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The Role of IL-12 Producing Antigen Presenting Cells in Bone Marrow Transplant Outcomes

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

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The Role of IL-12 Producing Antigen Presenting Cells in Bone Marrow Transplant Outcomes

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a treatment used to cure patients with high risk and relapsed cancers of the blood and bone marrow, such as acute leukemia or multiple myeloma, via a graft-versus-leukemia (GvL) effect. The GvL effect of the graft is mediated primarily by donor T cells that are activated by a combination of both donor and host antigen presenting cells (APCs). However, a strong GvL effect is often is accompanied by a phenomenon called graft-versus-host-disease (GvHD). A fundamental question in the field is that of how to separate the phenomenon of GvL activity from that of GvHD. Dendritic cells (DCs) are APCs that stand at the forefront of GvHD initiation and are a likely target in tipping the balance from GvHD towards GvL. Clinical studies have indicated that higher numbers of donor plasmacytoid DCs (pDCs) are correlated with improved survival and lower GvHD in patients undergoing unrelated donor allo-HSCT. Upon conducting murine studies, it was also found that mice receiving purified populations of HSCs, T cells, and pDCs had improved donor T cell expansion, enhanced T-helper-type-I (T_H1) polarization of donor T cells, enhanced GvL activity and survival, without an increase in GvHD. While sorting of purified populations enhanced survival in murine systems, the sorting of purified populations is not translatable to clinical practice. We aimed to develop a feasible method for enriching pDCs in BMT, while elucidating the mechanism by which donor pDCs separated GvL from GvHD. We found that by depleting myeloid DCs (mDCs) and in turn enriching for pDCs in total BM in a murine allogeneic model of BMT, we mirrored the results seen in transplants with purified populations. Mice receiving mDC depleted BM had enhanced GvL, donor T cell expansion, as well as an increase in serum IFN-γ compared with mice receiving unmanipulated BMT. We also found that the trends were dependent upon the ability of cells in donor BM to produce IL-12. The primary IL-12 producers were presumably pDCs. To confirm this, we transplanted mice with purified population of HSCs, T cells, and pDCs from either WT or IL-12 KO mice and found an increase in survival when mice received WT pDCs as compared with IL-12 KO pDCs. In addition to minimizing GvHD in transplant, graft failure is also of concern in certain transplant conditions. We aimed to elucidate the role of IL-12 producing host-APCs in engraftment in a murine model of allogeneic transplant. By creating radiation chimeras where host hematopoietic cells were incapable of producing IL-12, we found that IL-12 production played an important role in facilitating donor T cell engraftment. Taken together, the data shed light on fundamental questions in the field of allo-HSCT and support local production of IL-12 as a critical event necessary to improve GvL, minimize GvHD, and improve engraftment.

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Chapter 1

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a treatment used to cure patients with high risk and relapsed cancers of the blood and bone marrow, such as acute leukemia or multiple myeloma, via a graft-versus-leukemia (GvL) effect. The GvL effect of the graft is mediated primarily by donor T cells that are activated by a combination of both donor and residual host antigen presenting cells (APCs). However, a strong GvL effect is often accompanied by a phenomenon called graft-versus-host-disease (GvHD) in which donor T cells attack recipient tissues, leading to adverse symptoms including but not limited to dermatitis, intestinal inflammation, liver damage, pneumonitis, and death. Even though HSCT is routinely used for high-risk malignancies, there is also a potential for HSCT to be used for many other ailments, including autoimmune diseases and immune deficiencies provided that the risk of GvHD be managed. While there are measures available to reduce GvHD symptoms, these approaches operate at the expense of diminishing GvL activity, or reducing the probability of stable engraftment by donor hematopoietic cells. In settings where GvL is not of concern, the common route to reduce GvHD is via systemic immunosuppression that brings with it a risk of infection[1].

A fundamental question in the field is that of how to separate the phenomenon of GvL activity from that of GvHD. The current paradigm for the initiation of GvHD is a three step model proposed by Ferrara, et al.[2] It is thought that initially pre-transplant conditioning such as radiation and chemotherapy results in tissue damage and subsequent breakdown of the gut epithelial barrier leading to the activation of toll like receptors (TLRs) in recipient APCs. Next, these activated APCs release cytokines, and eventually

activate donor T cells. Cytotoxic donor T cells then expand and mediate the hallmark tissue damage of GvHD in the transplant recipient. [1, 2]

As APCs are at the forefront of GvHD initiation, they are a likely target in tipping the balance from GvHD towards GvL. Dendritic cells (DCs) are professional APCs of a variety of subsets that can induce different effector functions in T cells and drive the differentiation of T cells towards T-helper-type-1 (T_H1), T-helper-type-2 (T_H2), T-helper-type-17 (T_H17), and regulatory T cell (Treg) phenotypes[3]. DC subsets differ in their immune function and responses upon antigen encounter. Murine splenic plasmacytoid DCs (pDCs) have been shown to produce significant amounts of interleukein-12 (IL-12) upon activation and polarize T cells towards T_H1 responses[4, 5, 6, 7]. Murine mDCs tend to polarize T cells towards T_H2 responses[4, 5, 6, 7]. Host and donor derived APCs also have been known to have different effects on GvL and GvHD. GvL activity requires host DCs for full effects, however it has been known for some time that donor APCs are able to contribute to GvL when tumor levels are low and we have shown that specific subsets of donor DCs can have significant effects on GvL [8, 9, 10, 11, 12]. Host DCs likewise initiate GvHD, but donor DCs have been known to amplify the phenomenon.

The data presented in this dissertation builds upon previous knowledge of the role of APCs in bone marrow transplant outcomes and further elucidates the role of dendritic cell subsets in separating GvL from GvHD. A clinically translatable method for increasing GvL activity while limiting GvHD is presented, and the role of interleukin-12 (IL-12) in promoting GvL activity is illuminated. In addition to the phenomena of GvL and GvHD,

engraftment is considered, and the necessity of IL-12 producing host cells in facilitating engraftment is demonstrated. Together, these findings clarify mechanisms by which APCs tip the balance between GvL and GvHD and support the potential clinical application of IL-12 as a therapy not only to ameliorate GvHD in HSCT but also to promote engraftment and GvL activity of donor T cells.

A brief history of hematopoietic stem cell transplantation

Early days of transplantation

The early days of transplantation were of organs and skin-grafts, which date back to the early 1900s. Organ and skin allografts were attempted, however the grafts could not function for more than 2 weeks. The reasons for rejection of allografts were largely unknown, though many theories had been suggested, including but not limited to something intrinsic within the graft, a humoral factor, or role for lymphocytes in rejection as suggested by James Murphy as early as 1914[13]. An "immunity hypothesis" was suggested whereby allograft rejection was attributed to blood-group incompatibility, and that if blood groups were compatible that skin allografts would be successful[14, 15]. In 1943, however, Gibson and Medawar published an article in the Journal of Anatomy that discredited the blood compatibility theory[16]. Gibson and Medawar described a 22-year-old woman who had experienced "deep thermal burns" and was in "poor condition" that they had given two sets of skin grafts from her brother's thigh[16]. The patient and her brother were of the same blood type, O. Nevertheless, neither set of homografts had grown successfully and "was therefore scraped off". Medawar and Gibson also noted that "the 2nd-set homografts were even worse than their 1st-set contemporaries," having

degenerated more rapidly than the first set[16]. They could not find a cellular mechanism to explain this and determined that the timeline for rejection correlated with the "pattern of an antigen-antibody reaction."[16] At the time there were many theories for why allografts did not succeed, including blood group incompatibility, humoral factors, something intrinsic within the graft, as well as a cellular basis for rejection. Gibson and Medawar were the first to establish an immunological basis for allograft rejection.

Not long after Gibson and Medawar, Ray Owen found that dizygotic twin calves with different red blood cell (RBC) types each had their own RBCs as well as a set of RBCs from the other twin, and that this caused the twin calves to lack an immune reaction to the foreign RBC from the other twin[17]. This suggested that exposure to antigens early in life, presumably due to a shared placenta in the calves, could induce immunological tolerance to an antigen.

Billingham, Brent, and Medawar set out to test the hypothesis that tolerance could be induced. They used both mice and chickens to show that when an animal of one strain is inoculated with cells of another strain during the fetal stage, that the resulting adult animal is tolerant to skin grafts from the other strain, while animals that had not been inoculated *in utero* would reject skin grafts[18]. Not only did they show that the animals were tolerant to skin grafts, but they showed that this was induced in the animal, and not a property of the graft. Injection of chopped lymph node fragments from animals not conditioned *in utero* into the "tolerized" animals restored the ability to reject the skin graft[18]. Also, skin grafts from other mouse strains could be rejected by the "tolerized"

mice provided that they did not match the strain used to condition the mice *in utero*[18]. This set the stage for future research not only in tolerance for skin and solid organ transplantation, but also for the future of hematopoietic stem cell transplantation.

The ideas for the bone marrow transplants came from experiments at the same time in the late 40s and early 50s. Jacobson, et al., showed that mice could survive lethal irradiation if the spleen was shielded during the process[19]. Jacobson believed that there was a humoral basis for this protection[20]. Lorenz, et al., then showed that lethally irradiated mice or guinea pigs could be rescued by a subsequent i.p. or i.v. injection of cells from the bone marrow of syngeneic animals, which, while not discounting a humoral component to protection, suggested that other components were involved in hematopoiesis[21].

The study that linked skin-allografts, with Medawar's hypothesis, and bone marrow transplantation was one by Main and Prehn in 1955[22]. Main and Prehn expanded upon the Jacobson and Lorenz studies by lethally irradiating DBA/2JN mice, subsequently injecting them i.v. with BM from either DBA/2JN mice or hybrid BALB/cAnN x DBA/2JN donors. Twenty four to thirty days post BMT, mice were given skin grafts from BALB/cAnN donors. Upon analyzing skin graft retention, it was found that 92% of mice receiving BM from hybrid BABL/cAnN x DBA/2JN mice retained their grafts compared with 7% of DBA/2JN (syngeneic) BM recipients.

Of course, these experiments occurred prior to the seminal paper by Bruce Glick in *Poultry Science* in 1956 showing a role for the bursa of Fabricius, a hindgut lymphoid organ, for antibody production in chickens. Ten years later, Max Cooper combined the procedures of bursectomy and thymectomy, to show that both organs are important in development of the lymphoid system. It was shown that chickens that were bursectomized, yet retained their thymus, could not produce antibodies, and yet could reject skin grafts. On the other hand, thymectomized chickens have normal circulating antibody numbers, but they have low white blood cell counts and die early. This indicated that there were two distinct components of the lymphoid system, and that the thymus was responsible for cell mediated immunity, like in humans and mice. More importantly for the transplant world, the skin graft and graft vs. host experiments using bursectomized chickens suggested that humoral immunity plays a more minor role in skin-graft rejection, and that allogeneic bone marrow must contain some cellular component that tolerizes animals to skin-grafts from allogeneic donors.

BMT for leukemia

Leukemia was originally described in 1827 and named in 1845 by Velpeau and Virchow, respectively. Rudolf Virchow, a German pathologist, described leukemia as the proliferation of white cells and named it from the greek words, leukos – white and aima – blood. The first attempt at using a transplant to cure leukemia was in mice by Barnes, et al., in 1956[23]. In his paper he hypothesized:

"On the one hand, a dose of x-rays which might be sufficiently lethal to normal cells of the bone marrow and lymphatic tissues to cause the death of the animal might well be completely lethal to leukaemic cells: the irradiated animal could then be treated with normal isologous bone marrow from the same strain of mouse for the repopulation of the hematopoietic tissues."[23]

In fact, in this first mouse study, with a sufficiently high dose of radiation followed by syngeneic BMT, 25/35 mice were alive at 90 days post-transplant[23].

Not long thereafter, in 1957, E. Donnall Thomas performed the first bone marrow transplants in humans with leukemia[24]. He performed transplants on 6 patients, and while the safety of the transplants was confirmed, only one patient experienced transient engraftment[24]. Two years later, Thomas performed a successful transplant using identical twin donors and confirmed that humans, like mice, could be protected from lethal irradiation by BMT[25]. The first successful allogeneic transplant to treat leukemia occurred in 1963 by Mathe, et al. with a patient suffering from acute lymphoblastic leukemia (ALL) that received BM from three related donors in order to improve the number of administered BM cells[26]. Soon after transplant, the patient experienced symptoms including weight loss, diarrhea, nausea, vomiting, and erythrodermia, consistent with acute GvHD[26]. The symptoms subsided after treatment with isoniazid, streptomycin, Δ-1 cortisone, and ε-aminocaproic acid[26]. The patient was still alive, with mixed-chimerism eight months after the transplant[26]. This was the first successful

allogeneic transplant of a leukemia patient who did not show signs of disease 8 months after transplant and was considered in complete remission.

Most early attempts at using BMT as a treatment for leukemia were unsuccessful, either due to failure to engraft, complications from acute and chronic GvHD, or opportunistic infections. A review article by Bortin, et al. in 1970 described 200 allogeneic transplants, not one of which successfully engrafted. In the 1970s, when engraftment was successful in transplants used for the treatment of leukemia, few patients survived long term, partly because all transplant recipients were in late stage disease, often after a relapse and after conventional therapies had failed. By 1979, Thomas, et al., described a study that began in 1976 where 12 / 19 patients undergoing allogeneic transplants for acute non-lymphoblastic leukemia (AML) in the first remission had survived to 3 years post-transplant, a marked improvement in a short time[27]. The field was changing, and new knowledge about irradiation doses, human leukocyte antigens (HLA), sources of stem cells, GvHD, and preparatory regiments had paved the way for more successful future transplants.

Separating GvL from GvHD

GvHD

Graft versus host disease (GvHD) was first described as a "runt disease" in 1959 by Billingham & Brent in neonatal mice injected with spleen cells from an unrelated strain[28]. The first report of GvHD in a human patient was during the course of the first successful allogeneic BMT reported by Mathe, et al., in 1963, whereby the patient experienced

symptoms of acute GvHD including, but not limited to, weight loss, diarrhea, vomiting, and erythrodermia[26]. Billingham & Brent later described three condition required for the development of GvHD which included: 1) The donor and the recipient must be genetically different; 2) The donor graft must contain immunocompetent cells; 3) The recipient must be immunocompromised and unable to eliminate the donor cells[29].

There are two types of GvHD: acute and chronic. Acute GvHD (aGvHD) occurs soon after transplant (within 100 days) and involves a general state of inflammation due to cytokine release and non-specific tissue damage by donor effector cells. Severity of acute GvHD is correlated with the degree of HLA- mismatch between the donor and the recipient[30]. The clinical manifestations of acute GvHD include maculopapular skin rash, gastrointestinal tract symptoms such as diarrhea, nausea, and inflammation, liver disease, as well as pneumonia in the lungs[1, 31]. The most commonly experienced symptom is skin rash, seen in as many as 81% of acute GvHD patients at onset[32].

Chronic GvHD (cGvHD) occurs later, three to eighteen months post-transplant, and involves humoral and antigen-specific immune responses against host-tissues leading to signs and symptoms similar to those seen in patients with an autoimmune disease[1]. Chronic GvHD can occur in conjunction with acute GvHD or entirely independently in a patient that never experience acute GvHD symptoms[1]. However, presence of acute GvHD does put one at a higher risk for devleoping chronic GvHD as does older age[33]. More recently, other classifications have been suggested including late-onset acute

GvHD (after day 100) and an overlap syndrome that has aspects of both acute and chronic GvHD[34].

The current view on the initiation of acute GvHD is that the pre-transplant conditioning regimen of irradiation and chemotherapy leads to tissue damage, specifically in the gut epithelium[2]. This initial tissue damage leads to release of LPS across the gut epithelial barrier. In addition to LPS, damaged cells release danger signals including proinflamatory cytokines such as TNF-α and IL-1β following pre-transplant conditioning[35, 36, 37]. LPS has been shown to accumulate in the liver, spleen, and serum of mice as acute GvHD progresses[38]. LPS activates DCs and other APCs via TLR4 stimulation, leading to upregulation of costimulatory molecules and MHC II expression on APCs, and subsequent production of pro-inflammatory cytokines by APCs[35, 36, 37].

In the second phase of GvHD initiation activated APCS go on to activate donor T cells. Host APCs have long been known to play a large role in initiating GvHD and host DC have been found to be sufficient to initiate acute GvHD in murine models[9, 39, 40]. Donor T cells begin to produce large amounts of T_H1 cytokines, including IL-2, IFN-γ, and TNF-α2[1]. While some of these proinflamatory cytokines are targets for therapies aimed at minimizing GvHD, it is of note that T_H1 type immunity is critical for successful GvL responses. Also, some pro-inflammatory cytokines such as IFN-γ have been implicated in suppressing GvHD to a degree by regulating donor T cell apoptosis. While host APC initiate GvHD, the intensity of GvHD can be modulated by donor APCs[1, 41, 42].

After activation of donor T cells, the third phase of GvHD initiation is the effector phase, in which cellular and inflammatory effectors mediate tissue injury and inflammation characteristic of the condition[1]. The primary cellular effectors include cytotoxic T cells (CTLs) and natural killer (NK) cells[36]. CTLs lyse target recipient cells via the Fas/Fas Ligand pathways as well as the perforin –granzyme pathway[1]. Further secretion of proinflamatory cytokines such as TNF-α stimulated by LPS activation of TLR4 amplifies inflammation and lead to a “cytokine storm”.

GvL

The graft-versus-leukemia (GvL) effect in the absence of adverse effects such as GvHD is the ultimate goal of successful allo-HSCT. It is believed that GvL is the result of CTLs directed against host hematopoietic cells. In the setting of HLA-matched transplants, it is thought that the CTL response is directed against minor histocompatibility antigens (miHAs) and/or tumor specific antigens. Savage, et al., were able to generate CTLs against the leukemia antigen, Wilms tumor antigen, using B-cell bound HLA-A2 class I monomers[43]. Similarly, Marijt, et al., found that patients receiving DLI after relapsed leukemia or myeloma from donors that were negative for two separate miHAs that were expressed on the recipients malignancies developed CTLs specific for the miHAs[44]. In the latter study, the frequency of miHA-specific CTLs increased along with the regression of disease[44].

GvL and GvHD usually go hand in hand. It is known that the strength of GvL activity is correlated with the incidence of cGvHD but not aGvHD. While aGvHD is a broad

inflamed state leading to damage against host tissues such as the skin, gut, and liver, cGvHD involves antigen-specific cellular and humoral immunity. GvL is thought to be the result of antigen-specific CTLs directed against host hematopoietic cells and parallels the mechanisms behind cGvHD more closely than those of aGvHD. A major question in the field is how to separate GvHD from GvL. Are GvL and GvHD one and the same, or are there two separate mechanisms driving the phenomena, and can they be differentiated?

Preventing GvHD while preserving GvL

Ways to prevent GvHD while preserving GvL is a fundamental question pursued by ongoing research. Ways to limit GvHD are described extensively elsewhere in a review by Li and Waller[45]. In brief, strategies are categorized by prophylactic pharmacological therapies, modification of donor T cells, modification of other cell subsets in the graft, as well as vaccines that target tumor antigens.

Prevention of GvHD by pharmacological agents has long been an effective method of preventing aGvHD while still somewhat allowing for GvL effects. Calcineurin inhibitors such as cyclosporine A and tacrolimus, which prevent T-cell activation, are often used in conjunction with more general immunosuppressants, such as methotrexate (MTX), mycophenylate mofetil (MMF), and prednisolone. However, while clinical trials using a combination of cyclosporine, MTX, and prednisolone did show a decrease in aGvHD among patients, overall survival did not improve[46]. Another drug is anti-thymocyte-globulin (ATG), which is used in combination with total body irradiation (TBI) or

busulfan and is often accompanied by MTX and/or cyclosporin. Similarly though, use of ATG along with pre-transplant conditioning did reduce aGvHD in a clinical trial, however overall survival had not improved[47]. A potential explanation for reduced GvHD without improved survival could be a parallel reduction in GvL activity due to the overall immune suppression and reduction of T cell activation.

Another method of preventing GvHD is by manipulation of donor T cells. An early strategy in the 1980s and 1990s was T Cell Depletion (TCD) of the donor graft via either negatively selecting for T cells *ex vivo*, positively selecting for stem cells *ex vivo*, or via use of anti-T cell antibodies *in vivo*[1]. While TCD does reduce both aGvHD and cGvHD, there is a higher risk of graft failure, relapse, and infections post-transplant[48, 49]. Problems with immune reconstitution experienced with receiving TCD grafts can be overcome through the use of a donor lymphocyte infusion (DLI). DLI is often delayed in order to prevent early donor T cell activation in the inflamed state of the recipient resulting from tissue injury soon after transplant. While delayed DLI has been successful in decreasing GvHD while preserving GvL in murine models of MHC-matched, miHA-mismatched transplants, data from clinical trials in humans have not resulted in similar outcomes[10, 50, 51, 52]. DLI has also been used to treat relapse in CML and low-grade non-Hodgkin lymphoma, giving many escalating doses of DLI until either a clinical response is achieved or symptoms of GvHD are manifested[53]. Other T-cell manipulation methods including eliminating alloreactive T cells via 4,5-dibromorhodamide-123 incorporation followed by exposure to light, selection for memory T cells, the use of fludarabine to eliminate naïve T cells from DLI, *ex vivo* activation of γδ T cells, and infusion of *ex vivo*

expanded Tregs[45]. These methods have been tested in murine models and many have shown contradictory results depending on the type of transplant and malignancy, activation status of the T cells, and methodology[45].

