INTERLEUKIN 15 AND TRANSPLANTATION BIOLOGY: THE INTERFACE OF INNATE AND ADAPTIVE IMMUNITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for The Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Bradley W. Blaser, B.S.

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The Ohio State University

2006

Dissertation Committee:

Michael A. Caligiuri, M.D., Adviser

William E. Carson, M.D.

Sherif Farag, M.D., Ph.D.

Yang Liu, Ph.D.

Approved by

_____

Adviser
The Integrated Biomedical Sciences Graduate Program

ABSTRACT

Years of clinical and experimental evidence have shown that both the antigen-nonspecific innate immune system and the antigen-specific adaptive immune system can effectively eliminate malignant cells that remain after front-line therapy for cancer. Because the immune response to any given stimulus requires the coordinated activity of a large number of diverse cell types, elaborate communication networks have evolved that utilize direct cell-cell interactions as well as soluble growth factors, or cytokines, that can potentially travel great distances in the body. Knowledge of the mechanisms and effects of these cell-cell and cell-cytokine-cell interactions is of paramount importance as physicians and scientists advance the frontiers of cancer immunotherapy. Presented here is a series of studies that define roles for the cytokine interleukin 15 (IL-15) in acute graft versus host disease (GVHD) and graft rejection. Mediated by both the innate and adaptive immune systems, graft rejection and acute GVHD are the most common life threatening side effects of allogeneic bone marrow transplantation (BMT), a promising immunotherapeutic approach for aggressive and otherwise incurable hematopoietic malignancies. Also presented are studies evaluating a novel therapeutic antibody designed to interrupt the cell-cell signals that serve to prevent tumor cell lysis by natural killer (NK) cells, a critical part of the innate immune system. Together, these data

unravel a small portion of the complex interactions between immune effector cells and malignant cells and provide justification for future basic and clinical immunotherapeutic studies.

To Anne, Waldo, and Ginger.

ACKNOWLEDGMENTS

I thank my wife, Anne, for her undying support and devotion to our marriage.

I thank my parents for their love and commitment to my education.

I thank my adviser Michael Caligiuri for outstanding mentorship. I cannot thank him enough for the care he has taken in my training, professional development and personal growth. Nor can I adequately express my gratitude for the rare opportunity to be a part of his laboratory.

I wish to thank all current and former members of the Caligiuri Laboratory including Brian Becknell, Dennis Chang, Dave Couling, Aharon Freud, Martin Guimond, Seth Karol, Tom Liu, Charlene Mao, Sameek Roychowdhury, Noah Schwind, Samuel Shin, Rossana Trotta, Jeff Vandeusen, and Susan Whitman. In particular, I wish to thank Sameek and Martin for invaluable guidance and instruction.

I wish to thank Donna Bucci and Tamra Brooks for irreplaceable administrative support.

I thank all of my collaborators, including Donna Kusewitt, Amy Ferketich, Sherif Farag, Roger Briesewitz, Danilo Perrotti, Robert Lee, and Manohar Ratnam for their assistance and for extending my knowledge of the scientific world.

I thank Andrea Velardi and Bruce Blazar for their support and guidance of our work.

I thank my dissertation committee, William Carson, Sherif Farag, and Yang Liu for their repeated commitments of time and effort on my behalf.

I thank Dr. Yates and the faculty of the Integrated Biomedical Science Graduate Program for their unwavering guidance and commitment to my education.

Finally I wish to acknowledge the generous support of my education through the University Fellowship, the Medical Scientist Program Fellowship, and the multiple funding organizations that provide for the Caligiuri Laboratory.

VITA

July 10, 1978 ……Born, Columbus, Ohio

2000 ……..B.S., The Ohio State University, Summa cum Laude, with Distinction in Molecular Genetics and with Honors in the Liberal Arts

2000-present…….M.D./Ph.D. candidate and Medical Scientist Program Fellow, The Ohio State University

PUBLICATIONS

Peer-Reviewed Research Articles

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FIELDS OF STUDY

Major Field: Integrated Biomedical Sciences with an emphasis in Immunology

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LIST OF ABBREVIATIONS

ADCC.....antigen dependent cellular cytotoxicity
AML.....acute myeloid leukemia
APC.....antigen presenting cell
B6.....C57Bl/6
B6D2F1.....B6 x DBA/2 F1 hybrid
BM(T).....bone marrow (transplantation)
DC.....dendritic cell
GVH(D).....graft versus host (disease)
GVT.....graft versus tumor
HLA.....human leukocyte antigen
HSCT.....hematopoietic stem cell transplantation
IFN-.....interferon
IL-.....interleukin
KIR.....killer cell Ig-like receptor
LPS.....lipopolysaccharide
MHC.....major histocompatibility complex
MST.....median survival time
NK.....natural killer
R.....receptor
rh.....recombinant human
rm.....recombinant murine
s.e.m.....standard error of the mean
TNF-α.....tumor necrosis factor alpha

CHAPTER 1

Introduction

1.1 Allogeneic Hematopoietic Cell Transplantation: Clinical Applications

Hematopoietic stem cell transplantation (HSCT) serves as a means of rescue for patients with disseminated cancers such as leukemia, lymphoma and multiple myeloma after treatment with lethal doses of anti-neoplastic therapeutics. Most commonly, recipients of HSCT receive up to 15 gy total body irradiation plus cyclophosphamide (60 mg/kg) over two successive days (1, 2). In addition to eliminating the majority of tumor cells, this combined regimen destroys the normal hematopoietic (blood forming) stem cells found in the bone marrow; without further intervention, recipients of this so-called myeloablative regimen will invariably die of bone marrow failure. As a result, these patients receive allogeneic (derived from a genetically different individual) hematopoietic cells from one of three sources: umbilical cord blood, bone marrow, and peripheral blood into which stem cells have been mobilized from the bone marrow by administration of growth factors. Although the frequency of true stem cells varies from each of these sources, recipients of bone marrow and mobilized peripheral blood typically receive 2-3 x 10^6 $CD34^+$ hematopoietic stem cells per kilogram of body weight while recipients of umbilical cord blood receive one-tenth this amount due to limitations on the amount of blood available from this source (3, 4). (For this reason, recent clinical trials have

combined umbilical cord blood from multiple donors with improved results (5)). Engraftment with donor cells usually occurs between 3 and 12 weeks after transplantation before which patients frequently require transfusion of red blood cells and platelets and careful prophylaxis against infectious disease (6).

Over 7,000 patients received allogeneic HSCT in 2002 alone for the treatment of malignancy (7). This regimen is curative in 46-65% of acute myeloid leukemia (AML) patients transplanted in first remission, in up to 49% of patients with relapsed high-grade non-Hodgkin's lymphoma, and in 80% of chronic-phase chronic myeloid leukemia patients (8-14). Yet, overall transplant related mortality can reach 40% depending upon the original malignancy and the source of stem cells so while allogeneic HSCT can provide great and lasting benefit for aggressive and otherwise incurable malignancies it remains a second-line treatment in the majority of cases (7).

1.2 Acute Graft Versus Host Disease and Graft Versus Tumor Activity

The primary reason why patients do not receive allogeneic HSCT is the potential occurrence of severe acute graft versus host disease (GVHD). GVHD is an immunologic response of donor-derived lymphocytes contained in the allograft against an allogeneic transplant recipient that results in potentially lethal damage to the liver and the epithelia of the skin and gut (14). The incidence and severity of acute GVHD depends strongly upon the degree of human leukocyte antigen (HLA) mismatch between the donor and recipient. In those patients who receive HSCT from an HLA-matched sibling donor, the incidence of severe acute GVHD is approximately 30% while in donor-recipient pairs mismatched at two loci, the incidence is as high as 70% (14, 15). Lethal acute GVHD is

responsible for 15% of all deaths after allogeneic transplantation including relapse (7). An additional 17-21% of deaths occur as a result of infection, frequently secondary to skin necrolysis and/or prophylactic immunosuppression associated with acute GVHD (7). Because of the frequency and severity of this disease, the safety of allogeneic HSCT is critically dependent upon finding HLA-matched donor-recipient pairs. But in fact, only 25-30% of candidate patients have an HLA-identical sibling donor and those who lack a related donor have a 10-50% chance of finding an HLA-identical unrelated donor depending upon racial background (16). Thus, approximately one half of all patients who could benefit from allogeneic HSCT do not receive it and up to three out of four of those who do suffer from acute GVHD.

At the heart of acute GVHD is T cell alloreactivity. Alloreactive T cells form a subset of the donor T cell pool that can recognize recipient tissues as foreign and are directly responsible for the destruction of liver, skin and gut tissues. Consequently, a major goal of acute GVHD prophylaxis is the suppression of T cell allogeneic responses. Complete elimination of T cell alloreactivity can be achieved through rigorous T cell depletion of the allograft, by transplantation of autologous cells harvested from the patient while in disease remission, or by transplantation of syngeneic cells harvested from an identical twin. Yet these methodologies are clearly associated with a higher rate of malignant relapse (17); the conclusion is that there is a graft versus tumor (GVT) effect mediated by alloreactive T cells which is responsible for a significant portion of the overall benefit of allogeneic HSCT. A great deal of effort has been invested in discovering the biological differences between GVH and GVT alloreactivity and, more importantly, in finding therapeutic means to inhibit the former and promote the latter.

1.3 The Natural History of Acute GVHD

The pathogenesis of acute GVHD has been divided into three phases: 1) An initial priming phase in which the damage to the gastrointestinal tract by the conditioning regimen plays a central role 2) a phase of donor-T cell activation and expansion that is promoted by the cytokine milieu of phase 1 and is critically dependent upon recipient-derived antigen-presenting cells (APC) and 3) a phase of recipient tissue destruction as a result of the direct action of donor-derived T cells (18). A discussion of each of these phases follows.

The standard conditioning regimen of cyclosporine and total body irradiation is directly toxic to cell lineages with a high turnover rate, particularly the those found in the GI system: oral mucositis is a very common side effect that can necessitate total parenteral nutrition after transplantation (19). However, the cellular damage extends to the large bowel where epithelial cell death and loss of the tight junctions between them allows translocation of bacterial products such as lipopolysaccharide (LPS) across the intestinal mucosa and into the circulation (18). What results is a form of sepsis referred to as the "cytokine storm" of acute GVHD (20). LPS is a potent stimulator for the production of tumor necrosis factor (TNF)-α by irradiation- or interferon (IFN)-γ-primed monocytes {Nestel, 1992 #96}. LPS also induces the production of IL-1 and IL-12 by monocytes (20, 21). TNF-α and IL-1 have a vast spectrum of biologic activities that include increasing the chemotactic activity of monocytes and granulocytes, the maturation of dendritic cells and the expansion of T cells (22-26). Dendritic cell maturation is accompanied by increases in expression of major histocompatibility (MHC)-I and -II molecules (HLA gene loci in humans and H-2 and I- gene loci in mice),

as well as co-stimulatory ligands such as B-7.1 and B-7.2 that further contribute to T cell expansion (27-29). IL-12 produced by monocytes polarizes donor-derived T cells toward a pro-inflammatory Th1/Tc1 phenotype, producing IFN-γ and TNF-α that feed back and intensify the cytokine storm (30, 31).

As alluded to, the cytokine milieu established in phase 1 primes the host for donor T cell expansion in phase 2. But through what mechanism are the requirements for T cell expansion (contact with alloantigen in the context of cell-surface MHC plus co-stimulation with consequent production of IL-2) met in this model? More to the point, what is an alloantigen? Research in solid organ graft rejection models has identified two pathways whereby alloreactive T cells can be presented with alloantigen. In the so-called indirect pathway, T cells are presented foreign peptide in the context of self MHC-I (32). In terms of acute GVHD, donor-derived APC phagocytose cellular material derived from the host and present it via MHC-II (classical presentation) or MHC-I (cross-presentation) to $CD4^+$ and $CD8^+$ T cells, respectively. This has long been thought a minor pathway for T cell alloreactivity in acute GVHD, although a recent study has defined a role for the indirect pathway in acute GVHD (Figure 1) (33). In the major pathway, known as direct allorecognition, donor-derived T cells are presented with host-derived peptides in the context of host MHC-I and MHC-II by host-derived APC (Figure 1) (34). Current thinking suggests that this interaction is peptide-dependent but peptide-non-specific, i.e., certain donor-derived T cell clones directly recognize determinants on host MHC as foreign regardless of the peptide presented (35). The direct pathway results in a frequency of alloreactive T cells of 1-5 per 10^2 total T cells, three logs higher than the

frequency of T cells that respond to a typical foreign antigen (35). Although the exact molecular mechanisms of T cell allorecognition remain unsettled, clearly acute GVHD is driven in large part by the large number of anti-host T cells present in the allograft.

The cellular mechanisms of T cell alloreactivity in acute GVHD have received much attention and achieved clarity in recent years. Shlomchik et al. were the first to show that long-term murine survivors of T cell depleted allogeneic BMT, which retain their native MHC expression on all parenchymal tissues such as skin and gut but express allogeneic MHC on donor-derived hematopoietic tissues are highly resistant to GVHD induced by a second, T cell replete BMT from the same donor strain (36). The conclusion is therefore that allogeneic host-derived hematopoietic cells are necessary for alloreactive T cell activation. These findings were extended with the observations that host-derived dendritic cells (DC) are sufficient to initiate acute GVHD and that alloantigen expression by the target organs of GVHD themselves—liver parenchyma, gut and skin epithelia—was not required (37, 38). Thus the third phase of acute GVHD—target organ destruction—is mediated by donor-derived T cells in an MHC-independent manner. Studies have demonstrated a requirement for expression of FAS-L, TNF-α, and TRAIL by donor-derived T cells in this phase, yet a full mechanism for the tissue specificity and MHC-independence of this process has yet to be described (39-41).

Together, these groundbreaking experiments have identified host-derived antigen presenting cells and in particular host-derived DC as the mechanistic center of acute GVHD and have provided a rationale to target these cells for elimination with the aim of preventing the disease.

1.4 Role of NK Cell Alloreactivity in Acute GVHD and Anti-Tumor Responses

One means of targeting host-derived DC for elimination is through the action of alloreactive donor natural killer (NK) cells. NK cells share many receptor and signal transduction pathways with T cells and are developmentally related to T cells (42-47). Yet NK cells are functionally part of the innate immune system because they recognize foreign or transformed cells in an antigen non-specific way (48). NK cell responses are polyclonal in that many mature NK may respond to a given target while T and B cell responses are normally monoclonal—one cell recognizes one antigenic target and clones itself to eliminate it (49-51). This functional difference has established a long-standing paradigm in the biology of infectious disease: NK cells (and/or other elements of the innate immune system, depending on the nature of the pathogen) provide a rapid first line of defense for the body to allow time for monoclonal T and B cell expansions to occur and completely eliminate the disease.

NK cells recognize target cells, become activated and attempt to lyse the targets by means of a complex balance of activating and inhibitory signals (52). Primary among the inhibitory signals in human NK are those provided by killer cell immunoglobulin-like receptors (KIR) (53-55). The ligand for KIR is MHC-I, expressed on the surface of all healthy nucleated cells in the body (56). KIR form a highly polymorphic cluster of up to 14 genes on chromosome 19q13.4 (56). Expression of one or more inhibitory KIR is acquired at random in NK cell development and stably maintained in daughter cells through DNA methylation (57). A subset of NK cells, thought to be precursors of the major cytolytic subset are not known to express KIR, but do express a heterodimeric

inhibitory receptor for MHC-I known as CD94/NKG2A (58). Interestingly, KIR are a relatively recent evolutionary product and are completely absent in mice. In a remarkable example of convergent evolution, mice express a structurally unrelated family of Ly-49 inhibitory receptors which engage mouse MHC-I and perform the same function as KIR in humans (59). Thus it is accepted that NK cell self-tolerance toward healthy cells is maintained through inhibition of killing by contact with MHC-I. However, in infected and transformed cells, MHC-I expression is often lost or downregulated, inhibition of NK activity is decreased, and the balance of NK cell signaling shifts toward activation (60).

Whereas a loss or decrease in MHC-I expression by target cells resulting in decreased inhibitory KIR signaling is the single most important criterion for NK cell activation, a number of activating receptors can also contribute to shifting the balance toward activation. The most well studied among these is the lectin-like receptor NKG2D. NKG2D is expressed on most human and mouse NK cells and binds so-called "stress ligands" such as MIC-A, MIC-B, ULBP-1, ULBP-2 (human) or Rae-1 (mouse), expressed on the surface of potential target cells (61-64). These ligands are similar in structure to MHC molecules but their expression is largely restricted to infected, transformed, or otherwise stressed cells (61-65). Upon ligand binding, NKG2D in association with transmembrane adaptor molecules can activate the phosphatidylinositol-3 kinase/MAP kinase pathway and stimulate cytotoxic function by the NK cell (66). Other activating receptors include a family of natural cytotoxicity receptors (NCR's) for which ligand binding characteristics have yet to be fully described as well as activating

forms of KIR molecules which promote cytotoxic function in response to MHC-I (67-70). While activating KIR are not well-studied, clearly their activity is subordinate to inhibitory KIR in maintaining NK cell tolerance toward healthy cells and they perhaps only contribute as accessory activation receptors in the response to infection or tumor.

Understanding how the NK cell receptor repertoire controls NK cell function has allowed clinicians and researchers to take advantage of NK cell alloreactivity for the prevention of acute GVHD and tumor relapse. The concept of NK cell alloreactivity was introduced with the observation that NK cells from F1 hybrid animals are able to reject hematopoietic grafts from either parent (host versus graft reaction) (71, 72). In these so-called "hybrid resistance" experiments, there are subsets of F1 NK cells that do not express the corresponding inhibitory Ly-49 receptors for the MHC molecules on a particular parental strain and are therefore activated by those cells. Years later, Martelli pioneered the "haploidentical" BMT in humans to generate MHC/KIR mismatches and NK cell alloreactivity in the graft versus host direction (73). His group demonstrated that recipients of haploidentical BMT with predicted NK cell alloreactivity demonstrated a very low incidence of acute GVHD, despite the large degree of MHC and KIR mismatch between donor and recipient. Figure 2 presents a graphical representation of haploidentical transplantation with and without NK cell alloreactivity.

Subsequently, it was shown that NK graft versus host activity was restricted to elimination of elements of the recipient immune system, in particular recipient-derived DC (74). The fact that alloreactive NK cells can specifically target these critical initiators of acute GVHD and virtually ignore the classical targets of the disease—liver, gut and skin—has made haploidentical BMT an important component of transplantation research.

What makes haploidentical BMT even more attractive is the opportunity for donor selection. Whereas the overall likelihood of finding an HLA-matched unrelated donor in the in the National Marrow Donor Program tissue bank is 70%, this drops to 34% for those of African-American descent (75, 76). Because nearly all individuals have living relatives with haploidentical HLA types, many more patients of diverse genetic background may benefit from this modality compared with traditional HLA-matched transplantation (73).

Subsequent to the clinical observation of diminished acute GVHD in recipients of haploidentical BMT, it was found in experimental models and in clinical trials that KIR mismatching allowed for a significant enhancement of NK-mediated GVT (74, 77). In the first study, immunodeficient mice received adoptive transfers of leukemia plus alloreactive (KIR mismatched relative to the leukemia) or non-alloreactive (KIR matched relative to the leukemia) NK cells. Recipients of alloreactive NK cleared the leukemia and had 100% survival while recipients of non-alloreactive NK died rapidly with leukemic engraftment similar to control mice that did not receive NK cells (74). In the clinical study, patients receiving haploidentical transplants with NK alloreactivity demonstrated superior resistance to leukemic relapse compared with patients transplanted without NK cell alloreactivity (77). These reports demonstrate the potential clinical benefit for acute GVHD and the GVT effect which may be obtained by exploiting NK alloreactivity.

In the multi-faceted roles of T and NK cells in acute GVHD and in the GVT effect, allogeneic BMT provides a complex and powerful example of how the adaptive and innate immune systems can cooperate to destroy normal healthy tissues or to

eliminate residual tumor cells. But how can physicians hope to control these reactions and to lessen or delay GVHD while maintaining or improving the GVT effect? The following sections detail our current knowledge of the biology of an important growth factor, or cytokine, that may in part control T and NK cell responses to both normal self and transformed tumor tissues and thus impact the GVT effect and the pathobiology of acute GVHD.

1.5 IL-15: Signal Communication and Transduction

IL-15 is a pleiotropic, pro-inflammatory cytokine produced in response to infection or cellular activation. Although IL-15 mRNA is widely and abundantly expressed in multiple tissues it is inefficiently translated and secreted with multiple post-transcriptional checkpoints (78-80). Multiple start codons in the 5'-UTR and an inefficient signal peptide restrict IL-15 protein production largely but not exclusively to parenchymal cells such as bone marrow stroma and bone marrow derived cells such as monocytes, macrophages and dendritic cells. Studies in both human and murine systems have shown LPS, type 1 interferons and CD40 ligation to be important stimuli for IL-15 protein expression (81-83).

Recent advances in the biology of IL-15, conducted simultaneously in the Waldmann and Ma laboratories have convincingly demonstrated that IL-15 can function as a surface bound cytokine (84, 85). Although functional surface bound IL-2, TNF-α, and other cytokines have been described, IL-15 is remarkable in that physiologic concentrations of the soluble cytokine are orders of magnitude lower than those required for its function as a soluble molecule. In fact, IL-15 has been shown to bind with high

affinity (K_D 10-50 pM) to a unique IL-15 receptor alpha chain (IL-15 Rα) expressed by both myeloid cells and parenchymal cells in multiple tissues in the body, effectively concentrating the cytokine at potentially important sites of immune function (86, 87). Moreover, once bound, IL-15-IL-15 Rα internalizes and is recycled to the plasma membrane, further enhancing the half-life of the cytokine (85). Most remarkable, however, is that IL-15 Rα expressing myeloid cells have the potential to "trans-present" IL-15 to a third cell type that need not express IL-15 Rα itself (Figure 3). Elegant bone marrow transplant and adoptive transfer experiments have shown that such "trans-presentation" of IL-15 is sufficient to drive the development and homeostasis of IL-15 dependent cell lineages *in vivo* (84, 88, 89). While expression of IL-15 Rα is not necessary for the T or NK cell response to IL-15, expression of the shared, heterodimeric IL-2/15Rβγ$_c$ is. Although the IL-2/15Rβγ$_c$ is believed to contain all of the intracellular signal transduction machinery for the IL-15 receptor, in the absence of IL-15 Rα, it binds IL-15 only very weakly (K_d~500 pM) (90). These biochemical and transplantation experiments highlight the importance of trans-presentation and cellular interactions in the signaling mechanism of IL-15.

1.6 IL-15 and the Innate and Adaptive Immune Systems

IL-15 signaling influences multiple cells of both the innate and adaptive immune systems. IL-15 is required for the development and survival of NK cells and has an important role with IL-12 in NK-macrophage/monocyte interactions by co-stimulating IFN-γ production by NK cells (90-92). IL-15, produced by cells of the monocyte and DC lineages can also signal in an autocrine/paracrine fashion to promote monocyte and DC

maturation and expression of co-stimulatory molecules such as B7.2, MHC-II, and NKG2D ligands (82, 93). These IL-15-primed DC are more efficient stimulators of NK cytotoxicity and antigen specific T cell responses compared to unprimed DC. Further, IL-15 can act directly on DC increasing production of IFN-γ, IL-12, NO and upregulating expression of CD40, MHCII, and the survival molecule Bcl-2 (94, 95).

IL-15 is a critical factor for the adaptive immune system as well. Originally isolated because of its ability to permit the growth of IL-2 dependent T cell lines in the presence of neutralizing antibodies, IL-15 shares the ability of IL-2 to promote T cell growth *in vitro* (96, 97). In particular, at low concentrations (6 ng/mL), IL-15 promotes the expression of Bcl-2 and the survival of memory $CD8^+$ T cells while at higher concentrations (50 ng/mL), IL-15 promotes the survival of naïve $CD8^+$ T cells and the proliferation of memory $CD8^+$ T cells (98). However, the effect of IL-2 and IL-15 are strikingly different *in vivo*. Whereas mice deficient in IL-2 or IL-2Rα demonstrate a lethal lymphoproliferative disease at 10-25 weeks of age, IL-$15^{-/-}$ and IL-15 $R\alpha^{-/-}$ mice are lymphopenic with deficits in both NK and memory $CD8^+$ T cell lineages (91, 99, 100). Conversely, IL-15 tg mice have dramatic polyclonal expansions of these lineages (101-103). In T cells, IL-2 reportedly induces expression of Fas ligand (FasL) and downregulates expression of the inhibitor of Fas-mediated apoptosis, c-FLIP, sensitizing reactive T cells to Fas-FasL-interactions and limiting the size of the T cell pool through activation induced cell death (AICD) (104-106). Conversely, IL-15 inhibits IL-2-stimulated AICD in $CD4^+$ T cells *in vitro* and promotes the expansion of $CD44^{high}$ memory $CD8^+$ T cells in opposition to IL-2 *in vivo* (101, 107, 108). Indeed, IL-15 has been shown to inhibit Fas-mediated apoptosis in antigen-specific memory $CD8^+$ T cells

(109-111). IL-15 increases $CD8^+$ T cell function *in vivo* against tumor cell lines, active viral, and microbial infections, and augments the primary and memory $CD8^+$ T cell responses after vaccination (112-118). Moreover, blockade of IL-15 signaling has been shown to extend the life of cardiac and pancreatic islet allografts in murine models of solid organ transplantation (119, 120).

Until recently, however, the effect of IL-15 on the pathobiology of acute GVHD and bone marrow allograft rejection and the cellular and molecular mechanisms by which impacts these diseases have received little attention in the literature. Presented in the following chapters is a series of studies which begin to elucidate these questions.

Figure 1. The molecular basis of T cell alloreactivity. Two signals are required for the T cell response to antigen. Specificity is provided by signal one. An individual T cell expresses one T cell receptor (TCR) that is specific for one peptide presented by one particular major histocompatibility complex (MHC) molecule on an APC. Co-stimulation is provided by signal two. In the absence of co-stimulation, the T cell will become anergic, or unresponsive to antigen. In indirect allorecognition, host proteins are scavenged by donor-derived APC and presented to donor T cells in the context of donor-type MHC. In direct allorecognition, host-derived APC present endogenous peptides in the context of host-type MHC. In this mechanism, donor T cells are thought to directly recognize the foreign MHC in a peptide-non-specific way. This results in a higher frequency of alloreactive T cell clones than the indirect mechanism.

Figure 1

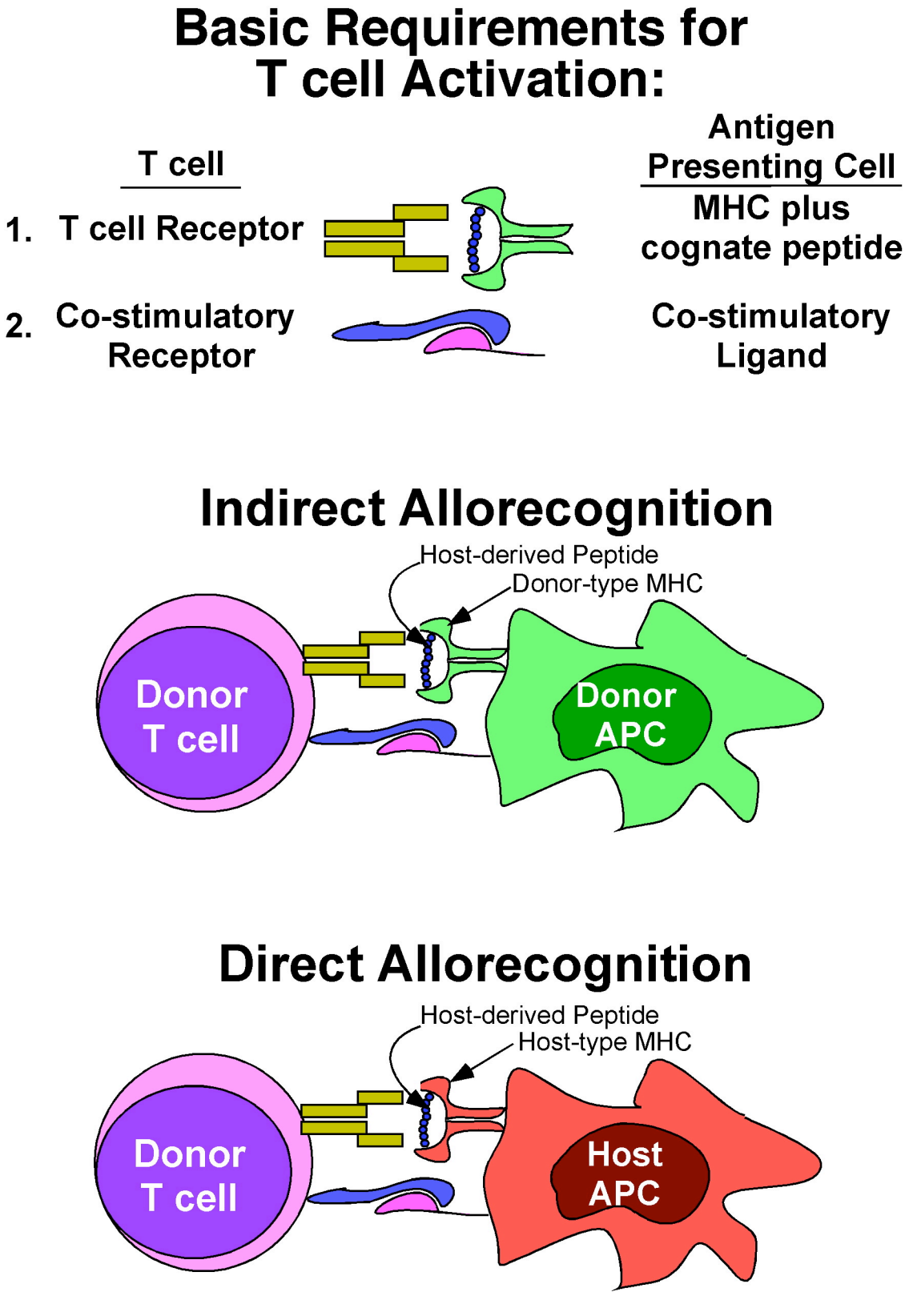

Basic Requirements for
T cell Activation:
T cell
Antigen
Presenting Cell
1. T cell Receptor
MHC plus
cognate peptide
2. Co-stimulatory
Receptor
Co-stimulatory
Ligand
Indirect Allorecognition
Host-derived Peptide
Donor-type MHC
Donor
T cell
Donor
APC
Direct Allorecognition
Host-derived Peptide
Host-type MHC
Donor
T cell
Host
APC

Figure 2. NK cell alloreactivity in hematopoietic stem cell transplantation. The most important determinant for NK cell alloreactivity is the interaction between KIR expressed by NK cells and HLA-C expressed by target cells. HLA-C alleles with an asparagine residue at position 80 (HLA-Cw2, Cw4, Cw5, Cw6, Group 1) and HLA-C alleles with a lysine residue at position 80 (HLA-Cw1, Cw3, Cw7, Cw8, Group 2) are distinctly non-cross-reactive with respect to a given KIR molecule. In the pictured example, the donor NK clone expresses KIR specific for Group 1 HLA-C. When transferred to Host 1, which is homozygous for Group 2 HLA-C, KIR on the donor NK will be unbound by ligand and NK alloreactivity in the graft versus host direction will be present. However, when transferred to Host 2, which expresses both Group 1 and Group 2 HLA-C, KIR will be engaged on the donor NK and no graft versus host alloreactivity will be present.

