Illumina TruSeq Stranded Total RNA library. Samples were sequenced on next generation sequencing at 2 X 151 bp in the paired ends. Fastq reads were trimmed and filtered for quality and adapter contamination with trimmomatic. Post-filtered reads were mapped against Ensemble mouse GRCm38/mm10 reference genome and gencode Release M16 gene annotation using STARaligner. Expression quantification was obtained using HTSeq counts, DESeq normalized and log2 transformed for further analysis.(126) Differential expression analysis for both human and murine samples was performed using a moderated t-test.(127) 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 an FDR cutoff of 0.05. Pathway analysis was performed using Cytoscape software v3.6.1 and ReactomeFI plugin (128, 129).

Statistical Analysis

Data were analyzed using Prism version 5 (Graphpad, San Diego, CA) for MAC and are displayed as mean+SD unless otherwise specified. Survival differences were calculated in a pairwise fashion using the log-rank test. Applicable data were compared by student-test and 1-way ANOVA. Significance was considered as a p-value of ≤ 0.05.

3-4-0 Results

Flt3L administration expands pDC content in bone marrow *in vivo*

To examine whether Flt3L treatment could enhance pDC content in vivo, we tested the effect of Flt3L administration on murine bone marrow donors due to the known effect of Flt3L in stimulating differentiation and expansion of pDC (164). We measured HSC, pDC, T cell, B cell and NK cell content in bone marrow allografts from mice treated with different dosing schedules of PBS or Flt3L (Table 1). Bone marrow grafts from mice treated with Flt3L had pDC content that increased with the number of Flt3L doses (Figure 1A). HSC, T cell, B cell, and NK cell content was not significantly affected in mice treated with less than 4 doses of Flt3L (Figure 1B-E). The schedule of 2 doses of Flt3L (schedule C) was chosen for all subsequent experiments because pDC content was increased 5-fold without significant differences in the content of other immune cells that might modulate GVHD, including HSC, T cells, B cells, and NK cells.

Flt3L administration to bone marrow donors may affect homing and lineage, but not phenotype of pDC

Since homing of donor pDC to GvHD target organs is dependent on their chemokine receptor expression (30) we measured expression of CCR4, CCR5, CCR7, CCR9, and CXCR4 on pDC from untreated BM and F-BM (133). Mice were treated with PBS or 300μg/kg of Flt3L on days -4 and -1 and bone marrow was harvested. There was significantly less CCR5 and CCR9 and more CXCR4 expression in pDC from F-BM compared with control pDC (Figure 2A), suggesting that ability of pDC to migrate to the gut and lymph nodes could be altered by treatment with Flt3L (83, 134).

To better understand the effect of pDC on T cell activation and polarization, we next examined the cytokine profiles and IDO production of pDC from BM and F-BM grafts. Flt3L treatment did

not result in substantive changes in the levels of IFNα, IL-12, IL-10 and IDO in F-BM pDC compared with pDC from untreated BM (Figure 2B-C).

Recipients of F-BM had increased survival and less GVHD compared to untreated BM recipients

We next performed a transplant 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-transplant survival and GvHD. Lethally irradiated (11 Gy) B10.BR mice were transplanted with 5 million T cell depleted (TCD) BM from C57BL/6 donor mice treated with PBS or 300μg/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 pDC and TCD F-BM contained ~250,000 pDC. Mice that received allogeneic TCD F-BM with the addition of donor T cells had significantly increased survival compared with recipients of TCD BM plus T cells (Figure 3A). Donor hematopoietic engraftment in all groups was near 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 for GvHD pathology in recipients of control TCD BM, without the addition of donor T cells. Using NIH criteria for histological diagnosis of GvHD (132, 161, 162), and euthanizing mice on day +30 post-transplant, GvHD histopathology was not significantly different comparing scoring sections of small intestine or liver between recipients of TCD BM + T cells and TCD F-BM + T cells (Figure 3D-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.

To isolate the role of pDC from F-BM in this transplant model, we transplanted B10.BR recipients with purified 5,000 HSC, 10^6 T cells, and 50,000 FACS-isolated pDC from F-BM or untreated

C57BL/6 BM. Recipients of F-BM pDC had 90% survival and lower GvHD scores compared to recipients of BM pDC, in which survival was 80% (p=NS; Supplemental Figure 1A-B).

Flt3L treated donors have different gene expression profiles than bone marrow donors

After observing a trend towards increased survival in the group that received an equal number of FACS isolated F-BM pDC compared with untreated BM pDC in a model system using purified HSC, T cell, and pDC transplants, we hypothesized that quantitative effects of Flt3L treatment on the increased number of pDC in donor marrow is not the sole determinant of improved transplant outcome following Flt3L treatment. Thus, to explore qualitative effects of Flt3L treatment on pDC we analyzed gene expression profiles of pDC from F-BM versus BM from untreated donor mice. Plasmacytoid dendritic cells were isolated by FACS from the bone marrow of mice treated with PBS or 300μg/kg of Flt3L on days -4 and -1. The isolated cells had greater than 95% purity and uniform plasmacytoid dendritic cell morphology when imaged following cytospin (Figure 4A). RNA was obtained from these purified pDC and sequenced using next generation sequencing. Pairwise analyses were conducted on individual genes and immunological pathways using a p-value cutoff of < .05. F-BM pDC have distinct gene expression profiles compared to pDC from untreated BM grafts (Figure 4B-C). Immunological pathways that showed significant differences (p-value < .05) include the adaptive immune pathways and immune checkpoint pathways. Upregulation of genes in the adaptive immune pathway include APRIL, Hmox1, and Etv5, all of which may affect the ability of donor pDC to regulate T cell polarization and activation (165). Immune checkpoint pathway genes, Bcl2, Cyclin D3, TIM-3, and ACK1, are upregulated in pDC from F-BM grafts, suggesting an increased ability for immune cell regulation (166). Finally, pDC from F-BM grafts upregulated Tox1 and Prss16, genes that regulate T cell selection in the thymus (Table 2) (167, 168).

In order to better interpret the translational relevance of these murine studies, gene expression profiles were compared between FACS-isolated human pDC from untreated bone marrow donors and pDC isolated from apheresis products of Flt3L-treated sibling donors. Because we did not have access to the bone marrow 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 (NCT022000380) that assessed the efficacy of Flt3L in mobilizing stem cells, and compared gene expression to pDC isolated from untreated BM acquired on a separate clinical study (NCT02485639). RNA from pDC samples were sequenced by Illumina Chip and differential gene expression along with immune pathway analysis was conducted. 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 upregulated in pDC from the Flt3L-treated donors compared with pDC from untreated bone marrow volunteers (Figure 4D-E) (Table 3). Additionally, toll like receptor (TLR) genes, including those in the TLR4 pathway, APP, MAP2K6, CD36, ACTG1, and IRF7, were downregulated in Flt3L-treated donor pDC, indicating decreased ability to stimulate innate immune pathways (Table 4) (169).

Flt3L bone marrow reduced T cell polarization post transplantation

Based upon the results of the gene array analyses of pDC from Flt3L treated mice and humans, we next studied the ability of pDC to interact with and influence donor T cell activation and immune polarization post-transplant in a non-paracrine cytokine fashion(88, 138). We examined the potential of pDC to stimulate or inhibit allo-activation of T cells by measuring surface expression of MHC II, CD86, and PDL1 on pDC. Plasmacytoid dendritic cells from Flt3L-treated donors expressed less MHC II and CD86 and expressed more PDL1 (Figure 5A). To determine whether changes in surface expression of co-stimulatory and co-inhibitory molecules affects T cell polarization, we performed a C57BL/6→B10.BR transplant with 5 million TCD BM or F-BM cells

and 4 million untreated T cells. On day 3 post-transplant, donor T cells were analyzed for their content of T cell transcription factors by flow cytometry. Mice that received TCD F-BM + 4 million T cells had donor T cells with significantly lower levels of Tbet and RORγT expression on day 3 post-transplant, consistent with decreased Th1 and Th17 polarization, respectively (Figure 5B). To better determine what role pDC played in the pattern of transcription factor expression in T cells post-transplant, we performed a C57BL/6→B10.BR using purified HSC, T cells, and purified populations of BM or F-BM pDC, and again measured T cell transcription factor expression on day 3. The same trend of lower transcription factor expression in T cells post-transplant was observed in this experiment as well along with lower production of cytokines (Supplemental Figure 2A-B). The effect of F-BM pDC on T cells does not appear to limit T cell proliferation, as T cell proliferation was equivalent comparing the addition of F-BM pDC versus untreated BM pDC to T cells in a mixed-lymphocyte reaction (Supplemental Figure 3A-B).