In addition to manipulation of the T cell content of the graft, an ongoing area of research is the manipulation of other cell subsets including the source of the donor graft, the NK cell and DC content of the graft, as well as mesenchymal stem cells in the graft[45]. Along with bone marrow, hematopoietic stem cells (HSC) can be obtained from sources such as G-CSF mobilized blood and umbilical cord blood (UCB) [45, 54]. Clinical studies have shown that GvL activity and aGvHD incidence is similar among all three sources of HSCs[49]. However, pediatric patients receiving BM-HSCT have lower cGvHD than patients receiving blood-HSCT[49], and pediatric patients receiving UCB-HSCT have lower cGvHD incidence than patients receiving BM-HSCT[55]. Mesenchymal stromal cells have been used to minimize GvHD both in murine studies as well as in clinical trials[45]. Mesenchymal stromal cells have been administered as a prophylactic treatment in clinical trials and have been shown reduce the risk of aGvHD though at the expense of an increased risk of relapse. Lastly, manipulation of APC subsets, namely DCs is a feasible method of separating GvL from GvHD. The role of different subsets of DCs from host and donor is controversial and of ongoing interest in the field.

Dendritic cells and their subsets

Dendritic cells (DCs), first characterized in 1973 in mice by Steinman and Cohn, are bone-marrow derived professional antigen presenting cells named for their characteristic

dendritic morphology[56]. They arise from BM derived HSCs which differentiate into both myeloid and lymphoid progenitors[57, 58]. GM-CSF, Flt3, and M-CSF all play a role in driving DC hematopoiesis[57, 58, 59, 60]. A majority of DCs in the body are in an immature state and provide immune surveillance, constantly taking up both self-antigens to maintain self-tolerance and monitoring for foreign antigens as a sign of danger or infection[61]. Upon encounter of a foreign antigen, pathogen associated molecular patterns (PAMPs) on the pathogen are recognized by pathogen recognition receptors (PRRs) such as Toll like receptors (TLRs) within the DC[62]. TLRs can also be activated by danger signals given off by dead or dying cells such as heat shock proteins, DNA, and high-mobility group protein B1. Upon activation, DCs upregulate Major Histocompatibility Complex II (MHC II), costimulatory molecules CD80, CD86, CD40, and CD83 (human DC), chemokine receptors, and begin migrating to lymph nodes and other secondary lymphoid organs[63]. Once within secondary lymphoid organs, DCs present antigen to T cells and initiate an adaptive immune response[63].

DCs are classified into distinct subsets based on function, location, progenitors, and expression of cell surface markers[64]. The two main subsets of DCs are "conventional" or myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Murine pDCs are characterized by the phenotype of Lineage- CD11c+ CD11b- B220+ PDCA1+. Murine mDCS are characterized as Lineage – Cd11c+ CD11b+ B220-. Of note, DCs can also be distinguished by their CD8α expression. Murine pDC are though to express CD8α, while mDC do not[65]. In the mouse, both pDC and mDC can arise from both common lymphoid progenitors as well as common myeloid progenitors[66]. Common lymphoid and myeloid

progenitors then differentiate into precursor DCs, which eventually go on to become mDCs, or "conventional" DCs (cDCs). Myeloid DCs can be subdivided further into more specific subsets including migratory Langerhans cells and dermal DCs which remain in the periphery and then present antigen in lymph nodes (LN) as well as lymphoid tissue resident DCs which remain in lymphoid organs and sample antigen while in the spleen, thymus and LN.[67] Another type of DC that does not arise from DC precursors, but rather a monocyte precursor, is a monocyte-derived DC or "inflammatory DC". Inflammatory DCs arise from monocyte precursors in the presence of GM-CSF and can drive T cell responses towards a T_H1 phenotype. Inflammatory DCs have a similar phenotype and function as mDCs, expressing CD11c, CD11b, and MHC class II, but they also express Ly-6C, a marker panel consistent with a precursor cell phenotype[64].

While all DCs are on patrol and uptake both self and foreign antigens to present to T cells, there are two pathways of antigen presentation: direct and indirect. Direct presentation is the uptake of antigen from the exterior followed by processing and presentation on MHC II on the surface of the DC. The indirect pathway is also called cross presentation and is the exhibition of endocytosed antigens on MHC I.

Upon uptake, processing of antigen, and activation of the DC, different DC subsets secrete distinct patterns of cytokines depending on their phenotype. It was initially thought that the differential expression of TLRs by different subsets of DCs and then subsequent signaling through those TLRs drove the immune response however it has since been found that stimulation of the same TLR can lead to different responses by

different DC subsets. For example, TLR7 is expressed by both human mDC and pDC, however when these DC are activated *in vitro* with the corresponding TLR7 ligand, imidazaquinoline, they produce IFN-α and IL-12, respectively[68]. In the mouse, it is generally thought that pDC (CD11b-CD8+) induce T_H1 responses in T cells while mDC (CD11b+CD8-) lead to T_H2 responses in T cells[7, 65, 69]. A major difference in the ability of DCs to polarize T cells is the capacity of DC subsets to produce IL-12p70. Plasmacytoid DCs tend to produce more IL-12p70 compared with mDCs. However, it is important to note that certain CD4-CD8- mDCs are capable of producing IL-12 when in certain conditions, just not to the level that pDCs normally produce IL-12[65]. Plasmacytoid DCs also can produce IFN-α and IFN-β when in certain laboratory conditions[65].

The cytokines that DC subsets produce can lead to effects on activated T cells and can alter the amount of cytokines that activated T cells secrete. Myeloid DCs lead to more production of IL-2, IFN-γ, GM-CSF, and IL-4 from both CD4 and CD8 T cells than pDCs, even though pDCs secrete more IL-12, which is T_H1 polarizing[70, 71, 72, 73]. Plasmacytoid DCs also can lead to apoptotic death in some activated CD4 T cells *in vitro* while mDCs do not have this capacity[74].

Human and mouse DCs have limited phenotypic and functional homology. Studies in human DCs have largely been limited to skin and blood DCs due to the rare nature of the subset and the ability to access sites. In addition to practical difficulties in studying human DC subsets, some markers, such as CD8, are not expressed at all on human DC subsets, making comparisons difficult[65]. Also unlike mouse pDCs, which are the primary

producers of IL-12, many human DC subsets secrete high levels of IL-12[75]. Currently, human pDCs are considered by be lineage-, MHC II+, CD11c-, CD123+, BDCA2+ (mouse pDCs are lineage -, MHC II+ CD11c+ PDCA1+ B220+)[76]. By contrast, human mDCs are lineage -, MHC II+, CD11c+, BDCA1, CD11c/b+ (mouse mDCs are lineage –, MHC II+, CD11c+, CD11b+). There is another subset of human mDCs which express BDCA3 (CD141) instead of BDCA1 and were thought to have functional correspondence to murine pDCs due to their capacity to secrete high levels of IL-12, although more recent studies found that human pDCs also have this ability[75, 77]. Due to the varied nature of DC subsets and their ability to produce cytokines and induce cytokine production in T cells, direct comparisons between mouse and human DC subsets are impractical.

The role of DCs in GvHD and GvL

The ability of DCs to direct adaptive immune responses has long been of interest in the transplant community. Most studies have focused on differential effects on GvHD and GvL by host-type and donor-type DCs, though more recent work has attempted to elucidate the contributions of different DC subsets to transplant settings.

Host DCs were initially found to be sufficient at inducing acute GvHD, and inactivation of host APCs by creating radiation chimeras in which host APCs lacked MHC II prevents aGvHD development[39, 40]. It has since been elucidated that host APCs are required for CD8 T cell dependent aGvHD, while both host and donor APCs can initiate CD4 T cell dependent aGvHD. Donor APCs can amplify CD8 T cell dependent aGvHD[8, 39, 40]. Chronic GvHD can be induced by both donor and host APCs, though donor APCs have a

larger role in intestinal cGvHD, while both donor and host APCs play a role in the development of skin cGvHD[78, 79].

The method of antigen presentation by DCs to T-cells can also influence initiation of GvHD. Acute GvHD is initiated by host DCs only via direct antigen presentation, and cannot be initiated by cross-presenting host DCs[8, 40]. Matte and Shlomchik reported that when donor APCs lack MHC class I, CD8 mediated GvHD is reduced, suggesting that cross presentation of donor APCs to CD8 T cells played a role in the development of GvHD[8]. To further investigate this, Wang and Shlomchik created mice that expressed defined MHC class I K^b restricted miHAs crossed to K^b deficient mice, leading to mice where the antigens could not be directly presented. It was found that donor CD4+ T cells were activated by host APCs (via MHC II), and then subsequently activated donor APCs. These activated donor APCs took up miHAs from host hematopoietic and non-hematopoietic transmembrane proteins and were able to cross present the antigens to CD8 T cells[80]. Markey, et al., have also recently found that donor cDCs in a murine model cross present antigen shortly after allo-HSCT, while donor pDCs and macrophages do not make a significant contribution in allo-antigen cross presentation[41].

Similar to GvHD, GvL activity requires host DCs for full effects, however donor APCs are able to contribute to GvL when tumor levels are low[8, 9, 10]. APCs are also required for effectiveness of DLI after transplant. In mice, GvL effects resulting from DLI require MHC II expressing host APCs for maximal activity[10].

Clinical studies suggest that the ratio or numbers of subsets of DCs present in the donor graft can affect survival. Rajasekar, et al., found that higher human pDC content of grafts correlates with more relapse and reduced long-term survival of patients undergoing peripheral blood mobilized HSCT for various hematological malignancies[81]. Similarly, Waller, et al., reported in 2001 that recipients of BM grafts from HLA-matched related donors that contained fewer precursors of pDCs (pre-pDC) had less relapse yet higher incidence of cGvHD compared with patients receiving higher numbers of pre-pDC[82]. More recently, Waller, et al., are reporting the results of the BMTCTN0201 clinical trial (unpublished, in submission)[83, 84]. The role of donor pDCs was reexamined yet this time using unrelated donors, 25% of which were HLA-matched, where donors and recipients were randomized to receive BM allografts or G-CSF mobilized peripheral blood HSC grafts[83, 84] Of note, the use of unrelated donors is associated with higher incidence of GvHD. Moreover, the previous 2001 study had a high percentage of patients with chronic myeloid leukemia (CML), which are highly sensitive to the GvL activity of donor T cells. In the recent analysis, it was found that a higher donor pDC content as well as higher naïve CD8+ T cell content in the graft was associated with improved survival though no difference in relapse rates[83, 84]. Transplantation with higher numbers of naïve CD8+ T cells was also associated with reduced risk of grade III-IV aGVHD, while pDC numbers did not correlate with GvHD incidence[83, 84].

Manipulation of specific subsets of DCs has been of interest as a way to separate GvL activity from the phenomenon of GvHD. Li and Waller took an approach of depletion of all CD11b+ cells from allogeneic BM grafts in a murine model, which included not only

DCs but also other cell subsets expressing CD11b including monocytes, macrophages, granulocytes, NK cells, myeloid suppressor cells, as well as effector and memory CD8 T cells[11, 85, 86, 87]. It was found that depletion of all CD11b+ cells from grafts led to enhanced survival of tumor bearing mice using a LBRM (a murine B10.BR leukemia cell line) model though with a slight increase in non-lethal GvHD[11]. Next, in order to determine whether CD11b+ DCs contributed to the increased GvL, Li, Darlak, and Waller performed transplants using purified cell subsets. B10.BR mice received allogeneic transplants from C57BL6/J donors of fluorescence-activated cell sorting (FACS) – purified HSCs (Lin- Sca1+C-kit+), MACS-sorted T-cells (negative selection column), and either mDCs or pDCs (CD11c+ and CD11b+ or CD11b-, respectively)[12]. Mice receiving grafts containing a combination of HSC, pDC and T cells had increased leukemia free survival without an increase in GvHD compared with mice receiving grafts of HSC, mDC and T cells or HSC and T cells alone[12]. Transplanting CD11b- DCs (pDCs) with HSC and T cells also led to an increased T_H1 polarization of donor T cells as evidenced by both intracellular (higher IFN-γ, lower IL-4, lower IL-10) and increased levels of T_H1 serum cytokines (IL-12, IFN-γ, IL-12) compared with mice receiving HSC, mDC, and T cells or HSC and T cells alone[12]. IFN-γ did not have direct tumoricidal acitivy, as shown by co-culture of LBRM (leukemia cell line) with IFN-γ *in vitro*[12]. We later examined the role of IFN-γ and indoleamine 2,3-dioxygenase (IDO) signaling between donor DCs and donor T cells. Mice received allogeneic transplants of FACS-purified HSCs, pre-pDCs (CD11c+CD11b-PDCA1+B220+) and T cells from either WT or IFN-γ KO mice[88]. It was found that when donor T cells were deficient in their capacity to produce IFN-γ, GVHD was more severe without enhanced GvL, regardless of whether

pre-pDCs had been added to the HSC graft[88]. We also found that IDO, an IFN-γ inducible gene, is upregulated in pDCs both in *in vitro* IFN-γ stimulated pDCs but also *in vivo* in donor pDCs transplanted with WT donor T cells and HSCs. IDO upregulation required the production of IFN-γ by donor T cells[88]. To determine whether IDO played a role in separating GvL from GvHD, we used IDO KO mice as a source for pre-pDCs. IDO expression by pre-pDC was found to be necessary to limit GvHD, as mice receiving IDO KO pre-pDCs along with HSCs and T cells had more severe GvHD than mice receiving HSCs, T cells, and WT pre-pDCs[88]. These data show that IDO expression by pre-pDC and IFN-γ production by donor T cells are critical regulators of the balance between sufficient GvL activity while limiting GvHD. We have suggested that initially, donor pDCs induce T_H1 polarization of activated donor T cells, which in turn secrete IFN-γ. This IFN-γ secretion provides negative feedback by inducing IDO expression by donor pre-pDCs, which then regulates GvHD by inducing Treg generation and down-modulation of inflammatory cytokines production by donor T cells. This model allows for just enough allo-reactivity of donor T cells to initiate GvL, while limiting the extent of GvHD.[88]

Interleukin-12

IL-12 is a heterodimeric pro-inflammatory cytokine that drives the differentiation of T_H1 cells. IL-12 was originally recognized in 1989 and was referred to as natural killer cell stimulatory factor (NKSF)[89]. It had been shown that human B cell lines could facilitate the growth of human NK cells and T cell clones, and had been observed that some B cell lines could stimulate NK cells to produce IFN-γ[89]. Michiko Kobayashi, while in the

Trinchieri lab, reported that that EBV-transformed B cell lines were producing a novel soluble factor, NKSF, that induced IFN-γ production by NK cells, enhanced NK cell cytotoxicity, as well as enhanced T cell responses to mitogens like PHA[89]. It was noted that the molecule was a heterodimeric cytokine that was composed of two disulfide-linked chains of 35 kDa and 40kDa, and while the p40 subunit was secreted by cell lines, only the heterodimer had activity on NK cell and T-cells[89]. Soon thereafter, Stern, et al. purified a cytokine that was named cytotoxic lymphocyte maturation factor (CMLF) from the supernatant of a human B cell line[90]. CLMF was found to be a 75 kDa heterodimer, with the same size 40kDa and 35 kDa subunits linked by a disulfide bond as NKSF. It was noted that Kobayashi, et al. had found a similar cytokine with similar activity, though the lack of sequence information of either cytokine made direct comparison impossible. It was later determined that the cytokines were identical, and Stern, et al. proposed the name IL-12 for the cytokine[91, 92].

The p35 (IL-12α) and p40 (IL-12β) subunits have homology to other cytokines. The p35 subunit has limited homology to the cytokines IL-6 and G-CSF[93]. The p40 subunit has extensive amino acid similarities to the extracellular portion of the IL-6 receptor as well as the ciliary neurotrophic factor receptor[93, 94, 95]. Two other cytokines, IL-23 and IL-27, have been found to share homology to subunits of IL-12 and are now part of the IL-12 cytokine family. IL-23 is composed of a p19 subunit heterodimerized to the IL-12 p40 subunit[96]. IL-27 is composed of a p40 related protein, EBV induced gene 3 (EBI3), and a p28 subunit, which is related to the IL-12 p35 subunit and is also known as IL-30.[97]

IL-12 production and other forms

Antigen presenting cells such as dendritic cells (DCs), macrophages, monocytes, and neutrophils are the primary producers of IL-12[98, 99]. However, other non-hematopoietic cell types such as keratinocytes, osteoblasts, epithelial cells, and endothelial cells are capable of producing IL-12[100, 101]. As early as the discovery of IL-12, it was noted that the p40 subunit of IL-12 is secreted as a monomer in abundant excess of the heterodimer, although only the heterodimer has biological activity to stimulate IFN-γ production by NK cells[89, 90]. When human monocytes are primed with IL-4 and IFN-γ and then stimulated with LPS, the longer the duration of stimulation, the more free p40 subunit is produced in relation to heterodimerized IL-12[102]. The p35 and p40 subunits are encoded by different genes on separate chromosomes in both humans and mice and are independently regulated[103, 104, 105]. It is thought that the transcription of the p35 chain is what regulates production of the heterodimer, as the p40 subunit is produced in such excess[106].

In addition to a p35 and p40 heterodimer, which forms IL-12p70 (74 kDa), the biologically active form normally referred to as IL-12, p40 is capable of homodimerizing, creating IL-12p80, in both mice and humans[107, 108]. Both human and mouse p40 homodimers can compete with the IL-12p70 heterodimer, however the homodimer does not have biological activity and does not signal through the IL-12 receptor[107, 108]. It is thought that the p40 subunit contains key binding epitopes for the IL-12 receptor, however p35 mediates biological activity[107, 108]. Of note, p35 alone does not bind the IL-12 receptor or mediate biological activity[107, 108]. The first reports showed that the IL-

12p40 homodimer can inhibit the IL-12 induced proliferation of mouse lymphoblasts[109] or PHA-activated human lymphocytes[108], as measured by tritiated thymidine incorporation, *in vitro,* as well as IFN-γ production *in vivo* in mice treated with an i.p. injection of LPS or KLH [109]. More accounts of IL-12p40 homodimer suppressing T_H1 differentiation in murine models have been described, including inflammation, transplantation, and cancer[109, 110, 111, 112, 113, 114, 115]. IL-12p40 can also be secreted as a monomer, which can also inhibit effects of the IL-12p70 heterodimer[116]. While the mouse IL-12p40 homodimer has been observed both in transfected cells *in vitro*[107] as well as in *vivo*[111], there are limited reports of IL-12p40 homodimer expression in humans. Walter, et al., reported in 2001 that the bronchioaveolar lavage fluid in asthma patients had an overproduction of IL-12p40 that was in homodimeric form[117]. Prostaglandin E_2 (PGE_2), an inflammatory mediator which drives formation of T_H2 cells, can inhibit IL-12p70 while inducing IL-12p40 subunit production in human DCs, though whether the IL-12p40 is in monomeric or homodimeric form is unknown due to poor stability of the human IL-12p40 homodimer[118]. The presence of IL-12p40 monomers in the serum of patients with systemic lupus erythematosus (SLE) has been correlated with disease activity[119].

While most research suggests a biologically inactive, purely antagonistic role of IL-12p40 monomer and homodimer to IL-12p70, there have been recent accounts of actual activity of IL-12p40 homodimers. Jana and Pahan, et al., have shown over the years that IL-12p40 homodimer is capable of inducing expression of iNOS, TNF-α, lymphotoxin-α and IL-16 in both human and mouse microglia and macrophages[120, 121, 122, 123, 124].

The first report of IL-12 production by DCs was *in vitro* by Macatonia, et al., in 1995[99], and then *in vivo* by Sousa, et al., in 1997, who showed that DCs in the spleens of mice stimulated with *Toxoplasma gondii* extract of LPS produced IL-12[125]. Production of IL-12 by phagocytes and DCs is stimulated by the activation of pathogen-recognition receptors (PRRs) in the cells. PRRs, such as toll-like receptors (TLRs), present in DCs and phagocytes bind pathogen-specific sequences and initiate innate immune responses. Stimulants such as bacteria, intracellular parasites, double stranded RNA, bacterial DNA, and CpG can stimulate production of IL-12 by APCS. Upon activation, DCs will upregulate CD40, which in turn binds to CD40L on T cells and enhances IL-12 production by DCs[126]. Of note, CD40 to CD40L binding is neither sufficient nor necessary for IL-12 production[126]. IFN-γ as well as the T_H2 cytokines IL-4 and IL-13 can also enhance IL-12 production by APCs[127, 128, 129]. While IFN-γ, IL4, and IL-13 can upregulate IL-12 production by DCs, IL-10 inhibits transcription of both subunits of IL-12[103, 130].

IL-12 activity on T cells and immune responses

IL-12 was shown early on to induce T_H1 cell development[131, 132, 133]. Macrophages activated by *Listeria monocytogenes* were shown to produce IL-12, which in turn drives the differentiation of T cells towards a T_H1 phenotype[131]. Seder, et al., found that IL-12 was not required for IFN-γ production by T_H1 cells, however that it enhanced IFN-γ production, and diminished the inhibition of IFN-γ production by IL-4[133]. This was one

of the initial reports that the balance of cytokines IL-12 and IL-4 could determine the fate of a T cell towards a T_H1 phenotype.