Figure 2

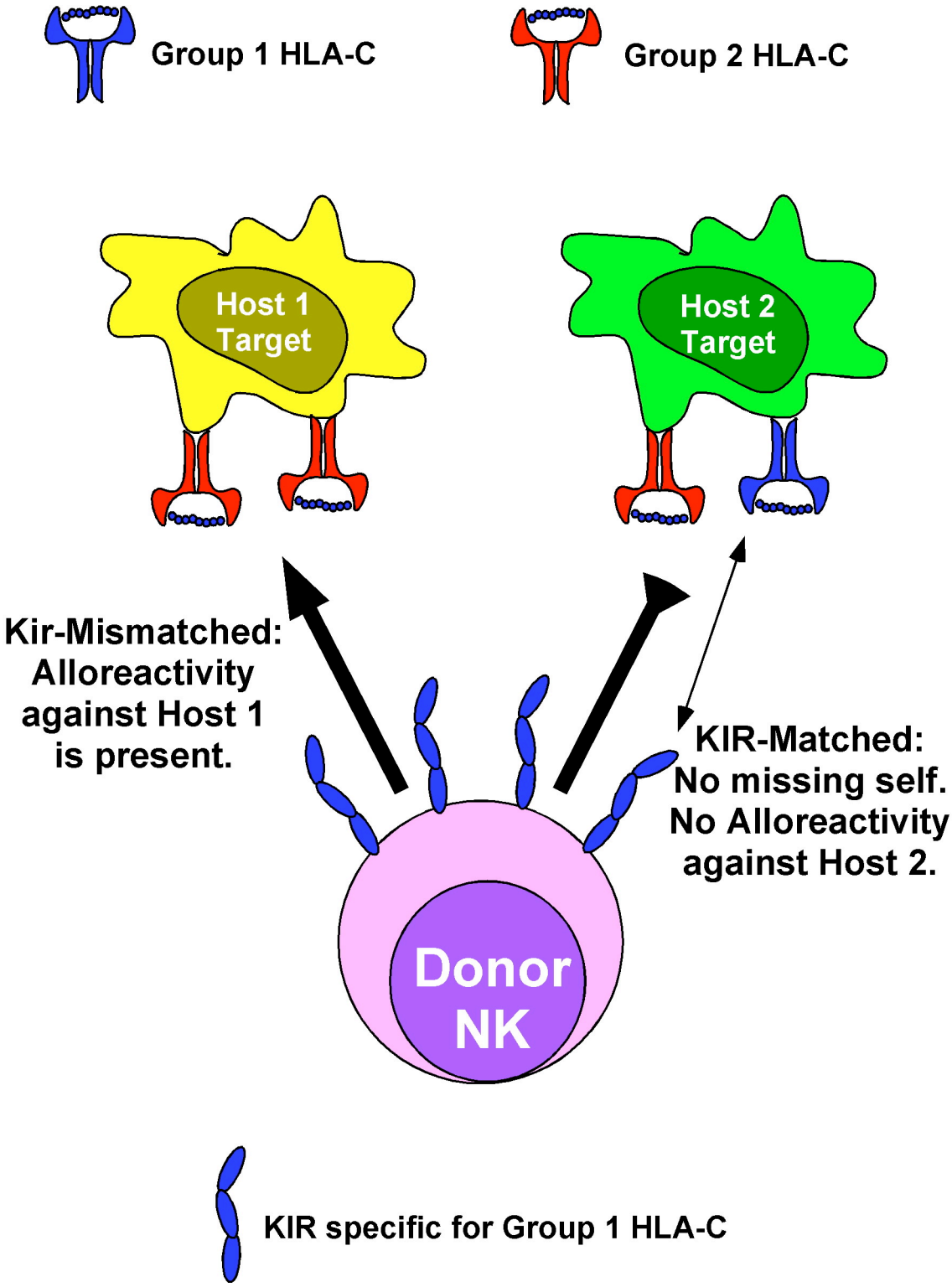
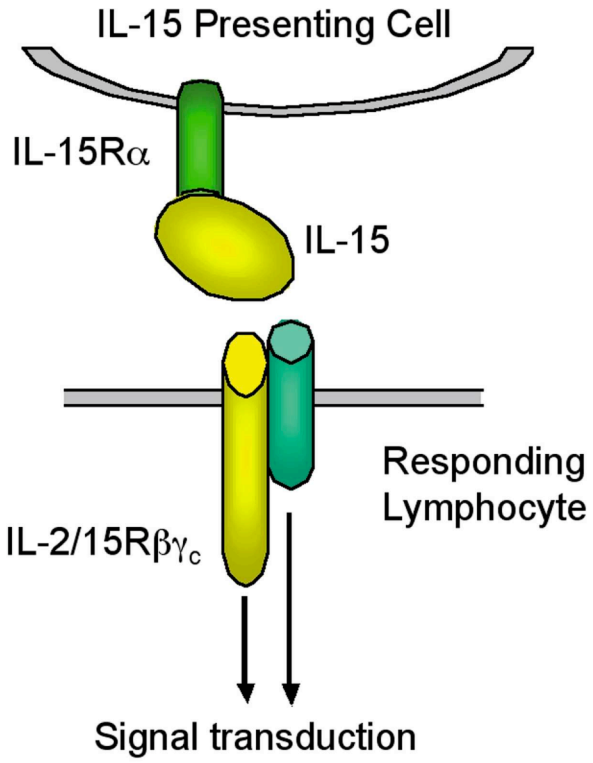

Group 1 HLA-C
Group 2 HLA-C
Host 1 Target
Host 2 Target
Kir-Mismatched: Alloreactivity against Host 1 is present.
KIR-Matched: No missing self. No Alloreactivity against Host 2.
Donor NK
KIR specific for Group 1 HLA-C

Figure 3. Trans-presentation of IL-15. IL-15 can potentially be secreted by an IL-15-producing cell and scavenged at some distance by an IL-15 Rα^+ IL-15 presenting cell. The high affinity of monomeric IL-15 Rα for IL-15 makes this possible in the absence of the IL-2/15 R$\beta\gamma_c$. More likely, however, IL-15 is produced and complexed intracellularly with IL-15 Rα before translocation to the plasma membrane. Once expressed as a surface bound molecule IL-15 can then be presented *in trans* to a responding cell, namely an IL-15 dependent lymphocyte, that need only express the IL-2/15 R$\beta\gamma_c$ shared heterodimeric receptor. This complex is sufficient to initiate signal transduction through the JAK/STAT pathway.

Figure 3


IL-15 Presenting Cell
IL-15Rα
IL-15
Responding
Lymphocyte
IL-2/15Rβγc
Signal transduction

CHAPTER 2

Donor-Derived IL-15 is Critical for Acute Allogeneic Graft Versus Host Disease

2.1 Introduction

Allogeneic BMT is a potentially curative therapy for patients with heritable immunodeficiencies and malignant diseases including leukemia, lymphoma, and myeloma (14). While the conditioning regimen for BMT leads to direct tumor destruction, donor-derived allogeneic T cells can exert an important GVT effect: recipients of allogeneic transplants have a decreased probability of relapse compared to recipients of autologous, syngeneic, or T cell-depleted BMT (17, 121). However, allogeneic BMT also carries a significant risk for GVHD, an immunologic attack of allogeneic donor T lymphocytes against normal recipient tissues, the magnitude of which depends in large part on the degree of HLA incompatibility between donor and recipient. Even with appropriate prophylaxis, the incidence of acute GVHD ranges from 30% in HLA-identical donor-recipient pairs to 70% for donor-recipient pairs HLA-incompatible at two loci (14, 15). Thus, GVHD remains the most significant obstacle to the wider application of BMT for the treatment of malignancy.

The role of IL-15, a T and natural killer NK cell growth factor, in allogeneic GVHD has not been addressed. We hypothesized that alteration of endogenous IL-15 production after allogeneic transplantation would affect the donor anti-host immune

responsiveness that is characteristic of acute allogeneic GVHD. To address this hypothesis, we employed the C57Bl/6 (B6)→(C57Bl/6 x DBA/2) F1 hybrid (B6D2F1) murine model of acute allogeneic GVHD using bone marrow (BM) cells obtained from IL-15$^{-/-}$ B6 mice or B6 mice with a ubiquitously expressed IL-15 transgene (IL-15 tg B6) that allows for efficient IL-15 protein production and secretion (91, 103, 122, 123). Our results indicate that eliminating endogenous IL-15 gene expression in donor BM cells prevents GVHD in host B6D2F1 wild type (wt) mice, and that deregulation of IL-15 gene expression in donor BM cells promotes acute GVHD in host B6D2F1 wt mice in a dose-dependent fashion. IL-15 mediated GVHD was associated with a dramatic expansion and activation of wt effector-memory CD8^{+} T cells with upregulated Bcl-2 protein whose depletion significantly improved survival. Collectively, these data identify donor bone marrow-derived cells as the predominant source of IL-15 critical for T cell function in acute GVHD.

2.2 Methods

Reagents

The following rat anti-mouse monoclonal antibodies (mAb's) were purchased from BD Pharmingen (San Diego, CA) as fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridinin chlorophyll (PerCP) or allophycocyanin (APC) conjugates: CD3ε, CD4, CD8α, CD43, CD44, CD62L, and TCRβ. The following mAb's were used for *in vivo* depletion of lymphocytes: anti-mouse CD4 (GK1.5), anti-CD8 (2.43) (National Cell Culture Center, Minneapolis, MN) and rat IgG isotype control (Sigma Chemical, St. Louis, MO). Bcl-2 protein was detected using a hamster

anti-mouse Bcl-2 antibody (BD Pharmingen). BrdU (Zymed, S. San Francisco, CA) and CFSE (Molecular Probes, Eugene, OR) were used according to the manufacturers' instructions. Anti-PE microbeads were purchased from Miltenyi Biotec (Auburn, CA). IL-15 transgene mRNA was reverse transcribed using standard techniques and cDNA was amplified using the following primers: Forward: 5'-CGCCGCGGACGCTGGTTAT-3' Reverse: 5'-GTAATCTTGCAACTGGGATGAA-3'. Annealing temperature: 55°C.

Flow cytometry

For surface staining, 5.0 x 10^5 cells were incubated with mAb for 25 min at 4°C, washed once with PBS containing 1% Fetal Bovine Serum, and fixed in PBS containing 1% formalin. Intracellular staining was performed with the Intracellular Flow Cytometry Kit according to the manufacturer's instructions (BD Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

Mice

Six to seven week female B6 (H-2^b) and B6D2F1 (H-$2^{b/d}$) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Six to seven week female IL-$15^{-/-}$ B6 mice were purchased from Taconic Farms (Germantown, NY). Murine IL-15 transgenic mice (IL-15 tg B6, C57Bl/6 background, H-2^b) were created as described for FVB mice and bred under specific pathogen-free conditions at The Ohio State University (103). Although measurements of serum murine IL-15 in the C57Bl/6 strain of IL-15 tg mice

are below our ELISA detection limits of 50 pg/mL, these mice consistently exhibit moderate expansions of NK and memory $CD8^+$ T cells early in life that are similar to other lines of IL-15 tg mice with measurable IL-15. Unlike the FVB strain, the IL-15 tg B6 mice do not exhibit evidence of malignant lymphoproliferation or leukemic transformation within the first year of life (103). All mice were between eight and twelve weeks old at the beginning of each experiment. Transplanted mice were maintained in sterilized microisolators and received irradiated rodent chow and acidified water plus oral antibiotic (Baytril, 0.2 mg/mL) for 21 days following transplantation. All animal research was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at The Ohio State University.

Bone marrow transplantation

The standard B6→B6D2F1 model of allogeneic BMT was used for these experiments (122). Briefly, B6D2F1 mice ($H\text{-}2^{b/d}$) were used as allogeneic recipients for a combined bone marrow and splenic T cell graft from the parental (B6) strain of mice. The signs and symptoms of acute GVHD in these mice are well described and include tissue damage to the liver, intestine and skin, weight loss, and death (122). In this model, wt B6 mice are used as syngeneic control recipients and display little tissue damage or weight loss and no mortality due to GVHD. Donor mice were euthanized by cervical dislocation and bone marrow and spleen were harvested by standard techniques. IL-15 tg B6 splenic T cells were not used to induce GVHD in any experiments because these mice display altered T cell populations and phenotypes early in life (103). Bone marrow cells were labeled with PE-conjugated rat anti-mouse monoclonal antibodies directed against

CD3ε, CD4, and CD8α followed by anti-PE microbeads. Bone marrow T cells were then depleted by passage through a MACS LD separation column according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Depletion efficiency was determined by flow cytometric analysis and was greater than 99%. After red blood cell lysis, 5 x 10^5 splenocytes were stained with anti-CD8α FITC and anti-CD4 PerCP and analyzed by flow cytometry. For independent experiments, T cell content of the transplanted splenocytes was standardized by flow cytometric analysis. T cell-depleted BM cells and splenocytes were mixed in serum-free RPMI 1640 at the appropriate concentrations to transfer 1 x 10^7 BM cells and 5 x 10^6 splenic T cells in 0.5 mL. In some experiments, IL-15 tg B6 BM cells and wt B6 BM cells were mixed such that the BM portion of the graft contained 50% or 25% IL-15 tg B6 BM cells. To prepare the recipients for transplantation, mice were given 1300 cGy gamma irradiation (Gammacell 40, 93.5 cGy/min) split into two fractions. Mice were then injected with 0.5 mL of the combined BM cell/splenic T cell graft via the lateral tail vein. Recipients were hydrated with 0.5 mL sterile RPMI 1640 medium, given intraperitoneally (IP), for the first five days post transplant.

***In vivo* lymphocyte depletions**

To deplete $CD4^+$ or $CD8^+$ T cell populations *in vivo*, mice were injected IP with 1 mg rat IgG, GK1.5, 2.43 or both GK1.5 and 2.43 mAb's, on the day of transplantation, followed by IP injections of 0.5 mg every other day thereafter. Depletion efficiency was verified by flow cytometry and was greater than 99%.

Assessment of GVHD

Mice were observed in a blinded fashion every three days after transplantation for the clinical symptoms of GVHD as described (124). Briefly, animals were scored from 0-2 for weight loss, inactivity, skin lesions, roughened coat and hunching and the scores combined to provide a clinical score from zero to 10 at each time point. Mice which were unresponsive or unable to obtain food and water were determined to be moribund and sacrificed in accordance with The Ohio State University ILACUC guidelines.

Histology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin by standard techniques. Blinded samples were submitted for semi-quantitative histopathologic analysis, performed by Donna Kusewitt, D.V.M, Ph.D., a board-certified veterinary pathologist who directs the Mouse Phenotyping Shared Resource of The Ohio State University Comprehensive Cancer Center. Acute GVHD lesions were graded as absent (0), minimal (1), mild (2), moderate (3), marked (4), or very marked (5) depending on their extent and severity.

Mixed lymphocyte reactions

1 x 10^8 wt B6 splenic T cells were primed *in vivo* in wt B6D2F1 allogeneic or wt B6 syngeneic hosts. After 6 days, spleens were harvested and splenocytes were isolated by standard techniques. 1 x 10^5 of these responder cells were restimulated *in vitro* with irradiated (30 Gy) naïve wt B6 or wt B6D2F1 stimulator cells at a ratio of 1:4 in the

presence of recombinant murine IL-15 (R&D Systems, Minneapolis, MN) at 10 ng/mL or 100 ng/mL or PBS control. Supernatants were harvested 24 or 72 hours after initiation of the culture and stored at -80°C until analysis.

Cytokine analysis

The Mouse Th1/Th2 Cytokine Bead Array (BD Pharmingen) was used to measure IL-2, IL-4, IL-5, IFN-γ, and TNF-α in mouse sera and culture supernatants. Samples were analyzed neat or diluted 1:2 or 1:4 with PBS. The FL-3 and FL-2 channels of a BD FACSCalibur flow cytometer were used to individually quantitate cytokine binding to multiple beads of known fluorescence. Samples were then analyzed using Cytokine Bead Array software (BD Biosciences.) In some experiments, IFN-γ was measured using the Quantikine Murine IFN-γ ELISA kit (R&D Systems). All assays were performed according to the manufacturers' instructions.

Statistics

Survival data were analyzed using the log rank test. All other data was compared using Student's two-tailed t-test. $P<0.05$ was considered statistically significant.

2.3 Results

The absence of IL-15 in donor bone marrow cells ameliorates acute GVHD mortality.

Because IL-15 is a pro-inflammatory cytokine, we hypothesized that decreasing endogenous IL-15 expression by reconstitution of allogeneic B6D2F1 recipients with

IL-15$^{-/-}$ B6 BM cells would reduce mortality from acute GVHD. B6D2F1 mice receiving IL-15$^{-/-}$ B6 BM cells and IL-15$^{-/-}$ B6 T cells had a significantly longer median survival time (MST) after transplantation compared to identical mice receiving wt B6 BM cells and IL-15$^{-/-}$ B6 T cells (44.5 days versus 62.5% survival 80 days post transplant, P=0.005, Figure 4).

Deregulation of endogenous IL-15 in donor BM cells increases the mortality and morbidity of acute allogeneic GVHD, and is dose-dependent.

We hypothesized that deregulation of endogenous IL-15 expression by transplantation of IL-15 tg B6 BM cells would promote acute GVHD. B6D2F1 mice receiving wt B6 T cells and IL-15 tg B6 BM cells had dramatically decreased MST compared to identical mice receiving wt B6 T cells and wt B6 BM cells (MST 12 days versus 30 days, P=0.0004, Figure 5A). Wt B6D2F1 mice receiving IL-15 tg B6 BM cells developed more weight loss (72.4±2.3% original weight versus 81.8±1.6% beginning on day 12 post transplant, P=0.008, Figure 5B) and worse clinical scores (3.3±0.4 versus 2.3±0.2 beginning on day 6 post transplant, P=0.029, Figure 5C) compared to B6D2F1 mice receiving wt B6 BM cells. Although *in vivo* detection of endogenous murine IL-15 protein was not possible, IL-15 transgene mRNA was detected in the bone marrow harvested from recipients of IL-15 tg B6 BM cells but not wt B6 BM cells (Figure 5D). Further, bone marrow mixing experiments indicated that IL-15-mediated mortality is significantly reduced when the fraction of IL-15 tg BM cells contained in the graft is

decreased below 50% (Table 1). B6 mice receiving either syngeneic wt B6 BM cells or syngeneic IL-15 tg B6 BM cells had 100% survival and did not develop clinical evidence of GVHD (Figure 5A-C).

Deregulation of endogenous IL-15 increases cholangiohepatitis and enteritis in recipients of allogeneic BMT.

B6D2F1 mice receiving IL-15 tg B6 BM cells or wt B6 BM cells were sacrificed 13 days post transplant and analyzed in a blinded fashion for histopathology characteristic of acute GVHD in the gut and liver (125, 126). B6D2F1 mice receiving IL-15 tg B6 BM cells had significantly more severe cholangiohepatitis (grade 3 versus grade 2, Figure 6A, upper panels; P=0.004, Figure 6B) and enteritis (grade 2 versus grade 1, Figure 6A, bottom panels; P=0.049, Figure 6B) compared to mice receiving wt B6 BM cells. No difference in pulmonary inflammation was noted between recipients of IL-15 tg B6 and wt B6 allogeneic BM cells (data not shown). B6 mice receiving syngeneic wt B6 or syngeneic IL-15 tg B6 BM cells had no evidence of cholangiohepatitis or enteritis (data not shown).

Deregulation of endogenous IL-15 expands activated effector-memory $CD8^+$ T cells after allogeneic BMT.

Analysis of recipient splenocytes at day 12 or 13 post BMT demonstrated an increase in alloreactive $CD8^+$ T cells co-expressing the GVHD activation marker CD43 in recipients of IL-15 tg B6 allogeneic BM cells compared to recipients of wt B6 allogeneic BM cells (73.0±0.5% versus 62.3±0.9%, P<0.0001, Figure 7A, B). There was

no difference in CD43 expression by $CD8^+$ T cells from recipients of IL-15 tg B6 and wt B6 syngeneic BM cells (Figure 7A, B); nor was there a difference in the proportion of alloreactive $CD43^+CD4^+$ T cells between recipients of IL-15 tg B6 and wt B6 allogeneic BM cells (data not shown). Further, splenocytes from recipients of IL-15 tg B6 allogeneic BM cells displayed a significant increase in the proportion of $CD44^{high}CD62L^{low}$ effector-memory $CD8^+$ T cells (44.4±4.1% versus 23.5±2.9%, $P<0.003$, Figure 7C) and a significant decrease in the $CD4^+$:$CD8^+$ T cell ratio (0.38±0.03 versus 0.80±0.07, $P<0.0001$, Figure 7D, E) compared to recipients of wt B6 allogeneic BM cells.

Deregulation of endogenous IL-15 after allogeneic BMT increases $CD8^+$ T cell survival.

We assessed the expression of Bcl-2 protein in donor-infused wt B6 allogeneic T cells of B6D2F1 recipient mice that had received either IL-15 tg B6 BM cells or wt B6 BM cells. $CD8^+$ T cells from the former had a mean fluorescence intensity (MFI) of 142±8 while $CD8^+$ splenocytes from the latter had a MFI of 80±4 ($P=0.001$, Figure 8A, B). There was no difference in Bcl-2 expression for $CD8^-$ splenocytes (Figure 8A). Further, T cell proliferation was assessed at days 4 and 6 post transplant by CFSE staining and from days 7-12 by BrdU incorporation. No difference in T cell proliferation was detected between allogeneic B6D2F1 recipients of IL-15 tg B6 and wt B6 BM cells by these methods.

IL-15 increases IFN-γ production by alloreactive T cells *in vitro.*

Wild type B6 splenocytes were primed for six days in wt B6D2F1 allogeneic hosts and restimulated *in vitro* with naïve irradiated B6D2F1 splenocytes in the presence of recombinant murine (rm) IL-15 or PBS as a control. Primed B6 splenocytes cultured in the presence of either 10 ng/mL or 100 ng/mL rm IL-15 produced higher amounts of IFN-γ compared to identical splenocytes cultured in the absence of exogenous IL-15 at 24 hours ($P \leq 0.03$) and at 72 hours post-initiation of the culture ($P \leq 0.03$; Figure 9). Splenocytes primed in wt B6 syngeneic hosts produced little IFN-γ upon stimulation in the presence or absence of rm IL-15 (Figure 9).

Deregulation of endogenous IL-15 affects the Th2/Tc2 cytokine profile in acute GVHD.

Analysis of serum collected from recipients of IL-15 tg and wt allogeneic BM cells at 12 or 13 days post transplant showed no significant difference in the amounts of the Th1/Tc1 cytokines IFN-γ (96.1±26.6 pg/mL and 40.9±2.8 pg/mL), TNF-α (166.5±11.4 pg/mL and 164.9±13.0 pg/mL) and IL-2 (1.5±0.7 pg/mL and 3.5±0.2 pg/mL). However, recipients of IL-15 tg allogeneic BM cells displayed lower serum concentrations of the Th2/Tc2 cytokine IL-5 (10.4±3.8 pg/mL versus 34.4±2.3 pg/mL, P=0.001, not shown). No IL-4 was detected in any of our assays.

IL-15-mediated exacerbation of GVHD requires $CD8^+$ T cells or $CD4^+$ T cells.

Depletion of $CD8^+$ T cells *in vivo* with anti-CD8 mAb extended survival of recipients of IL-15 tg B6 allogeneic BM cells compared to control treatment with rat IgG

(MST 64 days versus 13 days, P=0.002, Figure 10). Depletion with anti-CD4 mAb also provided protection from IL-15-mediated GVHD (MST 28 days versus 13 days, P=0.002, Figure 10) although the benefit was significantly less than that provided by CD8-depletion (MST 64 days versus 28 days, P=0.048, Figure 10). Finally, *in vivo* depletion of both $CD4^+$ and $CD8^+$ T cells provided complete protection from IL-15-mediated acute GVHD (100% survival compared to control injected recipients of IL-15 tg allogeneic BM cells, P=0.002, Figure 10).

2.4 Discussion

Our results indicate that endogenous IL-15 provided by donor wt BM cells is a critical component for the manifestations of acute allogeneic GVHD. Deregulation of IL-15 expression by transplanted BM cells resulted in an expansion of wt alloreactive ($CD43^+$) effector-memory ($CD44^{high}CD62L^{low}$) $CD8^+$ T cells in the spleen. Our demonstration of a skewed $CD4^+$:$CD8^+$ T cell ratio and unaltered frequency of alloreactive ($CD43^+$) $CD4^+$ T cells in recipients of IL-15 tg B6 allogeneic BM cells suggest that the effect of IL-15 in these experiments was specific to effector-memory $CD8^+$ T cells. Further, wt B6 splenocytes primed *in vivo* and restimulated for only 24 hours *in vitro* with naïve allogeneic splenocytes produced significantly more IFN-γ in the presence of exogenous recombinant IL-15 than in the absence of exogenous IL-15. It is likely, then, that IL-15 from donor derived BM cells promotes both the activation and expansion of alloreactive T cells after allogeneic BMT. These alterations in T cell phenotype and function were associated with a significant increase in tissue inflammation in the gut and liver and a striking increase in acute GVHD morbidity and mortality.

IL-15-mediated acute GVHD was completely abrogated by T cell depletion, indicating that this process was T cell-dependent. There are additional data in support of this notion that donor-derived IL-15 is not mediating its effect directly but rather via a T cell-dependent process. First, IL-15 tg B6 marrow alone was able to fully reconstitute hematopoiesis in wt B6D2F1 allogeneic hosts without morbidity and mortality (data not shown) and second, IL-15 tg B6 marrow had no deleterious effect when infused with wt B6 splenic T cells into irradiated wt B6 syngeneic hosts. Most surprisingly, however, transplantation of IL-15$^{-/-}$ B6 BM cells prolonged survival of wt allogeneic hosts compared to transplantation of wt B6 BM cells. For the first time, these data identify donor bone marrow-derived cells as a population wherein IL-15 expression predicts outcome after allogeneic BMT.

In acute GVHD, CD4^{+} Th2-polarized cells can prevent disease by reducing IFN-γ and TNF-α production and by inhibiting donor CD8^{+} T cell expansion (127, 128). Moreover, CD8^{+} Tc2-polarized cells demonstrate a reduced capacity for causing GVHD while maintaining a GVT effect (129). The data presented here demonstrate that deregulation of IL-15 significantly decreases serum concentrations of the Th2/Tc2 cytokine IL-5 after allogeneic BMT and significantly increases the production of the classic Th1/Tc1 cytokine IFN-γ by alloreactive T cells *in vitro*. These findings are in agreement with those of Ishimitsu et al. that IL-15 tg mice show reduced serum concentrations of IL-5 due to expanded CD8^{+} Tc1 cells in a murine model of asthma and extend work demonstrating the co-stimulation of IFN-γ production by IL-15 and IL-12 in NK and T cells (90, 114, 130, 131). Although we observed only sporadic increases of IFN-γ in the serum of recipients of IL-15 tg B6 allogeneic BM cells, collectively these

data indicate that deregulation of IL-15 after allogeneic BMT can cause a polarization away from the Th2/Tc2 cytokine response and toward the Th1/Tc1 type response. Importantly, TGF-β or other Th2/Tc2 cytokines such as IL-10 inhibit T cell responses to alloantigen and may also have been downregulated in recipients of IL-15 tg B6 allogeneic BM cells (132). Nevertheless, these findings represent, to our knowledge, the first *in vivo* evidence of a role for IL-15 in polarizing T cell responses in GVHD.

The T cell response to IL-15 was originally demonstrated to require expression of the IL-2Rβ and γ_c chains (96, 97, 133); a private receptor alpha chain (IL-15 Rα) was subsequently identified which binds IL-15 with high affinity (K_D 10-50 pM) and signals through the IL-2/15R$\beta\gamma_c$ complex (87). Dubois et al. reported that IL-15 functions primarily as a surface bound cytokine, presented by IL-15 Rα in "trans" to target cells expressing the IL-2/15R$\beta\gamma_c$ (85). Emerging evidence suggests that while IL-15 Rα expression by bone marrow-derived cells is necessary for the homeostatic proliferation of splenic memory $CD8^+$ T cells, expression of IL-15 Rα by $CD8^+$ T cells themselves is not (88, 134). In our experiments, IL-15 tg B6 bone marrow cells efficiently produced and secreted IL-15 protein that was presumably captured by IL-15 Rα on host APC in lymphoid tissue and presented *in trans* to donor-derived $CD8^+$ T cells. In contrast, IL-$15^{-/-}$ bone marrow cells were unable to produce the cytokine and as a result host APC stimulated donor-derived $CD8^+$ T cells less efficiently. This hypothetical scenario is supported by the elegant work of Teshima et al. who proved that the alloantigen stimulus for donor-derived $CD4^+$ and $CD8^+$ T cells is delivered by host-derived APC (37, 38).