GvL activity was not diminished in recipients of F-BM

To determine whether Flt3L treatment of marrow donors affected the GvL activity of the allogeneic transplant, we compared the growth of leukemia cells in recipients of F-BM compared with untreated BM using bio-luminescent imaging of luciferase+ leukemia cells and survival analyses of leukemia-bearing transplant recipients. First, C57BL/6 mice were irradiated (11Gy) on day -2, inoculated with 50,000 syngeneic luciferase^{+} 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 comparing recipients of untreated TCD BM plus 1 million T cells versus TCD F-BM plus 1 million T cells recipients (Figure 6A-C). Additionally, using the LBRM tumor line in C57BL/6→B10.BR transplant recipients, there was a significant prolongation of survival comparing recipients of TCD F-BM plus 4 million T cells to recipients of TCD BM plus 4 million T cells (Supplemental Figure 4A-C).

3-4-0 Discussion

GvHD remains the most significant complication following HSCT.(140) Although recipients of bone marrow or G-CSF-mobilized grafts from unrelated donors have equal survival up to 7 years post-transplant, there is a higher incidence of chronic GvHD in recipients of G-CSF-mobilized grafts (45, 131). Furthermore, the BMTCTN 0201 study showed that recipients of marrow grafts containing higher numbers of pDC had increased survival and lower treatment related mortality due to less GvHD (97). Results of this study also showed that pDC content of the marrow graft varied greatly amongst volunteer bone marrow donors, raising the question of how to increase the content of immune-regulatory donor pDC in all bone marrow allografts. We report herein that administration of Flt3L to bone marrow donors increased pDC content of the graft and graft recipients of Flt3L-treated bone marrow or purified donor pDC have increased survival and less GvHD after allo-transplantation.

In our previous studies, we have shown that murine BM donor pDC limit GvHD without attenuating GvL (136, 152). Furthermore, we have unpublished data confirming that with transplantation of allo-grafts containing highly purified HSC, T cells, and pDC, recipients of BM pDC have increased survival and decreased GvHD incidence compared to recipients of G-CSF-mobilized pDC (Hassan 2018, submitted). Thus, data from the current study showed that Flt3L administration to bone marrow donors increased pDC content 5-fold spurred further characterization of the effects of Flt3L treatment on the quality of pDC in bone marrow and HSCT outcomes. Transplanting 5 million T cell depleted bone marrow grafts 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 BM cells and T cells.

Interestingly, although pDC from untreated BM and F-BM have a similar phenotype, we show that F-BM pDC have an enhanced cell-intrinsic ability to limit GvHD and a gene expression profile that

supports greater immune-modulatory capacity compared with pDC 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 pDC from untreated BM (Figure 5B and Supplemental Figure 2A). Thus, the upregulation of adaptive immune and immune checkpoint pathways in pDC 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 pDC from untreated BM(34, 46, 131, 170). This coupled with increased expression of genes involved in positive and negative selection in the thymus, may enable F-BM pDC to induce tolerance post-transplant. Additionally, pDC from Flt3L treated human donors have downregulated the expression of genes involved in TLR cascades and innate immune cell pathways compared with pDC from untreated BM, with the most significant decrease seen in expression of TLR4. Downregulation of TLR4 may also play a role in limiting GvHD in the gut, because gut GvHD can activate the release of LPS from bacteria, ultimately activating TLR4 on pDC, which can further aggravate injury in the gut and augment GVHD (171-173). Thus, the downregulation of genes involved in TLR cascades and other innate immune pathways in F-BM pDC may also play a role in the attenuation of GvHD following transplantation of pDC from F-BM or unfractionated F-BM.

The present study has some limitations. Although the current studies focus on donor pDC and the effect of Flt3L administration to bone marrow donors on pDC, other donor cell types may contribute to the transplant outcomes following Flt3L treatment of BM donors. We have shown with our dose and schedule (day-4, day -1 with respect to marrow harvest), there is a significant change in content and quality of the pDC Although there was no significant change in content of HSC, NK cells, T cells, and B cells in Flt3L-treated donor bone marrow grafts, determining the effect of Flt3L administration on the quality of these cells will further clarify the mechanisms that

result in increased survival and decreased GvHD in F-BM recipients (131). Thus, the clinical utility of Flt3L treatment of human bone marrow donors could be studied by characterizing the effect on immune cell content and quality within the bone marrow graft. Among volunteers treated with Flt3L, some previous phase 1 studies have shown safety of daily administration for a week or more (57). Additionally, we did not test the ability of peripheral blood mobilized Flt3L grafts to affect transplant outcomes, because we observed that HSC and pDC mobilization was not equivalent to G-CSF mobilization (Supplemental Figure 5A-B). Furthermore, Flt3L as a single agent for stem cell mobilization is not efficient (Clinical trial NCT022000380) and Flt3L would need to be combined with other agents such as CXCR4 antagonists for this approach to be feasible for clinical practice. Additionally, we compared gene expression in different sources of Flt3L-stimulated pDC in mice and humans, isolating F-BM pDC from Ftl3L-treated murine BM and F-apheresis pDC from human donor apheresis products due to the lack of available human F-BM samples. Nevertheless, the similarities in gene expression between pDC 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 pDC from bone marrow of Flt3L-treated human donors is warranted in a planned clinical study to further validate the clinical translation potential of these findings.

To summarize, we report a novel method using Flt3L treatment of bone marrow donors to reduce the GvHD-promoting activity the marrow graft and marrow pDC, and improve survival of allogeneic BM transplant recipients. Flt3L treatment increased the content and immune-regulatory capacity of pDC in bone marrow 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, the present pre-clinical data provide an impetus to test the clinical effect of Flt3L treatment of bone marrow donors as a novel method to improve transplant outcomes.

3-5-0 Figure Legends

Figure 1. Flt3L administration to bone marrow donors increased pDC content in graft. Mice were treated with PBS or 300μg/kg of Flt3L according to the schedule in Table 1. (A) pDC, (B) HSC, (C) B cell, (D) T cell, and (E) NK cell content were measured by flow cytometry. n=3 per group. *P < .05, **P < .01, ***P < .001.

Figure 2. Phenotype of pDC from BM versus F-BM grafts are similar. Mice were treated with PBS or 300μg/kg of 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 50μg of CpG for 9 hours at 37°C. Intracellular staining for cytokine and (C) IDO expression was measured by flow cytometry. n=3-6 per group, from 2 independent experiments. **P < .01.

Figure 3. Grafts from Flt3L-treated donors increased survival and decreased GvHD. C57BL/6 donor mice were treated with PBS or 300μg/kg of Flt3L on days -4 and -1. B10.BR recipient mice were transplanted with 5 million T cell depleted (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 + 4 million T cells, and TCD FBM + 4 million T cells. *P < .05 represents significance using Kaplan-Meier survival analysis. (B) Chimerism of recipients 30 days post-transplant by groups as indicated. (C) Clinical GvHD scores of mice that received T cells. n=30 per group, from 2 independent experiments. *P < .05 represents significance between groups using two-way ANOVA. (D) Representative histopathological samples of small intestine and liver at day +30 from each treatment group photographed at 600X. Black squares denote apoptotic cells in the small intestine crypts. Histopathological grades of GvHD associated pathology. Graded 0-4. Score of 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). Grade 0-1. Grade of 0: no pathology, 1: Portal triad lymphocytic infiltration and apoptotic bile duct epithelial cells. (E) Average histopathological grade of GvHD-associated pathology in the small intestine (F) or liver. n=4-6 per group, from 2 independent experiments.

Figure 4. F-BM pDC have distinct gene expression profile from BM pDC. Mice were treated with PBS and 300μg/kg of Flt3L on days -4 and -1. Plasmacytoid dendritic cells were isolated by FACS. (A) Slides of pDC 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 upregulation and downregulation (BM vs F-BM). n=3 per group from one experiment. (E) Healthy human donors were untreated or treated with 75μg/kg of CDX-301 (recombinant Flt3L) for 5 consecutive days. Untreated donors underwent bone marrow harvest and Flt3L-treated donors underwent leukapheresis on day 6. Plasmacytoid dendritic cells were isolated by FACS. RNA sequencing was done with Illumina HumanHT-12 v4 beadchip. Heatmap depicting the significantly differentially expressed genes in the human samples using z-score scaling, Euclidean distance, and complete clustering. (F) Volcano plot depicting upregulation and downregulation of genes (BM vs F-Apheresis). n=4-5 per group from one experiment.

Figure 5. Flt3L treatment of bone marrow donors decreased expression of T helper cell transcription factors in recipients. Donor mice were treated with PBS or 300μg/kg of Flt3L on days -4 and -1. (A) CD86, MHC II, and PDL1 surface expression was measured by flow cytometry. (B) A C57BL/6→B10.BR transplant was performed where recipient mice were transplanted with 5 million T cell depleted BM or F-BM cells with the addition of 4 million T cells. Intranuclear staining of transcription factors Tbet, GATA3, RoRγT, and FoxP3 of T cells was assessed by flow

cytometry 3 days post-transplant. n=6 per group, combined data from 2 independent experiments. *P < .05, **P < .01.