IL-12 maintains T_H1 cell fate through binding to the IL-12 receptor (IL-12R). The IL-12 receptor is composed of two chains, IL-12Rβ1 and IL-12Rβ2, both of which have homology to the common receptor β-chain of the IL-6-like cytokine superfamily, gp130. Naïve T cells express IL-12Rβ1, but not IL-12Rβ2. Initially it was thought that IL-12 initiated T_H1 cell differentiation. It has since been revised that the initial signal for T_H1 differentiation is T-bet, which is induced in naïve T-cells by NK-cell derived IFN-γ or STAT1 signaling[134, 135]. T-bet in turn induces IL-12Rβ2 expression and IL-12 binding helps to maintain a T_H1 phenotype[134, 135]. IL-12Rβ2 contributes to signal transduction and it is required for IL-12p70 activity. When IL-12p70 binds to the IL-12R, IL-12Rβ1 is phosphorylated, which then initiates JAK-STAT signaling pathways. IL-12 induction of IFN-γ production is dependent on STAT4[136].

Other IL-12 family members

Since the discovery of IL-12 and the homology of its subunits to cytokines and receptors of IL-6 and G-CSF, other cytokines have been discovered that share homology to IL-12 and that share IL-12 subunits. In addition to IL-12, there are three more IL-12 family members: IL-23, IL-27, and IL-35.

IL-23 is a proinflamatory cytokine discovered in 2000 that is comprised of the p40 subunit of IL-12 in addition to a p19 subunit (approximately 19 kDa) and like IL-12, IL-

23 also activates STAT4[96] after binding to a receptor comprised of the IL-12Rβ1 chain and the IL-23R chain[137]. IL-23 is produced by activated DCs and macrophages upon PRR activation and can stimulate the proliferation of humans and mouse activated and memory T cells, but not naïve T cells *in vitro* as well as induce IFN-γ production by T cells[96]. IL-23 is distinct from IL-12 in that it promotes the maintenance of T_H17 cells. T_H17 cells are important in defense against extracellular bacteria and fungi and are characterized by the secretion of IL-17[138]. TH17 cells are generated from naïve T cells by the actions of IL-6 and TGF-β, and are then maintained by the presence of IL-23. Of note, TGF-β can lead to the generation of regulatory T cells (Tregs), but IL-6 can inhibit the generation of Tregs, and in combination with TGF-β will lead to T_H17 cell generation[138].

IL-27 is an inhibitory cytokine composed of two subunits, EBI3 and p28[97]. EBI3 shares homology with the p40 subunit of IL-12 and p28 is related to the IL-12 p35 subunit and is also known as IL-30.[97] IL-27 can induce the generation of Tregs[139, 140, 141]. IL-27 also enhances the production of IFN-γ as well as IL-10, which together along with STAT1 activation and subsequent downregulation of RORγt will suppress T_H17 generation[139, 140, 141, 142].

IL-35 is the last of the IL-12 family, and is a heterodimeric inhibitory cytokine composed of the same EBI3 subunit as IL-27 but is paired with the p35 subunit of IL-12[143]. IL-35 is produced by Tregs and inhibits the generation of T_H1 and T_H17 cells. IL-35 can also

produce iTr35 cells, an induced Treg population characterized by their lack of Foxp3 expression and their secretion of IL-35, yet not IL-10 or TGF-β[144, 145].

Antitumor activity of IL-12

IL-12 has been considered in clinical practice as an anti-tumor therapy. IL-12 secreted by DCs and phagocytic cells leads to activation of NK cells and T cells, TH1 polarization of T cells, IFN-γ secretion by T cells and NK cells, and enhancement of NK cell effector functions. IL-12 also leads to upregulation of MHC I and MHC II by APCs, which aids in the presentation of tumor antigens to T cells. Most of the anti-tumor properties that IL-12 has are due to its effects on downstream molecules. IL-12 has anti-angiogenic properties by inducing IFN-γ-inducible protein 10 (IP-10) and monokine-induced by IFN-γ (Mig) and the major anti-tumor effects that IL-12 produces are due to the induction of IFN-γ production by T cells and NK cells[146, 147, 148]. In addition to IFN-γ acting to promote T cell and NK cell function, IFN-γ may have direct effects against tumors by inhibiting angiogenesis. IFN-γ has also been shown to enhance the GvL activity of donor T cells while limiting GvHD by both inducing apoptosis of allo-reactive CD4 and CD8 T cells, contributing to the functional activity of alloantigen-reactive Tregs, and by altering the susceptibility of GvHD affected tissues to alloreactive T cells[139, 149, 150, 151].

Administration of IL-12 in tumor bearing mice has led to reduction or complete inhibition of tumor development in both solid tumor models and hematological malignanices[152]. Many preclinical murine studies have shown efficacy of recombinant

IL-12 against tumors[153, 154, 155], though IL-12 toxicity in humans is a significant problem and only low doses are tolerated (0.5 μg/kg)[156, 157]. Also, use of repeated doses of recombinant IL-12 in clinical trials has been shown to paradoxically lead to a shift from T_H1 type immunity to T_H2 type immunity in serum cytokines in humans, as seen by a decrease in IFN-γ and an increase in IL-10[158, 159]. As a result, groups have shifted to the possibility of using IL-12 in combination with other cytokines such as IL-2, IL-7, IL-21, IL-18, GM-CSF, and IFN-α[160]. These approaches are reviewed in detail elsewhere by Weiss, et al[160]. Heinzerling, et al., found that intra-tumoral injection of DNA coding for IL-12 in mice with a B16 model of melanoma leads to regression of tumor[161]. The same group conducted human trials with intratumoral injection of a plasmid encoding IL-12 and found complete remission or disease stabilization in 3/9 metastatic melanoma patients[162].

IL-12 and GvHD

Due to its potent effects on T cell activation and polarization, IL-12 has been of interest in the transplant community as a potential therapy for GvHD. As early as 1995, Sykes, et al., showed that a single injection of recombinant IL-12 inhibits acute GvHD in a fully-MHC-mismatched model of murine BMT[163]. It was found that IL-12 treatment led to increases in serum IFN-γ, reduced T cell expansion in the spleen on day 4 post-transplant compared with untreated mice, yet increased CD8+ T cells on day 7 post transplant[163]. The hypothesis was that CD8+ T cells were primarily mediating GvL, whereas CD4 T cells were mediating GvHD[163]. Sykes, et al., did later find that the single dose of IL-12

reduced CD4 dependent GvHD, but also preserve GvL effect that was CD8 dependent in the murine model used.[164] Reddy, et al., evaluated levels of IL-12 in HSCT patients with various hematological malignancies and found that high levels of circulating IL-12 were correlated with reduced relapse without increased GvHD[165]. Of note, the same group independently reported that high total DC numbers upon immune reconstitution were associated with lower relapse and decreased GvHD[166]. Taking together the research on IL-12 in GvHD as well as anti-tumor immunity suggests that IL-12 has potential use as a therapeutic in the BMT clinical setting, and plays an important role in GvHD as well as GvL.

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Chapter 2

Enrichment of IL-12 Producing Plasmacytoid Dendritic Cells in Donor Bone Marrow Grafts Enhances Graft-versus-leukemia Activity in Allogeneic Hematopoietic Stem Cell Transplantation

Abstract

A critical question in the field of allogeneic hematopoietic stem cell transplantation (HSCT) is how to enhance graft-versus-leukemia (GvL) activity while limiting graft-versus-host-disease (GvHD). We have previously reported that donor bone marrow precursors of plasmacytoid dendritic cells (pre-pDC) can polarize donor T-cells towards Th1 immunity and augment GvL activity of donor T-cells while attenuating their GvHD activity in a murine model of allogeneic HSCT. Clinical data on the role of donor pre-pDC and cDC on transplant outcomes has been conflicting. To test the effect of increasing the proportion of pre-pDC versus cDC in a bone marrow graft, we enriched CD11b- pDC by selectively depleting the CD11b+ myeloid dendritic cells (mDC) population from bone marrow using FACS sorting in a murine model of allogeneic bone marrow transplantation. Donor T-cell expansion and GvL activity in mice that received marrow depleted of mDC was greater than in mice transplanted with undepleted marrow. GvHD was not increased by depleting mDC. In order to examine the mechanism that depleting mDC enhanced the GvL activity of donor T cells, we used BM and pDC from IL-12p40KO mice and found that the increased GvL activity of mDC-depleted BM was IL-12 dependent. This study indicates that a clinically translatable strategy of engineering the DC content of the graft can improve allo-transplant clinical outcomes through the regulation of donor T cell activation and GvL activity.

Introduction

Donor T-cells mediate the graft-versus-leukemia (GvL) effect, but also cause graft-versus-host disease (GvHD) following allogeneic hematopoietic stem cell transplantation (HSCT). Both GvL and GvHD require antigen-presenting cells (APC) to activate T-cell effectors.[1, 2] In contrast to the requirement for host dendritic cells (DC) in the initiation of GvHD,[3] the role for donor DC on transplant outcomes is less clear. We have previously reported that recipients of bone marrow (BM) grafts from HLA matched related donors containing fewer precursors of plasmacytoid dendritic cells (pre-pDC) had less relapse and more chronic GvHD compared with patients that received larger numbers of pre-pDC. [4] More recently, in analysis of the BMTCTN0201 clinical trial we reexamined the role of donor pDC in the context of unrelated donor transplants[5]. Donors and recipients were randomized to receive either BM allografts or G-CSF mobilized blood stem cell grafts from unrelated donors, 25% of which were HLA-mismatched. In an analysis of subsets of immune cells in the BM grafts, we found that recipients of grafts containing more immature donor pDC and donor-naïve CD8+ T cells had improved survival compared with recipients of grafts containing fewer of these cell subsets[5,6].

The current study was undertaken to further explore the role of donor pre-pDC and classical DC in allogeneic transplant outcomes using established murine bone marrow transplant (BMT) models. Murine DC subsets have a functional correspondence but incomplete phenotypic identity with human DC subsets. Both human and murine pDC are important in initiating innate immunity, [7, 8]and murine BM-derived pDC produce significant amounts of interleukin-12 (IL-12) and polarize T-cells toward Th1 immune

responses *in vitro*.[8, 9, 10, 11] Human dendritic cell subsets are distinguished by different patterns of phenotypic markers than murine DC. Human conventional dendritic cells (cDC) produce proinflammatory cytokines when activated, and it has been suggested that human cDC are the equivalent of mouse mDC (Lin-CD11c+CD11b-CD8α+)[12]. While some studies have shown that human cDC produce large quantities of IL-12p70, suggesting that they are the functional analog of murine pDC, a recent examination by Mittag, et al. has found that both pDC and cDC from human spleen can produce IL-12p70, as well as perform functions primarily restricted to murine pDC such as cross presentation [13]. MacDonald *et al.* have previously showed that depleting $CD11c^+$ cDC reduced the severity of GvHD in mice,[14] and donor cDC are the most effective population in presenting alloantigen and stimulating naïve donor T-cells responses early post-BMT.[15]

Recently, our group has reported the addition of donor bone marrow cells enriched for pre-pDC to a graft composed of purified hematopoietic stem cell (HSC) and T-cells significantly improved long-term leukemia-free survival without increasing GvHD compared with recipients of donor HSC and T-cells.[16] Transplantation of FACS sorted donor pre-pDC added to purified HSC and donor T cells resulted in Th1 and Tc1 immune polarization of donor T cells with higher serum levels of IL-12, interferon-γ (IFN-γ), interleukin-2 (IL-2) at 3 and 10 days post-transplant compared with recipients of HSC and T-cells alone.[16] In order to establish the mechanism by which different donor DC subsets regulate post-transplant immunity, we explored the role of IL-12 produced by donor DC in the augmentation of the GvL activity of donor T-cells in a murine BMT model. Based upon the markedly higher serum levels of IL-12 in the recipients

transplanted with bone marrow enriched for pre-pDC, we hypothesized that passive enrichment of pDC by depleting mDC from a BM graft might enhance GvL activity of donor T-cells, and IL-12 synthesized by donor pDC may be necessary for enhanced activation and GvL activity of donor T cells,

This study was designed to test whether selective and specific depletion of a rare BM DC subset would have significant clinical outcomes in a murine model of BMT. To test this hypothesis we used two allogeneic murine BMT models (C57BL/6→B10.BR and C57BL/6→FVB). We report herein that recipients of mDC-depleted BM had increased donor T-cell proliferation, engraftment and survival post-transplant without increased GvHD compared with recipients of undepleted BM in tumor-bearing mice. Enhanced proliferation and engraftment of donor T-cells was not seen with mDC-depleted BM from IL-12p40 KO donors. These data show that selective pDC enrichment in BM allografts enhances donor T-cell engraftment, and GvL activity. The beneficial effects of mDC-depletion appear to be IL-12 dependent.

Materials and Methods

Mice

B10.BR (H-2K^{k}, CD45.2, CD90.2), C57BL/6 (B6, H-2K^{b}, CD45.2, CD90.2), FVB/NJ (H-2^{q}, CD45.1, CD90.1), PepBoy (B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ)(H-2K^{b}, CD45.1, CD90.2) mice on the B6 background and IL-12p40 knock out (IL-12 KO) mice (B6.129S1-*Il12b*^{tm1Jm}/J)(H-2K^{b}, CD45.2, CD90.2) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Congenic strains expressing CD90.1 and CD45.2 on a B6 (H-2K^{b}) background (BA), and CD90.1, CD45.2 on a B10 (H-2K^{k}) background (BA.B10) were backcrossed 10 generations to the parental strain and bred at Emory University

Animal Care Facility (Atlanta, GA, USA). The luciferase-expressing transgenic mice on the B6 background were bred at Emory University. Mice were used at 8-12 weeks of age. Procedures conformed to National Institutes of Health (NIH, Bethesda, MD, USA) animal care guidelines and were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Cell Preparations

Donor mice were killed in a humane manner and femurs, tibias, and spleens were removed aseptically. Bone marrow (BM) cells and splenocytes were harvested with sterile PBS. T-cells were purified by incubating splenocytes with biotinylated anti-CD11b, B220, CD49b, and TER119 antibodies (BD Biosciences), followed by anti-biotin microbeads, and negative MACS selection using an LS magnetic column (MACS, Miltenyi Biotech). For CD11b^{+} mDC depletion from T-cell depleted (TCD) BM grafts, BM cells were incubated with biotin-conjugated anti-CD3 antibodies (BD Biosciences) followed by anti-biotin microbeads (Miltenyi Biotech). TCD BM was then collected using LS magnetic columns (MACS, Miltenyi Biotech) and stained with a cocktail of anti-CD11c-FITC, CD11b-APC-Cy7, Lineage (IgM, CD19, CD49b and TER119)-PE Abs. Lineage^{-} CD11c^{+} CD11b^{+} cells were depleted by FACS using the FACSAria (Becton Dickinson, San Jose, CA, USA) cell sorter using Diva software (BD). After initial scatter-based gating for viability, the Lineage^{-} and Lineage^{+} populations were identified and further gated on CD11c and CD11b; CD11c^{+}CD11b^{+} double positive cells were discarded. All other Lineage^{-} and Lineage^{+} cells were sorted for transplant and comprise the CD11b^{+} mDC-depleted BM population. For FACS-sorting of HSC and

pDC, harvested BM was stained with cocktails of biotinylated Lineage (CD3, CD19, TER119, IgM, CD49B, and CD11b), PE-conjugated lineage (CD4, CD8, Gr1, Iab), anti-PDCA1-V450, anti-B220-PeCy5, anti-Ckit-APC, anti-Sca1-PeCy7, and anti-CD11c-FITC. HSC and pDC were sorted as described previously in Lu, et al.[17]

Tumor Cells

LBRM 33-5A4, a B10 T-cell lymphoma cell line,[18] was purchased from American Type Culture Collection (ATCC)(Manassas, VA, USA). Tumor cell lines were cultured according to ATCC recommendations. Tumor cell lines were tested to be free of lymphocytic choriomeningitis virus, mouse hepatitis virus, mouse minute virus, and mouse parvovirus by the University of Missouri Research Animal Diagnostic Laboratory (Columbia, MO, USA). This cell line was transfected to express luciferase in our laboratory and was used in an in vivo bioluminescent imaging experiment.

Transplantation

On day -2, recipient B10.BR mice received a total of 11 Gy irradiation divided into 2 doses, 5.5 Gy 3 hours apart.[19] On day 0, recipient mice were injected intravenously with combinations of 1×10^6 or 3×10^6 mDC-depleted TCD BM cells and 3×10^5 or 1×10^6 MACS-purified T-cells from B6 donors in a B6→B10.BR model. For tumor experiments, recipient B10.BR mice received an i.v. dose of 5×10^5 cells of viable luciferase$^+$ LBRM on day -1. For purified HSC, pre-pDC, and T cell transplants, on day 0 recipient mice were injected intravenously with combinations of 3000 FACS-sorted HSC, 50,000 FACS-sorted pre-pDC, and 3×10^5 MACS-purified T-cells from B6 donors. All

transplant recipients were monitored for survival and clinical signs of GvHD daily. Mice were scored for clinical signs of GvHD by weight loss, posture, activity, fur texture, and skin integrity twice weekly for the 30 days, and then weekly as described by Cooke, et al..[20] According to IACUC protocol, moribund mice, mice with greater than 25% weight loss, and mice surviving until the end of the experiment, were euthanized and considered to have died on the day following euthanasia for analysis of post-transplantation survival.

T cell proliferation, intracellular cytokine expression, and serum cytokines

The proliferation of donor T-cells in recipient spleen were analyzed by using the intracellular fluorescent label carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) to tag proliferating cells. Donor T-cells were stained with CFSE before transplant and recipient spleens were removed on day 3.5, and cell suspensions prepared. Proliferation of donor T-cells was determined by flow cytometric analysis of CFSE dilution profiles gated on donor T cells population.[21] Intracellular cytokine expression (IL-4, IL-10, IL-17, TNF-α, and IFN-γ; BD Biosciences) by $CD4^+$ and $CD8^+$ T cells was analyzed by using a Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA, USA) as described.[22] Appropriate isotype controls were used for all intracellular targets. All samples were analyzed on a FACS Canto (Beckon Dickinson, San Jose, CA, USA) and list mode files were analyzed using FlowJo software (Tree Star, Inc 2007, Ashland, OR).

Serum and secreted cytokine levels

Serum was harvested and stored at -20C until use for cytokine analysis by ELISA (OptEIA ELISA kit for IFN-γ; BD Biosciences, Ready-SET-Go ELISA kit for IL-13; eBIoscience). ELISA plates were read using a SpectraMax 340PC spectrophotometer (Molecular Devices, Sunnyvale, CA, USA)

In vivo bioluminescent imaging

In vivo bioluminescent imaging (BLI) was performed using a cooled charge-coupled device (CCD) camera system (IVIS Imaging System 100, Xenogen, Alameda, CA, USA). The mice received a subcutaneous injection of D-luciferin (0.15 mg/g body weight; Firefly luciferin potassium salt, Xenogen, Alameda, CA, USA). Ten minutes later, mice were placed in the light-tight chamber of the CCD camera system under rodent cocktail (ketamine 20 mg/ml and xylazine 2.5 mg/ml, 0.1 ml/25g body weight) anesthesia, and photographic and luminescent images in the ventral projection were acquired using a 3 minutes exposure time. Up to five mice were imaged simultaneously. On each *in vivo* BLI image, a region of interest (ROI) encompassing the entire mouse was created, and the whole-body signal in the ROI (photons/sec) was quantified using Living Image software (version 3.2, Xenogen, Alameda, CA, USA). The whole-body bioluminescent signal was used as a marker of the donor T-cell burden.

Statistical Analyses

Analyses of data were performed using SPSS (version 18 Mac, SPSS Inc. Chicago, IL, USA) or GraphPad PRISM (version 5 Mac, GraphPad Software, San Diego, CA, USA, www.graphpad.com). Data are presented as mean ± SD of all evaluable samples. Survival

differences between groups were calculated with the Kaplan-Meier or Mantel-Cox log-rank test in a pair-wise fashion. Differences in GvHD outcome between groups were compared using the Kruskal-Wallis test. Differences in the levels of T-cell CFSE division, bioluminescence ROI values, and numbers between groups and other parametric tests were performed using the one-way analysis of variance (ANOVA) followed by a Bonferroni post-test. P-values of less than 0.05 were considered significant. In Bioluminescent imaging quantification data, significant outliers were removed following a Grubb's test for outliers.