Thus our data identify a unique interaction between donor and host hematopoietic cells mediated by IL-15 and suggest that this molecule may be an important "third signal" for lymphocyte activation in acute GVHD.

While depletion of either $CD4^+$ or $CD8^+$ T cells improved MST in our model, $CD8^+$ T cell depletion had the more profound impact on survival, consistent with an expansion of effector-memory $CD8^+$ T cells in recipients of IL-15 tg B6 allogeneic BM cells. Thus, we hypothesized that IL-15 may promote the homeostatic proliferation or survival of effector-memory $CD8^+$ T cells after allogeneic BMT. Studies have demonstrated that IL-15 promotes phosphorylation of STAT5 and induces binding of STAT5 and c-myb to promoter elements of the anti-apoptotic molecule Bcl-2 (135, 136). *In vitro* and *in vivo* evidence has demonstrated that IL-15 upregulates Bcl-2 protein expression and maintains memory $CD8^+$ T cells by serving as a survival factor (98, 137-139). Consistent with this, recipients of IL-15 tg B6 allogeneic BM cells had an increase in wt effector-memory $CD8^+$ donor T cells that expressed more Bcl-2 protein than wt $CD8^+$ donor T cells from recipients of wt B6 allogeneic BM cells. Somewhat surprisingly, we did not observe any evidence for enhanced $CD8^+$ T cell proliferation in recipients of IL-15 tg B6 allogeneic BM cells by CFSE dilution on days 4 or 6 or by BrdU incorporation from days 7 to 12 post transplant. Therefore in our experiments, it is likely that the expansion of wt effector-memory $CD8^+$ donor T cells was predominantly the result of IL-15-induced survival.

Thus far, two studies have shown that in patients receiving allogeneic BMT for various hematological malignancies, those who developed GVHD had higher serum concentrations of IL-15 compared to those who did not develop GVHD (140, 141).

Although the etiology of increased serum IL-15 is not addressed in these reports, there may be polymorphisms that result in deregulated expression of IL-15 protein. Recently, Lin et al. reported polymorphisms in the IL-10 promoter region that result in high protein expression are associated with decreased incidence of severe acute GVHD in recipients of allogeneic BMT (142, 143). Because IL-15 expression is regulated primarily at the post-transcriptional level by multiple AUG's present in the 5'-UTR and an inefficient signal peptide, polymorphisms in these regions may result in efficient IL-15 protein production (78, 79). The data presented here suggest that potential bone marrow donors with such polymorphisms may confer increased risk of acute GVHD to recipients. Thus, a detailed analysis of donor IL-15 gene polymorphisms and post transplant IL-15 expression in patients with and without acute GVHD may allow for further risk stratification of potential allogeneic BMT donor-recipient pairs.

Because of its apparent role in the priming and survival of allogeneic $CD8^+$ T cells, blockade of IL-15 signaling may be an effective treatment or prophylaxis for acute GVHD. Clinical experience with anti-IL-2Rα antibodies has demonstrated the feasibility of modulating cytokine signaling for the treatment of this disease. Basiliximab and daclizumab, chimeric and humanized anti-IL-2Rα monoclonal antibodies, respectively, achieved response rates of 71% and 47% in steroid-resistant acute GVHD, although it is not clear whether therapeutic effect in these trials was achieved through interrupting IL-2 signaling or depletion of activated IL-2Rα^+ T cells (144, 145). However, many patients with GVHD failed to respond to IL-2Rα blockade, raising the possibility that trans presentation of IL-15 may provide redundant signaling through the IL-2/15R$\beta\gamma_c$ complex (146). A fully human anti-IL-15 antibody, HuMax-IL15, has shown efficacy in a

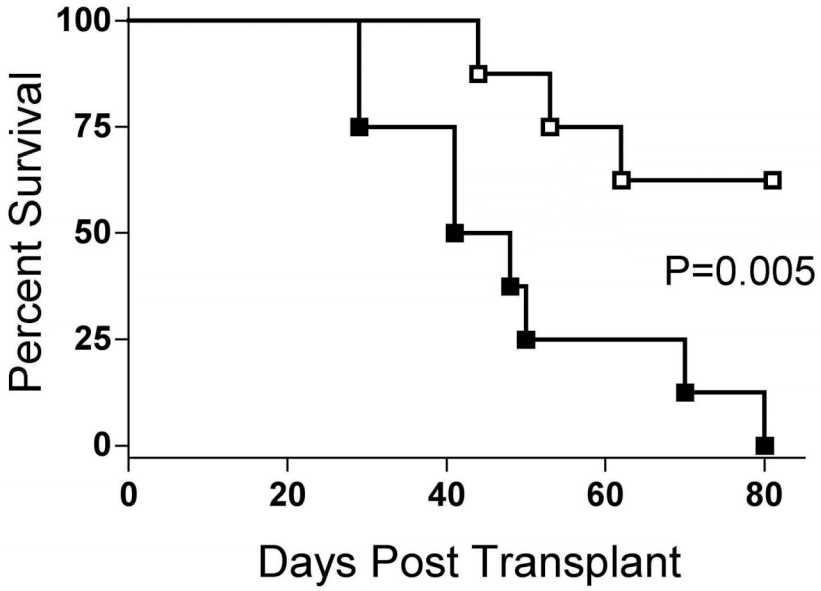
xenograft model of human psoriasis and is currently in clinical trials for the treatment of rheumatoid arthritis (147). Therefore, IL-15 may be an attractive target for neutralization, particularly in patients refractory to anti-IL-2Rα targeted therapy.

While blocking IL-15 signaling may be a promising therapy for acute GVHD, it is not clear how such treatment may alter the GVT effect. IL-15 has been shown to enhance the *in vivo* anti-tumor activity of polyclonal, antigen-specific and genetically targeted $CD8^+$ T cells after adoptive transfer (112, 116, 117, 148). Additionally, Katsanis et al. demonstrated a survival benefit for IL-15-treated lymphoma-bearing mice after transplantation with syngeneic bone marrow and activated T cells (149). Consequently, it is possible that blocking IL-15 signaling may result in a loss of tumor-specific memory $CD8^+$ T cells and diminish the GVT effect *in vivo*. To date, no studies have addressed the *in vivo* role of IL-15 in promoting or inhibiting $CD8^+$ T cell activity against allogeneic tumor targets.

Despite years of intense investigation, GVHD remains the most significant obstacle to the wider application of allogeneic BMT. The results presented herein suggest a role for IL-15 in promoting acute GVHD by activating and supporting the survival of alloreactive effector-memory $CD8^+$ T cells. Moreover, these data identify donor bone marrow-derived cells as the predominant source of IL-15 critical for acute GVHD. Further investigation is warranted to determine if IL-15 should be pursued clinically as a target for neutralization in GVHD prophylaxis and treatment.

Figure 4. The absence of donor bone marrow cell-derived IL-15 ameliorates acute GVHD mortality. Wild type B6D2F1 mice were transplanted with 5 x 10^6 IL-$15^{-/-}$ B6 splenic T cells and 1 x 10^7 wt B6 BM cells (■, n=8) or IL-$15^{-/-}$ B6 BM cells (□, n=8) and observed for acute GVHD mortality. Survival times were compared using the log rank test: P=0.005.

Figure 4



Donor BM Genotype	Donor T cell Genotype	Host
wt B6	IL-15$^{-/-}$ B6	B6D2F1
IL-15$^{-/-}$ B6	IL-15$^{-/-}$ B6	B6D2F1

Figure 5. Deregulation of endogenous IL-15 increases the mortality and morbidity from acute GVHD. B6 or B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 BM cells from either wt B6 or IL-15 tg B6 mice. (**A**) Survival of wt B6D2F1 mice receiving IL-15 tg B6 allogeneic (IL-15 tg allo, ●, n=12) or wt B6 allogeneic BM cells (wt allo, ■, n=15), P=0.0004. Survival of wt B6 mice receiving IL-15 tg B6 syngeneic (IL-15 tg syn, ○, n=7) or wt B6 syngeneic (wt syn, □, n=7) BM cells. Data represent combined results from two independent experiments. (**B**) and (**C**) Mice from each group described above were weighed and scored for clinical GVHD as described in Methods. Data are representative of results (mean ± s.e.m.) from two similar experiments. (**D**) IL-15 transgene expression in bone marrow of recipients of IL-15 tg B6 allogeneic BM cells. Positive control (+) cDNA was prepared from a known IL-15 tg B6 mouse and negative control cDNA (-) was prepared from a wt B6 mouse. Sample integrity was established by PCR amplification of 18s cDNA.

Figure 5

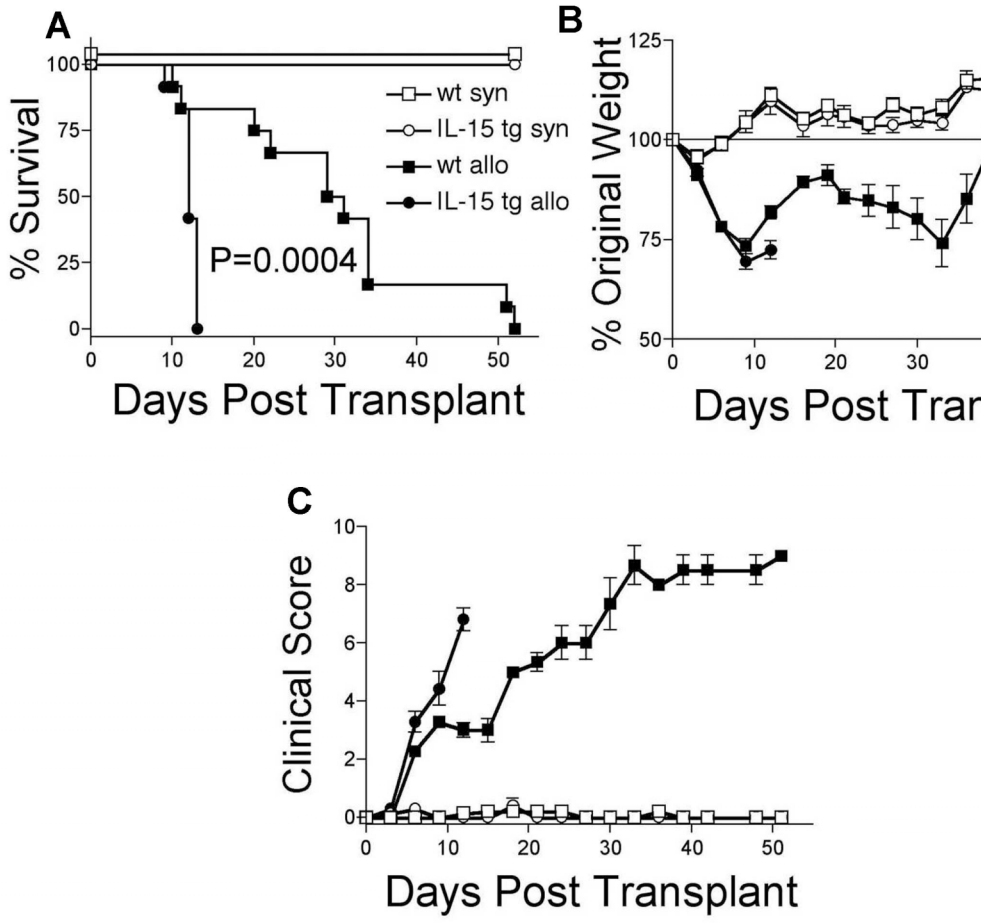

A
% Survival
100
75
50
25
0
0
10
20
30
40
50
Days Post Transplant
wt syn
IL-15 tg syn
wt allo
IL-15 tg allo
P=0.0004
B
% Original Weight
125
100
75
50
0
10
20
30
40
50
Days Post Transplant
C
Clinical Score
10
8
6
4
2
0
0
10
20
30
40
50
Days Post Transplant
D
wt allo
IL-15 tg allo
–
+
IL-15 transgene
18s

Figure 6. Deregulation of endogenous IL-15 increases cholangiohepatitis and enteritis in recipients of allogeneic BMT. (**A**) Upper panel: Cholangiohepatitis in animals with acute GVHD. Mononuclear inflammatory cells surround bile ducts (asterisks). Lesions shown in a liver harvested from a recipient of IL-15 tg B6 allogeneic BM cells (IL-15 tg allo) are more severe than those shown in a liver from a recipient of wt B6 allogeneic BM cells (wt allo) and include areas of hepatocyte necrosis (arrows). Bar=50 μm. Lower panel: Enteritis in animals with acute GVHD. There is a mononuclear inflammatory cell infiltrate in the lamina propria; crypts are hyperplastic and contain numerous mitotic figures. The intestine harvested from a recipient of IL-15 tg B6 allogeneic BM cells has more severe lesions than that harvested from a recipient of wt B6 allogeneic BM cells, including an area of crypt necrosis (arrow). Bar=100 μm. (**B**) Coded slides were scored by a veterinary pathologist in a blinded fashion as described in Methods. Mice receiving IL-15 tg B6 allogeneic BM cells (solid bars) had significantly worse GVHD histopathology in the gut (P=0.049) and liver (P=0.004) compared to recipients of wt B6 allogeneic BM cells. Results are representative of two independent experiments involving a total of 12 mice.

Figure 6

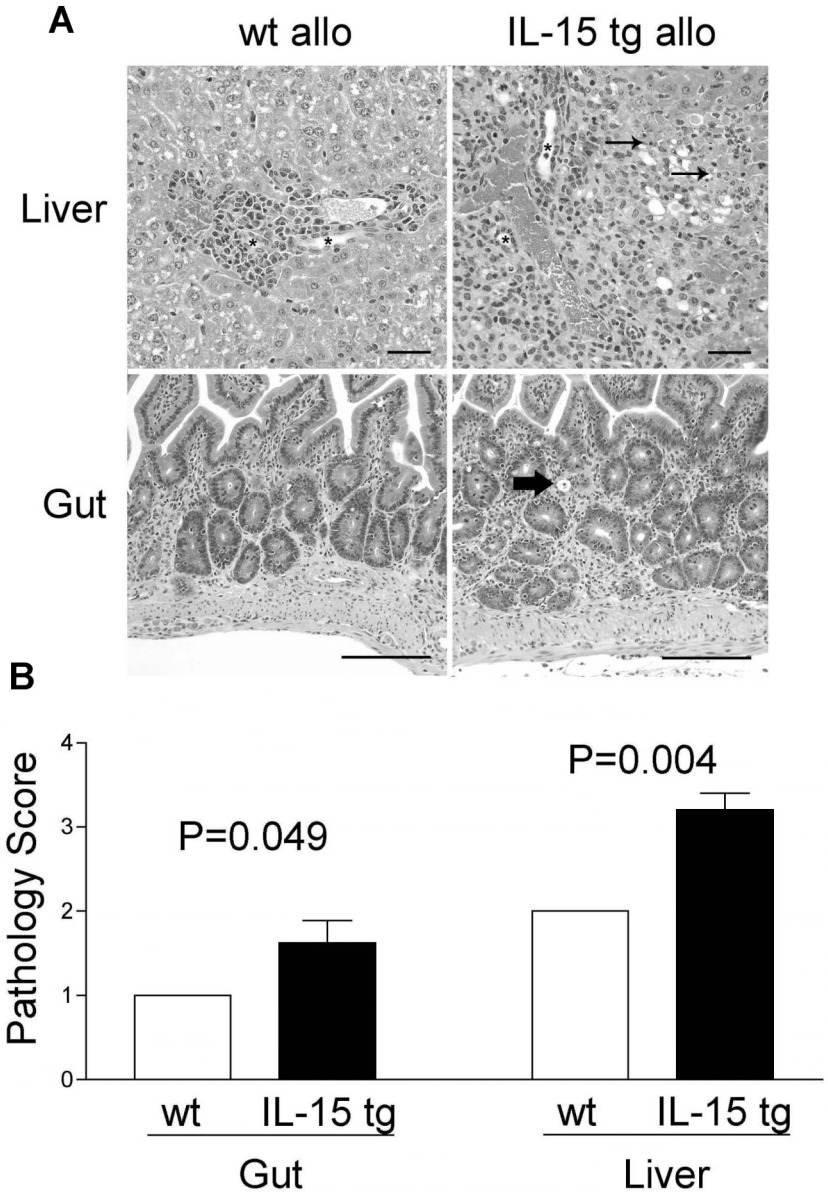

A
wt allo
IL-15 tg allo
Liver
Gut
B
Pathology Score
0
1
2
3
4
P=0.049
P=0.004
wt
IL-15 tg
wt
IL-15 tg
Gut
Liver

Figure 7. Deregulation of endogenous IL-15 alters the T-cell phenotype after allogeneic BMT. Spleens were harvested from syngeneic or allogeneic BMT recipients 12 or 13 days after transplantation. (**A, B**) The percentage of gated $CD8^+$ T lymphocytes that were also positive for the activation antigen CD43 was determined by two-color flow cytometric analysis. Values represent mean ± s.e.m. for all groups and are representative of two independent experiments involving a total of 17 mice. (**C**) The percentage of gated lymphocytes that were $CD8^+CD44^{high}CD62L^{low}$ was determined by three-color flow cytometric analysis. Values represent combined mean ± s.e.m. from three independent experiments involving a total of 24 mice. (**D, E**) The ratio of $CD4^+$:$CD8^+$ T lymphocytes was determined by two-color flow cytometric analysis. Values represent combined mean ± s.e.m. from three independent experiments involving a total of 24 mice.

Figure 7

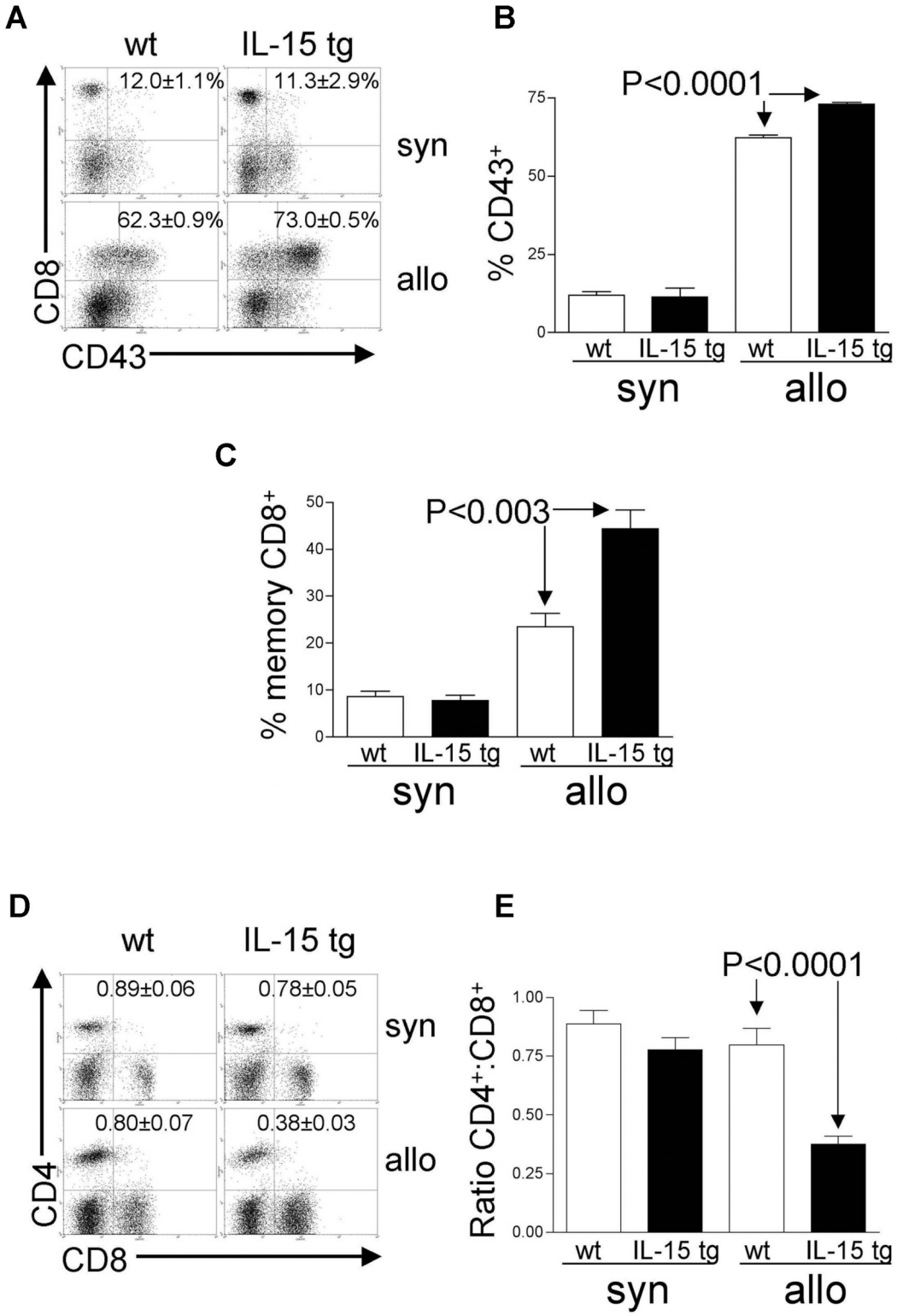

A
wt
IL-15 tg
12.0±1.1%
11.3±2.9%
syn
62.3±0.9%
73.0±0.5%
allo
CD8
CD43
B
P<0.0001
% CD43+
75
50
25
0
wt
IL-15 tg
wt
IL-15 tg
syn
allo
C
P<0.003
% memory CD8+
50
40
30
20
10
0
wt
IL-15 tg
wt
IL-15 tg
syn
allo
D
wt
IL-15 tg
0.89±0.06
0.78±0.05
syn
0.80±0.07
0.38±0.03
allo
CD4
CD8
E
P<0.0001
Ratio CD4+:CD8+
1.00
0.75
0.50
0.25
0.00
wt
IL-15 tg
wt
IL-15 tg
syn
allo

Figure 8. Deregulation of endogenous IL-15 increases Bcl-2 expression in $CD8^+$ T lymphocytes after allogeneic BMT. Spleens were harvested from recipients of IL-15 tg B6 or wt B6 allogeneic BM cells 12 or 13 days after transplantation. (**A**) Flow cytometry histogram showing Bcl-2 protein expression in gated $CD8^+$ or $CD8^-$ splenocytes taken from recipients of wt B6 allogeneic (open histogram) or IL-15 tg B6 allogeneic (solid histogram) BM cells. Mean fluorescence intensity (MFI) values for each group (mean ± s.e.m.) are indicated. (**B**) MFI values for Bcl-2 were obtained from gated $CD8^+$ lymphocyte populations. Mean Bcl-2 MFI values ± s.e.m. for recipients of wt B6 or IL-15 tg B6 allogeneic BM cells are shown. Data are representative of two independent experiments involving a total of 13 mice.

Figure 8

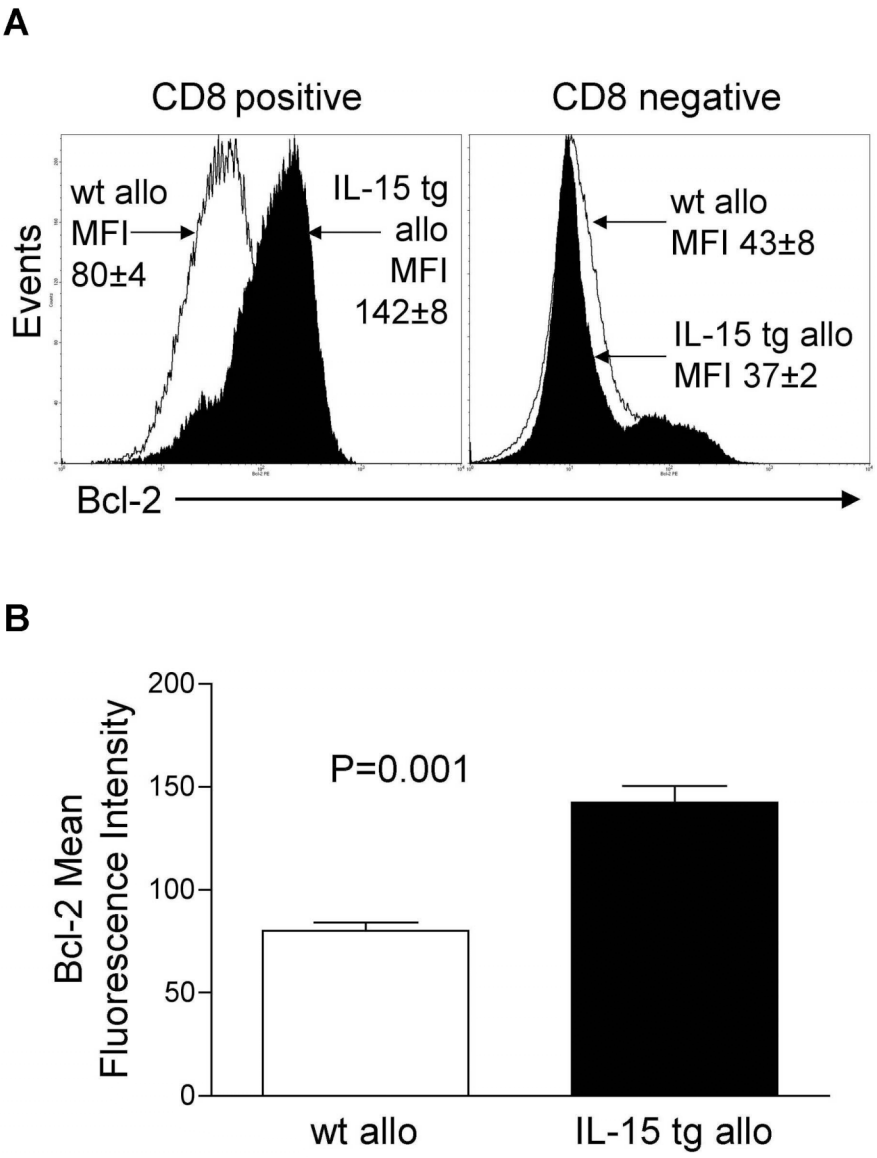

A
CD8 positive
CD8 negative
wt allo
MFI
80±4
IL-15 tg
allo
MFI
142±8
wt allo
MFI 43±8
IL-15 tg allo
MFI 37±2
Events
Bcl-2
B
Bcl-2 Mean
Fluorescence Intensity
200
150
100
50
0
P=0.001
wt allo
IL-15 tg allo

Figure 9. IL-15 increases IFN-γ production by alloreactive T cells *in vitro*. Wild type B6 splenocytes (1×10^8) were primed *in vivo* in allogeneic (wt B6D2F1, n=4) or syngeneic (wt B6, n=1) hosts for six days. Splenocytes were then harvested and restimulated *in vitro* with irradiated naïve B6D2F1 splenocytes for 24 or 72 hours. IFN-γ production by allogeneic- or syngeneic-primed splenocytes in the presence of PBS, or 10 ng/mL or 100 ng/mL rm IL-15 is expressed as the mean ± s.e.m. of triplicate cultures. ND=not detectable.

Figure 9

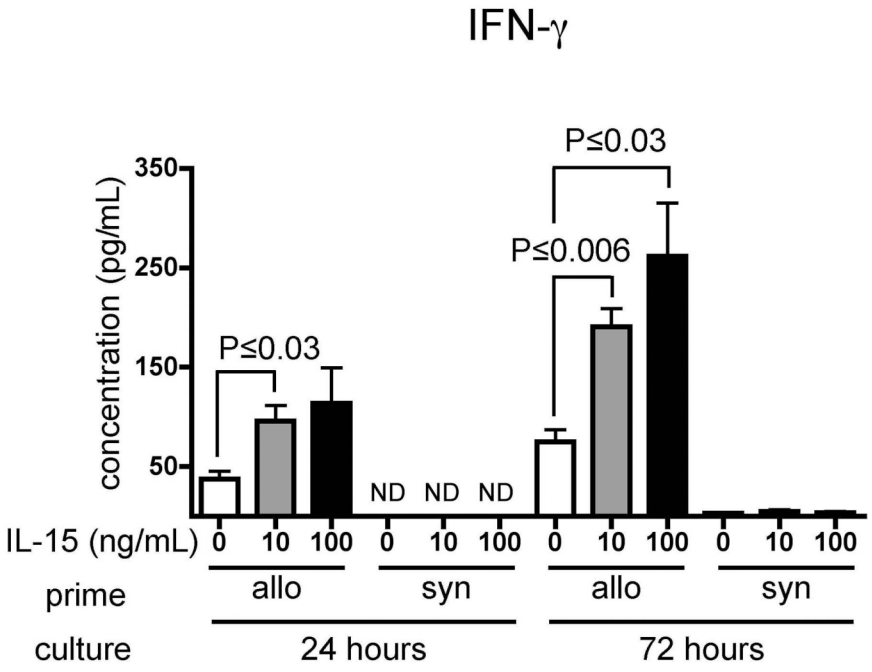

IFN-γ
concentration (pg/mL)
350
250
150
50
P≤0.03
P≤0.006
P≤0.03
ND ND ND
IL-15 (ng/mL) 0 10 100 0 10 100 0 10 100 0 10 100
prime
allo
syn
allo
syn
culture
24 hours
72 hours

Figure 10. IL-15-mediated exacerbation of acute GVHD is dependent upon $CD8^+$ or $CD4^+$ T lymphocytes. B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 BM cells from either wt B6 or IL-15 tg B6 mice. Survival of recipients of IL-15 tg B6 allogeneic BM cells treated with control rat IgG (●, MST=13 days, n=7) or depleting doses of rat anti-mouse CD4 (□, MST=28 days, n=5), CD8 (Δ, MST=64 days, n=5), or both (○, 100% survival, n=5). Median survival time for B6D2F1 mice transplanted with 5 x 10^6 wt B6 splenic T cells, and 1 x 10^7 BM cells from wt B6 mice treated with control rat IgG was 34 days (not shown). □ versus ●, P=0.002; Δ versus ●, P=0.002; ○ versus ●, P=0.002; □ versus Δ, P=0.048.