Figure 6. No loss of GvL effect in recipients of F-BM. 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. The # indicates censored subjects. (C) Quantification of tumor burden. n=10-15 per group, from 2 independent experiments. ***P < .01.

3-6-0 Figures

Figure 1.

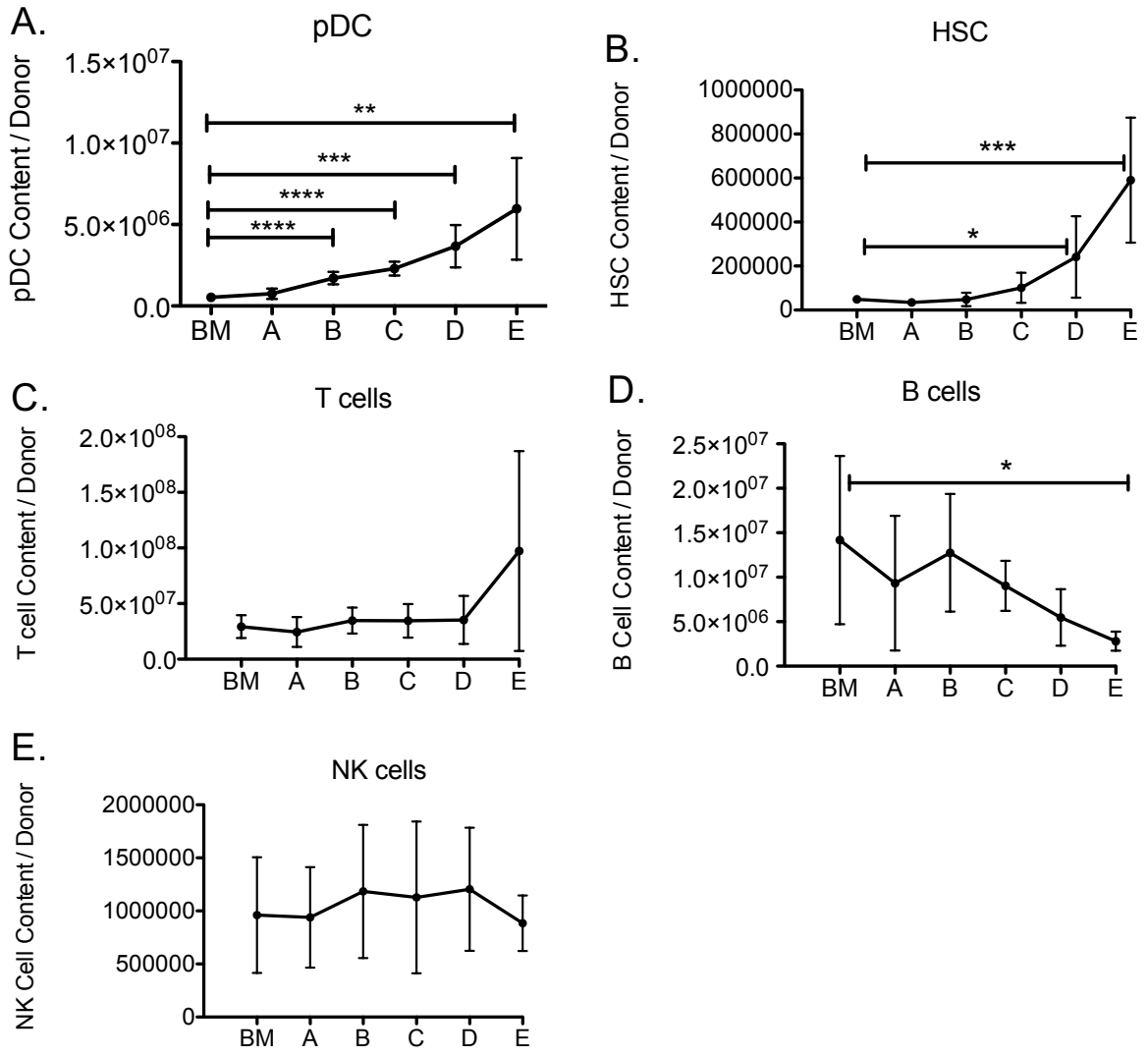


Figure 2.

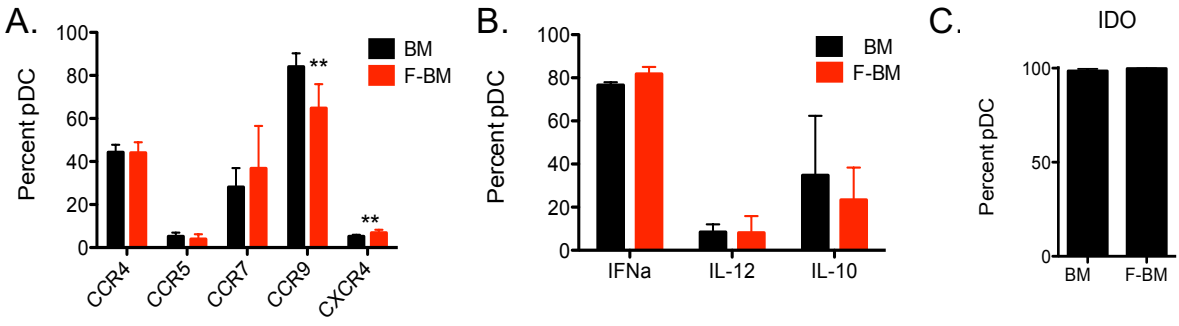

A.
Percent pDC
100
80
60
40
20
0
BM
F-BM
**
**
CCR4
CCR5
CCR7
CCR9
CXCR4
B.
Percent pDC
100
80
60
40
20
0
BM
F-BM
IFNa
IL-12
IL-10
C.
IDO
Percent pDC
100
50
0
BM
F-BM

Figure 3.