Results

Enriching the ratio of pDC to mDC enhances GvL

Our previous experiments have shown that tumor-bearing mice transplanted with purified HSC, T cells, and pre-pDC have increased survival compared with mice receiving purified HSC and T cells alone[16]. The strategy of transplanting multiple FACS- purified subsets from a BM graft is not practical for clinical practice. To model the selective depletion of a BM DC subset, a FACS gating strategy was developed to passively enrich lineage^{-} CD11c^{+} CD11b^{-} pDC by selectively depleting the lineage^{-} CD11c^{+} CD11b^{+} mDC from the BM graft while preserving the content of HSC, T cells, B cells, monocytes, NK cells, and pre-pDC (Figure 2-1A, B). The content of lineage-CD11b+CD11c+ mDC was depleted by 99%, while the recovery of the other cell subsets was > 95% (data not shown). The CD11b+CD11c+ mDC represent average frequencies of 0.3% and 0.4% (SD=0.03 and 0.17%) of the nucleated BM cells from WT and IL-12p40KO animals, respectively. The CD11c+CD11b- DC sub-population represents average frequencies of

0.7% and 0.8% (SD = 0.15 and 0.29%) of murine BM cells from WT and IL-12p40KO animals, respectively. Thus the average ratio of pDC to mDC is approximately 2-3 : 1 in both WT and IL-12p4KO mice (data not shown). To study the effect of selectively depleting mDC from the BM graft on the GvL activity of donor T cells we used an established C57BL/6 to B10.BR transplant model and a B10.BR tumor cell lines, LBRM, that was stably transfected with luciferase. B10.BR mice were lethally irradiated and given 1 x 10^6 WT B6 T cells along with 3 x 10^6 undepleted BM cells or mDC-depleted $^{-}$ BM cells from WT B6 or IL-12p40KO donors as we have previously described [16]. Clinical GvHD scores were monitored twice weekly after transplant, as described by Cooke, et al.[20]. Survival of mice was monitored daily. In non-tumor-bearing mice transplanted with allogeneic BM and T cells, no significant difference in survival or GvHD score was seen among different treatment groups (Figure 2-1C, D). Next we determined whether selective depletion of mDC from a murine BM allograft would augment donor T cells activation and GvL activity. B10.BR mice were lethally irradiated and given 5 x 10^5 LBRM cells one day after irradiation. Two days after irradiation, mice were transplanted with 1 x 10^6 WT B6 T cells along with 3 x 10^6 undepleted BM cells or mDC-depleted BM cells from WT B6 or IL-12p40KO donors. Mice receiving mDC-depleted BM had significantly increased survival (median survival 49 days) compared with mice receiving either undepleted BM (median survival 21.5 days) or IL-12p40 KO mDC-depleted BM (median survival 32 days) (Figure 2-1E). GvHD scores between groups were not significantly different, except on day 15 where mice receiving IL-12p40KO mDC-depleted BM had a significantly higher GvHD score than mice receiving WT mDC-depleted BM (Figure 2-1F). Mice receiving mDC-depleted BM from IL-12p40

KO recipients experienced significantly higher weight loss than mice receiving WT mDC-depleted BM from days 12-17 post-transplant, which correlates with the higher GvHD scores received by IL-12p40 KO recipient animals (data not shown). Weight loss could also be due to increased tumor burden, as the IL-12p40KO mice had the lowest median survival in a tumor model, yet not in a non-tumor model.

Depletion of mDC results in early increased proliferation and expansion of donor CD8+ T cells in an IL-12 dependent manner

We next used CFSE-labeled T-cells to test the effects of enriching pDC in BM grafts on early allo-reactive donor T-cell proliferation in the spleen. A large number of CD8+ T cells in the spleen at early time points post-transplant has been correlated with improved GvL activity in a murine leukemia model[23]. Lethally-irradiated B10.BR mice received 1 $\times 10^6$ CFSE-labeled T-cells from CD45.1$^+$ congenic Pepboy (B6 background) donors, in combination with FACS-sorted 3×10^6 undepleted BM cells or mDC-depleted BM cells from IL-12p40 KO or WT B6 donors. Figure 2-2 shows the level of donor T cell proliferation on day 3.5 post-transplant as assessed by CFSE dilution in the spleen of transplant recipients. Of note, there was a significant increase in the proliferation of donor CD8 T cells in the group of which the mDCs had been depleted from BM compared with recipients of undepleted BM ($p < 0.01$) (Figure 2-2A, B). This is similar to our previously reported findings where all CD11b+ cells in the BM were depleted and data in which pDC were added to purified HSC and T cells and indicates that the residual pDC component of the marrow augments T cell proliferation. The role of donor pDC-derived IL-12 in enhancing T cell proliferation was confirmed by results showing that

mice receiving IL-12p40KO BM did not have enhanced proliferation of donor T cells following selective depletion of the mDC component.

The expansion of donor T cells of recipients of undepleted BM, mDC-depleted BM, and IL-12p40KO mDC-depleted BM is shown in panel C. In this case luciferase transgenics were used as a source of donor T cells in order to track the *in vivo* expansion of donor T cells. We transplanted 3×10^5 luciferase$^+$ T-cells in combination with 3×10^6 mDC-depleted BM cells from either IL-12p40 KO or WT C56BL/6 mice, or undepleted BM cells from WT B6 donors into B10.BR mice following lethal irradiation. Whole body bioluminescent images of mice were taken at days 5, 7, 14, 21 and 30 and the total bioluminescence signal in photons/second was determined per mouse.

These data show homing of donor T cells initially to the spleen on day 5 post-transplant with subsequent expansion in the abdominal cavity on days 7-30 (Figure 2-2C). Comparing T cell proliferation as assessed by emitted photons, we found that there was a trend towards greater T cell expansion in the recipients of mDC-depleted BM on day 5 and a significant difference in the overall level of T cell expansion at day 30 post-transplant, with lower numbers of donor T cells in the group receiving IL-12p40KO BM ($p<0.05$) and undepleted BM (p=ns) (Figure 2-2D). These data indicate that IL-12 producing donor DC may augment the allo-reactivity of donor T cells similar to a previous report by Anderson, et al., which indicated a role for donor DC in CD4-mediated GvHD of the gut [24]. The lower levels of T cell proliferation seen in the recipients of IL-12KO mDC-depleted BM are consistent with the role of donor derived

IL-12 in promoting Th1 polarization of T cells and contributing to GvHD at later times post transplant.

Effect of mDC depletion on the production of inflammatory cytokines

We hypothesized that mDC depletion would also polarize donor T cells towards a Th1 phenotype and we examined intracellular cytokine production by splenic donor T cells and serum cytokines on day 10 post-transplant. Both the percentages and median fluorescence intensity of IFN-γ, TNF-α, and IL-10 producing CD4 and CD8 T cells was not significantly different between recipients of undepleted BM, WT mDC-depleted BM, and IL-12p40KO mDC-depleted BM (Figure 2-3A-C, data not shown). However, IL-12p40KO mDC-depleted BM recipients had significantly higher percentages of IL-17 producing CD4 and CD8 donor T cells in the spleen on day 10 post-transplant (Figure 2-3D, $p<0.01$ and $p<0.05$, respectively). IL-17 has been implicated in contributing to CD4-mediated GvHD, which is consistent with increased GvHD that the IL-12p40 KO mDC-depleted BM recipients experienced on D15 post-transplant (Figure 2-1F). The amount of IFN-γ in the serum was also significantly higher in mice receiving WT mDC-depleted BM (Figure 2-3E). IL-12 did not seem to play a role in this increased production, as when mice received mDC-depleted BM from IL-12p40KO mice, high serum levels IFN-γ were also seen. This correlated with previous findings where mice transplanted with purified population of HSC, pDC, and T cells had an increase in production of Th1 cytokines[16]. Lastly, serum IL-13 was significantly higher in mice receiving mDC-depleted BM compared with mice receiving undepleted BM (Figure 2-3F, $p<0.01$). Serum IL-13 was not measured in mice receiving IL-12p40 KO mDC-depleted BM. IL-13 has been shown

to generate MDSC that can reduce GvHD, without impairing GvL[25]. We have also seen this phenomenon in mice receiving purified populations of HSC, pre-pDC, and T cells, as compared with mice receiving HSC and T cells alone (unpublished data, Waller lab).

IL-12 production by pre-pDC prolongs survival in tumor-bearing mice receiving HSC, purified pre-pDC and T cells

While we had found that mice receiving mDC-depleted BM had increased survival in a tumor-bearing model, and this advantage seemed to be IL-12p40 dependent, we hypothesized that IL-12 producing pDC remaining in the BM were responsible for the enhanced GvL activity of donor T cells. To confirm this hypothesis, we transplanted mice with purified populations of HSC, pre-pDC (CD11c+CD11b-B220+PDCA1+), and T cells. Mice were lethally irradiated and received 1 x 10^6 luc-LBRM cell i.v. one day after irradiation. One day after tumor injection, mice were transplanted with FACS-sorted combinations of 3000 WT HSC, 50,000 pre-pDC from either WT or IL-12p40KO mice, and 3 x 10^5 WT T cells, or a combination of 3 x 10^6 BM cells with 3 x 10^5 WT T cells as a control. All radiation control mice died within 12 days post-transplant (data not shown). Mice receiving HSC alone experienced 0% survival, with a median survival of 14 days (Figure 2-4A). Mice transplanted with HSC, WT pre-pDC and T cells had a survival of 75% at 40 days post-transplant, slightly higher than mice receiving whole BM and T cells (Figure 2-4A). All mice receiving HSC, IL-12p40KO pre-pDC and T cells died, with a median survival of 12 days. We speculate that the lack of IL-12p40 being produced early in transplant may result in rapid death from acute-GvHD. Surviving mice were imaged at 36 days post transplant for luciferase-positive tumors. Of the surviving mice, only 15% of

mice receiving WT BM and T cells had no detectable tumor, as determined by photons emitted from the tumor, while 34% of mice receiving HSC, WT pre-pDC, and T cells had no detectable tumor. The tumor light signal emanating from each mouse was quantified and is shown in panel B (Figure 2-4). While not significant, mice that received BM and T cells had a higher average bioluminescent signal than mice receiving HSC, WT pre-pDC, and T cells (Figure 2-4B).

Discussion

Ineffective GvL and the development of acute and chronic GvHD limit the broad application of allo-BMT. Based upon the clinical reports that the content of donor pDC in allogeneic BM grafts is associated with survival,[4] we developed murine model systems to test whether selective alteration in the relative numbers of donor pre-pDC versus donor mDC favorably improves the balance between GvL and GvHD in allogeneic BMT. Initially we reported that depletion of all $CD11b^+$ cells (including $CD11b^+$ DC, $CD11b^+$ NK cells, and myeloid suppressor progenitor cells) from the donor BM enhanced the anti-tumor activity of allograft donor T cells without significantly increased GvHD in mice.[26] Then, using highly purified populations of donor pDC, we observed that transplanting purified pDC along with HSC and T-cells leads to Th1 polarization of donor T cells and improved GvL activity compared with transplanting HSC, mDC and T-cells, or HSC and T-cells alone,[16] and demonstrated the bi-directional signaling between donor T-cells and donor pDC with IFN-γ produced by donor T-cells inducing indoleamine 2,3-dioxygenase (IDO) synthesis by donor pDC limited GvHD.[17] However, the approach of sorting purified populations of cells is impractical from a clinical translation standpoint. In the

current study we sought to develop a clinically translatable approach of graft engineering that would result in the same GvL improvement.

We passively enriched the relative content of donor pDC: mDC in the BM allograft by selectively depleting CD11b+ DC with high speed FACS to test whether depletion of mDC from BM graft has similar effect as positive addition of pDC of grafts consisting of purified populations of HSC and T cells. We found that tumor-bearing mice receiving mDC-depleted BM had increased survival without an increase in GvHD. Mice receiving mDC-depleted BM recipients have increased donor T-cell proliferation at early time points (day 3) in the spleen *in vivo*, especially donor $CD8^+$ T-cell proliferation. Using BLI we have shown that on day 30 post-transplant, recipients of mDC-depleted BM had a greater number of $luciferase^+$ donor T cells overall. Enhanced expansion and proliferation of donor T-cells were not observed in the recipients transplanted with mDC-depleted BM from IL-12p40 KO donors, which suggest that the effects of mDC-depleted BM on the donor T-cell proliferation and expansion appear to be IL-12 dependent. In order to confirm that IL-12p40 produced by pDC is responsible for increase GvL, we transplanted mice with purified populations of HSC, pre-pDC from either WT or IL-12p40KO donors, and T cells. Mice receiving HSC, WT pre-pDC and T cells had increased survival as compared with mice receiving HSC, IL-12p40KO pre-pDC and T cells.[16]

These data are consistent with our previous reports relevant to the role of pDC in donor immunity following allogeneic HSCT. It has been known that donor $CD8^+$ T-cells are an important mediator of GvL effect.[27] Fowler *et al* have previously shown that allogeneic

CD8^{+} T-cells generated by in vitro allo-antigenic priming in the presence of IL-12 mediate more potent GvL effects than those primed in the presence of IL-4.[28, 29] In our previous studies, it was found that adding donor pDC to purified HSC and T cells increased T cell proliferation after transplant and survival in a tumor-model compared with mice receiving HSC, mDC, and T cells or HSC and T cells alone.[16] There are reports of CD11b+CD11c+ DC of having a tolerogenic phenotype. In a model of i.v. tolerance in experimental autoimmune encephalomyelitis (EAE), mice immunized i.v. with a MOG-peptide following induction of EAE have less severe EAE than mice immunized with PBS, and contained abundant CD11c+CD11b+ DC in the spleen and central nervous system. These CD11c+CD11b+ DC were capable of inhibiting proliferation of MOG-specific T cells, enhancing the development of Th2 cells, as well as enhancing regulatory T cell generation.[30] These tolerogenic CD11b+ DC also had reduced IL-12 production and enhanced IL-10 production upon coculture with T cells and LPS.[30] CD11b+CD11c+ DC that express high levels of IDO have also been found in orally tolerized mice in a collagen-induced arthritis model.[31] These CD11b+ IDO+ DC could suppress collagen-specific T cell proliferation as well as induce regulatory T cells.[31]

In previous studies, the result of adding donor pDC to purified HSC was polarization of donor T-cells toward Th1 and Tc1 immune responses with higher serum levels of IL-12, IFN-γ, IL-2 and higher numbers of IFN-γ-producing donor T-cells.[16] Based on significant Th1 polarization of donor T-cells induced by cotransplantation of FACS purified donor pDC, higher serum levels of IL-12 reported by our group[16], and the potent ability of IL-

12 to stimulate Th1/Tc1 cells,[32] we hypothesized that Th1 polarization of donor T-cells induced by mDC-depleted BM might be IL-12 dependent. In this study, however there were no significant differences in production of Th1/Tc1 cytokines by donor T cells with the presence or absence of IL-12 producing pDC.[33, 34, 35]

Of note, the IL-12KO mice used are IL-12p40 KO, which result in the absence of IL-12p70, IL-12p80 and IL-23 expression. IL-23 is produced by APCs following BMT and has been suggested to induce subsequent expression of IL-22, which is reduced by the presence of GvHD [36]. Deficiency of recipient-derived IL-22 leads to increased GvHD as measured by intestinal and liver pathology, but not the skin. Thus, interpretation of the results obtained with IL-12p40 KO mice as donors must account for the potential role of IL-23 as well.

Interestingly, mice receiving mDC-depleted BM had significantly higher levels of IL-13 present in the serum at 10 days post-transplant. Highfill, et.al. reported that MDSC generated in the presence of IL-13 can inhibit GvHD, migrate to sites of allo-priming, limit the activation and proliferating of donor T cells, yet do not reduce the GvL effect of donor T cells[25]. We propose that IL-13 might be a mechanism by which mice receiving mDC-depleted BM experience higher survival in a tumor-bearing model, without increased GvHD.

Another potential mechanism by which enrichment of pDC may augment GvL without increasing GvHD is through the production of IFN-α by pDC. Studies by Robb, et al.

using mice deficient in the IFNAR1 component of the type I-IFN receptor show that type I-IFN signaling prevented GvHD in CD4-mediated models of GvHD[37]. Interestingly, type I-IFN signaling in donor cells enhanced CD8-dependent GvHD and GvL and augmented donor CTL function.[37] While the source of IFN-α in a BMT setting is unclear, pDC are the most potent producers of Type I-IFNs following viral infection[38].

The current studies were designed to explore more a feasible and translatable graft engineering strategy to regulate the relative numbers of pDC: mDC and to improve the efficacy of allogeneic BMT. Our data consistently showed that pDC enrichment of BM allografts enhanced donor T-cells expansion, donor $CD8^+$ T-cell proliferation and GvL activity, and the beneficial effects of pDC enrichment were dependent of IL-12. Taken together, our data support that selective depletion of an immune-phenotypically defined DC subset from the murine BM graft is a novel and feasible method for manipulation of the BM graft to regulate donor T-cell activation and enhance GvL activity without leading to increased GvHD. Meanwhile, we demonstrated IL-12 is a key cytokine synthesized by donor pDC that is necessary for augmentation of GvL activity of donor T-cells in murine allogeneic BMT model.

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Figure legends

Figure 2-1. Depletion of mDCs enhances GvL in an IL-12 dependent manner. (A) Gates for sorting CD11b$^+$ mDC-depleted BM cells. CD3$^+$ T-cells were depleted from BM cells by incubating BM cells with biotinylated anti-CD3 antibody, followed by anti-biotin microbeads, and negative MACS selection using an LS magnetic column. Total nucleated cells were gated on lineage markers (CD3, Ter119, DX5, IgM, and CD19), followed by CD11b and CD11c. Lineage$^-$ cells were gated on CD11c and CD11b. CD11c$^+$CD11b$^+$ double positive cells were discarded. Gray bold line shows gating for excluding double positive cells. All other Lineage$^-$ and Lineage$^+$ cells were sorted for transplant and comprise the CD11b$^+$ mDC-depleted BM population. Percentage of lineage negative cells with an mDC phenotype (CD11b+CD11c+) is shown pre-sort **(A)** and post-sort **(B).** Survival **(C, E)** and mean GvHD clinical scores **(D, F)** of mice that received 1×10^6 MACS purified Pepboy splenic T-cells plus 3×10^6 undepleted BM cells or FACS sorted mDC-depleted BM cells from IL-12 KO or WT donors in C57BL/6J → B10.BR transplant model (n=6-10 mice per group). Recipients in panels (**E**) and (**F**) were injected with 5×10^5 viable luciferase$^+$ LBRM tumor cells on day -1, one day following radiation and one day before BMT. Panel E: *, $p < 0.05$ and ***, $p<0.001$ represent a log-rank comparison of survival of tumor-bearing transplant recipients of WT mDC-depleted BM with recipients of IL-12 KO mDC-depleted BM or undepleted BM, respectively. Panel F: *, $p < 0.05$ comparing IL-12 KO with WT mDC-depleted BM with a on day 15 post-trasplant using an unpaired two-tailed t-test. Survival and GvHD data includes n=10 per

group.

Figure 2-2. Donor T cell proliferation is increased in mDC-depleted BM recipients in an IL-12 dependent manner. Lethally irradiated CD45.2$^+$ B10.BR mice received 1 × 10^6 CFSE-labeled CD45.1$^+$ T-cells from Pepboy donors with 3 × 10^6 undepleted BM cells or FACS sorted mDC-depleted BM cells from IL-12 KO or WT B6 donors. Recipient splenocytes were prepared at day 3.5 after transplant and analyzed for the proliferation of donor T-cells. **(A)** Average percentage of cells in each division peak, *** $p < 0.001$, and ****, $p < 0.0001$ when comparing % undivided T-cells in different times in IL-12 KO vs. WT mDC-depleted groups; *, $p < 0.05$ when comparing % divided T cells in WT undepleted vs. mDC-depleted groups **(B)** Mean percentages (±SD) of T-cells that had undergone six to seven cell divisions are shown, *, $p < 0.05$ and **, $p < 0.01$ comparing in different groups. Data is representative from one of two independent experiments of n=3 per group. **(C)** Lethally irradiated CD45.2$^+$ B10.BR mice received 1 × 10^6 luciferase+ T-cells from C57BL6/J donors with 3 × 10^6 undepleted BM cells or FACS sorted mDC-depleted BM cells from IL-12 KO or WT B6 donors or undepleted BM cells. BLI images show luciferase$^+$ donor T-cells expansion in allogeneic recipients. BLI images were taken on days 5, 7, 14, 21 and 30 post-transplant. The color bar represents the scale used for the pseudocolor image. Images are representative from one experiment of n=5 per group. **(D)** Whole body images of mice were taken and total signal in the ROI (photons/sec) was determined for whole-body on post-transplant day 5, 7, 14, 21 and 30; **, $p < 0.01$ comparing ROI of WT mDC-depleted vs. IL-12p40KO mDC-

depleted at day 30. Data are compiled from two independent experiments of n=5 per group.

Figure 2-3. Serum IFN-γ and IL-13 is increased in recipients of mDC-depleted BM. Lethally irradiated CD45.2^{+} B10.BR mice received 3 × 10^{5} CD45.1^{+} T-cells from Pepboy donors with 3 × 10^{6} undepleted BM cells or FACS sorted mDC-depleted BM cells from IL-12 KO or WT B6 donors. Recipient splenocytes were prepared at day 10 after transplant and analyzed for the percentages of intracellular cytokine producing donor T-cells of **(A)** IFN-γ (p=ns), **(B)** TNF-α (p=ns), **(C)** IL-10 (p=ns), and **(D)** IL-17 (One way ANOVA of CD4 data resulted in p=0.0003, ** represents $p < 0.01$ from a Bonferroni post-test analysi comparing WT mDC depleted and IL-12p40KO mDC depleted recipients ; One way ANOVA of CD8 data resulted in p=0.0091, * represents $p < 0.05$ from a Bonferroni post-test comparing wt mDC depleted and IL-12p40KO mdC depleted recipients). Serum was also collected and analyzed for levels of **(E)** IFN-γ (One way ANOVA results in p=0.0284, * represents $p < 0.05$ from a Bonferroni post-test comparing WT undepleted and WT mDC depleted recipients) and **(F)** IL-13 (**, $p < 0.01$ comparing WT undepleted and WT mDC depleted groups using an unpaired two-tailed t-test). Data are compiled from 2-3 independent experiments (n=3 per experimental group).