Figure 10

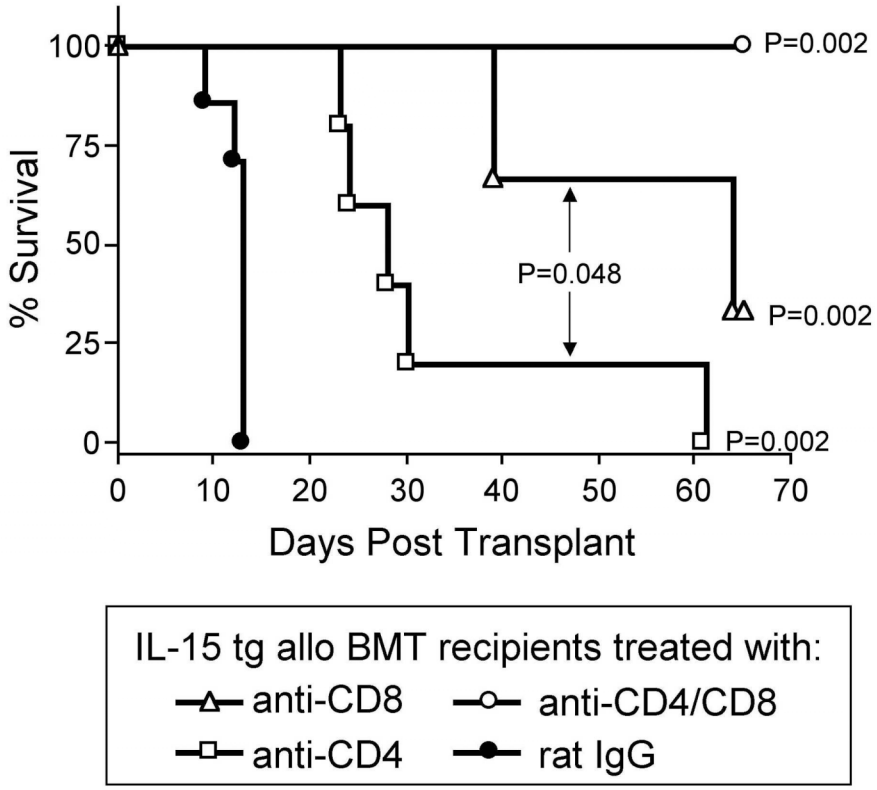

100
75
50
25
0
% Survival
0
10
20
30
40
50
60
70
Days Post Transplant
P=0.002
P=0.048
P=0.002
P=0.002
IL-15 tg allo BMT recipients treated with:
anti-CD8
anti-CD4/CD8
anti-CD4
rat IgG

Table 1. Assessment of survival following BMT with alterations in the fraction of IL-15 tg B6 donor BM cells transplanted into B6D2F1 recipients. For all recipients, the total number of wt B6 splenic T cells was 5 x 10^6, and the total number of BM cells was 1 x 10^7. Median survival times for mice transplanted with a given percentage of IL-15 tg BM cells were compared with the MST for mice transplanted with an immediately higher percentage of IL-15 tg BM cells as shown in the table. NA indicates not applicable; NS indicates not significant.

Table 1

Recipient	Total Number wt B6 Splenic T cells	Total Number BM cells	Percent IL-15 tg B6 BM cells	Median Survival Time (days)	P (logrank test)
B6D2F1	5×10^6	1×10^7	100	14.5	N.A.
B6D2F1	5×10^6	1×10^7	50	14	N.S.
B6D2F1	5×10^6	1×10^7	25	22	0.004
B6D2F1	5×10^6	1×10^7	0	30	0.01

CHAPTER 3

Trans-Presentation of Donor-Derived Interleukin 15 is Necessary for the Rapid Onset of Acute Graft Versus Host Disease but not for Graft Versus Tumor Activity

3.1 Introduction

Acute GVHD is the most common severe side effect of allogeneic BMT, reaching Grade III-IV status in 29-41% of transplant recipients (150). It is mediated by donor-derived T cells from the allograft and results in a shock-like "cytokine storm" within the first week after transplantation followed by $CD4^+$ and $CD8^+$ T cell mediated tissue destruction in the liver, gut, and skin (18). Acute GVHD can largely be averted by using syngeneic or T cell-depleted allogeneic grafts, although these methodologies are associated with higher rates of malignant relapse (17).

A major goal of BMT research, therefore, is to discover factors which, when modulated through pharmacologic means or by prescreening donors, can lessen acute GVHD lethality while maintaining GVT activity. IL-15 is a cytokine critical for the survival and homeostasis of memory $CD8^+$ T cells which are known to actively promote acute GVHD (107, 151, 152). Exogenously administered IL-15 can increase both autoimmune disease and memory $CD8^+$ T cell function against autologous tumor targets (116, 117, 153). Recently, we and others have shown that deregulation of endogenous IL-15 expression or administration of exogenous IL-15 can increase acute GVHD

lethality in the presence of donor-derived allogeneic T cells (154, 155). Yet the mechanism by which endogenous IL-15 can promote acute GVHD, alter post-transplant immune reconstitution and influence the GVT effect remain unclear. We report that in the absence of donor-derived IL-15 expression, acute GVHD lethality is significantly decreased yet donor T cell reconstitution and GVT effects are maintained. Further we demonstrate that donor-derived IL-15 is necessary for optimal type 1 T cell polarization in acute GVHD and provide evidence for cellular and molecular mechanisms by which this is accomplished. Taken together, these data support the notion that targeting IL-15 in allogeneic stem cell transplantation may move us closer to dissecting harmful acute GVHD from the beneficial GVT effect.

3.2 Methods

Reagents, monoclonal antibodies and flow cytometry

Fluorochrome conjugated anti-mouse antibodies were all purchased from BD Pharmingen (San Jose, California). Routine cell surface staining was performed using standard techniques; data was acquired on a Becton Dickinson FACScalibur flow cytometer using CellQuest software. Recombinant human (rh) IL-15 was generously provided by Amgen (Thousand Oaks, California); rm IL-15 was purchased from RND Systems (Minneapolis, Minnesota).

Mice

Female C57Bl/6 (B6, H-2^{b}), B6D2F1 (H-$2^{b/d}$), and T-$bet^{-/-}$ (B6 background, H-2^{b}) mice (6- to 7-week-old) were purchased from Jackson Laboratories (Bar Harbor, ME).

Female IL-15$^{-/-}$ B6 mice (6- to 7-week-old) were purchased from Taconic Farms (Germantown, NY) (91). IL-15 R$\alpha^{-/-}$ B6 mice were generously provided by Averil Ma (University of California San Francisco) and used to establish a breeding colony at The Ohio State University (100). IL-15 tg B6 mice were created as described and maintained at The Ohio State University (103). All mice were between 8 and 12 weeks old at the beginning of each experiment. Mice that underwent transplantation were maintained in sterilized microisolators and received irradiated rodent chow and acidified water plus oral antibiotic (Baytril, 0.2 mg/mL) for 21 days following transplantation. All animal research was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at The Ohio State University.

Bone marrow transplantation

The B6→B6D2F1 model of experimental acute GVHD has been described in detail elsewhere (122, 154). Briefly, T cells were depleted from BM cells harvested from wt B6, IL-15$^{-/-}$ B6, or IL-15 R$\alpha^{-/-}$ B6 mice by labeling with PE conjugated anti-CD3, anti-CD4 and anti-CD8 antibodies followed by anti-PE microbeads and passage through magnetic LD columns (Miltenyi Biotec, Auburn, CA). The efficiency of T cell depletion was >98%. Splenic T cells were purified by negative selection from wt B6 mice by labeling splenocytes with PE conjugated anti-Ter 119, anti-NK1.1, anti-Gr-1, anti-CD11b, anti-CD11c, anti-I-A/I-E and anti-B220 (BD Pharmingen), followed by anti-PE microbeads and passage through LD columns (Miltenyi Biotec). Negatively selected T cells were >95% CD3^{+}. Recipients were conditioned with 1300 cGy whole-body gamma irradiation (Gammacell 40, MDS Nordion, Ottawa, Ontario) split into two doses 24 hours

prior to intravenous infusion of the combined bone marrow and T cell graft. In most cases, recipients were given 1 x 10^7 T cell-depleted BM cells and 5 x 10^6 splenic T cells. Because of the losses associated with T cell purification and because of the limited number of animals available, experiments involving T-bet$^{-/-}$ B6 donors used whole splenocytes adjusted to contain 5 x 10^6 splenic T cells in all groups to induce GVHD. At sacrifice, spleens of recipient mice were removed and splenocytes prepared by standard techniques. IEL were isolated from gut tissue according to published methods (156). In some experiments, spleen and liver were preserved in 10% neutral buffered formalin prior to routine histologic processing.

***In vitro* T cell stimulation and intracellular flow cytometry**

Ninety-six well tissue culture plates were coated overnight at 4°C with hamster anti-mouse CD3 and CD28 (50 μL per well, each antibody at 10 μg/mL, BD Pharmingen). Splenocytes harvested from recipients of wt B6 splenic T cells and either wt B6 BM cells or IL-15$^{-/-}$ B6 BM cells 12 days after transplantation were pooled (N=3 per group) and cultured at 2 x 10^6 cells/mL in complete medium in the presence of 20 μg/mL Golgiplug (Brefeldin A, BD Pharmingen) for 6 hours. After incubation, splenocytes were stained for surface markers, washed, and then fixed and permeabilized using the BD Intracellular Flow Cytometry kit according to the manufacturer's instructions. Intracellular staining was performed with PE-conjugated rat anti-mouse IFN-γ (IgG_1) or control rat IgG_1 (BD Pharmingen).

Measurement of cytokines

Mouse serum samples were diluted 1:2 and assayed for a panel of cytokines using the mouse Th1/Th2 Cytometric Bead Array Kit (BD Pharmingen). The kit was used according to the manufacturer's instructions.

Quantitative real-time PCR

RNA was purified from PBMC using the Qiagen Rneasy RNA extraction kit. RNA was reverse-transcribed using Invitrogen reagents and quantitative real-time PCR was performed using an ABI 7600 Sequence Detector. Primers and probe specific for murine T-bet and spanning an intron were designed as follows: Forward 5'-CTAAGCAAGGACGGCGAATG-3'; Reverse 5'-CAAACATCCTGTAATGGCTTGTG-3'; Probe 5'-CTGTCCTTCACCGTGGCTGGGCT-3'. Amplification of 18s RNA served as an internal positive control in all samples.

Statistics

To compare the clinical evaluation and the percent of baseline weight data, a linear mixed effects model was fit to the data. The terms included group, time and the group*time interaction. The model assumptions include normality and equal variances of the residuals. These assumptions were checked for each model. Survival data were compared using the logrank test and all other data were compared using Student's t-test. $P \leq 0.05$ was considered statistically significant.

3.3 Results

Acute allogeneic GVHD is attenuated in the absence of endogenous IL-15 produced by donor bone marrow-derived cells.

Allogeneic recipients of IL-15$^{-/-}$ B6 T cell depleted bone marrow (TCD BM) cells and highly purified (>95% CD3^{+}) wt B6 T cells demonstrated increased survival compared to recipients of wt B6 TCD BM cells and wt B6 T cells (MST 25.5 days versus 46.5 days, P=0.0005, Figure 11A). Weight loss (Figure 11B) and clinical evidence of acute GVHD (Figure 11C) were also significantly less severe in recipients of IL-15$^{-/-}$ B6 TCD BM cells compared to recipients of wt B6 TCD BM cells (P=0.02 and 0.007, respectively) (124). To prove that the increased death rate due to acute GVHD in recipients of wt B6 BM cells was as a result of post-transplant IL-15 production by these cells and not by differences in the cellular constituency of the wt and IL-15$^{-/-}$ allografts, IL-15$^{-/-}$ B6 donor mice were pretreated with rh IL-15 or PBS as a control (91). Compared to wt B6 donors, control-treated IL-15$^{-/-}$ donors demonstrated significant decreases only in CD11c^{+}B220^{+} plasmacytoid dendritic cells and NK1.1^{+}CD3^{-} NK cells. Treatment with IL-15 normalized plasmacytoid dendritic cell numbers and increased NK cell numbers (5.5% versus 1.47%, P=0.0001) compared to control-treated wt B6 donors (Table 2). However, recipients of wt B6 splenic T cells and TCD BM cells from IL-15-pretreated IL-15$^{-/-}$ mice showed significantly longer survival compared to recipients of wt B6 splenic T cells and wt B6 TCD BM cells (MST 32 days versus 54 days, P=0.02, Figure 11D) and there was no difference in survival between recipients of BM cells from IL-15- and PBS-pretreated IL-15$^{-/-}$ B6 donors (Figure 11D).

Coordinate expression of IL-15 with IL-15 Rα is required in acute GVHD.

We hypothesized that expression of IL-15 in acute GVHD must occur by the same donor cell that presents IL-15 *in trans* via IL-15 Rα to responding lymphocytes; this so-called coordinate expression model has been described in immune-deficient mouse models but not in disease states (157). If this model holds in acute GVHD, then recipients of IL-15$^{-/-}$ and IL-15 Rα$^{-/-}$ BM cells should demonstrate similar survival advantages compared to recipients of wt BM cells. Further, because in the coordinate model IL-15 Rα$^{-/-}$ cells (which do express IL-15) cannot produce soluble IL-15 for capture and presentation by IL-15$^{-/-}$ cells (which do express IL-15 Rα), recipients of IL-15$^{-/-}$ and IL-15 Rα$^{-/-}$ BM cells mixed 1:1 should also demonstrate a survival advantage. The rationale for the mixing experiment is presented in Figure 12A. Wild type B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6, IL-15$^{-/-}$ B6 or IL-15 Rα$^{-/-}$ B6 donors. A fourth cohort received 1 x 10^7 TCD BM cells from IL-15$^{-/-}$ and IL-15 Rα$^{-/-}$ B6 mice mixed 1:1. Recipients of IL-15$^{-/-}$ B6 or IL-15 Rα$^{-/-}$ B6 BM cells lived significantly longer after transplantation compared to recipients of wt B6 BM cells (IL-15$^{-/-}$ B6 versus wt B6: MST 49 days versus 28 days, P=0.02; IL-15 Rα$^{-/-}$ B6 versus wt B6: MST 47 days versus 28 days, P=0.002, Figure 12B). There was no difference in survival between recipients of IL-15$^{-/-}$ or IL-15 Rα$^{-/-}$ B6 BM cells (Figure 12B). In addition, recipients of the mixed BM cells demonstrated significantly longer survival after transplantation compared with recipients of wt BM cells (MST 43 days versus 28 days, P=0.002, Figure 12B). There was no difference in survival between these mice and recipients of either IL-15$^{-/-}$ B6 or IL-15 Rα$^{-/-}$ B6 BM cells (Figure 12B).

Absence of donor-derived IL-15 does not decrease donor T cell chimerism or memory CD8$^+$ T cell reconstitution.

Splenocytes were harvested from allogeneic recipients of wt B6 splenic T cells and wt B6 TCD BM cells or IL-15$^{-/-}$ B6 TCD BM cells 5, 12, or 28 days after transplantation. There was no significant difference in donor CD4$^+$ or CD8$^+$ T cell chimerism at any of these time points (Figure 13). Further, in the absence of donor-derived IL-15 there was no significant decrease in absolute numbers of donor-derived CD8$^+$ CD122$^+$ memory T cells (Figure 14), CD8$^+$ CD44^{hi} CD62L^{lo} effector memory T cells (Figure 15) or CD8$^+$ CD44^{hi} CD62L^{hi} central memory T cells (Figure 16) harvested from the spleen. IL-15 is critical for the survival of NK cells and may contribute to the survival of monocytes and dendritic cells, yet there was no decrease in the absolute numbers of these cells in recipients of IL-15$^{-/-}$ B6 BM cells (Figures 17, 18) (91, 94). Finally, IL-15 is reportedly critical for the survival of intraepithelial lymphocytes (IEL) (89); IEL have been shown to contribute to intestinal epithelial cell apoptosis in acute GVHD (158). There was no difference in the percent of donor-derived TCR-β^+ CD4$^+$, TCR-β^+ CD8$^+$, TCR-γ^+ CD4$^+$, or TCR-γ^+ CD8$^+$ IEL in the presence or absence of donor-derived IL-15 (Figure 19).

Donor-derived IL-15 is necessary for optimal IFN-γ production and CXCR3 expression by T cells in acute GVHD.

B6D2F1 mice were lethally irradiated and infused with wt B6 splenic T cells and TCD BM cells from either wt B6 or IL-15$^{-/-}$ B6 mice. Serum and splenocytes were

harvested 5 and 12 days after transplantation. There was no difference in serum IFN-γ concentration between recipients of wt B6 and IL-15$^{-/-}$ B6 TCD BM cells at day 5 after transplantation (data not shown), while at day 12 after transplantation, recipients of wt B6 TCD BM cells demonstrated significantly higher serum IFN-γ compared to recipients of IL-15$^{-/-}$ B6 TCD BM cells (183 ± 13.6 pg/mL versus 67 ± 17.3 pg/mL, P=0.002, Figure 20A). Further, *in vitro* restimulation of splenocytes harvested at day 12 using immobilized anti-CD3 and anti-CD28 crosslinking antibodies revealed increased percentages of IFN-γ producing CD4$^+$ and CD8$^+$ T cells from recipients of wt B6 BM cells compared to CD4$^+$ and CD8$^+$ T cells from recipients of IL-15$^{-/-}$ B6 BM cells (data not shown). CXCR3 is a receptor for the chemokines CXCL9, CXCL10, and CXCL11 that is preferentially expressed on T_{h1} cells (159-161). Recipients of wt B6 splenic T cells and wt B6 BM cells demonstrated significantly higher expression of CXCR3 by donor-derived CD4$^+$ T cells at day 5 after transplantation compared to recipients of wt B6 splenic T cells and IL-15$^{-/-}$ B6 BM cells (mean fluorescence intensity (MFI) 10.99 ± 0.18 versus 9.11 ± 0.60, P=0.04, Figure 20B, C).

Expression of T-bet by donor splenocytes is necessary for IL-15 mediated acute GVHD.

T-bet is a member of the T-box family of transcription factors that is necessary for type 1 T cell lineage commitment, promotes IFN-γ production in both CD4$^+$ and CD8$^+$ T cells and is necessary for optimal expression of CXCR3 (162, 163). Highly purified wt B6 splenic T cells cultured in the presence of rm IL-15 demonstrated significantly increased levels of T-bet mRNA beginning 9 hours after stimulation (Figure 21A).

Further, peripheral blood leukocytes from IL-15 tg B6 mice that overexpress IL-15 using the H-2D promoter (103) expressed T-bet mRNA at 3.4 fold higher levels compared with wt B6 mice (P=0.0016, Figure 21B). Accordingly, compared with wt B6 mice, IL-15 tg mice were found to have a greater percentage of $CD8^+$ T cells constitutively expressing CXCR3 (60.0 ± 1.6% versus 23.7 ± 1.2%, P≤0.0001, Figure 21C) and a greater percentage of $CD8^+$ T cells that produced IFN-γ after anti-CD3/CD28 stimulation as measured by intracellular flow cytometry (18.7 ± 0.96% versus 3.0 ± 0.21%, P=0.0005, Figure 21D). Consistent with these *in vitro* findings, 86% of B6D2F1 recipients of 1 x 10^7 IL-15 tg TCD BM cells and whole T-$bet^{-/-}$ B6 splenocytes adjusted to contain 5 x 10^6 T cells survived more than 55 days after transplantation. In contrast, recipients of wt B6 splenocytes and either IL-15 tg B6 or wt B6 TCD BM cells demonstrated respective median survival times of 14.5 or 28.5 days after transplantation (P=0.0001, 0.001 respectively, Figure 21E). Further, recipients of IL-15 tg B6 TCD BM cells and T-$bet^{-/-}$ splenocytes had significantly less severe weight loss over the course of the experiment when compared with recipients of IL-15 tg B6 BM cells and wt B6 splenocytes (P<0.0001, Figure 21F).

GVT activity is preserved in the absence of donor-derived IL-15.

B6D2F1 mice were lethally irradiated and given 2000 P815 mastocytoma tumor cells ($H\text{-}2^{d/d}$), 5 x 10^6 wt B6 (allogeneic) splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 or IL-$15^{-/-}$ B6 mice. For controls, identical recipients were given 5 x 10^6 wt B6D2F1 (syngeneic) T cells and 1 x 10^7 wt B6D2F1 TCD BM cells plus or minus 2000 P815 cells. Recipients of syngeneic T cells plus P815 demonstrated rapid death from

tumor (MST 15 days, Figure 22A). Recipients of allogeneic IL-15$^{-/-}$ B6 TCD BM cells and wt T cells lived significantly longer compared to recipients of allogeneic wt B6 TCD BM cells and wt T cells (MST 47 days versus 21.5 days, P=0.0006, Figure 22A). Confirming death due to acute GVHD, all allogeneic recipients of wt or IL-15$^{-/-}$ B6 TCD BM cells demonstrated clinical signs of acute GVHD prior to death, had no evidence of hindlimb paralysis and no evidence of circulating P815 cells in the peripheral blood at 1, 2 or 3 weeks after transplantation (data not shown). Representative mice were sacrificed when moribund and liver and spleen harvested to examine for the presence of tumor cells. Only recipients of syngeneic TCD BM and syngeneic T cells showed evidence of tumor at these time points (Figure 22B). Taken together, these data show that not only is the GVT effect preserved in the absence of donor-derived IL-15, but overall survival after transplantation is dramatically increased as well.

3.4 Discussion

Using a well-described and clinically relevant murine model of allogeneic BMT, we have shown that IL-15 is produced and presented *in trans* by donor-derived cells in acute GVHD. Donor-derived IL-15 was necessary for full type 1 T cell polarization and promoted acute GVHD lethality in a manner dependent upon expression of T-bet in donor cells. Yet donor-derived IL-15 was dispensable for achieving full donor T, NK and myeloid cell reconstitution under myeloablative conditions. Finally, overall survival was substantially improved in the absence of donor-derived IL-15 in a commonly used

model of acute GVHD and GVT activity. Together we believe these data provide the mechanism by which donor-derived IL-15 signals and impacts T cell polarization and immune reconstitution in acute GVHD.

To rigorously prove that donor bone marrow-derived cells produce IL-15 in experimental acute GVHD with significant enhancement of morbidity and mortality, we have considered the possibility that the survival difference observed between recipients of wt B6 and IL-15$^{-/-}$ B6 BM cells was related not to *bona fide* differences in IL-15 production, but rather to differences in the cellular constituency of the bone marrow portion of the allografts (91). For example, there were significantly fewer CD11c^{+} B220^{+} plasmacytoid dendritic cells identified in control-treated IL-15$^{-/-}$ B6 donors compared to control-treated wt B6 donors. This shift could have contributed to the observed survival difference irrespective of differences in IL-15 production after transplantation. However, our data show that administration of recombinant IL-15 to IL-15$^{-/-}$ B6 donors normalizes the cell population distribution in the donor bone marrow compartment, yet preserves a survival advantage for the recipients of these cells after transplantation. Notably, NK cells were nearly absent in control-treated IL-15$^{-/-}$ B6 donors but increased to supraphysiologic levels by the administration of recombinant IL-15, consistent with their strict dependence on IL-15 for growth and survival (91). Because an increase in survival relative to recipients of wt donor cells remained in the presence or absence of donor NK cells in these experiments and because NK cell alloreactivity in our model has been demonstrated to be in the host versus graft direction, we believe that skewing of the donor NK cell compartment is not essential to the survival differences observed (74). Nevertheless, we cannot rule out the possibility that pre-treatment with exogenous IL-15

specifically induced a tolerogenic NK cell subset (not present or not sufficiently active in wt B6 donors to confer a survival benefit compared with IL-15$^{-/-}$ B6 donors) that precisely neutralized any pathogenic effect of donor IL-15 pre-treatment. We believe these data provide strong evidence that endogenous IL-15 production by donor-derived cells underlies the pathobiology of acute GVHD.

Studies in immunodeficient mouse models have linked production of IL-15 and its presentation *in trans* via IL-15 Rα to responding lymphocytes as events occurring coordinately by the same cell (157). Here, we have asked whether the dysregulation of pro-inflammatory cytokines and/or the rapid cell turnover (with potential release of IL-15 from dying cells) characteristic of acute GVHD are sufficient to circumvent this currently accepted mechanism of IL-15 signaling. Because recipients of mixed IL-15$^{-/-}$ and IL-15 Rα$^{-/-}$ BM cells demonstrate similar survival compared to recipients of either type of genetically deficient cells alone, it is unlikely that donor IL-15 Rα$^{-/-}$ cells can produce IL-15 for presentation by donor IL-15$^{-/-}$ cells. Thus the coordinate presentation model is not altered by the cytokine dysregulation in acute GVHD. Further, because recipients of IL-15$^{-/-}$ TCD BM cells (which could in theory scavenge IL-15 released by dying host or donor cells for presentation via IL-15 Rα) and IL-15 Rα$^{-/-}$ TCD BM cells (which neither scavenge nor present native IL-15) demonstrate similar survival curves, there is no evidence that IL-15 released as a result of cellular turnover contributes to acute GVHD.

These observations have significant consequences given the cellular mechanisms of alloreactive T cell activation. It is clear that the most important encounter by donor T cells with alloantigen occurs in the first five days after transplantation and is mediated by host-derived antigen presenting cells (36, 38, 164). Our data suggest that donor-derived

IL-15 does not contribute to this T cell-APC interaction because it remains bound to the surface of donor-derived cells. More likely, a donor-derived IL-15-producing and -presenting cell encounters alloreactive donor T cells some time after the initial contact with alloantigen. This conclusion is contrary to the proposed role of IL-15 trans-presentation as a third co-stimulatory signal between APC and T cells in addition to allogeneic MHC-TCR and CD28-CD86 (85). Alternatively, it is possible that the donor-derived IL-15-producing and -presenting cell has the ability also to present host alloantigens and contribute to acute GVHD via the indirect pathway as has been described (33, 165).

Nevertheless, our data have shown that donor BM-derived IL-15 is necessary for maximal type 1 T cell polarization. The role of IFN-γ, the prototypical type 1 cytokine, in acute GVHD remains controversial. IFN-γ is known to increase MHC-I, MHC-II and co-stimulatory ligand expression by antigen presenting cells and to compromise the integrity of the intestinal epithelial lining, all of which are necessary for optimal alloreactive T cell activation in acute GVHD (18, 29, 36, 38, 166, 167). In early studies, IFN-γ was found to be elevated in humans with acute GVHD while murine IFN-$\gamma^{-/-}$ donor T cells were shown to confer a prolonged disease course, consistent with this picture (168-170). Yet studies using lethal conditioning regimens have demonstrated that a critically-timed burst of IFN-γ production by donor-derived T cells in the first two days after transplantation can lead to apoptosis of donor T cells and protection from acute GVHD (171, 172). Enhanced Fas/FasL-mediated clearance of alloreactive donor T cells

likely explains these findings (173, 174). Thus donor-derived IFN-γ may play a bifunctional role in the pathogenesis of acute GVHD, promoting APC function and gut pathology while enhancing Fas-mediated apoptosis of alloreactive donor T cells

Yet while IFN-γ production is the hallmark function of pro-inflammatory type 1 polarized T cells, it is by no means their only distinguishing characteristic. Expression of receptors for adhesion molecules and chemokines that promote T cell migration to inflammatory sites such as PSGL-1 and CXCR3 are preferentially increased in type 1 T cells compared to type 2 T cells (159, 175). T-bet has been identified as the master control gene for this plurality of functions: $CD4^+$ T cells that overexpress T-bet produce more IFN-γ and express more cell-surface CXCR3 under both type 1 and type 2 polarizing conditions compared to $CD4^+$ T cells from wt mice (162, 163). Further, $CD4^+$ T cells from T-$bet^{-/-}$ mice have severe defects in IFN-γ production and CXCR3 expression when cultured in type 1 polarizing conditions (162, 163). The observations that IL-15 tg mice demonstrate increased expression of IFN-γ, CXCR3, and T-bet and that purified T cells rapidly upregulate T-bet *in vitro* in response to IL-15 identify T-bet as a downstream target of IL-15 in T cells. Although we cannot formally rule out an effect of IL-15 through T-bet on other donor-derived cells, the observations of decreased type 1 T cell functions in allogeneic recipients of IL-$15^{-/-}$ B6 BM cells and a dramatic reduction in mortality in recipients of IL-15 tg B6 TCD BM and T-$bet^{-/-}$ splenocytes strongly suggest that enhancement of type 1 polarization is a primary, non-redundant role for donor-derived IL-15 in acute GVHD.

To understand the consequences of decreasing endogenous IL-15 production in terms of the GVT effect, we have used a model of tumor occurrence after challenge at the

time of BMT that has been repeatedly validated in the literature (123, 176, 177). Here, we have shown for the first time that in the absence of donor-derived IL-15 GVT activity is maintained while GVHD mortality is substantially delayed. Because the vast majority of allogeneic HSCT in clinical practice is T cell replete, these findings are an advancement on a previous study using T cell depleted transplants to show exogenously administered IL-15 enhanced GVT activity (155). Thus our data highlight the potential clinical benefit of neutralizing IL-15 through pharmacologic or immunotherapeutic means. Moreover, because our data strongly support the notion that IL-15 is expressed in a coordinate fashion with IL-15 Rα in acute GVHD, synergistic antagonism of IL-15 signaling may be achievable by targeting both IL-15 and IL-15 Rα.