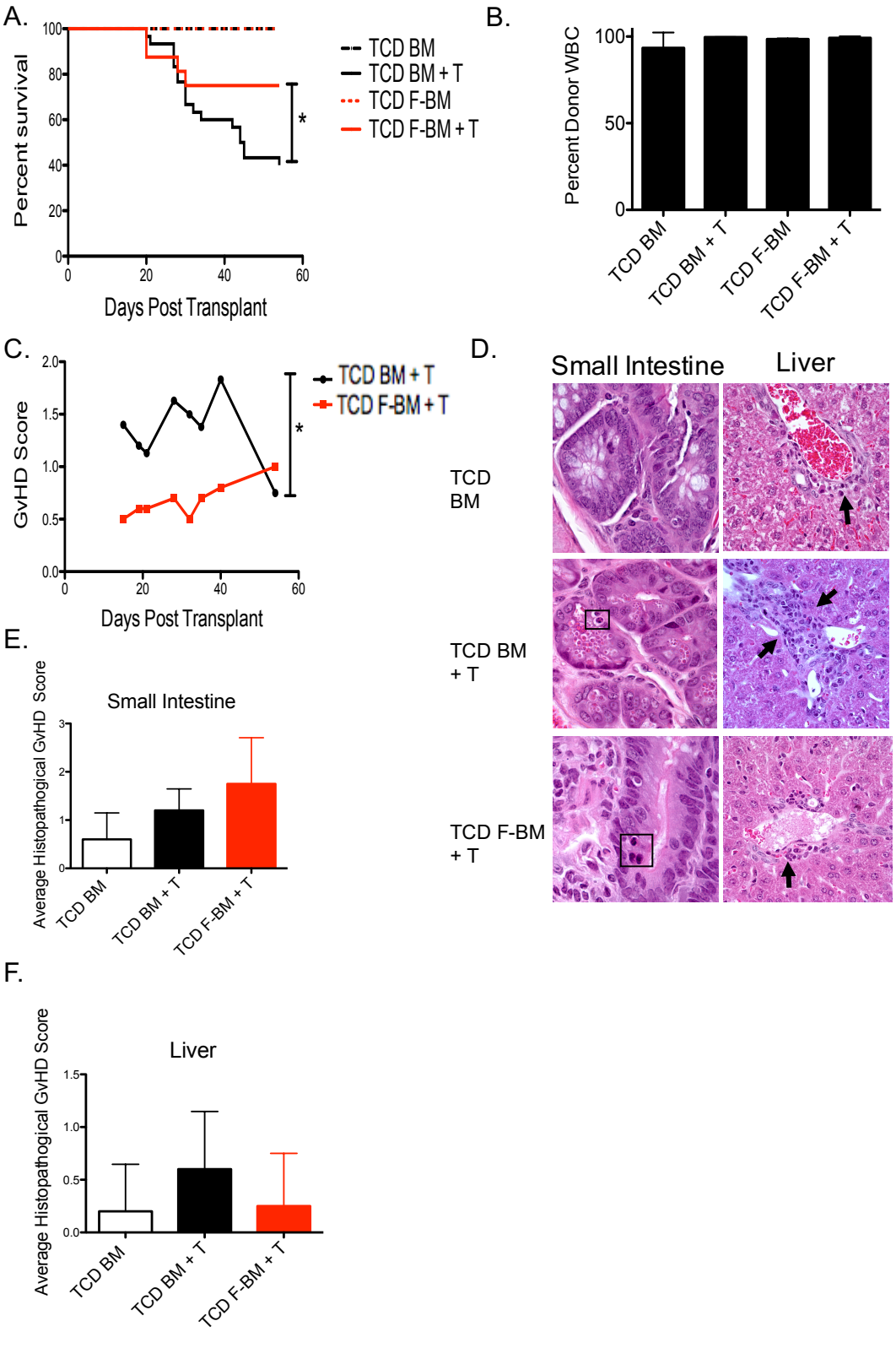

A.
Percent survival
Days Post Transplant
TCD BM
TCD BM + T
TCD F-BM
TCD F-BM + T
*
B.
Percent Donor WBC
TCD BM
TCD BM + T
TCD F-BM
TCD F-BM + T
C.
GvHD Score
Days Post Transplant
TCD BM + T
TCD F-BM + T
*
D.
Small Intestine
Liver
TCD BM
TCD BM + T
TCD F-BM + T
E.
Small Intestine
Average Histopathogical GvHD Score
TCD BM
TCD BM + T
TCD F-BM + T
F.
Liver
Average Histopathogical GvHD Score
TCD BM
TCD BM + T
TCD F-BM + T

Figure 4.

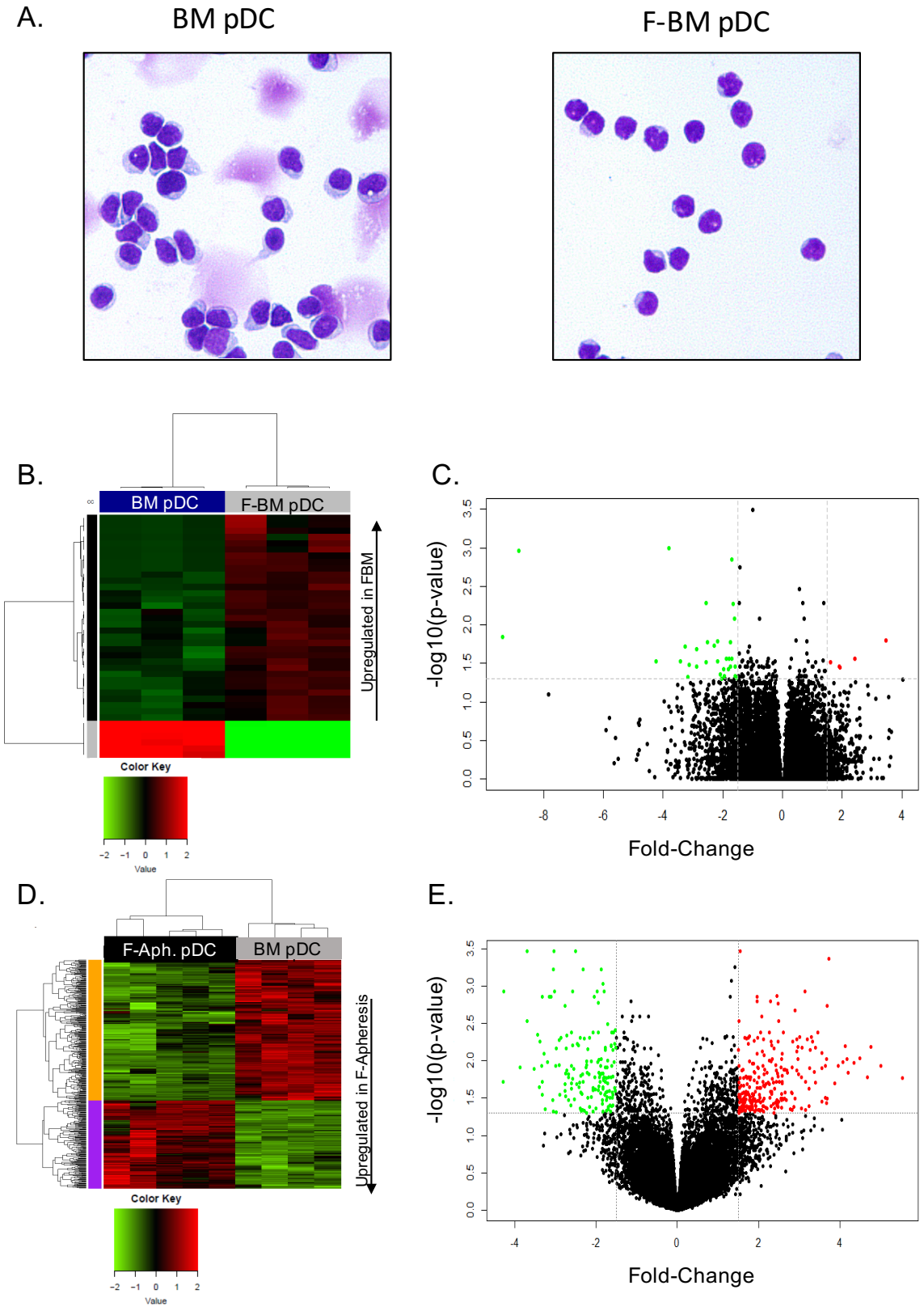

A.
BM pDC
F-BM pDC
B.
BM pDC
F-BM pDC
Upregulated in FBM
Color Key
Value
C.
-log10(p-value)
Fold-Change
D.
F-Aph. pDC
BM pDC
Upregulated in F-Apheresis
Color Key
Value
E.
-log10(p-value)
Fold-Change

Figure 5.