Figure 2-4. IL-12p40 producing pre-pDC confer a survival advantage to tumor-bearing mice transplanted with purified population of HSC, pre-pDC and T cells. CD45.2+ B10.BR mice were lethally irradiated and received 1 x 10^{6} luciferase-positive

LBRM cells i.v. one day after irradiation. Two days after irradiation mice were transplanted with 3000 FACS sorted HSC (Lin-Sca1+Ckit+), 50,000 WT (C57BL/6J) or IL-12p40KO pre-pDC (Lin-CD11c+CD11b-B220+Pdca1+), and 3 x 10^5 T cells. A control group received 3 x 10^6 total BM cells and 3 x 10^5 T cells **(A)** Mice receiving HSC, WT pre-pDC, and T cells had increased survival compared with mice receiving HSC, IL-12p40KO pre-pDC and T cells (*, $p < 0.05$ represents a log-rank comparison of survival). **(B)** Whole body images of surviving mice were taken on day 36-post transplant and total signal in the ROI (photons/sec) was determined for whole-body ($p = 0.56$ comparing recipients of BM + T cells and recipients of HSC + WT pDC + T cells using an unpaired two-tailed t-test). Data are representative from one of two independent experiments (n=4-7 per experimental group). Statistics were conducted on survival data combined from both replicate experiments.

Figure 2-1

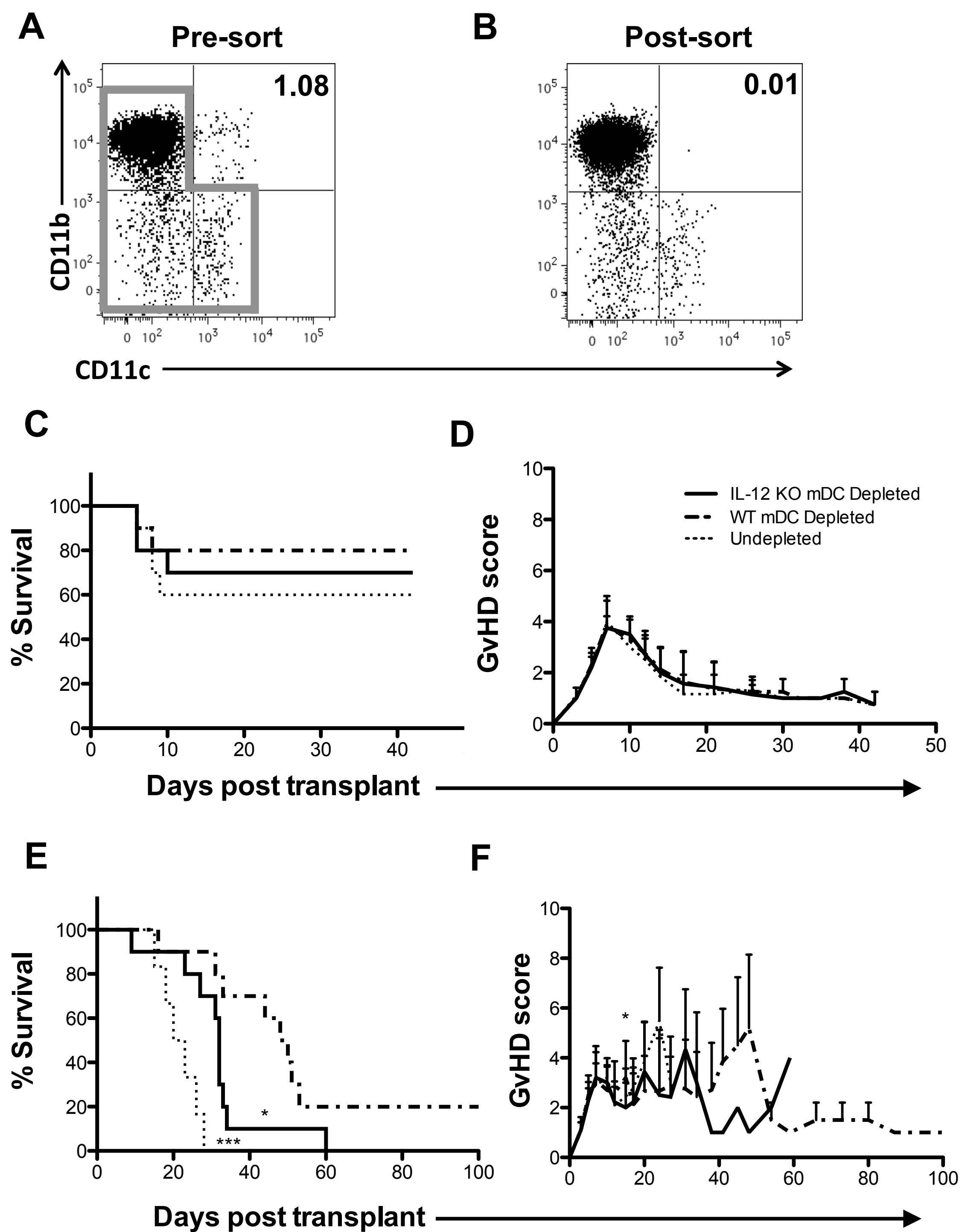


Figure 2-2

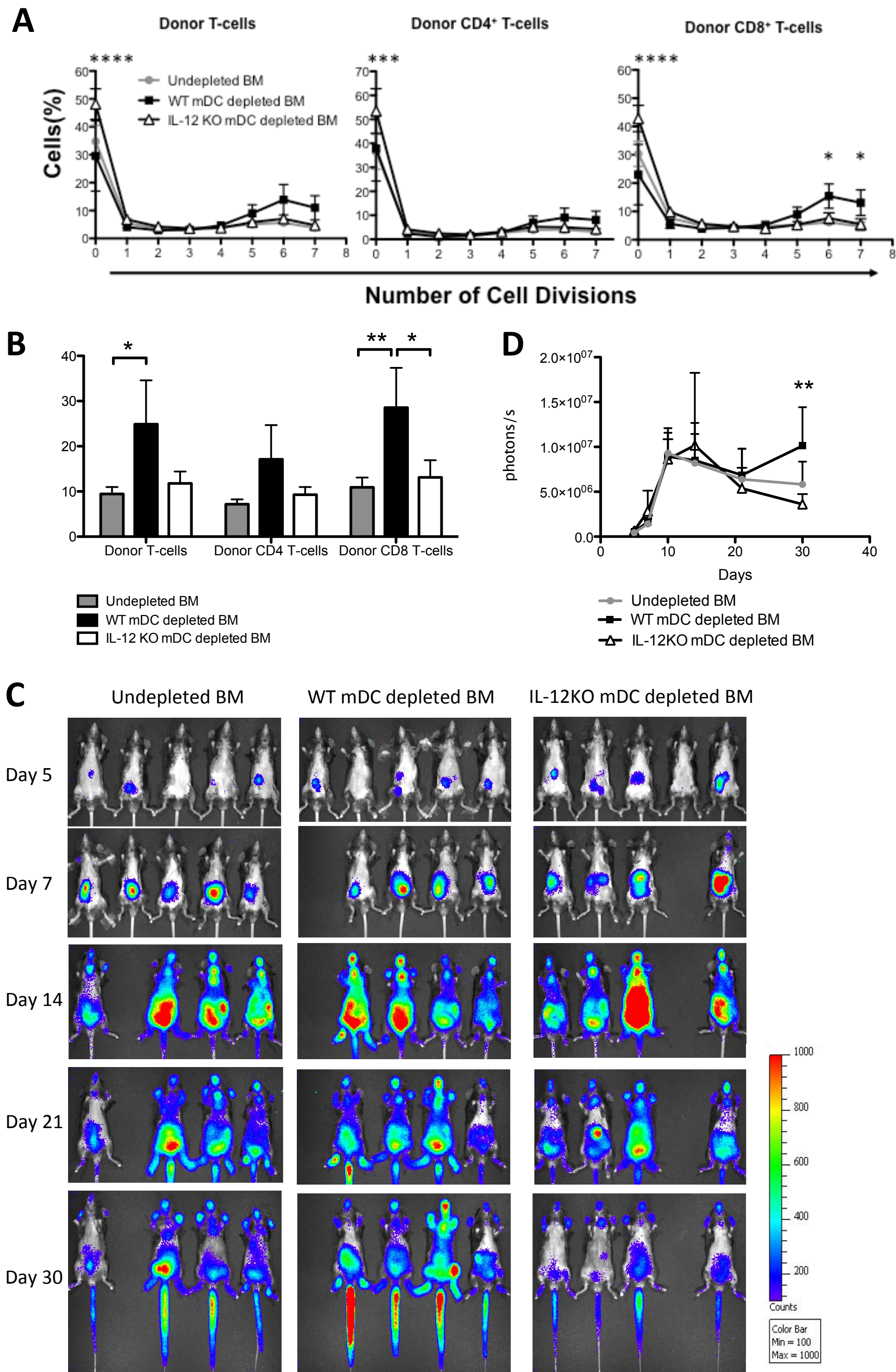


Figure 2-3

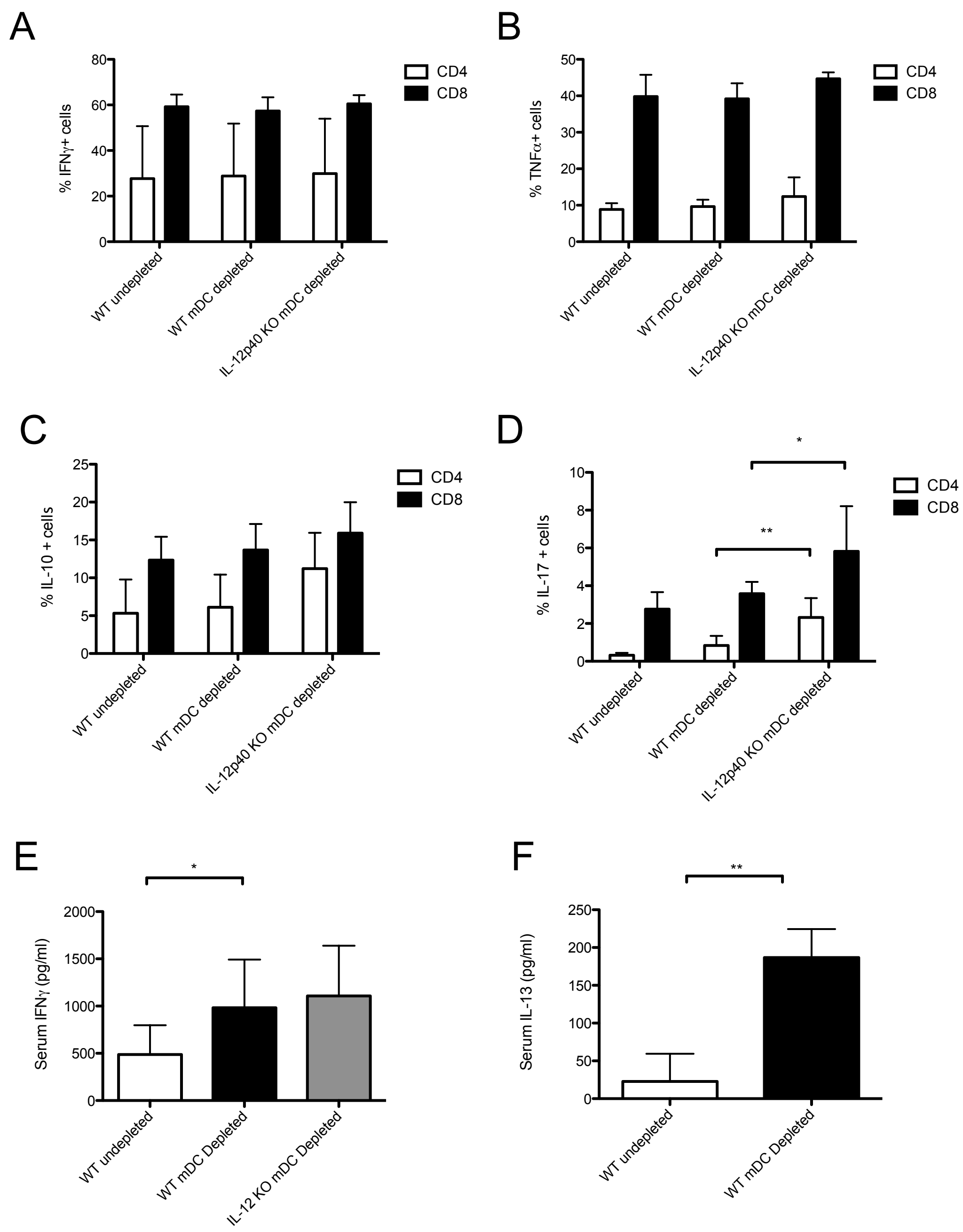
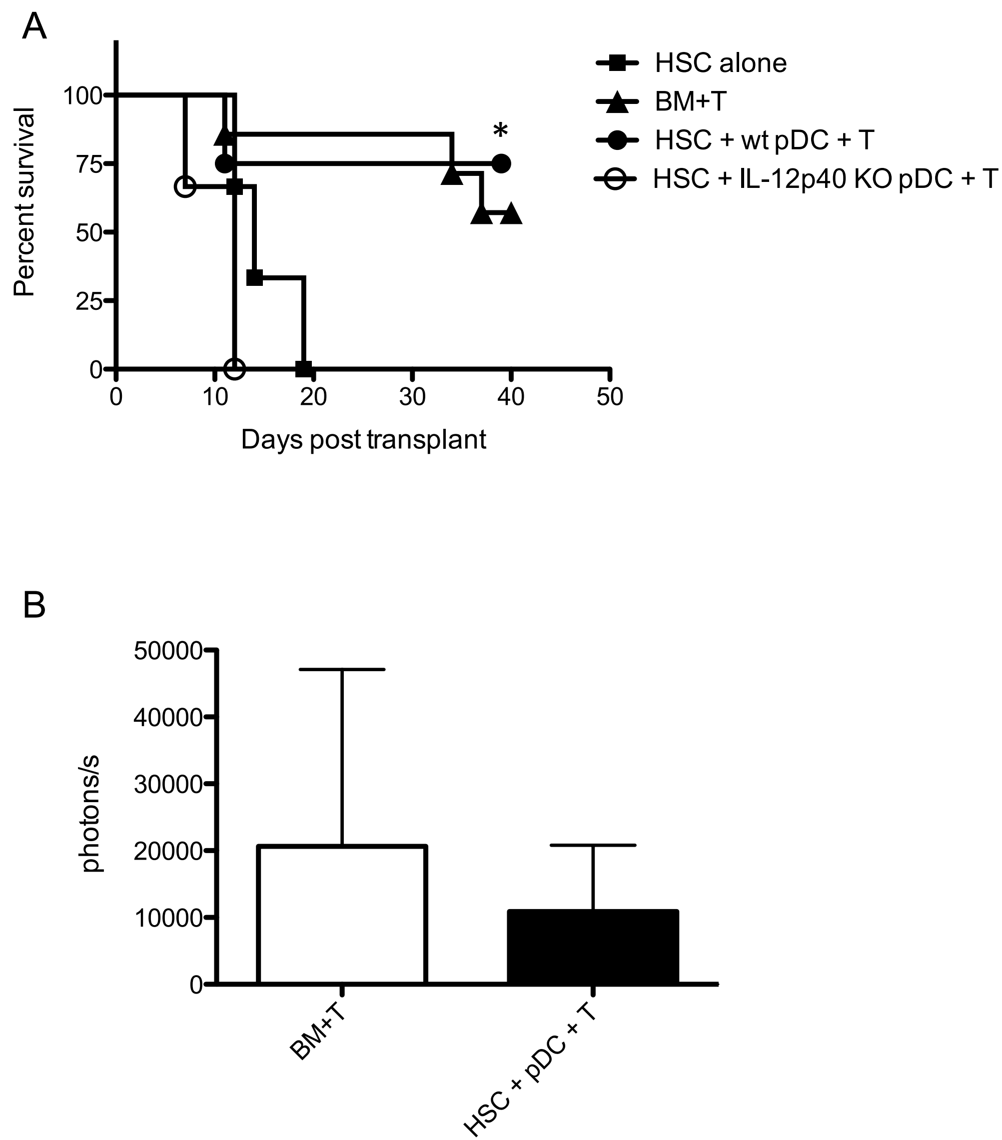


Figure 2-4



Chapter 3

Host Bone Marrow-derived IL-12 Enhances Donor T cell Engraftment in a Mouse Model of Bone Marrow Transplantation

Abstract

Donor cell engraftment is critical for the success of allogeneic bone marrow transplants. Graft failure is a result of donor cells either failing to engraft initially or being eliminated at later timepoints. Donor cell engraftment is facilitated by donor T cells, which eliminate residual host hemato-lymphoid cells such as NK cells and T cells. We aimed to explore the role of host hematopoietic cell derived IL-12 on donor cell engraftment in a murine model of BMT. We established radiation chimeras by transplanting C57BL6/J (B6) mice with BM from either congenic B6 mice or IL-12p40 KO mice. These WT → WT or IL-12 KO → WT chimeras then underwent a secondary transplant with allogeneic (FVB) BM. Survival, engraftment, donor T cell expansion, cytokine production by donor T cells, as well as expression of stimulatory markers on donor T cells was analyzed. Mice whose residual host hematopoietic cells were capable of producing IL-12 had higher survival, donor T cell engraftment, and erythroid engraftment. We have also found that an increased number of donor T cells in IL-12 KO → WT chimeras have a regulatory phenotype, expressing foxp3, producing lower levels of TNF-α, higher levels of IL-10, and expressing higher levels of ICOS as well as PD-1 on CD4 T cells. To our knowledge, this is the first report of a beneficial role of IL-12 production by host cells in the context of bone marrow engraftment in a murine model of BMT.

Introduction

Donor hematopoietic cell engraftment is the cornerstone of all successful allogeneic bone marrow transplantation (BMT). Allograft rejection occurs when donor cells fail to engraft initially, or when there is a loss of donor cells at a later timepoint following initial engraftment. While the overall frequency of graft failure in BMT is less than 5%, graft failure is still a major concern when the source of the allograft is a T cell depleted (TCD) human leukocyte antigen (HLA) – haploidentical donor, in cord blood transplants[1], in BM grafts from unrelated donors[2], or in patients where non-myeloablative conditioning is used. LeBlanc, et al. examined patients with various hematological malignancies or solid tumors that had received non-myeloablative conditioning or a higher-intensity conditioning regimen followed by a hematopoietic stem cell transplant from either HLA-identical siblings or unrelated donors[3]. It was found that 6/24 patients receiving non-meyloablative conditioning experienced graft failure, compared with 1/34 patients in the higher intensity conditioned group[3].

The addition of donor T cells to the BM graft has been shown to facilitate engraftment in animal models [4, 5]. The current model for hematopoietic cell engraftment in allogeneic BMT is that host dendritic cells (DC) activate donor T cells, which then promote engraftment by eliminating radio-resistant cytotoxic host immune cells, especially natural killer (NK) cells and host T-cells. Host DC have also been shown to initiate graft-versus-host disease (GvHD), and inactivation of host DC can prevent GvHD[6]. The interplay

between residual host DC and T cells presents a delicate balance between engraftment and GvHD.

We have recently shown that tumor-bearing mice receiving BM grafts which were depleted of myeloid DC (mDC) had enhanced survival and donor T-cell expansion compared with mice receiving unmanipulated BM grafts, which contained both mDC and plasmacytoid DC (pDC)[7]. Using interleukin-12 knockout (IL-12p40 KO) mice as BM donors, it was found that the increased survival in mice receiving mDC depleted grafts was dependent on the production of IL-12p40 by donor pDC[7]. IL-12 is produced by DCs that can drive the development of donor type 1 helper T-cells (Th1) and type 1 cytotoxic T-cells (Tc1). It has also been shown that administration of exogenous IL-12 in a mouse model of BMT can reduce GvHD while preserving the graft-versus-leukemia effect (GvL)[8].

While the role of IL-12 has been extensively studied in the context of GvL and GvHD, the role of IL-12 in graft failure has yet to be fully examined. Administration of exogenous IL-12 can protect the bone marrow from the effects of lethal irradiation, at the expense of sensitizing the intestinal tract[9]. Administration of exogenous IL-12 at certain doses has also been shown to facilitate hematopoietic engraftment following lethal irradiation[10]. We aimed to explore the role of IL-12 produced by residual host hematopoietic cells in donor cell engraftment. As IL-12 can be produced by non-hematopoietic cells, including keratinocytes, osteoblasts, epithelial cells, and endothelial cells, we established radiation chimeras in C57BL6/J (B6) mice in which only hematopoietic cells lacked the ability to produce IL-12p40[11, 12]. After confirmation of

donor hematopoietic chimerism, mice received lethal irradiation followed by an allogeneic BMT (FVB or B10.BR → B6 model). Survival, engraftment, donor T cell expansion (using luciferase positive T-cells), and cytokine production were examined. It was found that murine recipients that lacked production of IL-12p40 by hematopoietic cells had lower survival, a lower level of donor hematopoietic engraftment, a reduced percentage Th1 cytokine producing donor T cells, and an increased percentage of regulatory T cells in the spleen. This is the first report that host-derived IL-12 plays a significant role in engraftment and has implications for the use of IL-12 in translatable settings where graft failure is of concern.