While the timing, location, and specific cellular source of donor-derived IL-15 still await discovery, it is now clear that its absence provides concrete benefits in experimental acute GVHD: systemic inflammation is reduced with a concomitant decrease in morbidity and mortality, while T cell reconstitution and GVT effects are maintained. Further, our analysis of the signaling mechanism of IL-15 in acute GVHD has identified IL-15 Rα and T-bet as potential new therapeutic targets in the disease. Together, these findings compel further research into the role of IL-15 in acute GVHD at both basic and clinical levels.

Figure 11. Absence of IL-15 production by donor-derived BM cells decreases acute GVHD morbidity and mortality. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from wt B6 (■) or IL-$15^{-/-}$ B6 mice (□). Survival was monitored daily (**A**) and body weights (**B**) and clinical GVHD scores (**C**) were collected in a blinded fashion every three days after transplantation. Survival data are combined from two similar experiments; weight and clinical score data are from one of two similar experiments. N≥8 mice per group. **(D)** IL-$15^{-/-}$ B6 donors were pretreated with 10 μg/day rh IL-15 or PBS as a control for 7 days prior to harvest of bone marrow. Survival of allogeneic recipients (N≥12 mice per group) of 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells harvested from rh IL-15 treated IL-$15^{-/-}$ B6 donors (○), PBS treated IL-$15^{-/-}$ B6 donors (□) or PBS treated wt B6 donors (■) was monitored daily after transplantation. All survival times were compared using the logrank test. Mean group weight and clinical GVHD data were compared using a linear mixed effects model as described in Methods. Error bars represent standard error of the mean (s.e.m.).

Figure 11

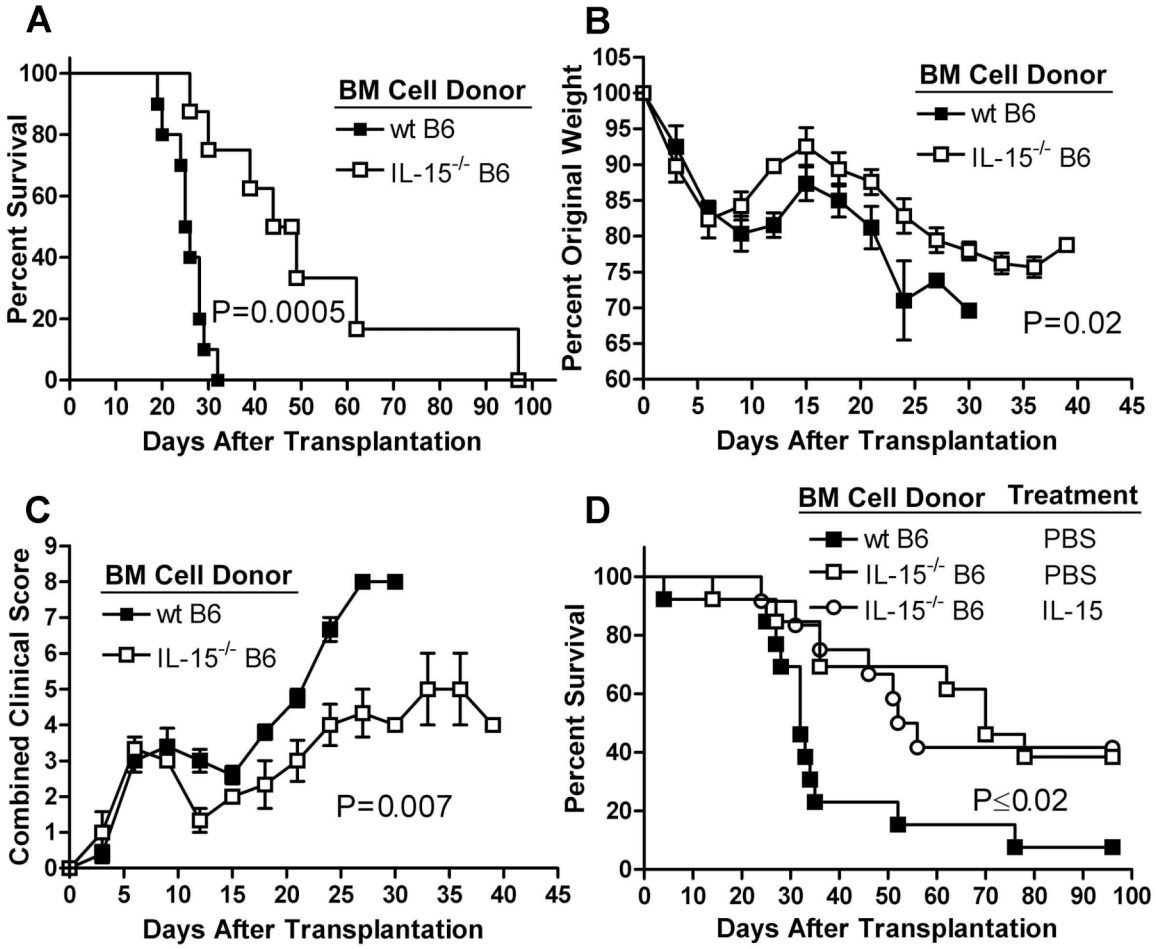

A
Percent Survival
Days After Transplantation
BM Cell Donor
wt B6
IL-15-/- B6
P=0.0005
B
Percent Original Weight
Days After Transplantation
BM Cell Donor
wt B6
IL-15-/- B6
P=0.02
C
Combined Clinical Score
Days After Transplantation
BM Cell Donor
wt B6
IL-15-/- B6
P=0.007
D
Percent Survival
Days After Transplantation
BM Cell Donor Treatment
wt B6 PBS
IL-15-/- B6 PBS
IL-15-/- B6 IL-15
P≤0.02

Figure 12. IL-15 is coordinately expressed with IL-15 Rα in acute GVHD. (**A**) Rationale behind the mixed BM cell experiment. Wild type B6 control donor BM cells can produce IL-15 for presentation by IL-15 Rα. IL-15$^{-/-}$ and IL-15 Rα$^{-/-}$ control donor BM cells are each genetically deficient in IL-15 signaling and each confers a survival benefit compared to wt B6 BM cells. In postulate 1, IL-15 Rα$^{-/-}$ donor BM cells produce IL-15 while IL-15$^{-/-}$ donor BM cells have IL-15 Rα. Because it is postulated that the same cell must produce and present IL-15, it is expected that survival will not be adversely affected. In postulate 2, coordinate production and presentation is not required. Thus, IL-15 Rα$^{-/-}$ donor BM cells produce IL-15 which is captured and presented by IL-15$^{-/-}$ donor BM cells, thereby restoring IL-15 signaling and reducing or eliminating any survival benefit compared to wt B6 control. (**B**) Lethally irradiated wt B6D2F1 mice (N=5 per group) were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 (■), IL-15$^{-/-}$ B6 (□) or IL-15 Rα$^{-/-}$ B6 mice (△). A fourth cohort of mice was transplanted with 1 x 10^7 TCD BM cells from IL-15$^{-/-}$ and IL-15 Rα$^{-/-}$ B6 mice mixed at a 1:1 ratio (○). Survival times were compared using the logrank test and results were consistent with postulate 1.

Figure 12

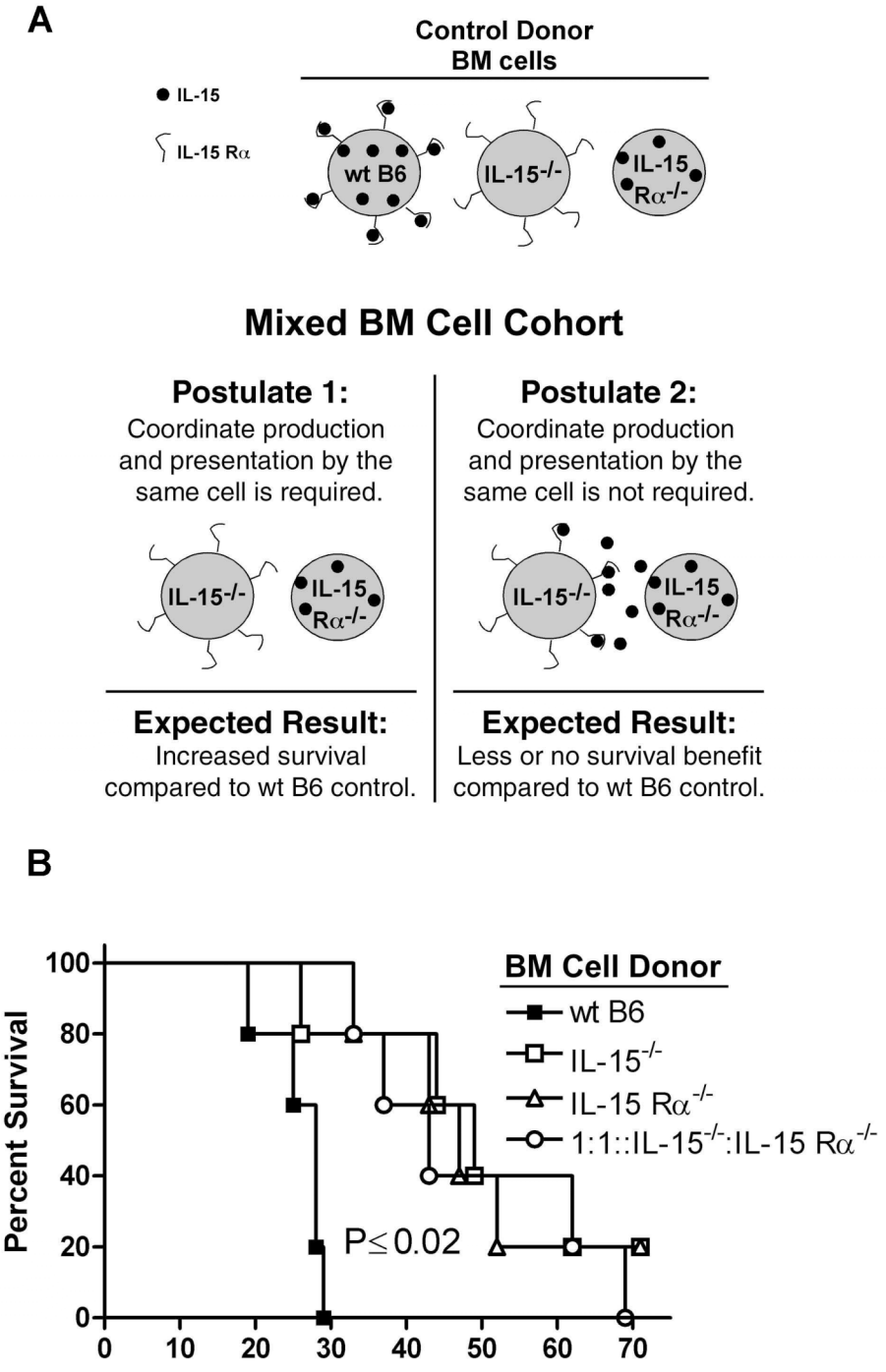

A
Control Donor
BM cells
IL-15
IL-15 Rα
wt B6
IL-15-/-
IL-15 Rα-/-
Mixed BM Cell Cohort
Postulate 1:
Coordinate production and presentation by the same cell is required.
Postulate 2:
Coordinate production and presentation by the same cell is not required.
IL-15-/-
IL-15 Rα-/-
IL-15-/-
IL-15 Rα-/-
Expected Result:
Increased survival compared to wt B6 control.
Expected Result:
Less or no survival benefit compared to wt B6 control.
B
Percent Survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Days After Transplantation
BM Cell Donor
wt B6
IL-15-/-
IL-15 Rα-/-
1:1::IL-15-/-:IL-15 Rα-/-
P≤0.02

Figure 13. Absence of donor-derived IL-15 does not decrease donor T cell chimerism. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and spleens harvested. Chimerism was calculated by dividing the number of $CD4^+H\text{-}2D^{d-}$ or $CD8^+H\text{-}D^{d-}$ events by the total number of $CD4^+$ or $CD8^+$ events. Values for individual mice from 1 of 3 to 5 similar experiments were averaged and plotted as mean ± s.e.m. The total number of mice analyzed in each group at each time point was between 9 and 14.

Figure 13

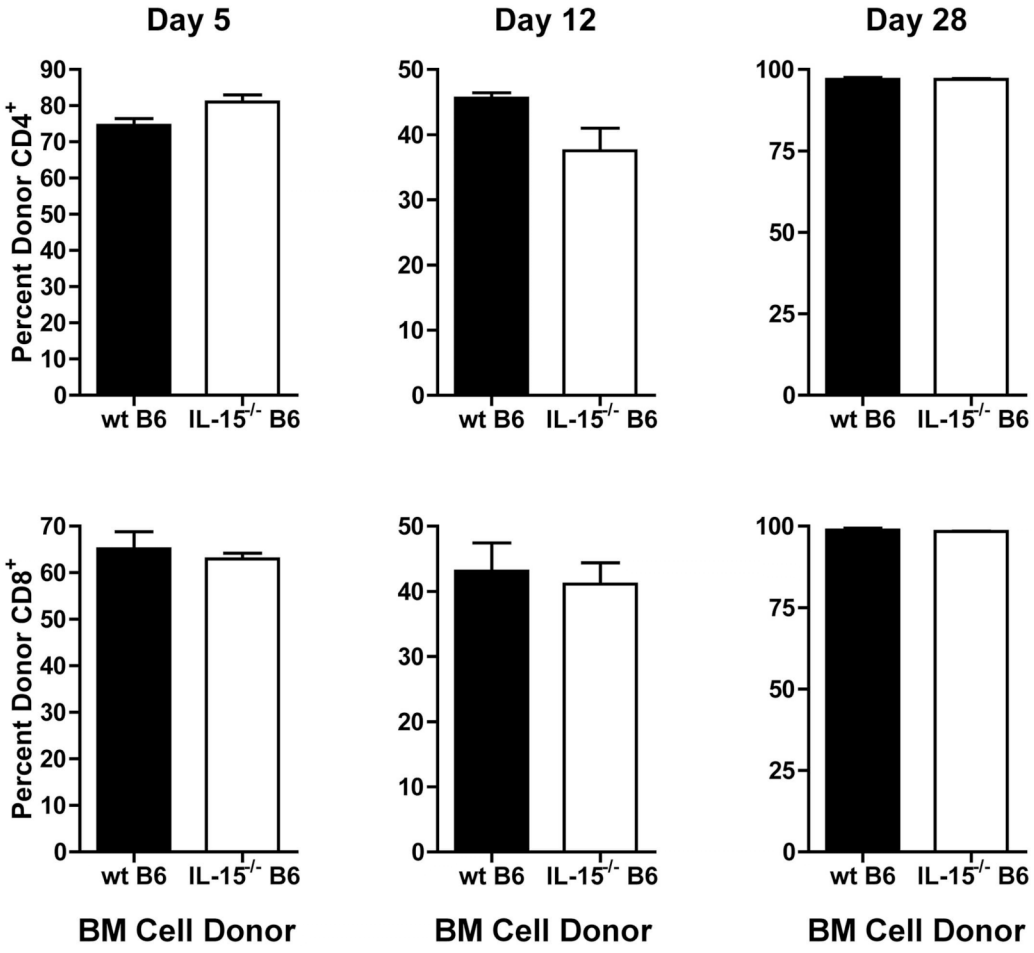

Day 5
Day 12
Day 28
Percent Donor CD4+
Percent Donor CD8+
wt B6
IL-15-/- B6
BM Cell Donor

Figure 14. Absence of donor-derived IL-15 does not decrease reconstitution of donor-derived $CD8^+$ $CD122^+$ memory T cells. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and spleens harvested. The number of $CD8^+$ $CD122^+$ H-$2D^{d-}$ events was divided by the total viable cell events collected and multiplied by the number of splenocytes recovered for each mouse. Values for individual mice from 1 of 3 to 4 similar experiments were averaged and plotted as mean ± s.e.m. The total number of mice analyzed in each group at each time point was between 9 and 14.

Figure 14

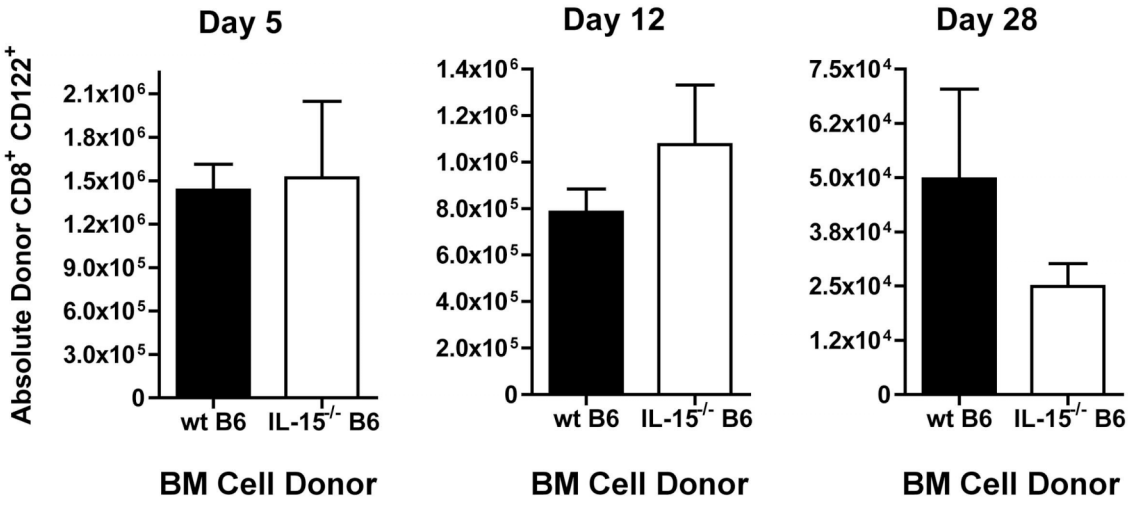

Day 5
Day 12
Day 28
Absolute Donor CD8+ CD122+
2.1x10^6
1.8x10^6
1.5x10^6
1.2x10^6
9.0x10^5
6.0x10^5
3.0x10^5
0
1.4x10^6
1.2x10^6
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
0
7.5x10^4
6.2x10^4
5.0x10^4
3.8x10^4
2.5x10^4
1.2x10^4
0
wt B6
IL-15-/- B6
BM Cell Donor

Figure 15. Absence of donor-derived IL-15 does not decrease reconstitution of donor-derived $CD8^+$ $CD62L^{lo}$ $CD44^{hi}$ effector memory T cells. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and spleens harvested. The number of $CD8^+$ $CD62L^{lo}$ $CD44^{hi}$ H-$2D^{d-}$ events was divided by the total viable cell events collected and multiplied by the number of splenocytes recovered for each mouse. Values for individual mice from 1 of 3 to 4 similar experiments were averaged and plotted as mean ± s.e.m. The total number of mice analyzed in each group at each time point was between 9 and 14.

Figure 15

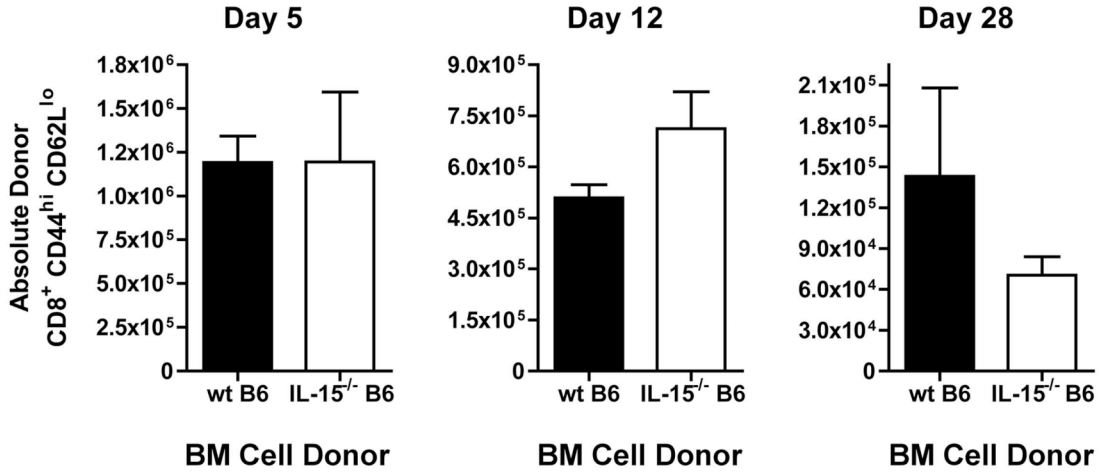

Day 5
Day 12
Day 28
Absolute Donor CD8+ CD44hi CD62Llo
1.8x10^6
1.5x10^6
1.2x10^6
1.0x10^6
7.5x10^5
5.0x10^5
2.5x10^5
0
9.0x10^5
7.5x10^5
6.0x10^5
4.5x10^5
3.0x10^5
1.5x10^5
2.1x10^5
1.8x10^5
1.5x10^5
1.2x10^5
9.0x10^4
6.0x10^4
3.0x10^4
wt B6
IL-15-/- B6
BM Cell Donor

Figure 16. Absence of donor-derived IL-15 does not decrease reconstitution of donor-derived $CD8^+$ $CD62L^{hi}$ $CD44^{hi}$ central memory T cells. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and spleens harvested. The number of $CD8^+$ $CD62L^{hi}$ $CD44^{hi}$ H-$2D^{d-}$ events was divided by the total viable cell events collected and multiplied by the number of splenocytes recovered for each mouse. Values for individual mice from 1 of 3 to 4 similar experiments were averaged and plotted as mean ± s.e.m. The total number of mice analyzed in each group at each time point was between 9 and 14.

Figure 16

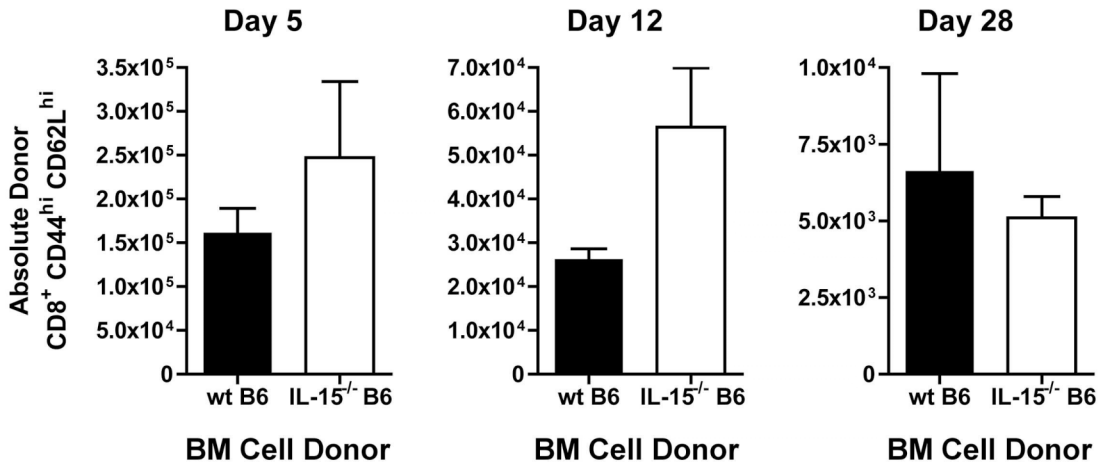

Day 5
Day 12
Day 28
Absolute Donor
CD8+ CD44hi CD62Lhi
3.5x10^5
3.0x10^5
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0
7.0x10^4
6.0x10^4
5.0x10^4
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
1.0x10^4
7.5x10^3
5.0x10^3
2.5x10^3
wt B6
IL-15-/- B6
BM Cell Donor

Figure 17. Absence of donor-derived IL-15 does not decrease chimerism or reconstitution of donor-derived NK cells. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and spleens harvested. Chimerism was calculated by dividing the number of $NK1.1^+$ $CD3^-$ H-$2D^{d-}$ events by the total number of $NK1.1^+$ $CD3^-$ events. Absolute numbers of donor-derived NK cells were calculated by dividing the number of $NK1.1^+$ $CD3^-$ H-$2D^{d-}$ events by the total viable cell events collected and multiplying by the number of splenocytes recovered for each mouse. Values for individual mice from 1 of 2 similar experiments were averaged and plotted as mean ± s.e.m. The total number of mice analyzed in each group at each time point was between 4 and 7.

Figure 17

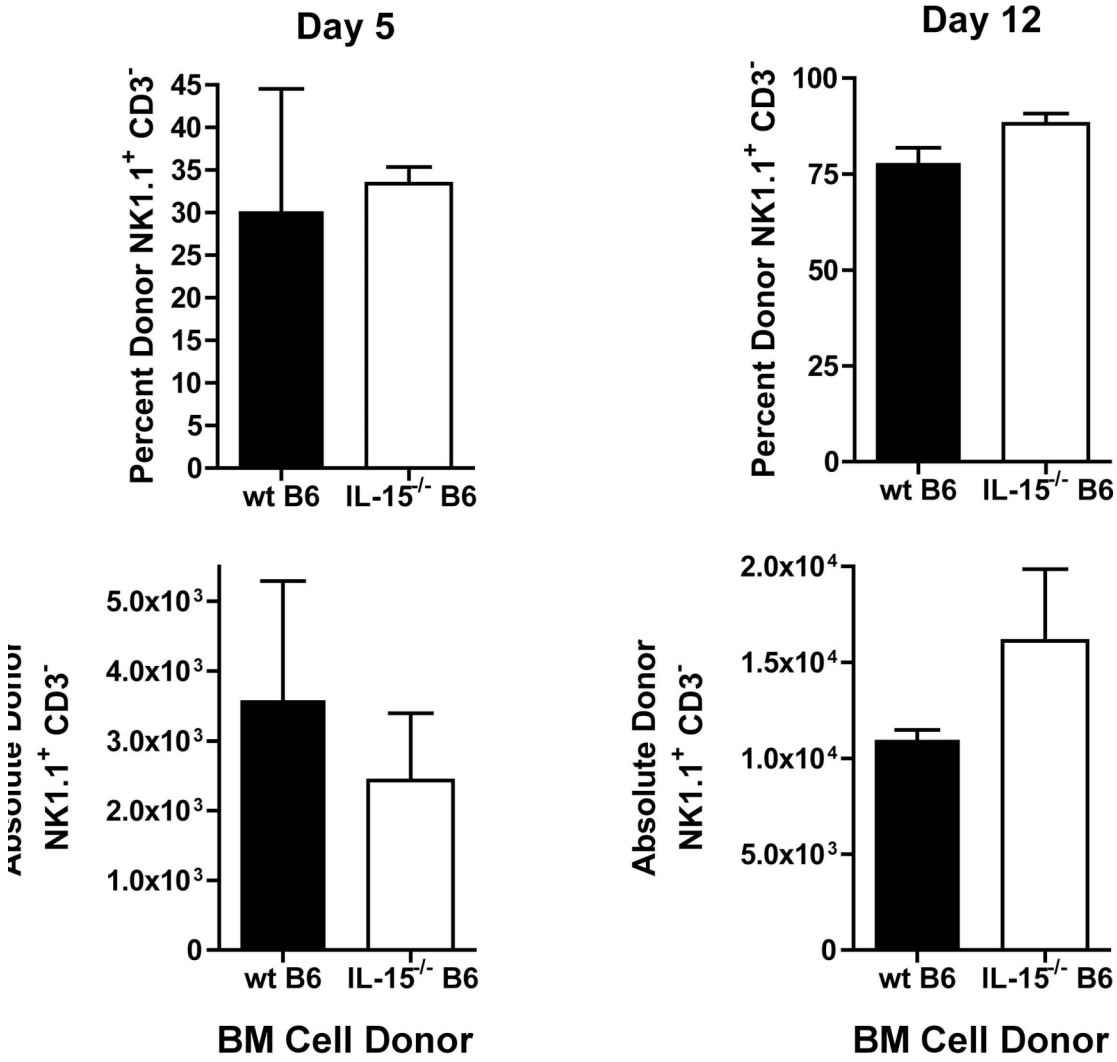

Day 5
Day 12
Percent Donor NK1.1+ CD3-
45
40
35
30
25
20
15
10
5
0
100
75
50
25
wt B6
IL-15-/- B6
Absolute Donor NK1.1+ CD3-
5.0x10^3
4.0x10^3
3.0x10^3
2.0x10^3
1.0x10^3
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3
BM Cell Donor

Figure 18. Absence of donor-derived IL-15 does not decrease myeloid reconstitution. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and spleens harvested. Absolute numbers of donor-derived monocytes/granulocytes ($CD11b^+$ $CD11c^-$ $H\text{-}2D^{d-}$) and dendritic cells ($CD11b^+$ $CD11c^+$ $H\text{-}2D^{d-}$) were calculated by dividing the number of monocyte/granulocyte or dendritic cell events by the total viable cell events collected and multiplying by the number of splenocytes recovered for each mouse. Values for individual mice from 1 of 2 to 3 similar experiments were averaged and plotted as mean ± s.e.m. The total number of mice analyzed in each group at each time point was between 5 and 13.