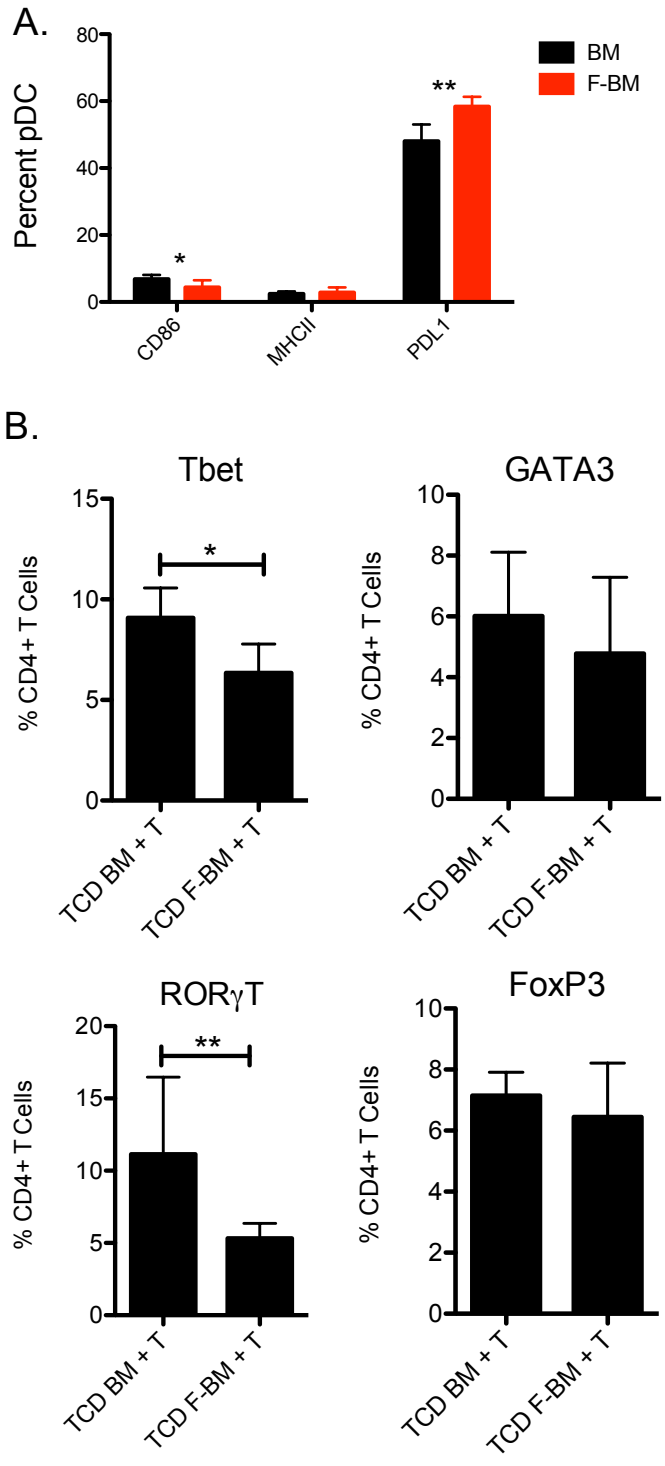
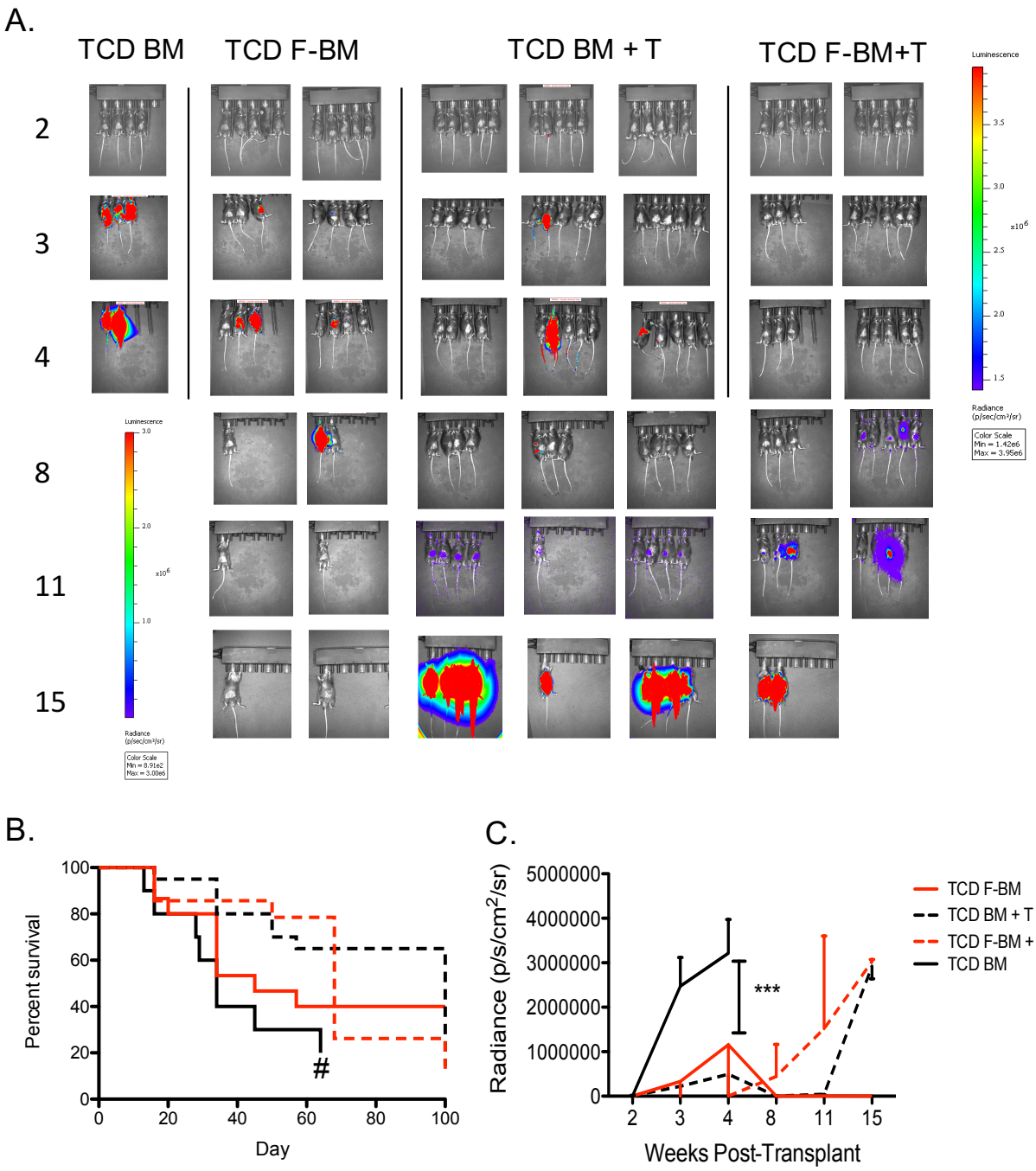

A.
Percent pDC
80
60
40
20
0
BM
F-BM
*
**
CD86
MHCII
PDL1
B.
Tbet
% CD4+ T Cells
15
10
5
0
*
TCD BM + T
TCD F-BM + T
GATA3
% CD4+ T Cells
10
8
6
4
2
0
TCD BM + T
TCD F-BM + T
RORγT
% CD4+ T Cells
20
15
10
5
0
**
TCD BM + T
TCD F-BM + T
FoxP3
% CD4+ T Cells
10
8
6
4
2
0
TCD BM + T
TCD F-BM + T

Figure 6.


A.
TCD BM
TCD F-BM
TCD BM + T
TCD F-BM+T
2
3
4
8
11
15
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

Supplementary Figure Legends

Supplemental Figure 1. Transplanting 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 MACS isolated T cells from untreated donor mice and 50,000 FACS isolated pDC from untreated donor BM or F-BM. (A) Survival curve of murine transplants. (B) GvHD scores of mice from figure S1A. n=10 per group, from one experiment. *P < .05 of both lines compared to one another using 2-way ANOVA.

Supplemental Figure 2. Transplantation of F-BM pDC decreased T helper cell transcription factors and function in donor T cells. 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, from one experiment. *P < .05.

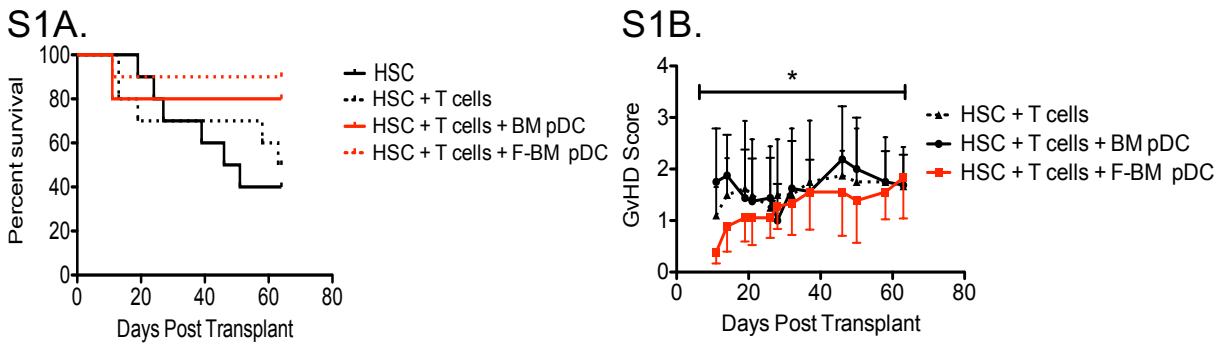
Supplemental Figure 3. F-BM pDC do not suppress T cell proliferation in MLR. (A) 50,000 FACS isolated C57BL/6 pDC from BM or F-BM grafts were added to an MLR containing 1 million C57BL/6 responder T cells and 1 million irradiated B10.BR stimulator cells. Proliferation was assessed by measuring CFSE dilution by flow cytometry. Histogram of CFSE fluorescence, showing T cells that underwent proliferation represented by the peak to the left. (B) 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, from one experiment.

Supplemental Figure 4. Transplantation of TCD BM from Flt3L treated donors prolong survival in a lymphoblastic leukemia model of GvL. C57BL/6→ B10.BR murine transplant recipients were lethally irradiated on day -2, received 2 million luciferase^{+} LBRM cells by tail vein injection on day -1, and were transplanted with 5 million T cell depleted bone marrow cells and 4 million untreated T cells on day 0. Recipient mice (A) Serial bioluminescent imaging of recipient mice. (B) Survival curve of murine transplant recipients. (C) Quantification of mean tumor burden by bioluminescent imaging in surviving mice. n=17 per group, from 2 independent experiments. *P < .05.

Supplemental Figure 5. 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, in one experiment **P < .01.

Supplementary Figures

Supplemental Figure 1.