Materials and Methods

Mice

FVB/NJ (FVB, H-2^q, CD45.1, CD90.1), C57BL6/J (B6, H-$2K^b$, CD45.2, CD90.2), B10.BR (H-$2K^k$, CD45.2, CD90.2, congenic B6 Pepboy (B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ, H-$2K^b$, CD45.1, D90.2, and IL-12p40KO (B6.129S1-*Il12b*^{tm1Jm}/J, H-$2K^b$, CD45.2, CD90.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Congenic strains expressing CD90.1 and CD45.2 on a B6 (H-$2K^b$) background (BA), and CD90.1, CD45.2 on a B10 (H-$2K^k$) background (BA.B10) were backcrossed 10 generations to the parental strain and bred at Emory University Animal Care Facility (Atlanta, GA, USA). The *luc*+ transgenic mice (FVB-L2G85) on the FVB background were a gift from Dr. Robert Negrin (Stanford Univeristy)[13]. All transplant recipients were monitored daily for survival. According to the Institutional Animal Care and Use Committee (IACUC) protocol, moribund mice, mice with greater than 25% weight loss, and mice surviving

until the end of the experiment, were euthanized and considered to have died on the day following euthanasia for analysis of post transplant survival. Mice were scored for clinical signs of GvHD by weight loss, posture, activity, fur texture, and skin integrity twice weekly for the first 30 days post transplant, and once weekly thereafter, using a GvHD scoring system described by Cooke, et al[14]. Procedures conformed to the National Institutes of Health (NIH, Bethesda, MD) animal care guidelines and were approved by the Emory University IACUC.

Cell Preparation

Donor mice were killed in a humane manner and femurs, tibias, and spleens were removed ascetically. BM cells and splenocytes were harvested with sterile phosphate buffered saline (PBS). T cells were purified by incubating splenocytes with a cocktail of biotinylated anti-CD11b, anti-B220, anti-DX5, and anti-TER119 antibodies, followed by anti-biotin microbeads, and negative magnetic activated cell sorting (MACS) selection using an LS magnetic column (MACS, Miltenyi Biotec). CD3+ T cells were depleted from BM cells by incubation of BM with biotinylated CD3 antibodies, followed by anti-biotin microbeads, and negative MACS selection using an LS magnetic column (MACS, Miltenyi Biotec).

Radiation chimeras

On day-1 prior to transplantation, recipient congenic B6 Pepboy mice (CD45.1) received a total of 11Gy irradiation, divided into two doses of 5.5Gy given 3 hours apart[15]. On day 0, mice were injected i.v. with 5 million BM cells from WT B6 donors (CD45.2) or IL-

12p40 KO donors (CD45.2). Chimerism was confirmed a minimum of 50 days post-transplant using flow cytometric analysis of blood stained with anti-CD3-PE-Cy7, anti-CD19-APC-Cy7, anti-CD45.1-PE, anti-CD45.2-FITC, anti-CD90.1-PerCP, and anti-CD90.2-APC (BD Biosciences, San Diego, CA)

Secondary transplant

On day-1 prior to transplantation, recipient chimeric mice (B6/BA or IL-12p40KO) (CD45.2, CD90.2) received a total of 9 Gy irradiation, divided into two doses of 4.5Gy given 3 hours apart.[15] On day 0, mice were injected i.v. with 5 x 10^6 T-cell depleted (TCD) BM cells from FVB donors (CD45.1, CD90.1) along with 3 x 10^5 MACS-purified T-cells from *luc*+ FVB donors. FVB mice that had not undergone an initial radiation chimera transplant were used as syngeneic control mice, and received a secondary transplant identical to the radiation-chimera mice. All transplant recipients were monitored for survival and clinical signs of GvHD daily. Mice were scored for clinical signs of GvHD by weight loss, posture, activity, fur texture, and skin integrity twice weekly for the 30 days, and then weekly as described by Cooke, et al..[14] According to IACUC protocol, moribund mice, mice with greater than 25% weight loss, and mice surviving until the end of the experiment, were euthanized and considered to have died on the day following euthanasia for analysis of post-transplantation survival.

Flow cytometry

For chimerism analysis, peripheral blood was collected on days 30, 60 and 100 (+/- 5 days) in tubes containing 20ul heparin form the tail vein of transplant recipients. For flow

cytometric analysis, red blood cells were lysed via incubation in an ammonium chloride lysis buffer. Host- and donor-derived leukocytes and T-cells were measured by flow cytometry using mAbs for specific leukocyte markers expressed on B6, congenic B6 Pepboy, IL-12p40 KO, FVB, B10.BR, and BA.B10 strains (anti-mouse CD3-APC, CD45.1-APC-Cy7, CD45.2-FITC, H2k^{b}-PE; BD Biosciences, San Diego, CA). Spleen samples were obtained on day 10 post transplant and intracellular cytokine expression of IFN-γ, TNF-α, IL-10, and IL-17 by donor CD4+ and CD8+ T-cells was analyzed by using a Cytofix/Cytoperm Kit (BD Biosciences, San Diego, CA). Presence of donor regulatory T cells in the spleen on day 10 post transplant was analyzed by surface staining using anti-mouse CD3-PE-Cy7, CD90.2-APC, H-2K^{k}-FITC, CD4-Alexa-700 and CD25-APC-Cy7, followed by intracellular staining of FoxP3-PE (BD Biosciences, San Diego, CA). All samples were analyzed on a FACS Canto (Beckton Dickinson, San Jose, CA) and list mode files were analyzsed using FlowJo software (Tree Star, Inc 2007, Ashland, OR).

In vivo bioluminescent imaging

In vivo bioluminescent imaging (BLI) was performed using a cooled charge-coupled device (CCD) camera system (IVIS Imaging System 100, Xenogen, Alameda, CA, USA). Mice received a subcutaneous injection of D-luciferin (0.15 mg/g body weight; Firefly luciferin potassium salt, Xenogen, Alameda, CA, USA). Ten minutes later, mice were placed in the chamber of the CCD camera system under rodent cocktail (ketamine 20 mg/ml and xylazine 2.5 mg/ml, 0.1 ml/25g body weight) anesthesia. Photographic and luminescent images in the ventral projection were obtained using a 3-minute exposure

time. Up to five mice were imaged simultaneously. On each *in vivo* BLI image, a region of interest (ROI) encompassing the entire mouse was created, and the whole-body signal in the ROI (photons/sec) was quantified using Living Image software (Version 3.2, Xenogen, Alameda, CA, USA). The whole-body bioluminescent signal was used as a marker of the donor T-cell engraftment.

Statistical Analyses

Analyses of data were performed using Prism version 5 for Mac (GraphPad Software, San Diego, CA, www.graphpad.com). Data are presented as mean ± standard deviation (SD) of all evaluable samples. Survival differences between groups were calculated with the Kaplan-Meier log rank test in a pair-wise fashion. Differences in the numbers of cells present in blood, bioluminescence ROI values, percentages of FoxP3+, cytokine-producing, and PD1 or ICOS expressing cells were performed using a two-tailed unpaired Student's t-test or a one-way analysis of variance (ANOVA) followed by a Bonferroni post-test to adjust for multiple comparisons. P-values of less than 0.05 were considered significant.

Results

Host-hematopoietic-derived IL-12 enhances survival after BMT

In order to examine the role of host-immune cell derived IL-12, radiation chimeras were established in order to create mice that lacked IL-12 in the hematopoietic compartment only. One day prior to transplant, congenic B6 mice were lethally irradiated with 11Gy total body irradiation (TBI) and transplanted i.v. with 5 x 10^6 BM cells from B6 or IL-

12p40KO mice. A minimum of 50 days after transplant, donor chimerism of total nucleated cells in both B6 and IL-12KO radiation chimeras was confirmed by flow cytometric analysis using congenic markers (CD45.1, CD45.2, CD90.1, CD90.2) (Figure 1A, B). Chimerism was found to be greater than 95% in all radiation chimeras (data not shown).

Radiation chimeras underwent a secondary transplant following irradiation of 9Gy TBI one day prior to transplant. In B6 radiation chimeras, radioresistant host-hematopoietic cells would be capable of producing IL-12 following irradiation and transplant, along with donor hematopoietic cells. In IL-12 KO radiation chimeras only the donor-derived hematopoietic cells would produce IL-12, as residual host hematopoietic cells were of IL-12 KO origin.

One day after the second irradiation course, chimeras were transplanted i.v. with 5 x 10^6 TCD BM cells from FVB donors along with 3 x 10^5 *luc*+ FVB T-cells. Control transplant mice (syngeneic) were FVB mice transplanted i.v. with 5 x 10^6 TCD BM cells from FVB donors along with 3 x 10^5 *luc*+ FVB T-cells. Survival of mice was monitored daily. Weight loss and clinical GvHD scores were monitored twice weekly after transplant, as described by Cooke, et al.[14]. IL-12 KO → WT mice had a median survival of 65 days post-transplant (41% survival at day 105 post-transplant), which was lower compared with WT → WT mice (median survival day undefined, 75% survival at day 105 post-transplant), though not significant (p=0.24)(Figure 1C). All syngeneic-transplanted mice survived to day 105. Percent weight loss from initial starting weight and GvHD scores

were similar between WT → WT and IL-12 KO → WT radiation chimeras (Figure 1D, E, F). Control transplanted mice did not experience weight loss after transplant. (Figure 1D).

Host-hematopoietic-derived IL-12 enhances donor T-cell engraftment after BMT

Next we determined the effect of host hematopoietic derived IL-12 on the engraftment of leukocytes, red blood cells, and platelets. On day 30 post-transplant, we measured the red blood cell (RBC) count, white blood cell (WBC) count, platelet number, and hemoglobin levels in the blood of recipient mice. Recipient mice in which host immune cells were capable of producing IL-12 had significantly higher erythroid engraftment as seen by significantly higher RBC counts and hemoglobin levels (Figure 2A, B respectively). WBC counts in the blood of recipients previously engrafted with WT BM was slightly higher, though not significant (Figure 2C). Platelet counts were not significantly different among groups (Figure 2D). We also measured the percentage of T cells of donor (FVB) origin as a percentage of total T cells. Radiation chimeras previously engrafted with WT BM had a higher percentage of donor T cells (37.87 ± 13.25) on day 30 post-transplant compared with IL-12 KO → WT chimeras (23.69 ± 10.98) (Figure 2E). Standard deviation in Figure 2E is very high in both groups, as most mice had engrafted primarily with FVB (80% or higher donor T cells of FVB origin), or had failed to engraft (lower than 40% donor T cells of FVB origin). On day 30 post-transplant, 40% of WT→WT chimeras had greater than 50% donor T cell engraftment, while only 10% of IL-12 KO → WT chimeras had greater than 50% donor T cell engraftment (Figure 2F). Mice that had failed to engraft died. Among surviving mice on day 60 post-transplant, 50% of WT →

WT chimeras had greater than 50% donor T cell engraftment, compared with 40% of IL-12 KO → WT chimeras (Figure 2F).

Since the radiation chimeras were transplanted with FVB TCD BM and luciferase-positive FVB T-cells, donor T-cell engraftment could be tracked using *in vivo* bioluminescent imaging at multiple time points post-transplant. Mice were imaged every week beginning at 7 days post-transplant and continuing until 42 days post-transplant. Representative imaging data from one experiment is shown in Figure 3A. A greater percentage of the WT → WT radiation chimeras had donor T cell bioluminescent signals, and the signals were of greater intensity, than that of IL-12 KO → WT radiation chimeras (Figure 3A). At day 42, only one IL-12 KO → WT radiation chimera had a strong bioluminescent signal, and the signal from donor T cells in 2/4 IL-12 KO → WT chimeras was unable to be seen visually. The strength of the signal from each mouse in photons/second was quantified using Xenogen LivingImage software. WT → WT chimeras had a higher average signal overall compared with IL-12 KO → WT chimeras (Figure 3B). The signal from WT → WT radiation chimeras was significantly higher on D14 post transplant ($p<0.05$), as well as slightly higher on days 28 and 35 post-transplant ($p=0.092$ and $p=0,098$, respectively) (Figure 3B). WT → WT mice experienced bioluminescent signal strength similar to or even higher than that of FVB mice receiving a syngeneic transplant.

The absence of host-hematopoietic-derived IL-12 drives donor CD4 T-cells towards a tolerogenic phenotype

While survival and donor-T cell engraftment was higher among WT radiation chimeras compared with IL-12 KO radiation chimeras, we next aimed to determine whether the phenotype of engrafted T cells was different between groups. On day 10 post-transplant, percentages of T cells expressing Foxp3, producing TNF-α or IL-10, and expressing ICOS or PD-1 were determined. IL-12 KO → WT chimeras had a higher percentage of donor CD4 T cells expressing the regulatory T cell marker Foxp3, as determined by intracellular staining for Foxp3 ($p<0.05$, Figure 4C). Representative flow plots for WT → WT and IL-12 KO → WT radiation chimeras are shown in Figure 4A, B, respectively. The ability of host hematopoietic cells to secrete IL-12p40 also had an impact on the percentage of donor FVB CD4 T cells that produce IL-10 and TNF-α. A greater percentage of donor CD4 T cells from WT chimeras produced TNF-α compared with IL-12 KO chimeras ($p<0.01$, Figure 4D), while a slightly higher percentage of CD4 T cells from IL-12 KO chimeras produced IL-10 compared with WT chimeras (p=ns, Figure 4E).

Signaling through ICOS has been shown to contribute to BM graft rejection and GvHD[16]. We measured ICOS expression in WT and IL-12 KO chimeras on donor CD4 and CD8 T cells 10 days post-transplant. IL-12 KO chimeras (gray dashed line) had higher expression of ICOS on CD4, but not CD8 T cells compared with WT chimeras (black solid line) (representative histograms, Figure 5A – CD4, 5B – CD8). Isotype control staining is indicated by a gray dotted line. The percentage of CD4 T cells expressing

ICOS was higher in IL-12 KO chimeras ($p<0.01$, Figure 5C), as well as the median fluorescence intensity of the signal ($p<0.05$, Figure 5D), compared with WT chimeras.

The PD-1/PDL1 pathway is critical for inducing peripheral deletional tolerance of anti-donor CD8 T cells in BMT[17]. We analyzed the expression of PD1 on CD4 and CD8 T-cells on day 10 post-transplant. Surprisingly, the level of PD-1 on the surface of CD8 T cells was similar between WT (solid black line) and IL-12 KO chimeras (gray dashed line) (Isotype control, gray dotted line)(Figure 5F). CD4 T cells in WT→IL-12 KO chimeras, however, had higher PD-1 expression (Figure 5E). The percentage of CD4 T cells expressing PD-1 was significantly higher in WT → IL-12 KO chimeras ($p<0.05$, Figure 5G), and the MFI was slightly higher (Figure 5H), compared with WT→WT chimeras. The percentages of CD8 T cells expressing PD1 and the MFI of the PD1 signal were not different comparing donor cells in WT → WT vs. IL-12 KO → WT chimeras (Figure 5G, H).

Discussion

We aimed to examine the role of IL-12 in engraftment of BM in a murine model of allogeneic BMT. These results are most applicable to transplants using reduced-intensity conditioning in which many host cells remain following transplant conditioning, and graft-failure is of a concern. Radiation chimeras were established where all cells (host and donor) were capable or producing IL-12 (WT→WT chimeras), as well as chimeras in which only the host hematopoietic cells were incapable of IL-12 production (IL-12 KO → WT chimeras). A dose of 9Gy was chosen for the second BMT using radiation

chimeras as recipients, as this is generally the highest tolerated dose for a second transplant. Since IL-12 producing APC are somewhat resistant to radiation, we hypothesized that the production of IL-12 by residual host APC would aid in the process of engraftment by promoting T_H1 immunity while not increasing GvHD severity. Our results show that that mice whose host hematopoietic cells were capable of IL-12 production had increased survival without higher levels of GvHD or increased weight loss. Donor T cell engraftment in mice whose residual host-hematopoietic cells lacked the ability to produce IL-12 was reduced compared with WT → WT mice, as seen by both flow cytometric analysis of chimerism, as well as in *in vivo* bioluminescent images of mice engrafted with *luc*+ donor T cells (Figure 2E, F, Figure 3). WT→WT chimeras had a higher percentage of donor CD4 T cells producing TNF-α and a lower percentage of CD4+ T cells producing IL-10 on day 10 following transplant compared IL-12 KO → WT chimeras (Figure 4D,E). This suggests that host derived IL-12 is promoting the maintenance of donor T cells of a T_H1 phenotype. These host cells can prime donor CD8+ T cells to eliminate cytotoxic host effectors that prevent engraftment.

WT → WT chimeras also had a lower percentage of donor CD4+ T cells expressing the T-reg associated transcription factor FoxP3+ compared with IL-12 KO → WT chimeras (Figure 4A-C). If a large percentage of donor CD4 T cells are of a regulatory phenotype, they are unable to prime donor CD8 T cells to eliminate cytotoxic host cells. We also found that induced costimulator (ICOS) was present in higher levels on CD4 and CD8 T cells in mice whose host-hematopoietic cells were incapable of producing IL-12.

ICOS is a CD28 superfamily member whose expression is induced on CD4 and CD8 T cells following T cell activation[18]. ICOS on T cells binds ICOS ligand (ICOSL), which is upregulated on APCs activated by TNF-α or TLR triggering by LPS[19, 20]. ICOS binding with ICOSL can stimulated IL-10 production by T cells[18]. Taylor and Blazar found that ICOS blockade (via either ICOS KO mice or ICOS monoclonal antibodies) led to higher engraftment rates in mice receiving TCD BM following non-myeloablative irradiation (5.5Gy). Our data coincides with these findings, as mice whose host-hematopoietic cells did not produce IL-12 had lower engraftment, and higher levels of ICOS on CD4 and CD8 T cells (Figure 5A-D).

We also found higher percentages of PD-1+ CD4 T cells in mice lacking the capacity to produce IL-12 by host immune cells (Figure 5G). Programmed Death-1 (PD-1) is also a member of the CD28 superfamily and is expressed by activated T cells[21, 22, 23]. PD-1 binds to PD Ligand 1 (PDL1), which is expressed on all hematopoietic and many non-hematopoietic cells, and PD Ligand 2 (PDL2), which is found on DCs and macrophages[24, 25, 26, 27]. PD1 binding with ligands leads to negative regulation of activated T cells. The PD1/PDL1 pathway is known to induce deletional tolerance of alloreactive CD8 but not CD4 T cells in BMT[17]. While we did not see a difference in the PD-1 expression of CD8 T cells, there were differences among CD4 T cells. We speculate that the higher percentages of PD1+ CD4 T cells would suggest a reduced capacity for CD4 T cells to activate donor DC, which in turn could activate CD8 T cells capable of eliminating cytotoxic host-derived effectors, leading to reduced engraftment.

In order to achieve stable engraftment in the presence of cytotoxic host cells not eliminated by irradiation, it is thought that host DCs activate donor T cells, which then eliminate the remaining cytotoxic host cells. Wang, et al., showed that host DC can activated donor CD4+ T cells, which in turn activate donor APC[16]. These donor APCs are then capable of cross presenting minor histocompatibility antigens (miHA) in an MHC-matched, miHA-mismatched, model to donor CD8+ T cells[16]. Production of IL-12 by DCs promotes maintenance of T cells in a T_H1 phenotype after commitment to a T_H1 lineage by T-bet and subsequent upregulation of the IL-12β2 subunit of the IL-12 receptor on T cells[28, 29].

A low dose of IL-12 has been shown to protect the hematopoietic system from irradiation as well as promote hematopoietic recovery after a reduced intensity irradiation[10]. Administration of a single dose of recombinant IL-12 has also been shown to protect mice from GvHD while preserving the GvL effect in a donor-derived IFN-γ dependent mechanism[8, 30, 31, 32]. Sykes, et al. found that upon administration of a single dose of IL-12 prior to transplant, the percentages of donor CD4 and CD8 T cells were reduced on day 4 post-transplant compared with mice that did not receive IL-12[30]. This was consistent with a reduction in GvHD seen in mice receiving IL-12[30]. However, on day 7 post-transplant, the opposite phenomenon was seen, where mice receiving IL-12 had higher percentages of donor-derived CD4 and CD8 T cells in the spleen compared with mice not receiving exogenous IL-12, suggesting that at later timepoints IL-12 promotes CD4 and CD8 T cell expansion responsible for enhanced GvL effects[30]. It was also found that mice receiving IL-12 had higher serum levels of the T_H1 cytokine IFN-γ[30].

The role of host-derived IL-12 in engraftment has not been examined previously. IL-12 promotes the maintenance of T_H1 cells that are necessary for elimination of residual host hematopoiesis cells in the process of engraftment. While increased T_H1 polarization might be thought to increase GvHD, administration of exogenous IL-12 has also previously been shown to prevent GvHD while preserving GvL effects[8, 30, 31, 32]. Proper engraftment is a paramount concern in clinical settings such as when the donor is HLA-haploidentical to the recipient, in cord blood transplants, in patients where non-myeloablative conditioning is used. Engraftment is also a challenge in treatment of sickle cell anemia with HSCT, due to prior host immunity resulting numerous blood transfusions in the patients history. While IL-12 has been examined as a therapeutic agent in reducing the severity of GvHD, we propose that these findings could be translatable to promoting engraftment in settings where graft failure is of concern.