Figure 18

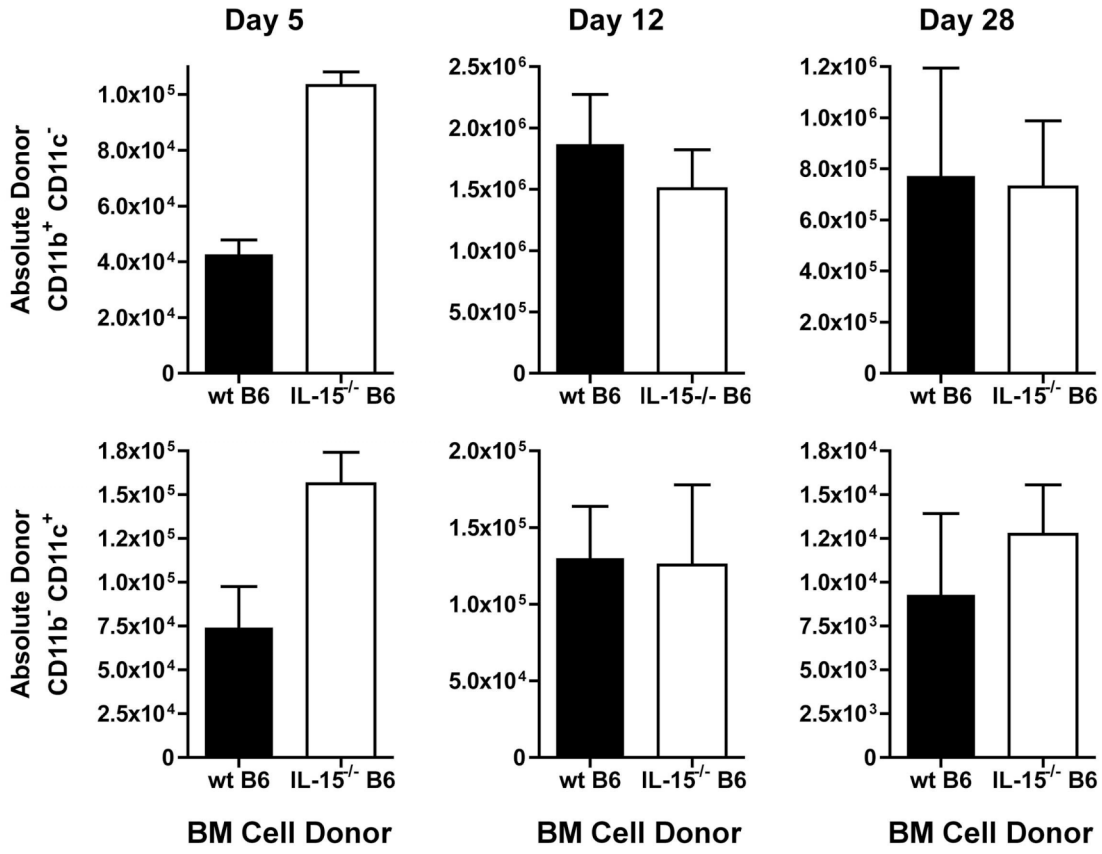

Day 5
Day 12
Day 28
Absolute Donor CD11b+ CD11c-
Absolute Donor CD11b- CD11c+
1.0x10^5
8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0
2.5x10^6
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
1.2x10^6
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
1.8x10^5
1.5x10^5
1.2x10^5
1.0x10^5
7.5x10^4
5.0x10^4
2.5x10^4
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
1.8x10^4
1.5x10^4
1.2x10^4
1.0x10^4
7.5x10^3
5.0x10^3
2.5x10^3
wt B6
IL-15-/- B6
IL-15-/- B6
BM Cell Donor

Figure 19. Absence of donor-derived IL-15 does not alter IEL phenotype. Lethally irradiated wt B6D2F1 mice were transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 mice or IL-$15^{-/-}$ B6 mice, sacrificed at the time points indicated, and gut (duodenum to rectum) was harvested. IEL were harvested as described in Methods and phenotyped by flow cytometry. Data represent mean ± s.e.m. from 1 of 2 similar experiments. Between 4 and 6 mice were analyzed at each time point.

Figure 19

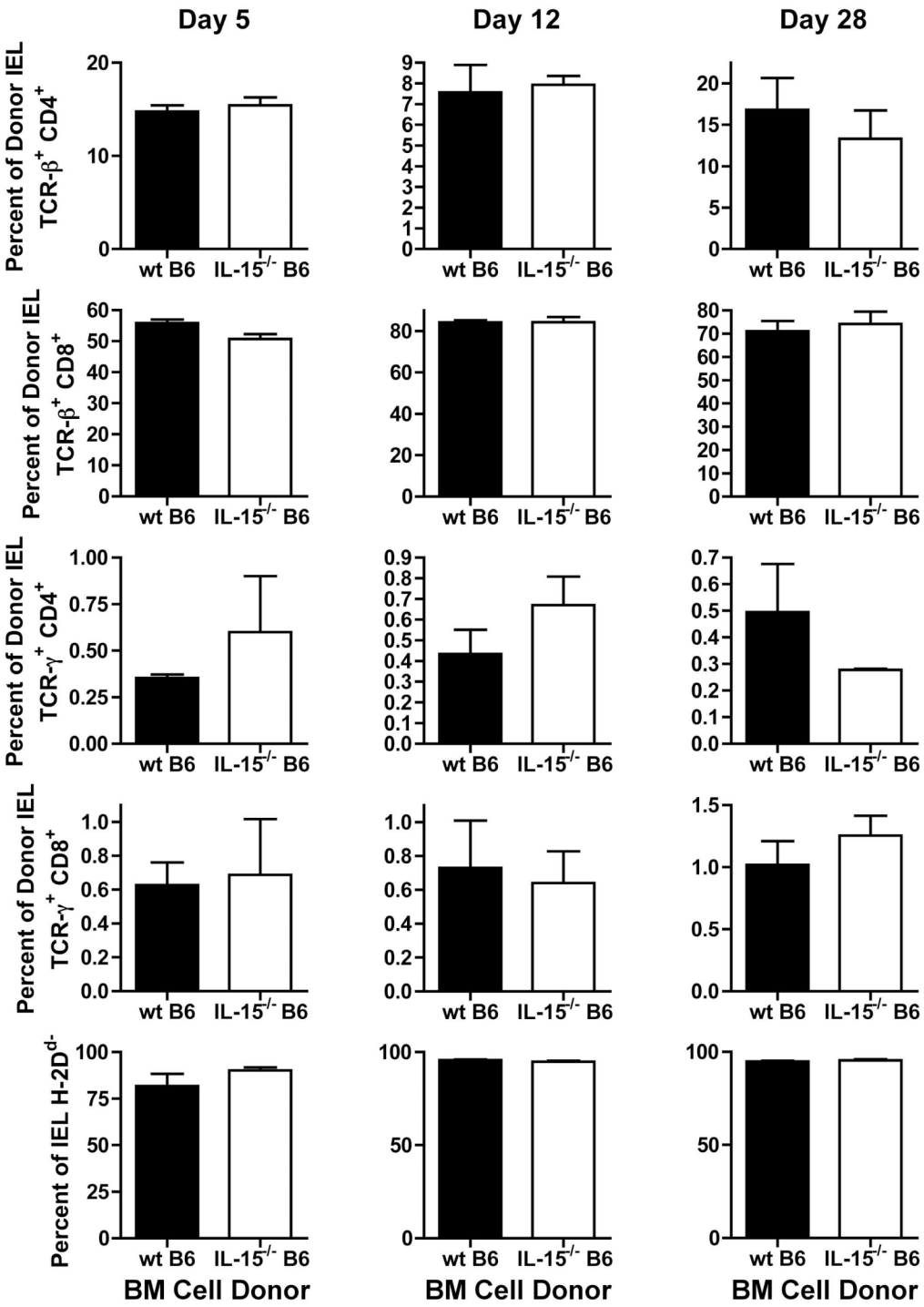

Day 5
Day 12
Day 28
Percent of Donor IEL TCR-β+ CD4+
Percent of Donor IEL TCR-β+ CD8+
Percent of Donor IEL TCR-γ+ CD4+
Percent of Donor IEL TCR-γ+ CD8+
Percent of IEL H-2Dd-
wt B6
IL-15-/- B6
BM Cell Donor

Figure 20. Donor-derived IL-15 is necessary for optimal IFN-γ production and CXCR3 expression by T cells in acute GVHD. (A*)* Wild type B6D2F1 mice were lethally irradiated and transplanted with 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 or IL-$15^{-/-}$ B6 mice. Mice were euthanized 12 days after transplantation, serum harvested, and IFN-γ concentrations measured by cytometric bead array. Data represent mean ± s.e.m. from one of two similar experiments with a total of N=8 mice per group. (**B*)*** Splenocytes were harvested from recipients of wt B6 or IL-$15^{-/-}$ B6 BM cells 5 days after transplantation, gated on $CD4^+$ donor-derived (H-$2Dd^-$) cells and co-stained for cell surface expression of CXCR3. Data represent average (mean ± s.e.m.) mean fluorescence intensity (MFI) values from N=3 mice per group. The experiment was performed twice with similar results. (**C**) Representative flow cytometric plots from allogeneic or syngeneic recipients of wt B6 splenic T cells and either wt B6 BM cells or IL-$15^{-/-}$ B6 BM cells are shown.

Figure 20

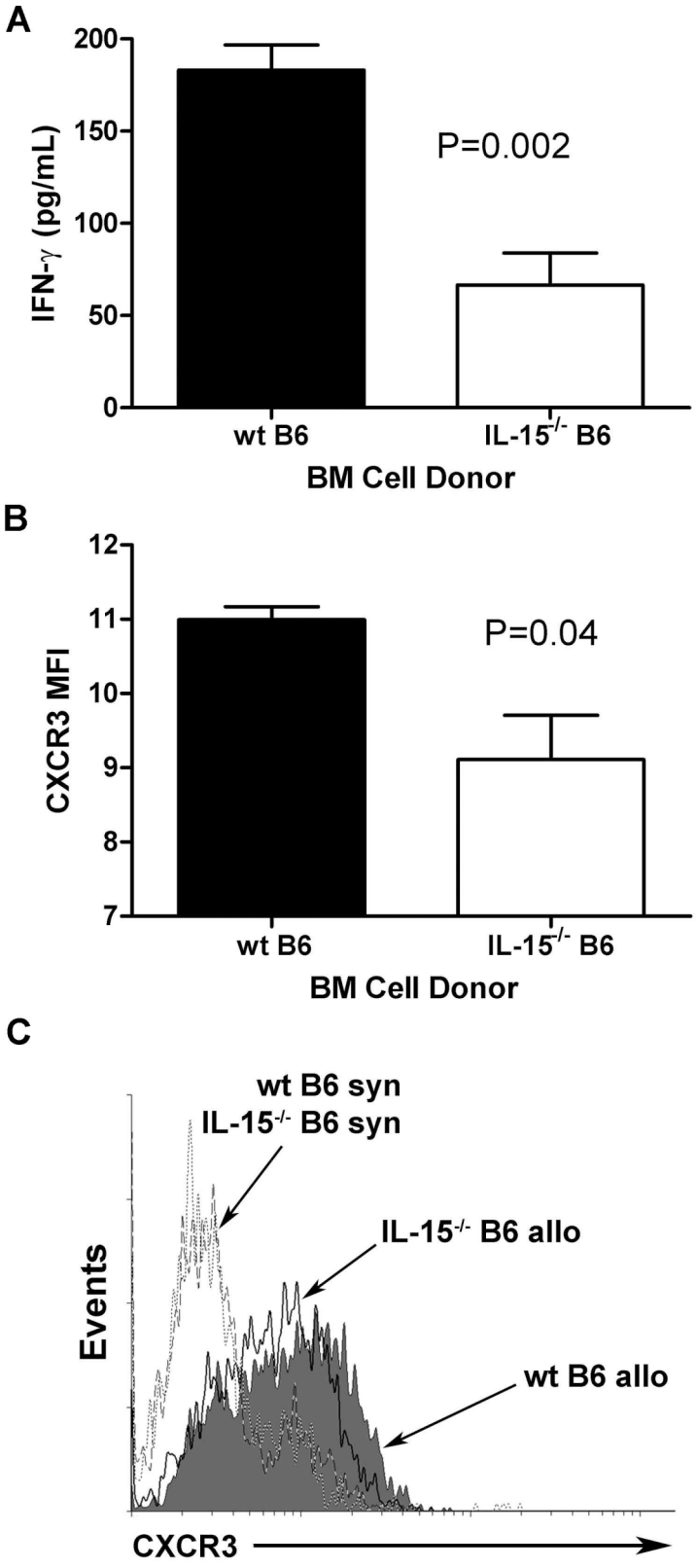

A
200
150
100
50
0
IFN-γ (pg/mL)
P=0.002
wt B6
IL-15-/- B6
BM Cell Donor
B
12
11
10
9
8
7
CXCR3 MFI
P=0.04
wt B6
IL-15-/- B6
BM Cell Donor
C
wt B6 syn
IL-15-/- B6 syn
IL-15-/- B6 allo
wt B6 allo
Events
CXCR3

Figure 21. IL-15-mediated acute GVHD requires expression of T-bet by donor splenocytes. (**A**). Highly purified wt B6 splenic T cells were cultured in the presence of rm IL-15 (10 μg/mL) for the times indicated. Mean fold induction of T-bet relative to time 0 ± s.e.m. is shown. The experiment was performed three times with similar results. (**B**) Peripheral blood mononuclear cells were harvested from 4-6 week old wt B6 or IL-15 tg B6 mice. Expression of T-bet was measured by quantitative real-time PCR. N≥6 mice per group. Data represent mean fold induction of T-bet relative to wt B6 mice ± s.e.m. (**C, D**) Splenocytes were harvested from wt B6 or IL-15 tg B6 mice (N=4 per group) and stained for cell surface expression of CXCR3 (C) or stimulated for 6 hours using immobilized anti-CD3/28 antibodies followed by fixation and staining for intracellular IFN–γ (D). Data represent mean percent positive cells ± s.e.m. (**E**) Lethally irradiated wt B6D2F1 mice were transplanted with 1 x 10^7 wt B6 TCD BM cells and 5 x 10^6 wt B6 unpurified splenic T cells (●, N=9), 1 x 10^7 IL-15 tg B6 TCD BM cells and 5 x 10^6 wt B6 unpurified splenic T cells (□, N=10), or 1 x 10^7 IL-15 tg B6 TCD BM cells and 5 x 10^6 T-bet$^{-/-}$ B6 unpurified splenic T cells (◊, N=7). Survival was monitored daily and was compared using the logrank test. Data are combined from two similar experiments. (**F**) Body weights from animals in **E** were measured, normalized to day 0, and plotted mean ± s.e.m. Groups were compared as described in Methods.

Figure 21

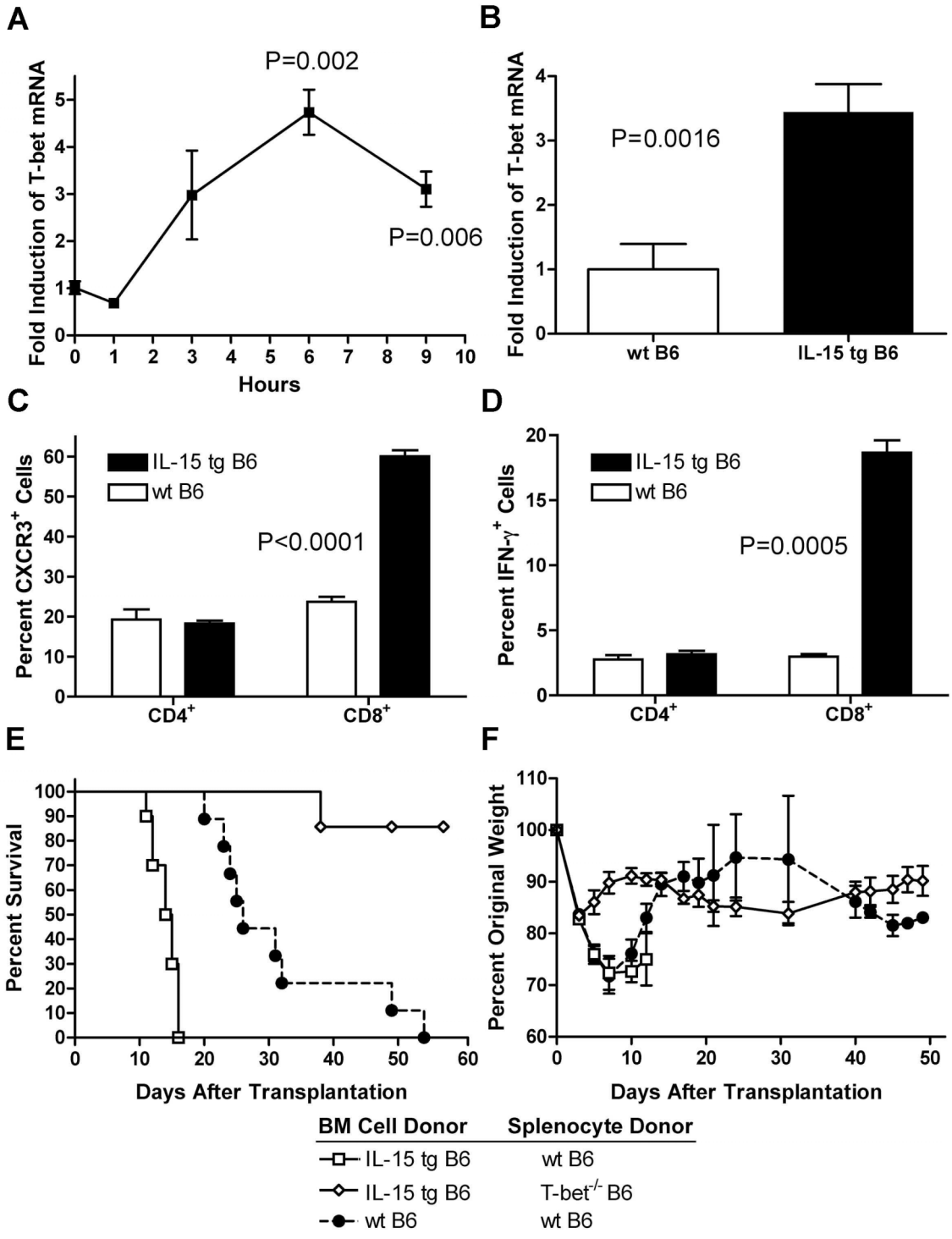
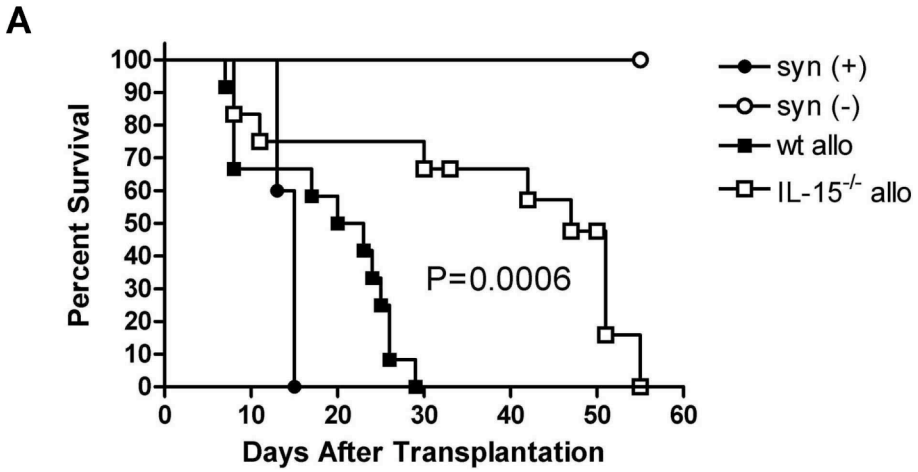

A
P=0.002
P=0.006
Fold Induction of T-bet mRNA
Hours
B
P=0.0016
Fold Induction of T-bet mRNA
wt B6
IL-15 tg B6
C
IL-15 tg B6
wt B6
P<0.0001
Percent CXCR3+ Cells
CD4+
CD8+
D
IL-15 tg B6
wt B6
P=0.0005
Percent IFN-γ+ Cells
CD4+
CD8+
E
Percent Survival
Days After Transplantation
F
Percent Original Weight
Days After Transplantation
BM Cell Donor
Splenocyte Donor
IL-15 tg B6
wt B6
IL-15 tg B6
T-bet-/- B6
wt B6
wt B6

Figure 22. GVT activity is preserved in the absence of donor-derived IL-15. (**A**). Lethally irradiated wt B6D2F1 mice received 2000 log-phase P815 cells, 5 x 10^6 wt B6 splenic T cells and 1 x 10^7 TCD BM cells from either wt B6 (wt allo, ■, N=12) or IL-$15^{-/-}$ B6 (IL-$15^{-/-}$ allo, □, N=12) mice. As controls, identical mice received 5 x 10^6 wt B6D2F1 (syngeneic) T cells and 1 x 10^7 wt B6D2F1 TCD BMC plus (syn (+), ●, N=5) or minus (syn (-), ○, N=5) 2000 P815 cells. Animals were monitored daily after transplantation and survival times were compared using the logrank test. Results are combined from three similar experiments. (**B**). Livers were harvested from representative recipients in the above experiment. Paraffin sections were stained with toluidine blue. Slides were reviewed by a board certified veterinary pathologist in a blinded fashion. Tumor rests are indicated by the white vertical arrows. Magnification=40x.

Figure 22


A
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Days After Transplantation
P=0.0006
syn (+)
syn (-)
wt allo
IL-15-/- allo

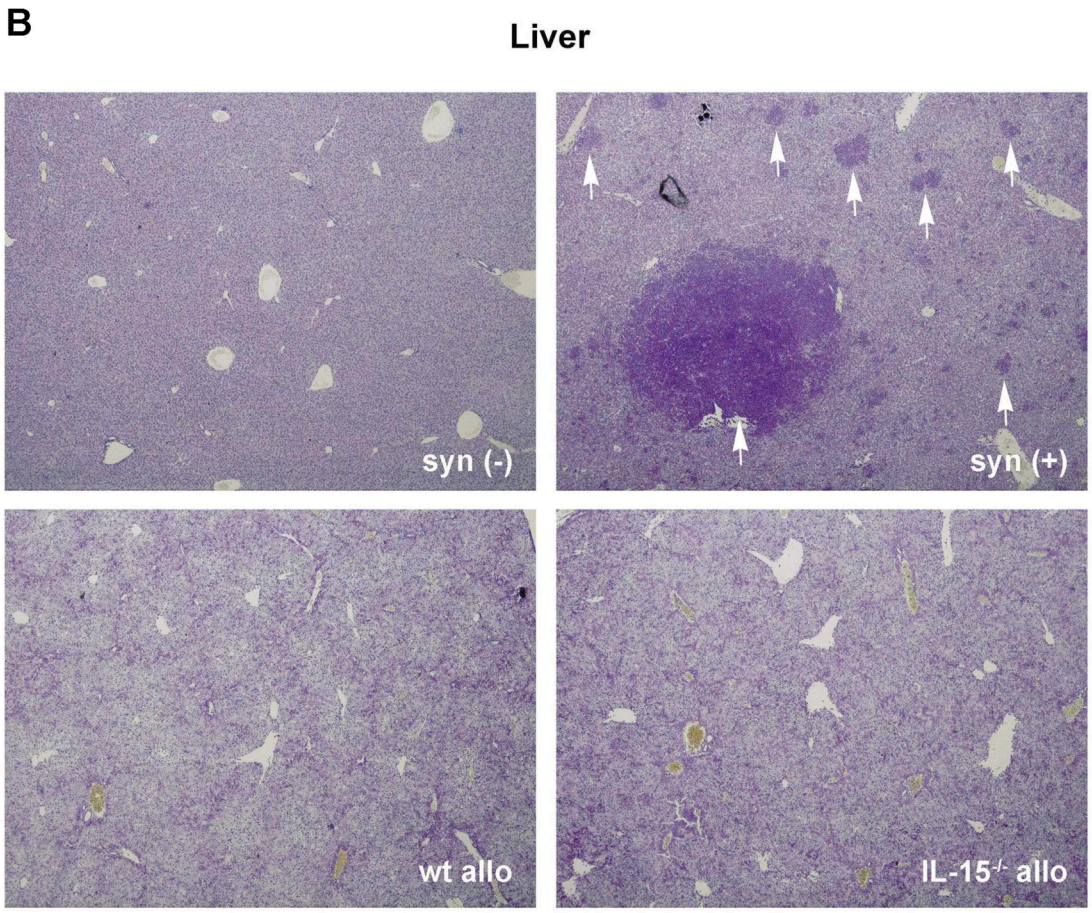

B
Liver
syn (-)
syn (+)
wt allo
IL-15-/- allo

Table 2. Flow cytometric analysis of IL-15-pretreated IL-15$^{-/-}$ B6 BM cell donors. Donor mice were pretreated with rh IL-15 or PBS as described in the text. Values represent mean percent of bone marrow cells that were CD11b^{+}GR-1^{+} monocytes/granulocytes, CD11c^{+}B220^{+} plasmacytoid dendritic cells, CD11c^{+}B220^{-} myeloid dendritic cells, CD11c^{-}B220^{+} B cells or NK1.1^{+}CD3^{-} NK cells. P values (in parentheses) were calculated using Student's t-test for comparisons between wt B6 and IL-15- or control-treated IL-15$^{-/-}$ B6 donors. N=10 donors per group.

Table 2

Percent Bone Marrow Cells (P value)

BM Cell Donor	WT B6	IL-15$^{-/-}$ B6	IL-15$^{-/-}$ B6
Pre-Treatment	PBS	PBS	rhIL-15
Monocytes/Granulocytes	31.7	31.3 (0.76)	29.3 (0.16)
Myeloid Dendritic Cells	1.84	1.28 (0.1)	2.01 (0.41)
Plasmacytoid Dendritic Cells	2.72	2.26 (0.009)	2.63 (0.74)
B Lymphocytes	22.3	25.9 (0.12)	18.6 (0.04)
Natural Killer Cells	1.47	0.16 (<0.0001)	5.5 (<0.0001)

CHAPTER 4

IL-15 but not IL-2 Rapidly Induces Lethal Xenogeneic Graft Versus Host Disease

4.1 Introduction

Xenogeneic graft versus host disease (X-GVHD) is the only experimental model of human allogeneic GVHD that incorporates human T cells and recapitulates the T cell expansion and tissue destruction seen in patients. Severe combined immunodeficient (SCID) mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID) demonstrate, albeit infrequently, lethal X-GVHD associated with high levels of human T cell engraftment and inflammation of the liver, kidneys, spleen and lung (178). We have previously demonstrated that administration of continuous, low dose rh IL-2 decreases human T cell engraftment in hu-PBL-SCID mice although how this affects X-GVHD mortality is unknown (179). Recently, data from our laboratory and others have shown that endogenous or exogenous administration of a structurally and functionally related cytokine, IL-15, increases T cell engraftment and mortality in murine models of acute allogeneic GVHD (154, 155). Therefore, we have hypothesized that exogenous rh IL-15 would enhance human T cell engraftment and X-GVHD mortality in hu-PBL-SCID mice in contrast to the effects of rh IL-2.

4.2 Study Design

Hu-PBL-SCID mouse model

Human peripheral blood lymphocytes (PBL) were obtained from healthy donors under an IRB-approved protocol and transferred via intraperitoneal injection to SCID mice pretreated with murine IL-2Rβ antibodies as described (179, 180). Each mouse received 5 x 10^7 viable human PBL. Mice were euthanized when they developed symptoms of weight loss, lethargy, dehydration, and dyspnea. All animal research was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Cytokines

Animals were dosed with 10 μg/day of rh IL-15 (Amgen, Inc., Thousand Oaks, CA), rh IL-2 (Proleukin, Chiron Corporation, Emeryville, CA) or PBS containing 0.05% human albumin via subcutaneous Alzet mini-osmotic pumps (Model 1007D, DURECT Corporation, Cupertino, CA) beginning one day after injection of human PBL.

In vivo antibody treatment

Animals were treated with anti-human CD3 (50 μg/mouse, Orthoclone-OKT3, Ortho Biotech Products, Raritan, NJ) or control mouse IgG (Sigma, St. Louis, MO) via intraperitoneal injection on days 1 through 5 after injection of human PBL (181).

Histopathology

Blinded histologic samples were prepared as described, reviewed by a board-certified veterinary pathologist, and scored for lymphocytic infiltration and tissue damage characteristic of X-GVHD (154, 182).

Cytokine bead array

The Human Th1/Th2 Cytokine Bead Array (BD Pharmingen, San Diego, CA) was used to measure IL-4, IL-5, IL-10, IFN-γ, and TNF-α in plasma samples as described for mice (154).

Statistics

Median survival times were compared using the log rank test. Student's t-test and the Exact Wilcoxon's Rank Sum test were used for the remaining comparisons.

4.3 Results and Discussion

Administration of rh IL-15 to hu-PBL-SCID mice resulted in rapid morbidity and mortality compared to IL-2 (Figure 23A, MST 8.4 days versus 25.0 days, P=0.005) or PBS control treatment (Figure 23A, MST 8.4 days versus 30.7 days, P<.0001). No statistically significant survival difference was observed between IL-2- and PBS-treated groups. Consistent with earlier reports, morbid mice developed hunched posture, inactivity, and labored breathing (178). Control SCID mice that received rh IL-15 (10 μg/day) but no human PBL displayed 100% survival (data not shown). Blinded analysis of histopathology in mice treated with rh IL-15 revealed an increase in lymphocytic

infiltrates in liver, kidney, spleen and lung compared to PBS-treated mice (Figure 23B, P=0.008, P=0.05, P=0.05, and P=0.03, respectively). Tissue infiltrates were minimal to absent in IL-15-treated animals that did not receive hu-PBL (data not shown). Early mortality in IL-15-treated mice was associated with elevations in serum human IFN-γ and IL-5 on day 10 after inoculation with human PBL compared to PBS-treated mice (Figure 23C). Differences in serum human IL-10, IL-4 and TNF-α levels were not observed. Finally, *in vitro* stimulation of splenocytes isolated from IL-15-treated mice with rh IL-15 and/or anti-human CD3/CD28 cross-linking beads resulted in significantly higher levels of IFN-γ compared to similarly cultured splenocytes from PBS-treated control mice (Figure 23D).

Analysis of chimerism in hu-PBL-SCID mice at day 10 post-engraftment by flow cytometry revealed a significant increase in the absolute number of human T cells (Figure 24A, P=0.02), human $CD4^+$ T cells (Figure 24A, P=0.043), and human $CD8^+$ T cells (Figure 24A, P=0.012) in the spleens of mice receiving rh IL-15 compared with mice receiving PBS. Consistent with the role of IL-15 in memory $CD8^+$ T cell activation and homeostasis, $CD8^+$ human T cells in IL-15-treated mice displayed memory-activated phenotypes with expression of CD25, CD69, CD45RA(dim) and CD45RO (Figure 24B, representative plots) (107, 183). Given the significant expansion of human T cells with IL-15 treatment, we hypothesized that *in vivo* depletion of human T cells would protect these mice from X-GVHD mortality. In fact, treatment of hu-PBL-SCID mice with mouse anti-human CD3 (OKT3) depleting antibody prevented IL-15-associated mortality compared to mice treated with rh IL-15 and control antibody (Figure 24C, P=0.003). Animals treated with PBS and OKT3 also had 100% survival (Figure 24C).