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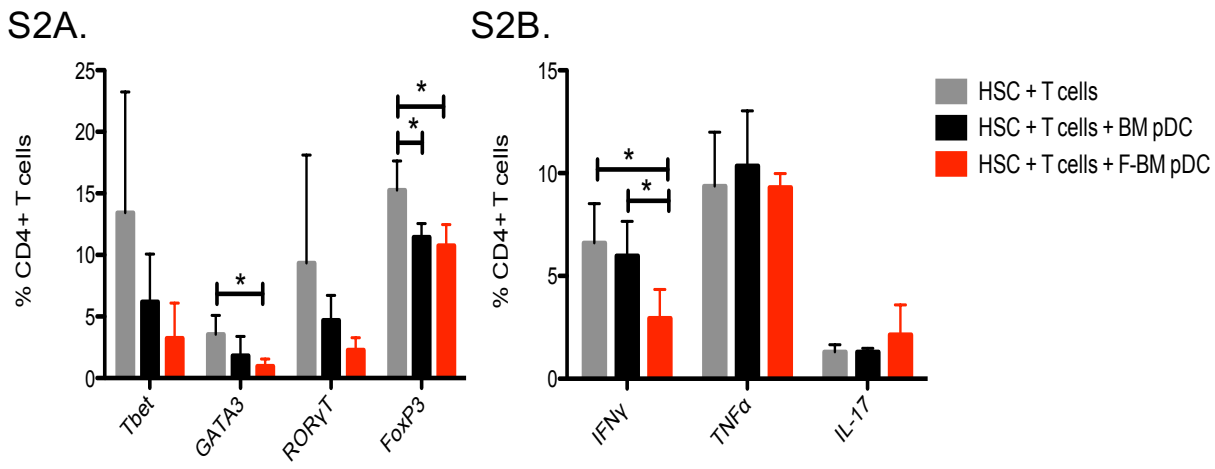
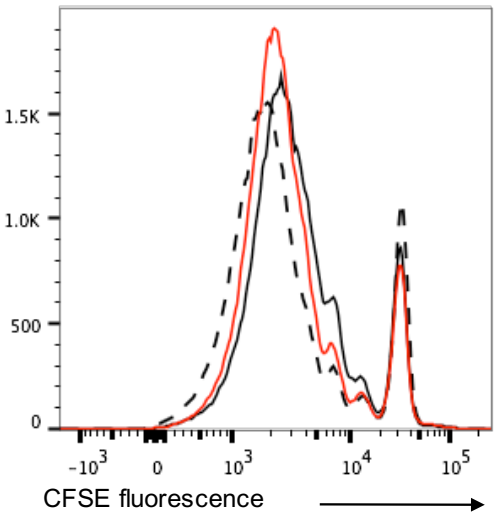


Supplemental Figure 3.

S3A.



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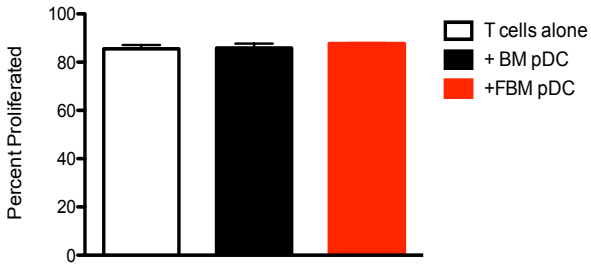

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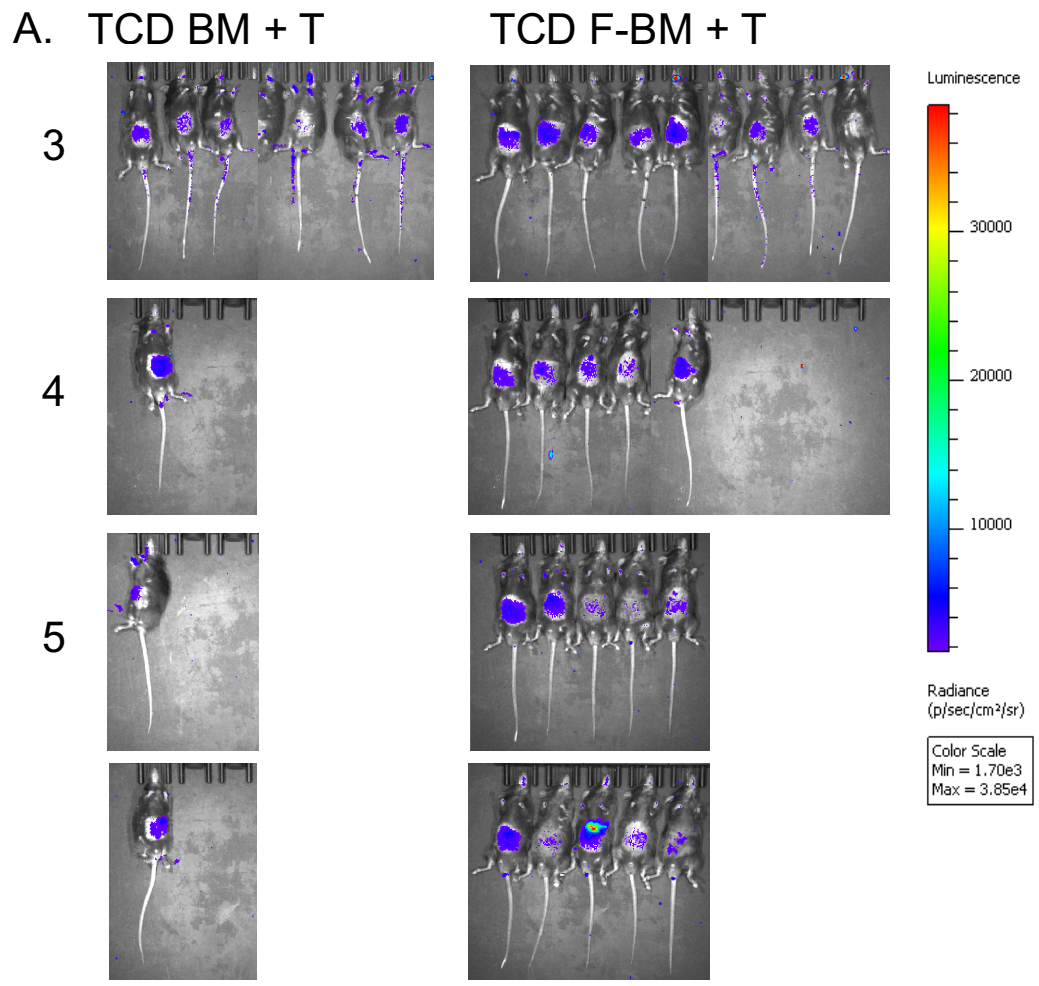
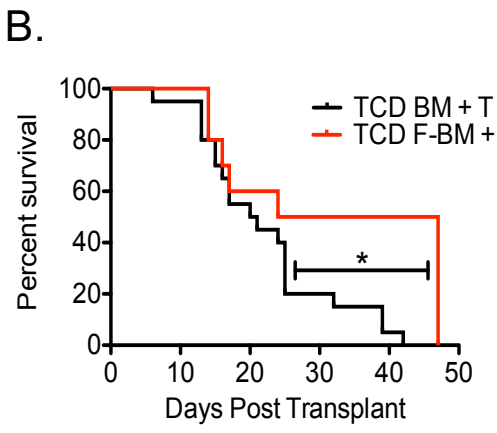
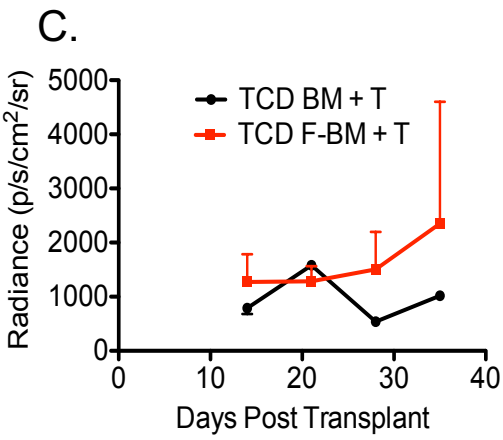
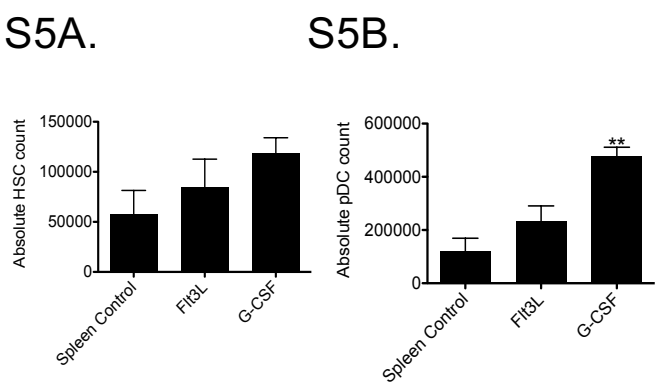






Supplemental Figure 5.



3-6-0 Tables and Table Legends

Table 1

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

Table 3-1-0. Flt3L administration schedule. Bone marrow controls were treated with PBS according to the schedule in the table. Experimental mice were treated with 300μg/kg of Flt3L following the schedule in the table.

Table 2

Gene	Pathway	logFC	NegLog10pvalue
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

Table 2. Genes upregulated in F-BM pDC. Upregulated genes F-BM vs BM in adaptive immune, immune checkpoint, and thymic induced T cell selection pathways that are present in Figure 4B plotted Fold Change (logFC) by –log10(p-value).

Table 3

Gene	Pathway	logFC	NegLog10pvalue
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

Table 3. Genes upregulated in F-Apheresis pDC. Upregulated genes F-apheresis vs. BM in adaptive immune and immune checkpoint pathways that are present in Figure 4D plotted Fold Change (logFC) by –log10(p-value).