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Figure legends

Figure 3-1. Host-derived IL-12 enhances survival following BMT Radiation chimeras were established by lethally irradiating (11Gy) B6 Pepboy (C57BL6/J congenic, CD45.1) mice followed by transplant with 5 x 10^6 BM Cells from either B6 (or BA – B6 congenic) or IL-12-p40 KO (B6 background) mice. Chimerism was confirmed a minimum of 50 days post-transplant by flow-cytometric analysis of congenic markers on total nucleated cells (CD45.1, CD45.2) for both B6 **(A)** and IL-12p40KO **(B)** chimeras. Flow plots are data from one representative mouse per group. Chimeric mice were then conditioned with 9Gy irradiation and transplanted with 5 x 10^6 FVB TCD BM cells along with 3 x 10^5 MACS-purified *luc*+ FVB T-cells. A syngeneic transplant was also performed using non-radiation chimera FVB mice as recipients. Survival **(C)**, percent weight loss from initial starting weight **(D)**, and combined GvHD scores **(E)** were monitored after transplant. Data shown is combined from 3 independent experiments of 4-5 mice per group (WT and IL-12p40 KO) or 3 mice per group (syngeneic).

Figure 3-2. Host-derived IL-12 enhances erythroid engraftment 30 days post-transplant. Radiation chimeras (B6 or BA and IL-12p40KO) were conditioned with 9Gy

irradiation and transplanted with 5 x 10^6 FVB TCD BM cells along with 3 x 10^5 MACS-purified *luc*+ FVB T-cells. A syngeneic transplant was also performed using non-radiation chimera FVB mice as recipients. The levels of red blood cells (RBC) (**A**), hemoglobin **(B)**, white blood cells (WBC) (**C**), and platelets **(D)** were measured in the blood 30 days post-transplant. The percentage of cells of donor–origin (FVB) in the T-cell compartment on day 30 post-transplant was determined by flow-cytometric analysis of congenic markers (CD45.1, CD45.2, CD90.1, CD90.2) after gating on CD3+ cells (**E**). The percentage of mice in each group (WT or IL-12p40KO) that exhibited greater than 50% FVB chimerism are shown in **(F)**. **, $p < 0.01$ comparing RBC of WT vs. IL-12p40KO at day 30; *, $p < 0.05$ comparing Hemoglobin of WT vs. IL-12p40KO at day 30. Data shown is combined from 2 independent experiments of 4 mice per group (WT and IL-12p40 KO) or 3 mice per group (syngeneic).

Figure 3-3. **Donor T cell engraftment is increased and occurs more rapidly in mice producing host immune-derived IL-12.** Radiation chimeras (B6 or BA and IL-12p40KO) were conditioned with 9Gy irradiation and transplanted with 5 x 10^6 FVB TCD BM cells along with 3 x 10^5 MACS-purified *luc*+ FVB T-cells. A syngeneic transplant was also performed using non-radiation chimera FVB mice as recipients. **(A)** In-vivo bioluminescent images of mice were taken weekly beginning 7 days post transplant and ending 42 days post transplant. The color bar represents the scale used for the pseudocolor image. Images are representative from one of two independent experiments of 4-5 mice per group (3 mice per group, syngeneic). **(B)** Total signal in the region of interest (ROI) (photons/sec) was determined for the whole-body on days 7, 13,

21, 28, 35, and 42 post-transplant. ROI data has been log transformed, however statistical analyses were completed on raw data. Data are combined from two independent experiments of 4-5 mice per group (3 mice per group, syngeneic); *, $p < 0.05$ comparing WT vs. IL-12p40KO on day 14 post-transplant; $p = 0.092$ and $p = 0.098$ comparing WT vs. IL-12p40KO on days 28 and 35 post transplant, respectively.

Figure 3-4. Host immune-derived IL-12 drives donor-T cells towards a Th1 phenotype. Radiation chimeras (B6 or BA and IL-12p40KO) were conditioned with 9Gy irradiation and transplanted with 5 x 10^6 FVB TCD BM cells and 3 x 10^5 MACS-purified FVB T-cells. Recipient splenocytes were prepared 10 days post transplant and analyzed via flow-cytometry for the percentages of regulatory T cells as well as TNF-α and IL-10 producing donor T-cells. Donor-T cells were gated on using congenic markers (CD45.1, CD45.2, CD90.1) and CD3. Regulatory T cells were defined as T cells expressing both CD4 and FoxP3. Representative flow plots for B6 **(A)** and IL-12p40KO **(B)** radiation chimeras are shown. Percentages of FoxP3+ T cells in the CD4 T cell compartment are shown in **(C)**; *, $p < 0.05$ comparing WT vs. IL-12p40 KO. Data shown are from two independent experiments of 4-5 mice per group. Percentages TNF-α and IL-10 producing CD4+ donor-T cells are shown in **(D)** and **(E)**, respectively. IL-10 data are representative from one of two independent experiments with 4-5 mice per group using pre-transplant irradiation of 9Gy or 10Gy (9Gy is shown). TNF-α data is combined from two independent experiments with 4-5 mice per group. **, $p < 0.01$ comparing WT vs. IL-12p40KO.

Figure 3-5. Host immune-derived IL-12 limits ICOS and PD1 expression by CD4+ donor T cells. Radiation chimeras (B6 or BA and IL-12p40KO) were conditioned with 9Gy irradiation and transplanted with 5 x 10^6 FVB TCD BM cells and 3 x 10^5 MACS-purified FVB T-cells. Recipient splenocytes were harvested 10 days post transplant and analyzed via flow-cytometry. Donor T cells were gated on using congenic markers (CD45.1, CD45.2, CD90.1) as well as CD3, and were then further differentiated using CD4 and CD8. Representative histograms are shown for ICOS expression in CD4+ (**A**) and CD8+ **(B)** donor T-cells and PD1 expression in CD4+ **(E)** and CD8+**(F)** donor T-cells (Isotype – fine dotted line, WT – black solid line, IL-12p40KO – gray dashed line). ICOS and PD-1 expression by CD4+ and CD8+ donor T cells was analyzed; Percentages of donor T-cells expression ICOS (**C**) and PD1 (**G**) are shown as well as the median fluorescence intensity (MFI) of the signal for ICOS **(D)** and PD1 **(H)**. **, $p < 0.01$ comparing percentages of ICOS+ CD4+ donor T-cells in WT vs. IL-12p40KO; *, $p < 0.001$ comparing MFI of ICOS signal in CD4+ donor T-cells in WT vs. IL-12p40KO; *, $p < 0.05$ comparing percentages of PD1+ CD4+ donor T-cells in WT vs. IL-12p40KO. Data shown for ICOS are representative from one of two independent experiments with 4 mice per group using pre-transplant irradiation of 9Gy or 10Gy (9Gy is shown). Data shown for PD1 are combined from two independent experiments of 4 mice per group.

Figure 3-1

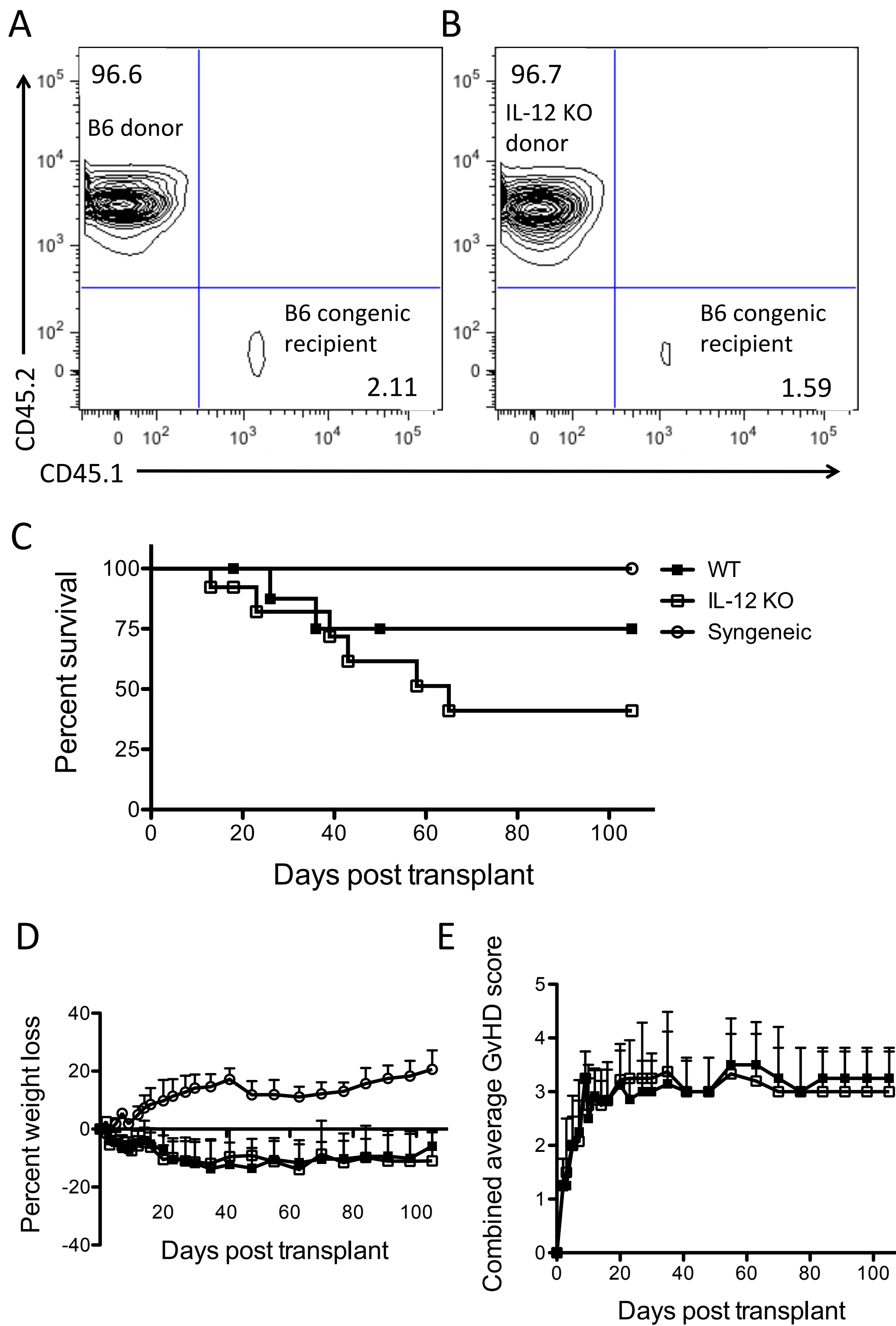


Figure 1

Figure 3-2

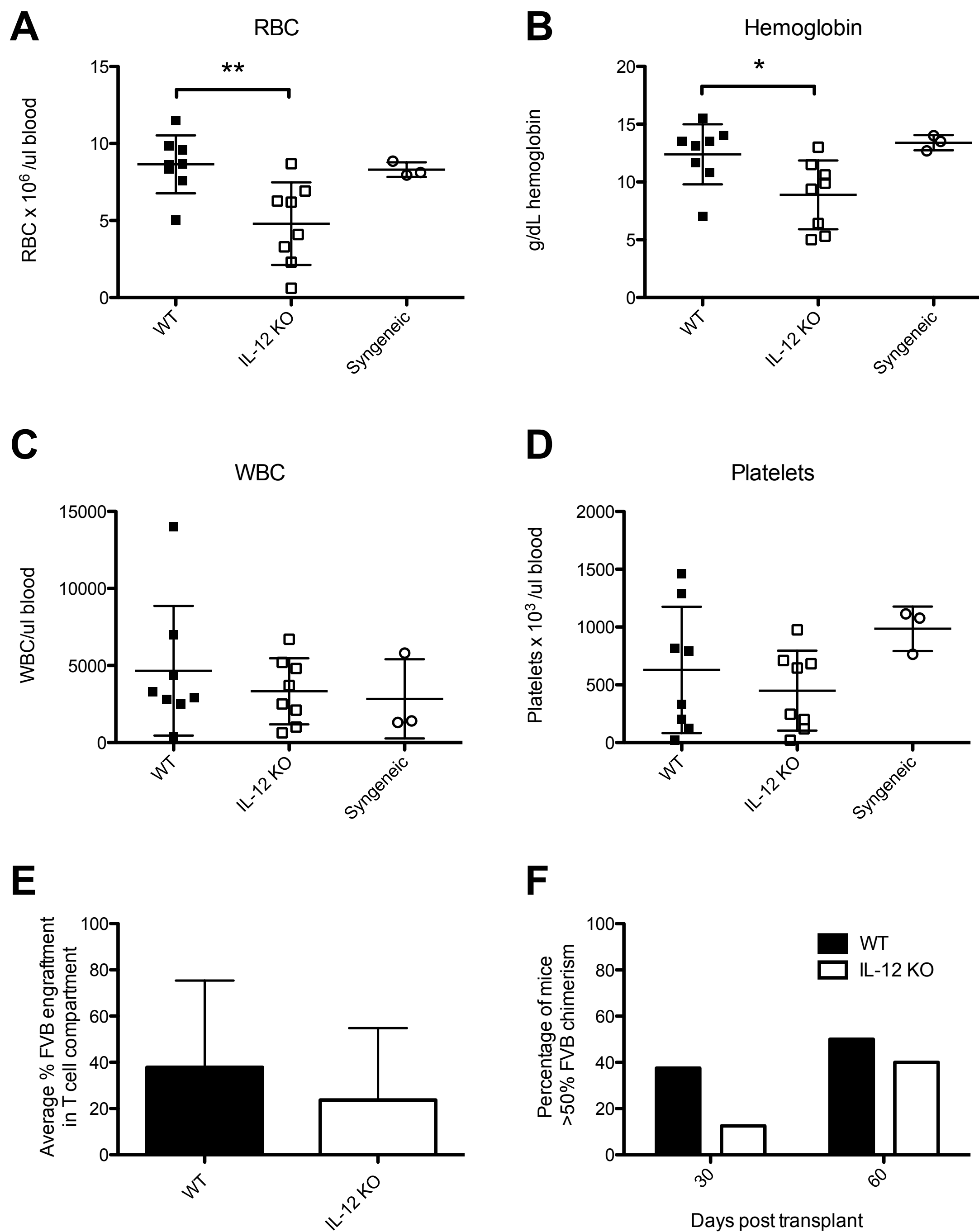
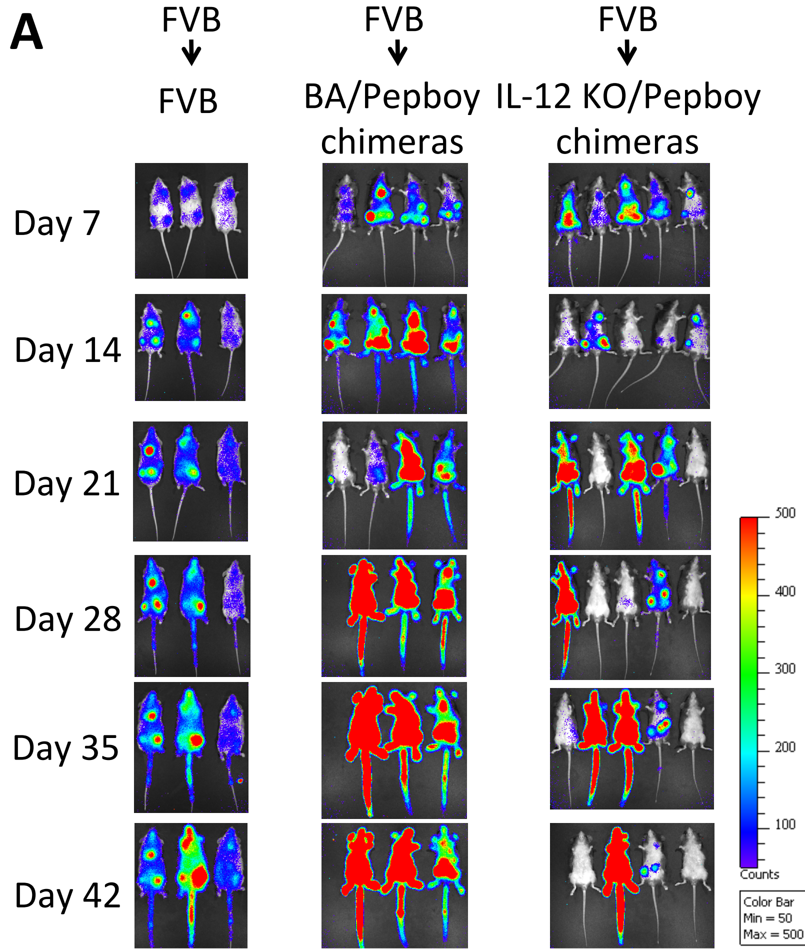


Figure 3-3



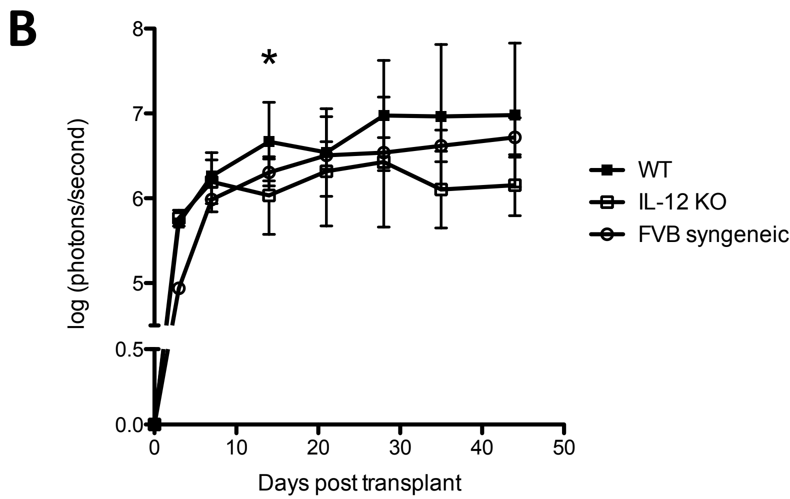


Figure 3

Figure 3-4

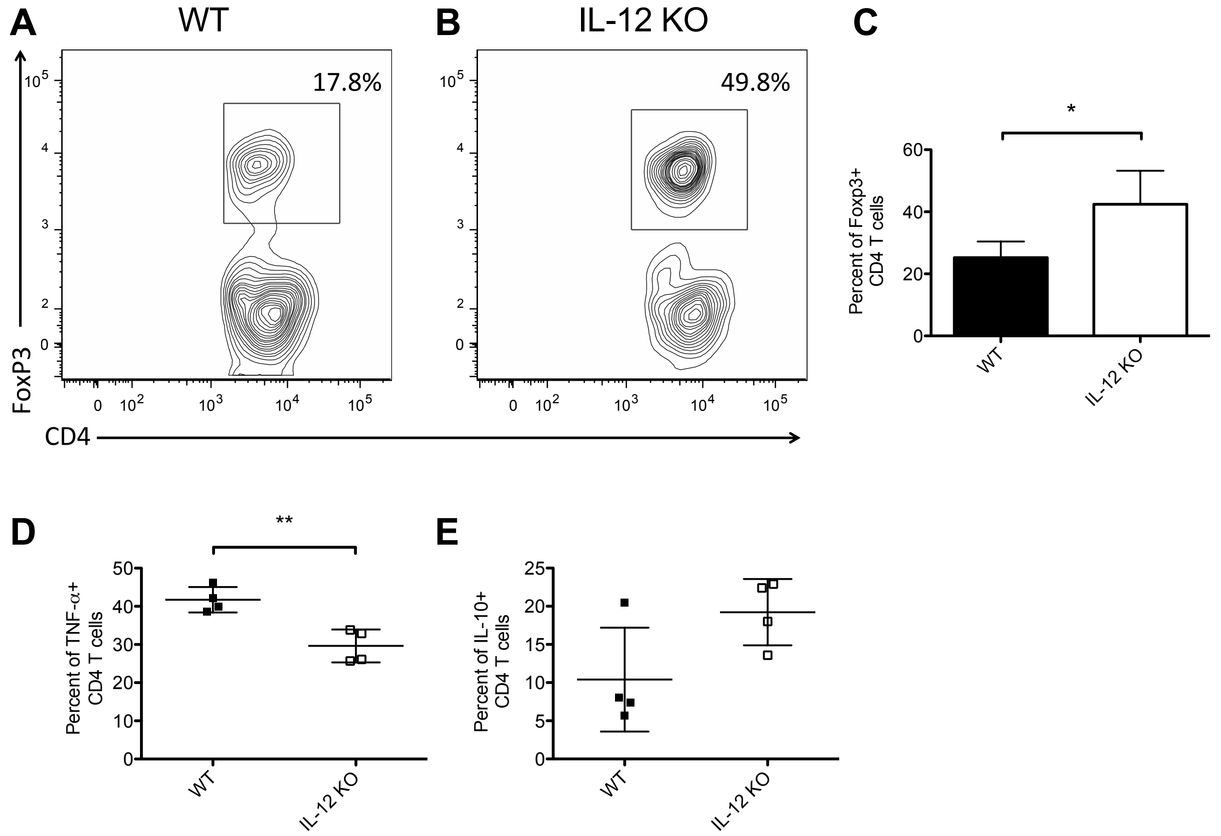
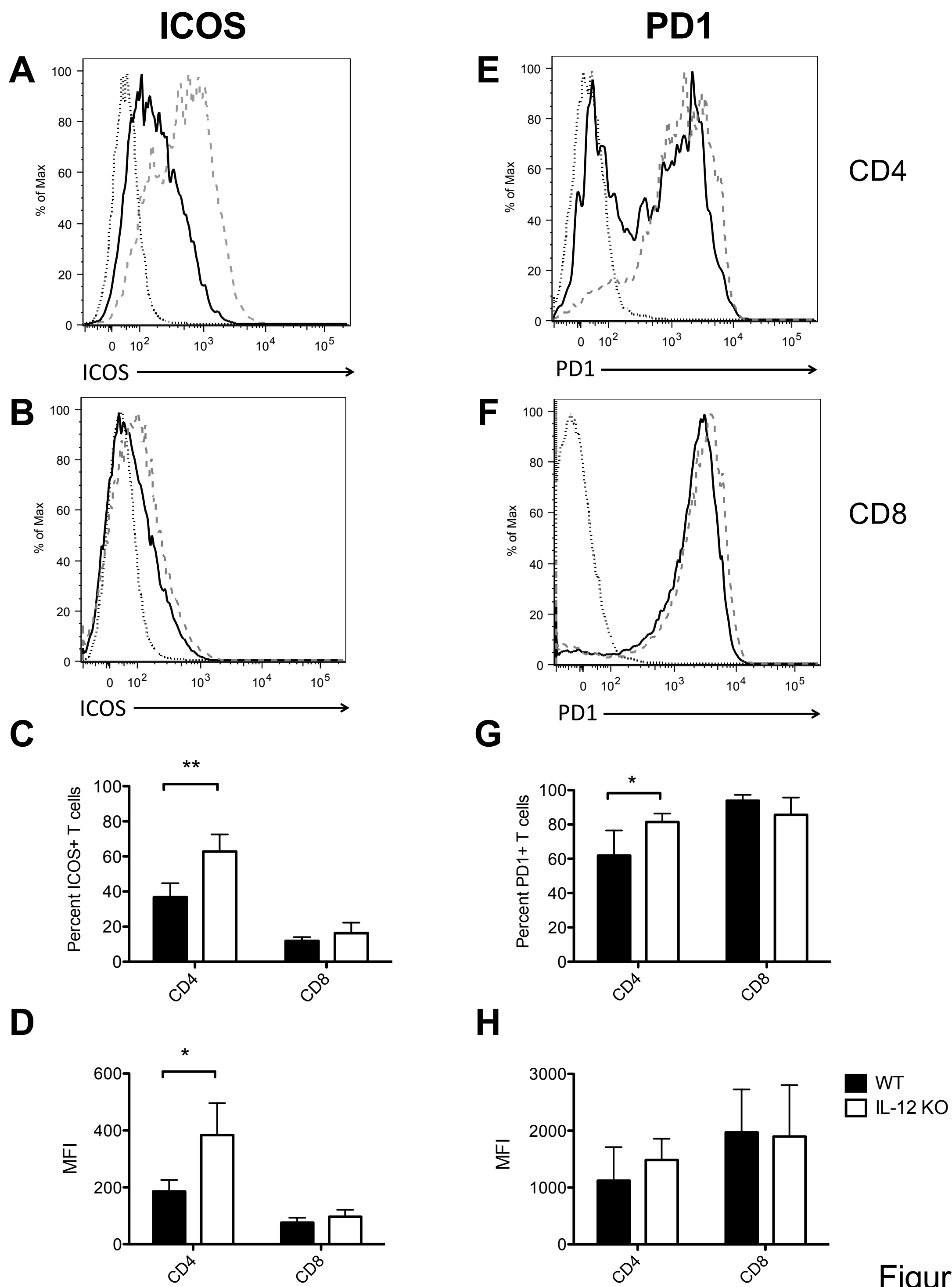