To our knowledge, these experiments are the first to demonstrate that *in vivo* administration of rh IL-15 potently activates and expands pathogenic human T lymphocytes with the capacity to induce X-GVHD. Remarkably, 100% of 27 mice tested in 5 independent experiments developed lethal X-GVHD. This finding is in contrast to the historically low incidence of X-GVHD in the hu-PBL-SCID mouse which is fundamentally due to low T cell chimerism (178, 184). Consistent with earlier reports demonstrating increased human T cell infiltration in xenografted synovial tissue from patients with rheumatoid arthritis, rh IL-15 increased human T cell infiltration in the target organs of X-GVHD (153, 185). Our results also show that IL-15-mediated X-GVHD can be prevented by complete T cell depletion. These data support the recent findings of Alpdogan et al. that exogenous IL-15 can be safely given in murine models of T cell-depleted allogeneic BMT (155). We have now extended these findings to an *in vivo* xenogeneic model that incorporates elements of a competent human immune system.

IL-2 and IL-15 are structurally related cytokines that share receptor components and display similar activity *in vitro* (97, 133). However, the effect of IL-2 and IL-15 on T cells differ strikingly *in vivo*. IL-15$^{-/-}$ mice demonstrate profound lymphopenia while IL-2$^{-/-}$ mice develop polyclonal T cell expansions and lethal autoimmune disease, likely due to an absence of activation induced cell death (91, 186). IL-15 transgenic mice that overexpress a highly secreted form of IL-15 show polyclonal expansions of memory CD8^{+} T cells while IL-2 transgenic mice demonstrate normal T cell populations (103, 187). Our results here show that administration of stoichiometrically equivalent doses of rh IL-2 and rh IL-15 results in significantly different survival rates of hu-PBL-SCID mice and extend the dichotomy of IL-2 and IL-15 function to a model of human GVHD.

The significant increase in human IFN-γ production *in vivo* seen in mice treated with rh IL-15 may provide one explanation for the rapid onset of X-GVHD seen in this model. IFN-γ is elevated in acute allogeneic GVHD while murine IFN-$\gamma^{-/-}$ donors fail to induce acute GVHD in allogeneic recipients (168, 169). Additional murine studies have shown that IFN-γ directly alters the barrier function of intestinal epithelial cells, increasing LPS release into the bloodstream (188). Finally, IFN-γ primes macrophages for the production of TNF-α and increases expression of MHC and co-stimulatory molecules by antigen presenting cells, enhancing T cell activation (21, 166, 189).

The results shown here demonstrate that xenografted human T cells respond to human IL-15 by expansion, activation, and tissue damage in SCID mice. As such they strengthen the importance of murine transplantation models and further provide caution against the use of rh IL-15 as an immune adjuvant after T cell-replete allogeneic BMT.

Figure 23. Recombinant human IL-15 increases X-GVHD mortality, morbidity, and serum IFN-γ. (**A**) Hu-PBL-SCID mice treated with rh IL-15 demonstrated a median survival time of 8.4 days compared to 25 days for mice treated with rh IL-2 ($P=0.005$) and 30.7 days for mice treated with PBS ($P<0.0001$). There was no statistically significant difference in survival between IL-2- and PBS-treated mice. (**B**) Blinded analysis of X-GVHD histopathology revealed a significant increase in mononuclear cell infiltration and tissue damage in the livers ($P=0.008$), kidneys ($P=.05$), spleens ($P=0.05$) and lungs ($P=0.03$) of IL-15-treated mice compared with PBS treated mice. (**C**) Serum was harvested from hu-PBL-SCID mice at day 10 and analyzed for the presence of human cytokines. Mice treated with rh IL-15 demonstrated increased serum human IFN-γ ($P=0.007$) and IL-5 ($P=0.02$) compared to PBS-treated mice. (**D**) Splenocytes were harvested from hu-PBL-SCID mice treated with either IL-15 or PBS and cultured (2×10^6/mL) in 24-well plates for 48 hours in the presence of anti-CD3/CD28 beads, rh IL-15 (5 ng/mL), both, or media alone. Human IFN-γ was measured in culture supernatants by cytometric bead array. Survival data were compared using the log rank test and all other data were compared using Student's t-test or the Exact Wilcoxon's Rank Sum test.

Figure 23

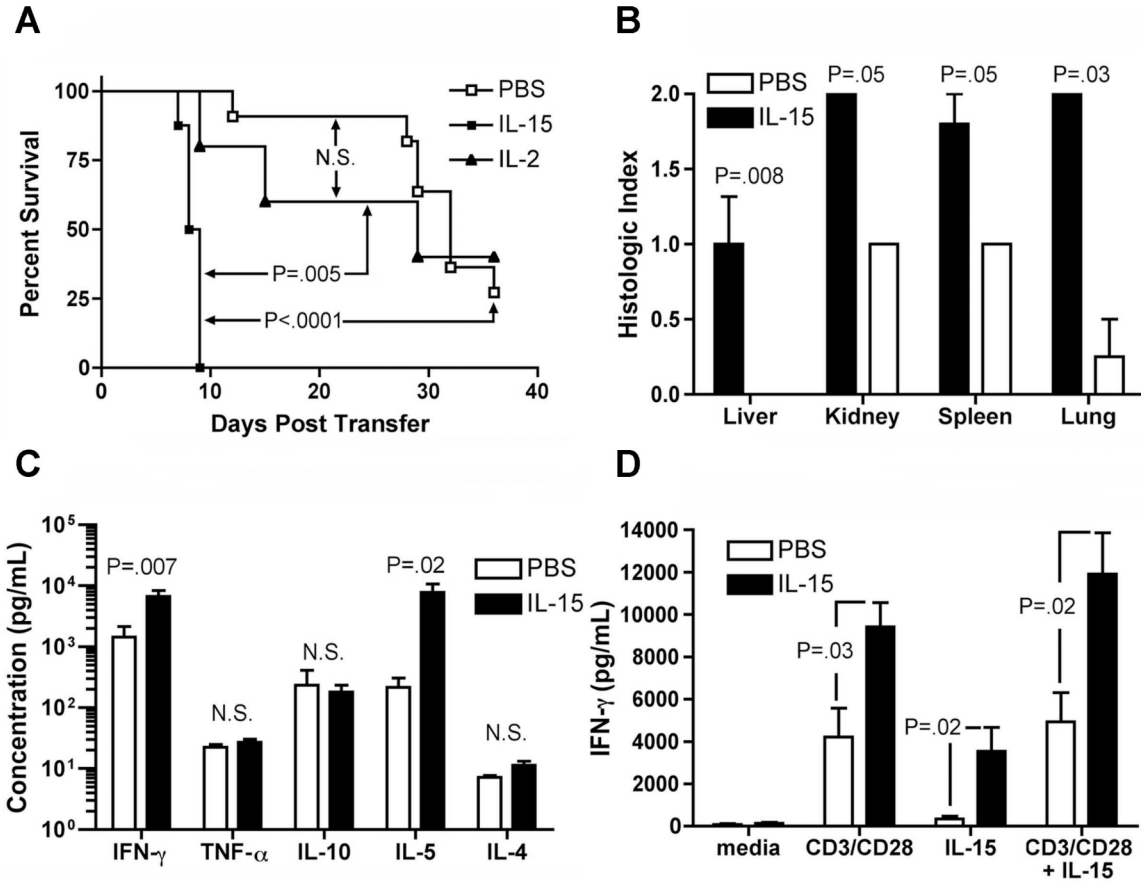
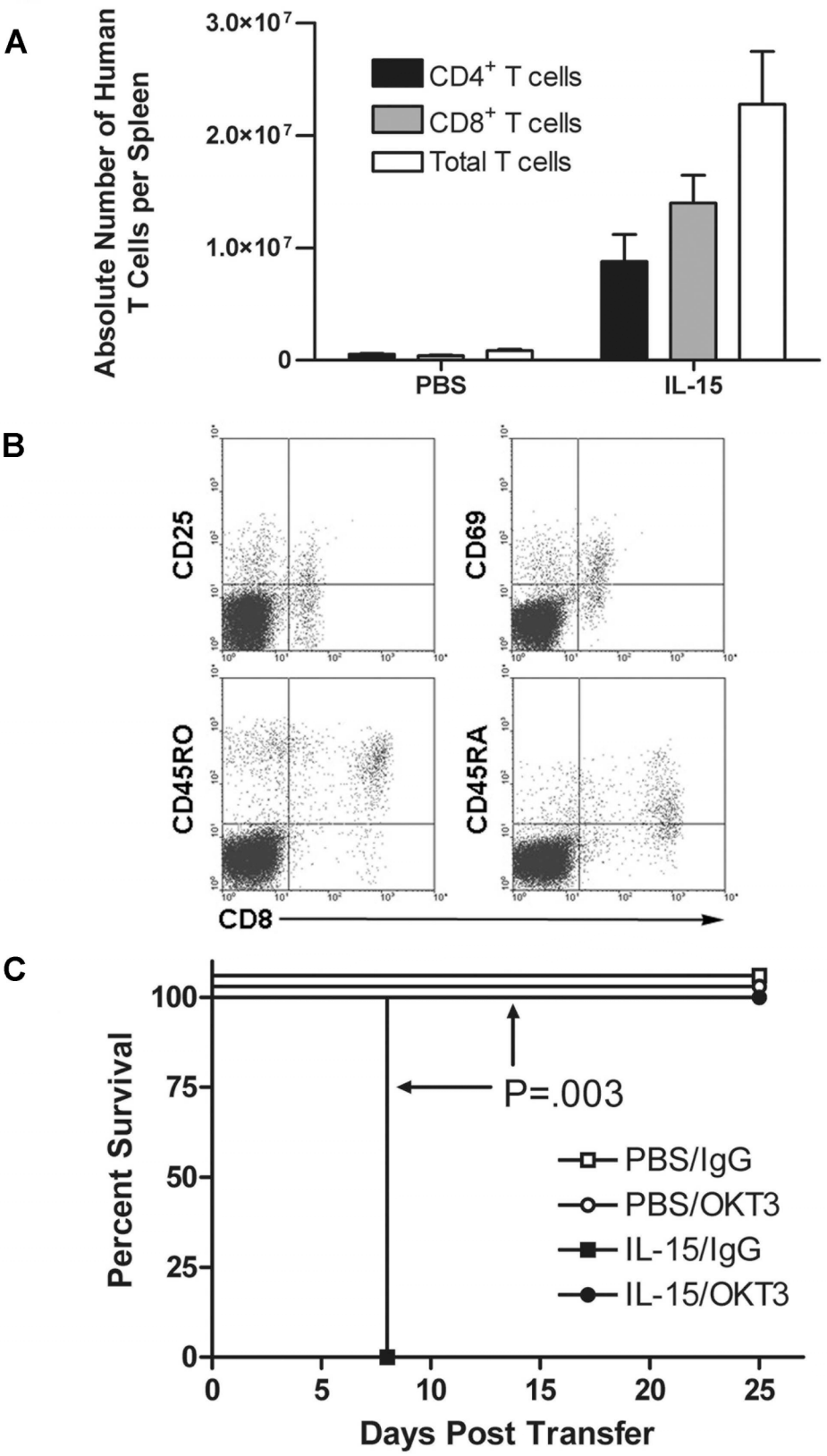

A
Percent Survival
Days Post Transfer
PBS
IL-15
IL-2
N.S.
P=.005
P<.0001
B
Histologic Index
PBS
IL-15
P=.008
P=.05
P=.05
P=.03
Liver
Kidney
Spleen
Lung
C
Concentration (pg/mL)
P=.007
N.S.
N.S.
P=.02
N.S.
PBS
IL-15
IFN-γ
TNF-α
IL-10
IL-5
IL-4
D
IFN-γ (pg/mL)
PBS
IL-15
P=.03
P=.02
P=.02
media
CD3/CD28
IL-15
CD3/CD28 + IL-15

Figure 24. IL-15 increases T cell engraftment in hu-PBL-SCID mice. (**A**) Spleens were harvested from hu-PBL-SCID mice treated with either rh IL-15 or PBS for 10 days. Mice receiving IL-15 demonstrated significantly higher engraftment with respect to the absolute number of both $CD4^+$ and $CD8^+$ T cells. (**B**) $CD8^+$ T cells demonstrated an activated memory phenotype. Data from one representative mouse is shown. (**C**) Hu-PBL-SCID mice were treated with either PBS or rh IL-15 and then each group was randomized for *in vivo* human T cell depletion with OKT3 antibody or control IgG. Depletion of human T cells completely abrogated IL-15-mediated X-GVHD lethality (P=0.003). Survival data were compared using the log rank test and all other data was compared using Student's t-test.

Figure 24


A
Absolute Number of Human T Cells per Spleen
3.0×10^7
2.0×10^7
1.0×10^7
0
CD4+ T cells
CD8+ T cells
Total T cells
PBS
IL-15
B
CD25
CD69
CD45RO
CD45RA
CD8
C
Percent Survival
100
75
50
25
0
P=.003
PBS/IgG
PBS/OKT3
IL-15/IgG
IL-15/OKT3
0
5
10
15
20
25
Days Post Transfer

CHAPTER 5

Deregulation of Endogenous IL-15 Promotes NK Cell Expansion and Graft Rejection in a Murine Model of Allogeneic Bone Marrow Transplantation

5.1 Introduction

We and others have demonstrated a role for IL-15 in acute graft versus host disease (154, 155). Deregulation of endogenous, donor-derived IL-15 expands alloreactive memory $CD8^+$ T cells and increases morbidity and mortality, consistent with the known function of this cytokine (154). Yet allogeneic bone marrow transplantation is a complex interaction of the donor immune system which can respond to host alloantigens and cause acute GVHD and the host immune system which may, through multiple mechanisms, attempt to reject donor-derived cells (190, 191). Although the host immune system is depleted over time by the transplant conditioning regimen and by the direct action of donor T cells, deregulation of endogenous IL-15 may provide additional growth factor support for host elements and enhance graft rejection. Graft rejection may become more significant as the number of donor T cells in the allograft is reduced.

NK cells are at the same time exquisitely dependent upon IL-15 as a growth factor and poised to reject foreign cells (71, 91). NK cells from lethally irradiated F1 hybrid mice retain the ability to reject transplantation of parental type bone marrow cells because the parental type cells do not express both MHC alleles present in the hybrid.

This observation, termed "hybrid resistance" was first noted by Kumar et al. and formed the basis for the "missing-self hypothesis" of NK cell activation (71). Here we have hypothesized that deregulation of endogenous IL-15 production after allogeneic BMT would increase hybrid resistance in a transplantation model where donor T cells are present in limited numbers.

5.2 Study Design

Mice and bone marrow transplantation

Eight to twelve week old female B6 and B6D2F1 mice were purchased from Jackson Laboratories. IL-15 tg B6 mice expressing a highly secreted IL-15 protein using the H-2D promoter were created as described and maintained in a breeding colony at The Ohio State University (103). The bone marrow transplantation protocol used here has been described in principle elsewhere (154). Briefly, bone marrow cells were harvested from wt B6 and IL-15 tg B6 mice, T cell depleted, and infused at the indicated doses into lethally irradiated (1300 cGy) wt B6 or wt B6D2F1 recipient mice.

Reagents

Monoclonal antibodies for flow cytometry were purchased from BD Pharmingen. Magnetic beads and columns were purchased from Miltenyi Biotec.

Histology

Sterna were harvested from moribund mice and preserved in 10% neutral buffered formalin prior to paraffin embedding and staining with hematoxylin and eosin. Images were captured as described previously (154).

Statistics

Survival times were compared using the logrank test. For all other data, Student's two-tailed t test was used. $P \leq 0.05$ was considered statistically significant.

5.3 Results and Discussion

Lethally irradiated wild type (wt) B6D2F1 mice received 2 x 10^6 wt B6 unpurified splenocytes and 5 x 10^6 T cell depleted bone marrow (TCD BM) cells from either wt B6 or IL-15 tg B6 mice. Recipients of wt TCD BM cells demonstrated 100% survival greater than 70 days after transplantation with no evidence of acute GVHD whereas recipients of IL-15 tg TCD BM cells had an MST of 25 days (P=0.0021, Figure 25A). Syngeneic recipients of wt B6 splenocytes and wt or IL-15 tg B6 TCD BM cells demonstrated 100% survival suggesting that death in allogeneic recipients of IL-15 tg TCD BM was not due to an intrinsic failure of the cells to home or engraft in the marrow. Allogeneic recipients of IL-15 tg TCD BM cells were sacrificed when moribund and liver, skin, gut and sterna were harvested. Compared to normal controls, blinded analysis of histopathology revealed diffuse marked hypoplasia of sternal bone marrow (Figure 25B). In contrast, there was no evidence of acute GVHD histopathology in the liver, skin or gut (data not shown).

We hypothesized that deregulation of endogenous IL-15 production after transplantation would expand NK cells. At 15 days after transplantation, recipients of wt B6 splenocytes and IL-15 tg B6 TCD BM cells demonstrated a significantly increased percentage of $NK1.1^{+}$ $CD4^{-}$ $CD8^{-}$ NK cells in the spleen compared to recipients of wt B6 splenocytes and wt B6 TCD BM cells (allogeneic: 0.64±0.04% versus 0.16±0.02%, P=0.0005, syngeneic: 8.6±0.2% versus 3.6±0.1%, P=0.01, Figure 26). While it is not yet clear whether these data represent expansions in donor or host NK, nor is it clear whether the expanded NK are responsible in whole or in part for the rejection of the donor allograft, future phenotyping and lymphocyte depletion studies will address these issues.

NK cell activity is primarily inhibited by interaction of MHC-I with inhibitory receptors expressed on the NK cell: the Ly-49 family in the mouse and KIR in man (59, 192). In order to maintain self-tolerance, NK cells must express at least one inhibitory receptor (56). Cytolytic function and cytokine production is permitted when a given NK cell is faced with targets that do not engage its repertoire of inhibitory receptors. In the hybrid resistance model, a subset of hybrid recipient NK cells express inhibitory receptors only for one of the parental MHC alleles; these NK cells are activated in the absence of that allele on bone marrow cells from the other parental strain (71). It is also very likely that a battery of activating receptors contribute to the activity of NK cells in hybrid resistance. Recently, the activating receptor NKG2D was shown to contribute critically to the lysis of parental bone marrow cells by F1 hybrid NK cells (193).

Although the missing-self hypothesis and the emerging role of activating receptors have identified a great deal of the mechanism behind hybrid resistance, comparatively less is known about the cytokine control of the process. IL-15 is a

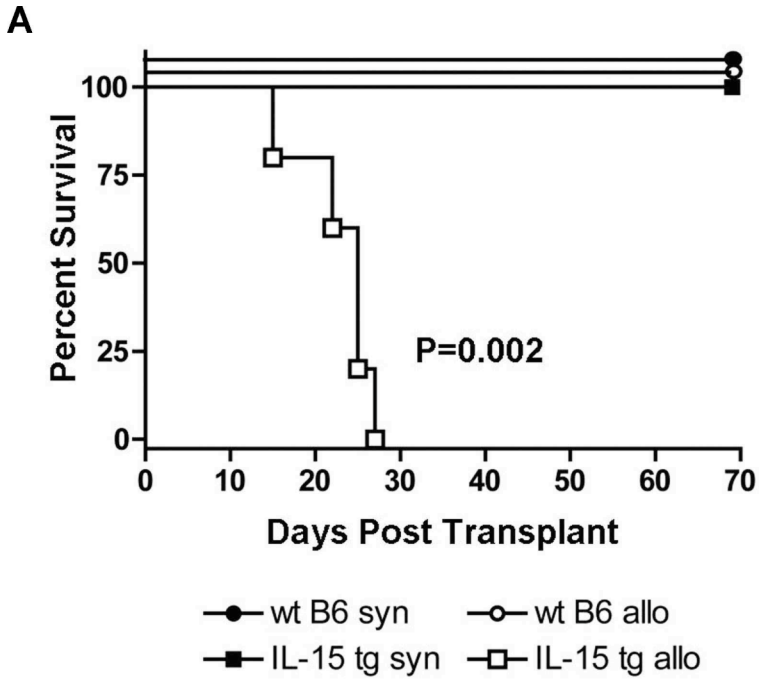
cytokine that is critical for development, survival and proliferation of NK cells *in vitro* and *in vivo*, but also contributes to NK cell activation (90, 91, 194). As a result, we have hypothesized that deregulation of IL-15 would increase NK-mediated graft rejection in the B6→B6D2F1 model of hybrid resistance. Our data show a dramatic increase in severe graft rejection in the presence of elevated expression of endogenous IL-15. These data are consistent with early findings that administration of IFN-α/β to lethally irradiated F1 mice increased hybrid resistance (195). IFN-α/β has subsequently been found to be a potent inducer of IL-15 production *in vivo* (107).

Administration of exogenous IL-15 to lethally irradiated mice has been shown to be an effective means to enhance immune reconstitution by donor T cells in an MHC-matched model of T cell depleted allogeneic BMT (155). Nevertheless, no NK alloreactivity is predicted by the missing self hypothesis in this model. The data presented here argue against this application of IL-15 in donor-recipient pairs where NK alloreactivity in the host versus graft direction is predicted. Enhancement of NK mediated graft rejection by exogenous IL-15 may be particularly problematic when conditions already favor rejection such as when a limited dose of post-thymic donor T cells is contained in the allograft or when non-myeloablative conditioning regimens are used (14).

With our previous studies describing a role for IL-15 in T cell mediated acute GVHD, these data showing an enhancement of graft rejection highlight the pleiotropic nature of IL-15 and hint at the complexity of cytokine signaling in allogeneic BMT. Nevertheless, it is becoming clear that IL-15 serves to narrow the window of beneficial cross-tolerance between donor and host which is the ultimate goal of clinical BMT.

Figure 25. Deregulation of endogenous IL-15 promotes graft rejection and early mortality after allogeneic BMT. (**A**) Allogeneic recipients of IL-15 tg B6 BM cells and wt B6 splenocytes had significantly shorter survival after transplantation when compared with allogeneic recipients of wt B6 BM cells and wt B6 splenocytes (MST 25 days versus 100% survival, P=0.002). Syngeneic recipients of wt or IL-15 tg B6 BM cells and wt B6 splenocytes demonstrated 100% survival. (**B**) Representative allogeneic recipients of IL-15 tg B6 BM cells were sacrificed when moribund and sterna harvested. Blinded analysis of histopathology demonstrated marked diffuse hypoplasia of the sternal bone marrow in these animals.

Figure 25


A
Percent Survival
100
75
50
25
0
0
10
20
30
40
50
60
70
Days Post Transplant
P=0.002
wt B6 syn
wt B6 allo
IL-15 tg syn
IL-15 tg allo

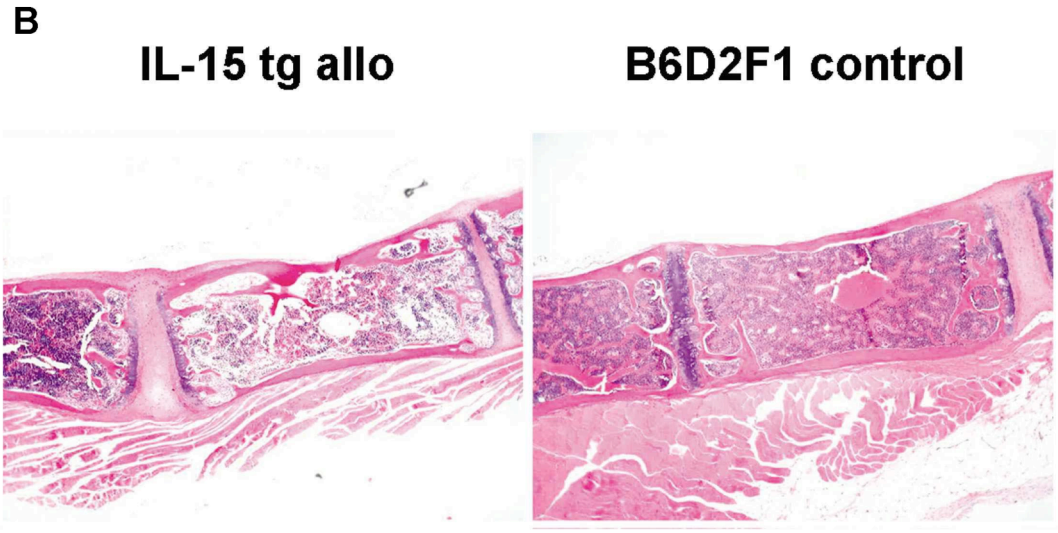
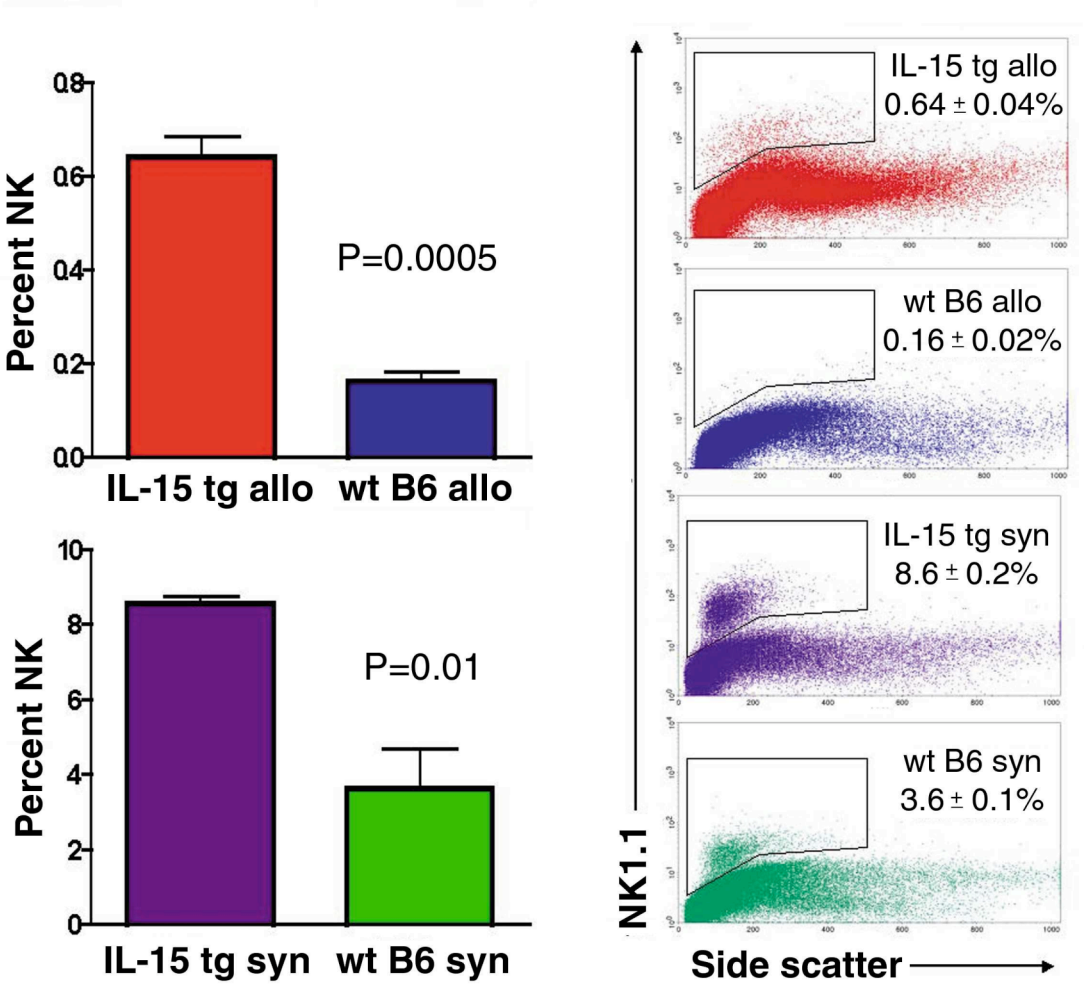

B
IL-15 tg allo
B6D2F1 control

Figure 26. Deregulation of endogenous IL-15 expands NK cells after allogeneic BMT. Allogeneic and syngeneic recipients of wt B6 splenocytes and either wt or IL-15 tg B6 BM cells were sacrificed 15 days after transplantation and spleens harvested. Histograms representing N=4 mice per group (mean ± s.e.m., left panels) and flow cytometric dot plots from representative mice (right panels) are shown.

Figure 26


Percent NK
0.8
0.6
0.4
0.2
0.0
P=0.0005
IL-15 tg allo
wt B6 allo
Percent NK
10
8
6
4
2
0
P=0.01
IL-15 tg syn
wt B6 syn
IL-15 tg allo
0.64 ± 0.04%
wt B6 allo
0.16 ± 0.02%
IL-15 tg syn
8.6 ± 0.2%
wt B6 syn
3.6 ± 0.1%
NK1.1
Side scatter

CHAPTER 6

A Novel Anti-KIR Antibody Increases NK Cytolytic Function Against Autologous Tumor

6.1 Introduction

Haploidentical HSCT is an emerging modality in the treatment of AML that relies on multiple mismatches between donor and host to generate NK cell graft versus leukemia activity (73). Graft rejection and graft versus host disease are avoided by an intense preparative regimen, high doses of transplanted stem cells and rigorous T cell depletion. While haploidentical HSCT has had success, there remains up to a 54% rate of transplant related mortality and as a result, it is both risky and not suitable for elderly patients (196).