Table 4

Downregulated in human F-Apheresis pDC	logFC	NegLog10pvalue
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

Table 4. Genes downregulated in F-Apheresis pDC. Downregulated genes in toll-like receptor cascades including the TLR4 pathway that are present in Figure 4D plotted Fold Change (logFC) by –log10(p-value).

Chapter 4: Discussion

4-1-0 Summary

Strategies to prevent graft-versus-host disease (GvHD) is of utmost importance in the field of hematopoietic stem cell transplantation (HSCT). Many methods have been studied, with the predominant clinical approach being the use of immunosuppressive drugs (1). Strategies of grant engineering, including T cell depletion of HSC grafts, have largely fallen out of favor (22, 23). A novel promising approach to this challenge is the enrichment of allogeneic HSCT facilitating immune cells. One cell type of interest in this regard is plasmacytoid dendritic cells (pDC), specifically enrichment of the donor pDC in bone marrow HSCT graft as opposed to the similar cells found in peripheral blood stem cell grafts.

Encouraging data in both murine models and humans have shown improved outcomes when greater than median quantities of pDC are present in bone marrow HSCT grafts (97, 142). Although these exciting results have been documented, details pertaining to the mechanisms by which bone marrow pDC improve allo-HSCT outcomes are lacking. Furthermore, the benefit of increased donor pDC has not been seen when recipients of granulocyte-colony stimulating factor (G-CSF) blood mobilized hematopoietic stem cell grafts have been studied, suggesting that egress from the bone marrow prior to transplantation in conjunction with the effects that G-CSF has on pDC alters pDC functions. We investigated the differences in the cell-intrinsic immunosuppressive activities and potential to modulate GvHD of the donor pDC found in bone marrow and G-CSF mobilized (G-mobilized) stem cell grafts in a murine allo-HSCT transplant model. We hypothesized that the observed difference in the activity of donor pDC in limiting GvHD was due to loss of tolerogenic functions of pDC during the G-CSF mobilization process. The work presented here show that BM pDC have both pro-inflammatory and anti-inflammatory properties that work in tandem with T cells to aid in donor stem cell engraftment and to maintain GvL in an IL-12 dependent manner, and that donor BM pDC limit GvHD in an IDO dependent

manner. We have shown that pDC isolated from donor bone marrow increase survival and decrease GvHD when compared to pDC isolated from G-mobilized grafts.

The quantity of pDC in a human BM graft varies considerably from donor to donor (97, 142, 152). Thus, we evaluated methods to increase the content of pDC in all BM grafts to achieve improved transplant outcomes in clinical practice. FMS-like tyrosine kinase 3 ligand (Flt3L) plays a significant role in pDC differentiation and proliferation (57, 59-61, 159, 174) and increases the pDC content of *in vitro* bone marrow cultures (60, 61). Thus, we sought to determine the effect of Flt3L treatment of bone marrow donors on post-transplant outcomes. We initially determined the differences in the effects of BM and G-mobilized pDC on transplant outcomes using grafts consisting of highly purified HSC, T cells, and pDC in murine BMT models. In order to increase the clinical relevance of the approach of increasing the pDC content of bone marrow grafts, we used unfractionated bone marrow from FLT3L-treated marrow donors, similar to what would be done in clinical practice. The ensuing graft contained increased numbers of pDC and recipients of bone marrow grafts from Flt3L treated donors (F-BM) had increased survival and decreased GvHD due to downregulation of pro-inflammatory pathways and upregulation of immunoregulatory pathways including pathways involved in T cell selection. These data confirm the beneficial role of bone marrow pDC in HSCT and provide a method to improve clinical HSCT outcomes.

In summary, the content of immune cells in an allogeneic HSC graft has a complex relationship with post-transplant outcomes. Herein, the role of pDC in HSCT has been evaluated thoroughly and discussed in detail. Enhancing mechanistic knowledge of how donor allo-HSCT graft composition and the immune function of pDC has the potential to increase the success rate of HSCT while reducing adverse effects such as graft rejection, GvHD, and relapse of the underlying malignancy.

4-2-0 Conclusions

In conclusion, we show that pDC play a significant and beneficial role in allogeneic hematopoietic stem cell transplantation. Acting alone or in conjunction with other immune cells as a facilitating cell population, pDC improve bone marrow transplant outcomes by enhancing hematopoietic stem cell engraftment and reducing GvHD (120). This occurs through the ability of pDC to affect the immunological milieu of the bone marrow environment in which donor hematopoietic stem cells engraft. Additionally, pDC induce the generation and proliferation of antigen-specific regulatory T cells, which suppress activation of allo-specific T cells, ultimately reducing GvHD (25, 121). The central roles of pDC in regulating GvHD and facilitating donor cell engraftment identifies this rare donor cell population as a novel target for cell-based therapies to improve the efficacy and decrease the morbidity of allogeneic hematopoietic stem cell transplants and thus increase access to this life saving procedure.

We used pDC isolated from the bone marrow of untreated mice and from the splenocytes of G-mobilized pDC. G-mobilized pDC were compared to pDC from splenocytes of untreated mice to confirm that the differences observed were due to G-CSF mobilization rather than the hematolymphoid organ from which they were harvested. Surface marker characterization and gene expression data confirmed that pDC from the spleen of untreated mice are more similar to pDC from the bone marrow of untreated mice than to pDC from the spleens of G-mobilized pDC. Thus, we conclude that the change in character and function of G-mobilized pDC is due to the effect of granulocyte-colony stimulating factor (G-CSF) mobilization of pDC form the bone marrow.

Because antigen presentation plays a vital role in GvHD, we assessed the ability of BM and G-mobilized pDC to present antigen in a series of T cell assays in which T cell polarization was

measured. In the control group where BM pDC were added to T cells in culture, BM pDC induced Th1 polarization due to increased production of IL-12 in BM pDC at baseline, which allows pDC to aid in engraftment and GvL post-transplant. With increased production of IL-12 coupled with a pro-inflammatory environment in the MLR there was only a slight increase in Th1 polarization confirming that pDC also contain the ability to suppress and/or reverse their pro-inflammatory responses. Finally, using the α model system in which transgenic T cells specifically recognize the TEα peptide presented by $H2^b$ MHC (175), we evaluated pDC antigen presentation *in vitro*. With the addition of BM pDC to TEα T cells with peptide in the culture medium, there was not a significant increase in Th1 polarization compared to the other two culture conditions. Thus, because cytokine production results in the biggest difference in T cell polarization and antigen presentation did note, antigen presentation is most likely not the major source of BM pDC:T cell interaction.

In contrast to the beneficial effects of donor bone marrow pDC, G-CSF administration to donors mobilized donor pDC that caused increased GvHD clinical and histopathological scores. In accordance with G-mobilized pDC limited ability to produce IL-12 at baseline, there was significantly less Th1 polarization in the control culture (G-mobilized pDC co-cultured with T cells) when compared to BM pDC under the same conditions. The addition of G-mobilized pDC to an MLR resulted in significantly less Th1 polarization compared to BM pDC. Alternatively, when G-mobilized pDC were co-cultured with TEα T cells and TEα peptide, there was a significant increase in Th1 polarization, indicating G-mobilized pDC ability to present peptide. Thus, the increased incidence and severity of GvHD in recipients of G-mobilized pDC is likely a consequence of activation of allo-reactive T cells following indirect antigen presentation by G-CSF mobilized pDC of host-derived peptides sequences. Therefore, the pro-inflammatory properties of G-mobilized pDC coupled with their limited ability to suppress or reverse immune

system activation results in the inability of donor G-mobilized pDC to limit GvHD or increase survival.

Confirming these results, gene expression data showed that BM pDC have downregulated expression of gene pathways associated with T cell polarization and co-stimulation, consistent with their ability to limit GvHD post-transplant. Following an initial pro-inflammatory effect of donor bone marrow pDC, the sustained upregulation of IDO leads to the induction of an immunotolerant environment. A clue as to the effect of BM pDC in decreasing GvHD is their expression of higher levels of CCR9 than G-mobilized pDC. CCR9 is a marker of tolerogenic pDC and a chemokine receptor necessary for pDC migration to the gut (134). When using wild-type (WT) BM, G-mobilized, and CCR9KO mice as pDC donors, we showed that donor T cell proliferation was lowest in the gut of mice that received WT BM pDC and highest in the mice that received CCR9KO pDC. Of note, T cell proliferation was also increased in the skin of mice that received G-mobilized pDC adding mechanistic insight into the occurrence of skin changes in recipients of G-mobilized pDC.