Figure 4

Figure 3-5



Chapter 4

Discussion

Allogeneic bone marrow transplantation is curative for many hematological malignancies including high risk and relapsed cancers of the blood and bone marrow, such as acute leukemia or multiple myeloma, via a graft-versus-leukemia (GvL) effect. The GvL effect of the graft is mediated primarily by donor T cells that are activated by a combination of both donor and host APCs. GvL is often accompanied by GvHD, in which donor alloreactive T cells mount an immunological response against the hematolymphoid cells in the recipient. In addition to the complication of GvHD, another complication during BMT is that of graft failure. After irradiation, some residual host cells remain, especially after reduced intensity conditioning, and clearance by cytotoxic donor T cells is necessary for proper engraftment of donor BM.

Elucidation of mechanisms that drive engraftment, GvL, and GvHD is a fundamental objective in the field of experimental hematology. APCs are some of the first responders in immune responses. Dendritic cells are professional APCs of a variety of subsets that can induce different effector functions in T cells and different DC subsets can drive the differentiation of T cells towards T_H1 or T_H2 responses[1]. Also, host and donor derived APCs also have been known to have different effects on GvL and GvHD. While GvL activity requires host DCs, donor APCs also play a significant role in certain settings. Host DCs initiate GvHD, but donor DCs amplify the phenomenon.

While it is apparent that host DCs are critical in the initiation of both GvHD and GvL, we became interested in the role of donor DCs on transplant outcomes. In 2001, Waller, et al., conducted a clinical study examining the cellular constituents of donor BM on

transplant outcomes in HLA-matched related donor BMT[2]. It was found that higher numbers of pDCs in the donor BM were associated with more relapse and less chronic GvHD[2]. Of note, more recently, Waller, et al., analyzed the clinical outcomes of patients transplanted on BMT CTN 0201 (unpublished data)[3, 4]. The more recent study involved patients who were receiving transplants from unrelated donors, many of which were HLA-mismatched. Also, as opposed to the previous study, very few patients had chronic myeloid leukemia (CML), which is known to be sensitive to GvL[5]. This time it was found that more donor pDCs in the graft were associated with less graft rejection, fewer deaths from GvHD, and no effect on relapse. Taken as a whole, the fact that the cellular makeup of donor BM could significantly affect the outcome of a transplant led us to examine the role of pDCs in transplant outcomes in murine models with the overall goal of engineering graft constituents to improve outcomes.

Initially, we aimed to elucidate the difference between grafts containing mDCs vs. pDCs. We first transplanted mice with FACS sorted purified populations HSCs, T cells, and either pDCs or mDCs[6]. Initial examinations used a non-tumor model. DCs were sorted and defined as being negative for lineage markers (CD3, Ter119, CD49b, CD19 and IgM), CD11c+, and either CD11b+ (mDCs) or CD11b- (pDCs) [6]. We found that mice receiving grafts containing HSCs, pDCs and T cells had higher donor T cell proliferation as compared with mice receiving HSCs and T cell alone or HSCs, mDCs, and T cells[6]. Transplanting pDCs along with HSCs and T cells increased IFN-γ and decreased IL-4 and IL-10 production by donor T cells as compared with HSCs and T cells alone, or HSCs with mDCs and T cells[6]. Serum T_H1 cytokines were also increased in mice

receiving HSC, pDCs, and T cells as compared with other groups[6]. Last, we examined the outcome of these transplants in a tumor model using a murine leukemia cell line, LBRM[6]. We found that mice receiving HSCs, pDCs, and T cells had significantly enhanced leukemia free survival as compared with mice receiving HSCs, mDCs, and T cells or HSCs and T cells alone[6].

Sorting purified populations of pDCs, HSCs, and T cells is not practical in the clinical setting. While transplants of purified HSCs do occur, there are significant problems associated with transplanting HSCs as opposed to BM transplants including a slow immune reconstitution and a lack of heterogeneity in reconstitution, which leads to a slower GvL effect and a possibility of infections prior to full reconstitution. Moreover, while isolation of purified populations via FACS for the purpose of transplantation has been performed and patients were transplanted, in order to obtain enough CD34+ HSCs for a transplant, the sorting process can take days. We aimed to develop a more clinically feasible method of altering the pDC content of the graft in order to enhance GvL without an increase in GvHD.

In order to mirror the results of the murine studies where mice receiving HSC, pDCs, and T cell had enhanced survival in a tumor model without increased GvHD, however more practically, we took an approach of enriching pDCs in total BM by way of depleting mDCs, a cell subset which comprised less than 1% of total BM. Depleting a population from whole BM is more practical than attempting to sort rare subsets such as HSCs and pDCs and could potentially be performed using a MACS column as opposed to a flow

cytometer. Murine BM was first T cell depleted, after which BM was stained with antibodies for lineage markers (CD49b, Ter119, CD19, IgM) as well as CD11c and CD11b. FACS was used in order to depleted Lineage –, CD11c+, CD11b+ cell, which comprised le than 1% of the total BM population. The other 99% of the BM was sorted and transplanted into mice. Mice received either control-sorted BM (run through the flow cytometer on forward- and side-scatter gates) or mDC-depleted sorted BM.

Similar to the previous studies using purified population, it was found that mice receiving mDC depleted BM had higher survival in a tumor model without increased GvHD (Figure 2-1E, F). Mice receiving mDC depleted BM had higher total T cell and CD8+, but not CD4+, T cell proliferation as measured by CFSE dilution on day 3.5 post-transplant (Figure 2-2A, B). T cell expansion following transplant was measured using luciferase positive T cells and *in vivo* bioluminescent imaging of anesthetized mice. Mice receiving mDC depleted BM had more donor T cells on day 30 post-transplant as measured by the photons / second bioluminescent signal emanating from the mouse (Figure 2-2 C, D). It has long been known that donor $CD8^+$ T-cells are an important mediator of GvL effect[7]. Mice receiving mDC depleted BM also had significantly higher levels of IFN-γ on day 10 post-transplant as well as higher serum levels of IL-13 (Figure 2-3E, F), consistent with previous experiments with purified populations of cells.

We also aimed to determine the mechanism by which the presence of pDCs as opposed to mDCs led to enhanced survival in a murine leukemia model. We had determined previously that addition of pDCs to grafts of HSCs and T cells led to an increase in IFN-γ

production by donor T cells, and this IFN-γ production was responsible for GvL activity, as evidenced by the lack of enhanced survival in mice receiving grafts of HSC, pDC, and IFN-γ KO T cells[8]. We had also determined that IFN-γ production by donor T cells led to an upregulation of IDO by donor pDCs[8]. This IDO upregulation was directly dependent on the ability of pDCs to respond to IFN-γ, since when IFN-g receptor KO mice were the source of donor pDCS in *in vitro* studies, IDO was not upregulated by pDCs[8]. Of note, when IDO KO mice were the source of donor pDCs in transplant with HSC and T cells, there was an increase in GvHD, as well as a modest reduction in the number of Tregs in recipient mice, suggesting that IDO expression by pDCs induced Treg generation, which then limited GvHD and continued T-cell activation[8].

Whereas we knew that donor pDC drove T_H1 polarization of donor T cells, and IFN-γ production by donor T cells in turn led to IDO upregulation that could limit GvHD while preserving GvL, it was not directly known how specifically donor pDCs, as opposed to mDCs, were driving T_H1 polarization and enhancing GvL. Knowing that pDCs are strong producers of IL-12 compared with mDCs, and that IL-12 can help maintain a T_H1 phenotype in T cells, we hypothesized that IL-12 production by pDCs is what drove donor T cell T_H1 polarization and enhancement of GvL activity.

In order to test this hypothesis, we took the approach of transplanting mice with either undepleted control-sorted TCD BM, mDC depleted TCD BM (pDC enriched), or mDC depleted TCD BM from IL-12p40 KO donors. While the mDC depleted BM from IL-12p40 KO donors was then enriched for pDCs, these pDCs were incapable of producing

IL-12p40, and thus IL-12p70. We found that when mice received mDC depleted BM from IL-12 KO donors, that the effect of survival enhancement disappeared, and in fact mice died slightly sooner than mice receiving undepleted BM grafts (Figure 2-1 E). In addition to IL-12 production being necessary for the survival difference seen between mice receiving undepleted and mDC depleted BM, IL-12 was necessary for T cell proliferation. Mice receiving IL-12 KO mDC depleted BM had similar CD8+ T cell proliferation on day 3.5 post-transplant as measured by CFSE dilution as mice receiving undepleted BM (Figure 2-2-A, B) and had a similar total body bioluminescent signal over time as mice receiving undepleted BM grafts (Figure 2-2 C, D). Interestingly, the increase in serum IFN-γ that was seen in mice receiving mDC depleted BM compared with undepleted BM was not abrogated when mDC depleted BM originated from IL-12 KO donors (Figure 2-3E).

While pDCs are main producers of IL-12, other cell types can produce IL-12 including other APCs such as macrophages and B cells, and some non-hematopoietic cells, including keratinocytes, osteoblasts, epithelial cells, and endothelial cells[9, 10, 11, 12]. In order to confirm that it was in fact the production of IL-12 by pDCs specifically that was improving survival, we transplanted mice that had been injected i.v. with LBRM with purified populations of HSCs, T cells, and either WT or IL-12 KO pDCs. As controls, we also transplanted mice with BM and T cells, as well as HSC alone. All radiation control mice and mice receiving HSCs alone died (Figure 2-4A). A majority of mice receiving either BM and T cells, or HSC and T cells with WT pDC survived (75% survival) (Figure 2-4A). When pDCs originated from IL-12 KO mice, all recipient mice died (Figure 2-

4A). The reason for the rapid death was presumably due to a high level of acute GvHD, as IL-12 has been known to reduce GvHD[7, 13]. At 36 days post-transplant, remaining mice were imaged. It was found that 85% of surviving mice receiving BM and T cells had detectable levels of tumor, while only 66% of mice receiving HSC, pDC, and T cell had tumor as measured by bioluminescent signal emanating from luciferase positive LBRM (Figure 2-4B). While HSC and BM transplants are not directly comparable, this data does support that a transplant enriched for pDCs has improved GvL activity. In summary, the data support that selective depletion of mDCs from the murine BM graft is a novel and feasible method for manipulation of the BM graft to regulate donor T-cell activation and enhance GvL activity without leading to increased GvHD. Moreover, we show that IL-12 is a key cytokine synthesized by donor pDCs that is necessary for the enhanced survival and donor T cell proliferation seen when mice receive mDCs depleted grafts.

The role of IL-12 in mDC depletion experiment was of interest in order to determine the mechanism by which pDC enrichment contributed to improved transplant outcomes. While we knew that IFN-γ production by donor T cells was important, and that IFN-γ production led to IDO expression by pDC, which in turn presumably enhanced GvL yet minimized GvHD by driving Treg induction, it not directly known why mice receiving transplants enriched for pDCs drove IFN-γ production by T cells. We hypothesized that IL-12 production by pDCs was the initial driving force in the improved GvL effects and did in fact find that mice receiving mDC depleted transplants experienced improved GvL with an increase in serum IFN-γ.

We propose a two-part model to explain the mechanism by which pDC enrichment drives enhanced GvL without increased GvHD. Initially, donor pDCs produce IL-12 in response to danger signals released by damaged cells from radiation. This IL-12 production leads to the polarization of donor T cells towards a T_H1 phenotype and production of IFN-γ. The IFN-γ production contributes to enhanced GvL effects, but also causes IDO to be upregulated by donor pDCs. IDO has been known by induce Treg formation, which can then limit GvHD (Figure 4-1). However this is only part of the story. We hypothesize that cross presentation contributes to the lack of increased GvHD. It is well documented that while host APCs initiate GvHD, donor DCs amplify it. It is presumed that host DCs initiate the process by activating donor CD4 T cells, which then activate donor DCs via cytokines and CD40/CD40L activation. These donor DCs will then cross present to donor CD8 T cells, leading to cytotoxic alloreactive responses against host tissues. However, only cDCs have been reported to cross present antigens, while pDCs have not been shown to cross present efficiently in the literature. By enriching for pDCs, and depleting mDCs, which are capable of cross presentation, one limits the amplification of GvHD by donor DCs (Figure 4-1).

While the role of IL-12 in GvHD and GvL has been examined previously using administration of recombinant IL-12, the role of IL-12 in BM engraftment has been largely unexamined. While the overall frequency of graft failure in BMT is less than 5%, graft failure is still a major concern when the source of the allograft is a T cell depleted (TCD) human leukocyte antigen (HLA) – haploidentical donor, in cord blood

transplants[14] or in settings where non-myeloablative conditioning is used[15]. Having seen the effects that a lack of IL-12 can have on GvL activity in Chapter 2, and knowing that administration of exogenous IL-12 can alleviate GvHD, we aimed to determine the role of IL-12 producing host APCs on transplant outcomes.

In order to establish a system in which host APCs were unable to produce IL-12, we created radiation chimeras by transplanting B6 mice with BM from congenic B6 donors or IL-12 KO donors, creating mice where host hematopoietic cells were unable to produce IL-12. It is important to note that there are non-hematopoietic cell types that are capable of synthesizing IL-12 in certain conditions[9, 10]. We then transplanted mice with allogeneic BM (FVB) and found that mice whose host APCs lacked the capacity to produce IL-12 had reduced survival and donor T cell engraftment as measured by *in vivo* bioluminescent imaging of luciferase positive T cells (Figure 3-1, 3-3). We also found higher levels of erythroid engraftment in mice whose host-APCs produced IL-12 as measured by RBC counts and hemoglobin levels (Figure 3-2). TNF-α production was reduced in mice that lacked host-APC IL-12 production (Figure 3-3D). Mice whose host APCs were capable of producing IL-12 had higher percentages of Foxp3+ CD4 T cells on day 10 post transplant, as well as higher levels of ICOS and PD1 on CD4 but not CD8 T cells (Figure 3-4A, B, C, 3-5). These data are the first to report that IL-12 production by host hematopoietic cells, presumably APCs such as pDCs, is a critical mediator of engraftment in allogeneic BMT.

While manipulation of IL-12 producing host-cells is not translatable to a clinical setting, the use of exogenous IL-12 as a therapeutic has been examined. While toxicity to the patient is of concern, doses that are tolerated have been evaluated (0.5 μg/kg)[16, 17]. IL-12 has also been examined as a potential therapy in combination with other cytokines[18] and clinical trials in melanoma patients using an IL-12 plasmid have been conducted with limited success (complete remission in 3/9 patients)[19]. Our data in the engraftment model sheds light on the potential of IL-12 as not only an anti-tumor therapy, but also as a way to promote engraftment in settings where graft failure is of concern.

Future directions include further elucidations of mechanisms, as well as pre-clinical models for the use of IL-12, and possibilities of translating research into the clinical setting. While we have demonstrated the role of IL-12 producing pDCs in GvL enhancement, we have not yet examined the theory of cross presentation contributing to increased GvL without increased GvHD. Mice could be transplanted with HSCs, T cells, and a combination of pDCs and mDCs, where subsets of DCs originated from WT or MHC I KO donors (beta 2m KO). If mice receiving transplants where mDCs were incapable of cross presenting had similar outcomes as mice receiving grafts enriched for pDCs, this would support the hypothesis that the lack of cross presentation was critical for improved outcomes. For engraftment studies, it would be of interest to examine the role of exogenous administration of IL-12 to prevent graft failure. A MHC-mismatched or MHC-matched and miHA mismatched murine system where mice were preconditioned to reject grafts could be used as a model for graft failure. Administration of exogenous IL-12 could be utilized to determine whether IL-12 would improve

engraftment in this setting. Lastly, graft engineering could be explored in human subjects. While FACS sorting of purified populations is not feasible, the use of a MACS column is possible. A limitation is that current knowledge of mDCs and pDCs has not isolated a single marker that is isolated to the mDC subset. CD11b is expressed on approximately 40% of BM cells, so depletion would require two separate markers, CD11c and CD11b. While automated MACS systems enhance practicality in clinical settings, a two-step two-column depletion would not be feasible. A novel approach could be the use of pharmacological agents along with G-CSF mobilization of HSC from peripheral blood to enrich for pDCs in the collection. Plerixafor alone or in combination with G-CSF has been shown to mobilize large numbers of pre-pDCs and presents an approach for mobilizing HSCs along with pre-pDCs from peripheral blood of donors. [20]

Overall, this data contributes significant knowledge to the field of allogeneic transplantation about the mechanisms by which IL-12 and IL-12 producing cells affect GvL, GvHD, as well as engraftment. While numbers of donor pDCs have been correlated with improved outcome in patients with MHC-mismatched BMT, the mechanisms by which pDCs could enhance GvL had not been elucidated. We found that similar phenomena hold true in a murine setting, where pDC can enhance donor T cell proliferation, T_H1 polarization, and GvL activity, all while limiting GvHD. These observations are dependent upon IFN-γ production by donor T cells, which then upregulate IDO expression on pDCs. We have shown that the production of IL-12 by donor pDCs is critical for GvL activity, presumably due to the T_H1 driving activities of IL-12, and we have supported the use of IL-12 as an anti-tumor therapeutic. We have also

presented a novel method by which graft content could be manipulated in a clinical setting by depleting a small population of the BM, myeloid DCs. Lastly, we are the first to report that host-derived IL-12 plays a significant role in engraftment in allogeneic BMT. Taken together, the data shed light on fundamental questions in the field of allogeneic hematopoietic stem cell transplantation and support IL-12 as a therapy to improve GvL, minimize GvHD, and improve engraftment.

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Figure Legends

Figure 4-1. Model for GvL enhancement by pDC enrichment without increased GvHD. The first step of GvHD initiation involves damage to the host gut epithelium and tissues due to pre-transplant conditioning, releasing LPS and cytokines such as TNF-α and IL-1. Host APCs are activated and then activate donor T cells, which mediate both GvHD and GvL activity. Cytokine secretion as well as CD40/CD40L ligation by donor T cells activates donor DCs. Activated donor pDCs secrete IL-12, which further activates donor T cells and leads to IFN-γ secretion and further T_H1 polarization, promoting the GvL effect. Secretion of IFN-γ by donor T cells leads to upregulation of IDO by pDCs, which then promote generation and maintenance of Tregs that limit GvHD. Activated donor mDCs can cross prime donor T cells, which can amplify GvHD. When donor mDCs are depleted, cross priming of donor T cells does not occur, as pDCs have not been shown to cross present.

Figure 4-1