We have exploited this mechanism of NK cell activation in the hopes of bringing the benefits of haploidentical HSCT to patients without the inherent risks of transplantation. Interaction of self MHC molecules with KIR is the primary means by which NK cell activity is inhibited; in haploidentical HSCT, KIR on donor-derived NK cells fail to recognize allogeneic MHC and are activated against host targets (74, 77). In collaboration with NovoNordisk, we have developed a novel, fully human antibody designated KIR1-7F9 intended to block the normal inhibitory signals delivered by KIR. Here, we show that KIR1-7F9 binds to NK cells harvested from human patients with

AML and multiple myeloma. Further, we show that this antibody increases both natural cytotoxicity against autologous AML blasts and antibody dependent cellular cytotoxicity (ADCC) against autologous chronic lymphocytic leukemia (CLL) tumor cells but not normal hematopoietic progenitor cells. Together, these data strongly support the further evaluation of KIR1-7F9 as an alternative to HSCT in both myeloid and lymphoid malignancies.

6.2 Study Design

Generation of KIR1-7F9

KIR1-7F9 was generated by NovoNordisk by immunization of a proprietary mouse strain carrying the full human Ig locus knocked-in to the mouse Ig locus. Hybridoma clones were made using standard techniques and supernatants screened for KIR binding and enhancement of natural cytotoxicity. Large scale preparations of KIR1-7F9 were made according to GMP standards.

Sample procurement

Fresh samples were procured from informed and consented individuals according to IRB protocol. PBMC and bone marrow mononuclear cells were obtained by density-gradient centrifugation. Cryopreserved PBMC and tumor were obtained from the cancer and leukemia group B (CALGB) tissue bank at The Ohio State University according to IRB protocol.

NK cell expansion

Due to the limited numbers of cyropreserved PBMC from AML patients available for use, before performing the cytotoxicity assays NK cells from these patients were expanded *in vitro* according to the method of Velardi (197). Briefly, NK cells were seeded on day 0 at 40 cells per well with 1.2 x 10^5 irradiated PBMC feeder cells in medium containing PHA. IL-2 (500 U/mL) was added on day +1 and cells were expanded for up to 2 months.

Natural cytotoxicity assays.

A flow cytometry-based cytotoxicity assay was used for these studies (198). Targets were labeled with the membrane dye PKH-26 according to the manufacturer's instructions (Sigma, Pittsburgh PA). Targets and effectors were co-incubated in v-bottom plates for 4 hours and then placed on ice. 10 uL of the viability dye To-Pro3 iodide (1:10,000 dilution, Molecular Probes, Eugene OR) were added to each well immediately prior to flow cytometric analysis. Percent specific lysis (P.S.L.) was determined as described (198).

ADCC assays

CLL tumor samples were thawed and cultured overnight prior to PKH-26 labeling as described above. Freshly isolated PBMC were mixed with target cells at various ratios as for natural cytotoxicity assays. KIR1-7F9, control $hIgG_4$, Rituximab and/or Herceptin were added at 30 μg/mL final concentration at the beginning of incubation. Detection of dead cells and calculation of P.S.L. was performed as for natural cytotoxicity assays.

6.3 Results and Discussion

PBMC were collected from AML patients in remission and multiple myeloma patients currently undergoing standard treatment and were analyzed for binding of KIR1-7F9 by flow cytometry. $CD56^+$ $CD3^-$ total NK cells from AML patients and multiple myeloma patients were positive for binding of KIR1-7F9 (representative flow plots from 4 patients and 1 normal donor, Figure 27). In agreement with previous findings, the majority of KIR expression was observed in the major cytolytic subset of $CD56^{dim}$ NK cells (58). When added to natural cytotoxicity assays using *in vitro* expanded NK cells as effectors and autologous AML tumor cells obtained at diagnosis as targets, KIR1-7F9 was highly bound by effector cells and significantly improved tumor lysis in 4 patients compared to isotype-matched control IgG (data from one representative patient, Figure 28).

To establish a safety profile for KIR1-7F9, $CD34^+$ hematopoietic progenitor cells (HPC) were purified from the peripheral blood of normal healthy donors and cryopreserved while NK cells were expanded using the same protocol used for patient samples. The expanded NK from healthy donors demonstrated binding of KIR1-7F9 equivalent to that of NK expanded from AML patients (data not shown). After 30 days of expansion, $CD34^+$ HPC were thawed and used as targets for the expanded autologous NK cells in the presence of KIR1-7F9 or isotype matched control IgG. KIR1-7F9 did not increase cytolysis of the normal $CD34^+$ HPC compared to control antibody (Figure 29).

Through its activation of SHP-2, KIR signaling can cause the dephosphorylation of SYK/ZAP 70, a critical downstream signaling intermediate of FCRγIII (CD16) (53).

CD16 is co-expressed with KIR on the $CD56^{dim}$ subset of NK cells and is absolutely required by these cells to function in ADCC (58, 199). We hypothesized that blockade of KIR signaling could increase NK-mediated ADCC against autologous tumor targets as well. Rituximab (anti-CD20) is an established targeting antibody for therapy and for *in vitro* ADCC reactions in chronic lymphocytic leukemia (CLL) (200). Whole PBMC from a CLL patient in treatment were used as effectors in an ADCC assay against CLL tumor cells collected at diagnosis. Co-incubation with KIR1-7F9 increased Rituximab-mediated ADCC of autologous CLL tumor when compared with control $hIgG_4$ plus Rituximab (Figure 30). No significant ADCC activity was observed with KIR1-7F9 or hIgG4 using an irrelevant targeting mAb, Herceptin (Figure 30). These data show that blockade of KIR signaling can enhance ADCC function against autologous CLL tumors.

The data presented here show increased NK function against autologous tumor in the presence of KIR1-7F9, consistent with the notion that the antibody blocks inhibitory KIR signaling. However, we cannot yet rule out the possibility that KIR1-7F9 acts an agonist for activating KIR molecules as well. The ligand binding properties of activating KIR remain ill-defined, yet current thought is that they bind classical MHC molecules with lower affinity when compared with inhibitory KIR (201). Activating KIR can enhance NK cell function under certain conditions but are not believed to be sufficient to activate NK cytolysis (201). Nevertheless, the majority of experiments demonstrating activation of KIR molecules (either inhibitory or activating) with anti-KIR antibodies require crosslinking of the receptors with a secondary antibody or use redirected lysis assays in which the activating antibody is captured and fixed by a target cell (53, 201). In these scenarios, KIR molecules are forced together and activating or inhibitory signaling

is potentiated. In our studies, no crosslinking antibodies were used and because KIR1-7F9 is an IgG_4 molecule that poorly binds F_c receptors, it is unlikely that the primitive blasts used as target cells can fix the antibody via the F_c portion and allow redirected lysis. The most facile explanation of the data is therefore that the anti-KIR antibody KIR1-7F9 blocks the inhibitory interactions between NK cells and MHC-I on autologous target cells resulting in increased lysis.

The data presented here suggest that KIR blockade by administration of KIR1-7F9 or other broad-spectrum anti-KIR antibodies in disease remission could be a safe and effective means to simulate NK alloreactivity in older patients or others not eligible for haploidentical transplantation. Moreover, because the frequency of alloreactive NK declines over time after haploidentical transplantation as the mature NK from the allograft are replaced by nascent NK tolerant to host MHC, long-term administration of KIR1-7F9 may generate a lasting but controlled state of NK alloreactivity against the tumor (202). Finally, it may be possible to potentiate this induced state of NK alloreactivity by adjuvant cytokine therapy with IL-2 or IL-15 (203, 204). Chronic administration of low-dose IL-2 with pulses at intermediate doses can expand and activate NK cells in patients with cancer (203); there is little clinical experience with IL-15 in this regard, although similar results might be expected with respect to NK cell activity. However, it is also possible that administration of KIR1-7F9 with adjuvant cytokines may reduce the specificity of NK activity and allow for the increased lysis of normal hematopoietic precursor cells. Our unpublished data showing increased bone marrow allograft rejection in response to IL-15 in a murine model of haploidentical

transplantation warn of this scenario. Nevertheless, KIR1-7F9 represents a promising new immunotherapeutic approach in the treatment of myeloid and lymphoid malignancy which should undergo further clinical development.

Figure 27. KIR1-7F9 binds to NK cells harvested from patients with cancer. PBMC were harvested from AML patients in remission or multiple myeloma patients undergoing standard therapy and stained with KIR1-7F9. Representative flow cytometry dot plots are shown.

Figure 27

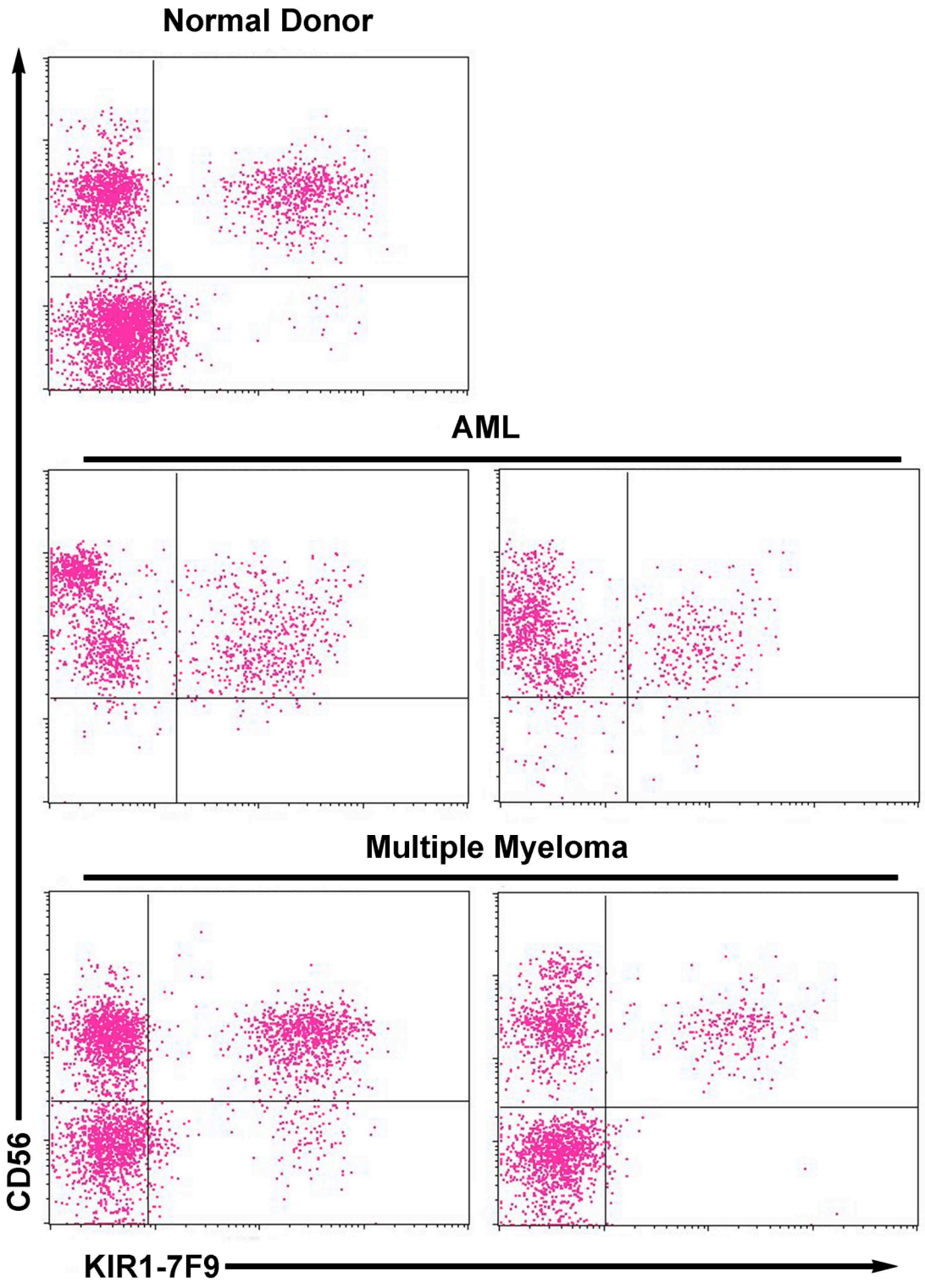
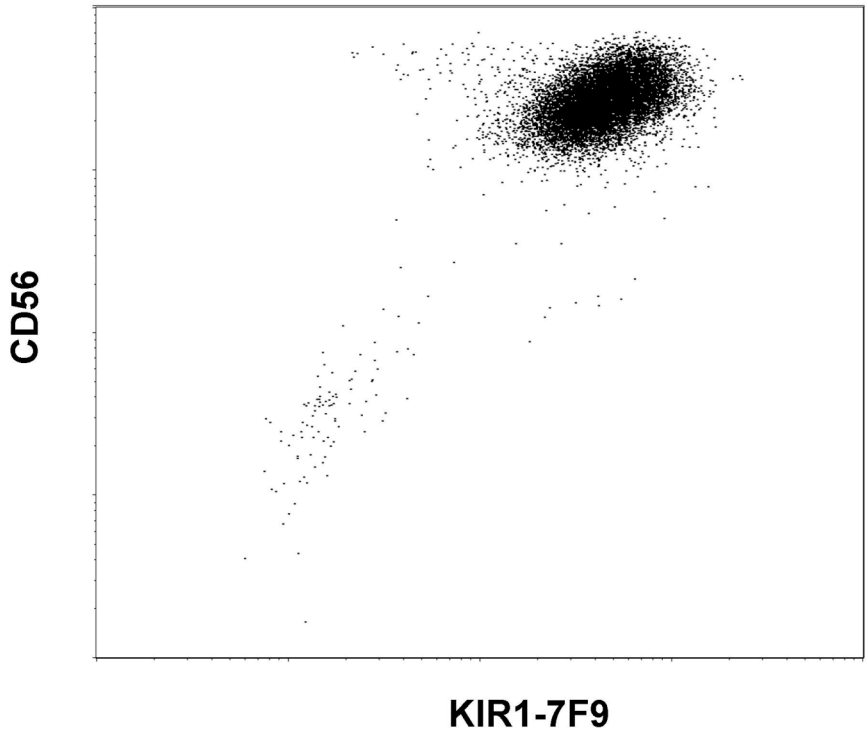


Figure 28. KIR1-7F9 increases natural cytotoxicity against autologous AML blasts. NK were expanded from PBMC and stained with KIR1-7F9 (top panel). Expanded NK were mixed with freshly thawed autologous AML blasts obtained at diagnosis (bottom panel). KIR1-7F9, control $hIgG_4$, or a mixture of mouse anti-human KIR Fab_2 fragments were added to each well (each at 30 mg/mL final concentration) and after four hours percent specific lysis was measured as described in Study Design.

Figure 28


CD56
KIR1-7F9

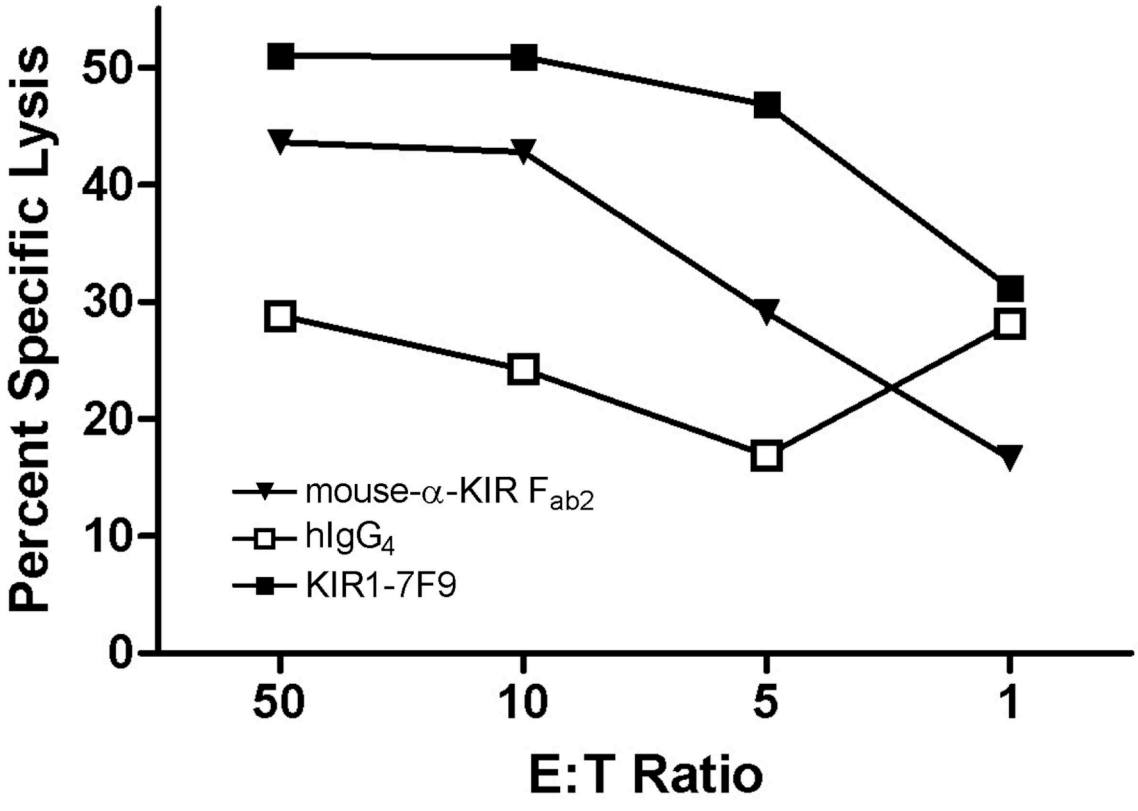

Percent Specific Lysis
50
40
30
20
10
0
mouse-α-KIR Fab2
hIgG4
KIR1-7F9
50
10
5
1
E:T Ratio

Figure 29. KIR1-7F9 does not increase lysis of autologous CD34$^+$ hematopoietic stem cells from healthy donors. NK and CD34$^+$ hematopoietic stem cells were isolated from peripheral blood from healthy donors. NK were expanded *in vitro* using the same protocol used for AML patients. CD34$^+$ cells were viably cryopreserved and thawed approximately 1 month later for use as targets for *in vitro* expanded NK. KIR1-7F9 or control hIgG$_4$ were added at 30 μg/mL and natural cytotoxicity assayed at an E:T ratio of 50:1 as described in Study Design.

Figure 29

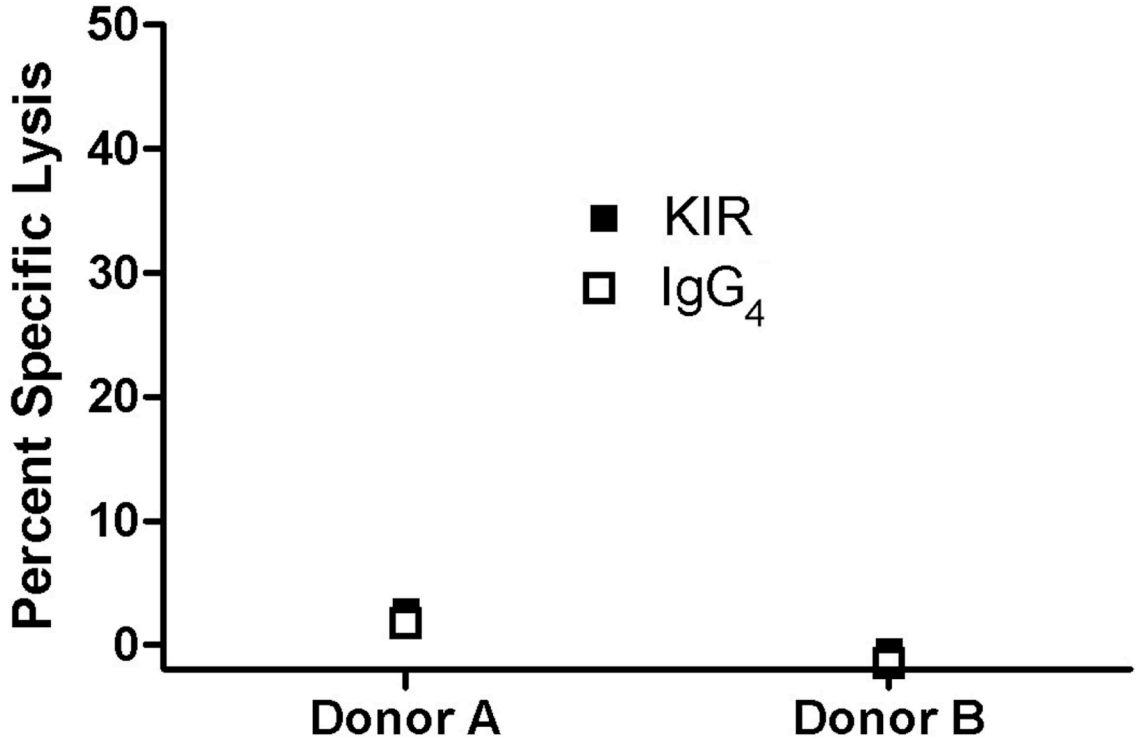
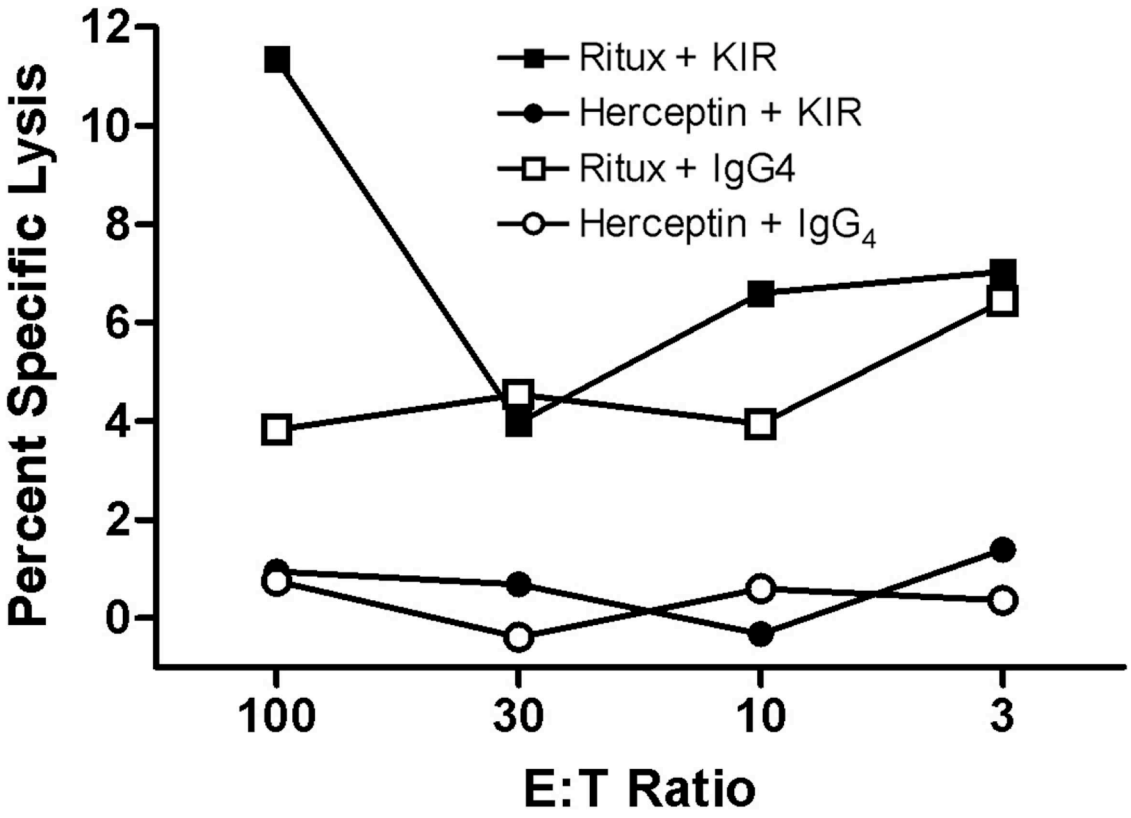

Percent Specific Lysis
50
40
30
20
10
0
KIR
IgG4
Donor A
Donor B

Figure 30. KIR1-7F9 increases ADCC against autologous CLL tumor cells. PBMC were harvested fresh from CLL patients undergoing treatment and mixed with freshly thawed CLL cells obtained at diagnosis. ADCC was performed in the presence of Rituximab (anti-CD20) or Herceptin (irrelevant control) to target CLL tumors and in the presence of KIR1-7F9 or control $hIgG_4$ (each antibody at 30 mg/mL). Target cell death was assayed after a 4 hour incubation as described in Study Design.

Figure 30


Percent Specific Lysis
12
10
8
6
4
2
0
100
30
10
3
E:T Ratio
Ritux + KIR
Herceptin + KIR
Ritux + IgG4
Herceptin + IgG4

CHAPTER 7

Synthesis and Conclusion

The work presented herein allows the following major conclusions to be drawn about IL-15 and the interface of innate and adaptive immunity in allogeniec BMT.

1. The production and trans-presentation of IL-15 by donor-derived cells predicts survival in a model of experimental acute allogeneic GVHD.
2. The absence of donor-derived IL-15 does not appreciably decrease T, NK or myeloid cell reconstitution after transplantation.
3. Type one T cell polarization is decreased in the absence of donor-derived IL-15.
4. The graft versus tumor effect is not decreased in the absence of donor-derived IL-15.
5. Donor-derived IL-15 can expand NK cells and increase graft rejection.
6. NK alloreactivity can be mimicked using a novel KIR-blocking antibody.

These findings have clear implications for future clinical experimentation. It has been proposed to develop IL-15 as a tool to enhance immune reconstitution after BMT (155). While under a limited set of conditions this may be feasible, our data suggest that IL-15 serves to enhance both graft versus host and host versus graft reactions. Whereas in the absence of exogenous IL-15 a beneficial cross-tolerance may develop between

donor and host, IL-15 administration would likely boost both T and NK cell alloreactivity and could increase the likelihood of either GVHD or graft rejection. On the contrary, partial or full neutralization of IL-15 with fully human antibodies already in phase II clinical trials for rheumatoid arthritis may provide multiple benefits in terms of delayed onset/decreased intensity of acute GVHD with maintained T cell reconstitution (147).

It is remarkable that donor-derived IL-15 can have such a significant impact on T cell polarization and survival in this model of acute GVHD, considering that donor-derived cells represent, at least initially, a very small proportion of the total cells in the recipient that are capable of producing IL-15. It may be worthwhile, then, to specifically discover means to eliminate this very potent IL-15 producing cell from the donor allograft. This would necessitate identification of the IL-15 producing cell—a challenging experimental goal given the post-transcriptional control of IL-15 production and secretion combined with the (poor) quality of anti-murine IL-15 antibodies. While this experiment may become feasible with the introduction of improved IL-15 detection reagents, the data presented here suggest that the IL-15 producing cell must also express IL-15 Rα. Depletion of IL-15 $R\alpha^+$ cells from the donor allograft may accomplish the goal of disrupting IL-15 signaling by donor-derived cells without knowing *a priori* what type of cell it is necessary to deplete. Because IL-15 producing and presenting cells would likely be regenerated as donor cells engraft, stable transduction of the allograft with small interfering RNA (siRNA) molecules specific for IL-15 and/or IL-15 Rα may provide a durable means to downregulate IL-15 signaling in the recipient.

Another intriguing possibility, alluded to in chapter 2 is that there may be genetic polymorphisms in IL-15 promoter and enhancer regions that can predispose individuals

to more or less efficient production of IL-15 (205). These polymorphisms were first identified in a sample of 200 individuals in Germany and showed a significant correlation with the incidence of bronchial asthma and atopy (205). Although there is no data provided to show that these polymorphisms result in differences in IL-15 at the protein level, based on the large body of evidence provided here and elsewhere that IL-15 contributes to pro-inflammatory type 1 responses as opposed to anti-inflammatory/allergic type 2 responses, it is straightforward to hypothesize that polymorphisms associated with allergic hypersensitivity would also be associated with less efficient production of IL-15. It follows, then, that donors with these low-IL-15 secreting polymorphisms could confer a survival advantage to allogeneic BMT recipients compared with donors with high-IL-15 secreting polymorphisms. In a landmark paper, Lin et al. demonstrated proof of this concept. Polymorphisms in the anti-inflammatory cytokine IL-10 were identified that resulted in increased protein expression; recipients of these cells demonstrated improved overall survival and decreased acute GVHD compared to recipients of cells with low-IL-10 producing polymorphisms (143). Future translational and clinical studies should address a similar role for IL-15 polymorphisms in allogeneic BMT.

Clinical studies are currently being designed and implemented to address the efficacy of the KIR-blocking antibody KIR1-7F9 in reducing tumor burden or extending survival when administered to patients with AML or multiple myeloma. The rationale behind such studies is clear: can NK alloreactivity against autologous tumors be induced without the hazard of stem cell transplantation? Our preliminary data would suggest an answer in the affirmative. Yet would augmentation of simulated NK alloreactivity with

exogenous IL-15 therapy be justified? Our data showing increased rejection of hematopoietic allografts in the presence of elevated IL-15 would at least warn of the possibility of toxicity in patients treated with both KIR1-7F9 and recombinant IL-15. It would be sensible to harvest and cryopreserve autologous bone marrow prior to any Phase I trial where induction of NK alloreactivity might result in lethal hematopoietic toxicity.

At the core of these studies is the strong proclamation by Mother Nature that the mechanisms of disease are often more complex than science can begin to understand. While donor-derived IL-15 is shown here to be a potent driving force behind acute GVHD, one would be hard-pressed to argue that it is the only critical signal in a disease where the expression of uncounted cytokines is altered and multiple cell types from host and donor interact continuously over time. Further, while NK cell alloreactivity may efficiently eliminate some tumor types it has proven utterly ineffective in other malignancies. And so these findings represent a small portion of the ever-expanding boundary of our knowledge of the innate and adaptive immune systems. Future studies will again deconstruct innate and adaptive immunity to learn how newly discovered cytokines control T cell function in acute GVHD, to find new ways to separate acute GVHD from GVT activity, or to determine why certain neoplasms are resistant to alloreactive NK cells. Yet in their own way, the data and conclusions presented here will likely influence the thinking in experimental cancer immunotherapy for years to come.

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