Moreover, we determined that these observed differences were dependent on a pDC:T cell feedback loop. With higher amounts of IL-12 production from BM pDC, there is ensuing IFNγ production from T cells. Initially, this inflammatory environment aids in engraftment and graft-versus-tumor (GvT). Because BM pDC have a sustained increase in indolamine 2,3-deoxygenase synthesis in response to IFNγ signaling compared to G-mobilized pDC, this results in increased numbers of regulatory T cells at day 10 post-transplant that aid in maintaining tolerance. Surprisingly, BM pDC limitation of GvHD does not diminish GvT because there is equal survival and tumor burden in recipients of BM pDC compared to recipients of G-mobilized pDC.

Next, we began to devise and evaluate a method to increase pDC content in BM. We focused on Flt3L due to its role in pDC differentiation and proliferation and its ability to increase pDC content *in vitro* (60, 61, 159). We showed that administration of Flt3L to bone marrow donors significantly increased pDC content without significantly affecting the content of hematopoietic stem cells and other immune cells in the graft although it remains to be studied whether Flt3L administration to donors alters the immunological properties of other donor immune cell subsets.

Although F-BM pDC had a similar phenotype to BM pDC, recipients of F-BM grafts had increased survival and decreased GvHD clinical scores compared to recipients of BM pDC. Interestingly, gene array analyses revealed differences between pDC from untreated donors and Flt3L treated donors isolated from both mice and humans. Plasmacytoid dendritic cells from Flt3L treated donors had upregulated expression of adaptive immune and immune checkpoint pathways. Furthermore, genes involved in T cell selection in the thymus were upregulated in F-BM pDC, suggesting a novel role for donor pDC in regulating T cell immunity post-transplant including intrathymic T cell selection and differentiation. As a result, post-transplant Th1 cell polarization is decreased in recipients of F-BM. Additionally, toll-like receptors are downregulated in human pDC. Therefore, stimulation of pDC from common side effects of the post-transplant inflammatory environment in the gut, such as LPS secretion from bacteria, are potentially diminished (171-173). Finally, decreased GvHD in recipients of F-BM does not diminish the GvT effect of allo-tranpslantation, but prolongs survival compared to recipients of BM due to decreased GvHD mortality.

In conclusion, we report that BM pDC increase survival and decrease GvHD without diminishing GvT effect compared to G-mobilized pDC. The mechanism of action of donor pDC is IL-12 dependent and requires cross-talk between pDC and T cells. Because BM pDC content varies between BM donors, we assessed methods to increase pDC content in all bone marrow grafts.

Herein, we describe a novel method to increase pDC content by treating BM donors with Flt3L. Additionally, Flt3L treatment not only increases pDC content, but also improves the ability of pDC to decrease GvHD thereby increasing post-transplant survival. Therefore, comparing the 3 sources of pDC, BM, G-mobilized, and F-BM, F-BM pDC have the most the greatest ability to positively impact the field of HSCT. Thus, further studies should be conducted to determine the effect of Flt3L treatment in healthy, human BM donors.

4-3-0 Future Directions

The clinical benefit of increased pDC in the grafts of BM donors compared to G-mobilized pDC was confirmed in our well established murine allogeneic HSCT model. Because G-mobilized grafts are so commonly used (9), we assessed alternative methods of cytokine mobilization to affect pDC content and function with the goal of increasing the content of donor pDC that function like BM pDC. Because, Flt3L plays a vital role in pDC differentiation and proliferation and has been previously studied as a hematopoietic stem cell mobilizing agent (57, 59, 159), we examined the effect of the combination of G-CSF and Flt3L for mobilization. This combination was comparable to G-CSF administration alone in mobilizing hematopoietic stem cells and pDC. However, grafts mobilized with this cytokine combination were not effective in increasing survival or reducing GvHD compared to BM or G-CSF graft recipients. Due to these results, and the finding that donor BM pDC increased survival and reduced GvHD compared to G-CSF mobilized donor pDC, we tested whether single-agent Flt3L treatment of BM donors would yield a donor graft capable of improving transplant outcomes. Our results of increased survival and decreased GvHD in recipients of F-BM compared to BM recipients allows us to report a novel method to increase pDC content in the BM grafts by administering just two doses of Flt3L to donors.

F-BM pDC also upregulate and downregulate genes that regulate the adaptive immune system, immune checkpoints and T cell selection in the thymus. Plasmacytoid dendritic cells have been shown to play a role in T cell selection in the thymus (94) and upregulation of these genes post-transplant play a role in the increased survival and decreased GvHD that is observed in recipients of F-BM. To further elucidate the importance of this gene pathway upregulation, post-transplant migration of pDC to the thymus will need to be evaluated. First, post-transplant migration of donor pDC to the thymus will be compared among recipients of BM, G-mobilized and F-BM pDC.

Additionally, PRSS16, a gene involved in T cell selection in the thymus is a gene that is most commonly known to be expressed by cortical thymic epithelial cells, is upregulated in pDC from F-BM grafts (176-178). Further studies must be conducted to confirm this finding. Due to the lack of commercial antibodies to TSSP that can be used in flow cytometry, Western blots will be used to verify that thymus-specific serine protease (TSSP), the protein encoded by PRSS16 (179), is expressed in pDC. Because gene expression is altered by many factors, it is also important to determine whether TSSP expression is modulated post-transplant. If pDC migrate to the thymus post-transplant as posited above, and TSSP is expressed in pDC in the thymic microenvironment, we will perform further studies to define the role of TSSP expression by donor pDC using murine transplant models.

First, we will determine whether PDCA1, a pDC marker (64), and TSSP are co-expressed by donor pDC in the thymus using immunohistochemistry. Subsequently, we will examine the spatial interaction between donor pDC and developing donor stem-cell-derived T cells in the thymus by immunohistochemistry. Due to the difficulty of recovering pDC post-transplant, it is important to use larger numbers of donor pDC and, if possible, transgenic GFP+ donor mice as the source of pDC. After confirming pDC presence in the thymus, we will determine the location of donor pDC in the thymus, as positive and negative selection occur in the cortex or medulla, respectively. Additionally, we will evaluate whether pDC that have homed to other lymphoid organs express TSSP.

While our findings are promising to the field of HSCT, the majority of results reported herein are from murine transplant models. Thus, clinical studies pursuing verification of these findings is important. Mechanisms that must be studied in human pDC include cytokine profiles at baseline and in response to immune activation, most importantly IL-12 production comparing BM to G-mobilized pDC. Furthermore, studies aimed at manipulating G-mobilized pDC to function in the

same manner as BM pDC would be beneficial to the field due to the predominant use of G-mobilized donor grafts in allogeneic transplants.

In addition, determining the effect of Flt3L on other cell types in bone marrow grafts is critical to fully understanding the results reported herein. The content, character, and function of pDC following Flt3L treatment of healthy human volunteers will need to be assessed. Additionally, using healthy volunteers, the effect of this method on other cells types in the grafts that may affect transplant outcomes will advance our understanding of the possible clinical translation of our results. Ultimately, a randomized clinical study examining HSCT post-transplant outcomes using grafts from Flt3L treated BM donors versus untreated donors will be needed to further determine the utility and benefits of this method of increasing pDC content in BM grafts.

Based on the benefits observed with increased pDC content and the ability of Flt3L to increase pDC content, the use of pDC as immunotherapy should be examined as it pertains to preventing or treating GvHD. Across preclinical and clinical studies, larger numbers of donor BM pDC have been associated with a reduction in GvHD. In our lab, a barrier to using pDC as immunotherapy include inability to obtain sufficient numbers of pDC either through cytokine induced proliferation or ex vivo expansion. The use of FLT3L treatment of marrow donors detailed here provides one method to increase pDC content of the donor graft. The post-transplant administration of Flt3L should also be examined as another method to increase pDC content in transplant recipients and hasten pDC reconstitution, which is associated with improved transplant outcomes (180-182). As was done in the experiments discussed here, the optimal schedule of Flt3L administration post-transplant will need to be determined. Additionally, the effect of Flt3L treatment on hematopoiesis and hematopoietic system reconstitution should be examined. Furthermore, assessment of the effects of post-transplant administration of Flt3L on GvHD and survival is of utmost importance. Alternatively, targeted in vivo depletion of pDC with antibodies or selective drugs should be

examined as a means to eliminate pDC tolerogenic that may contribute to an immunosuppressive tumor microenvironment or pDC that have pathological functions in autoimmune diseases (183, 184).

While strategies of improved HSCT primarily benefit patients with hematological malignancies, the approaches we have developed may also benefit patients with non-malignant hematological disorders such as sickle cell anemia and thalessemias who can be cured by allogeneic HSCT. Currently, the use of allogeneic HSCT in these populations is typically limited to those patients with the most severe disease who have already suffered from major complications including end-organ failure. While HSCT can greatly improve the lives of these individuals, the ability to receive an HSCT before life threatening complications occur will decrease mortality and greatly reduce morbidity. Our ultimate goal of improving HSCT is to make this life saving treatment available to many more people who can benefit.

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