Tim-3/Gal-9 and B7-H3 act as negative regulators of Graft versus Host Disease

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Abstract

Allogeneic HSCT is a treatment option for many malignant and nonmalignant hematologic disorders with potential to treat a variety of diseases. However, a very significant limiting factor for its use is the high risk of graft versus host disease (GVHD) that limits its efficacy and use in broader applications. GVHD occurs when there is an antigen disparity between the donor and the recipient. This disparity leads to activation of donor T cells that migrate to GVHD target organs and mediate damage. Current therapies involve broad immune suppression that can lead to toxicity and increased relapse rates for cancers. By elucidating the positive and negative regulatory pathways in GVHD biology, new therapeutic targets can be identified for translation into the clinic. Research presented here investigates the role of the Tim-3/gal-9 pathway as well as the B7-H3 pathway in acute GVHD. Recipients of Tim-3 deficient donor T cells had accelerated GVHD lethality while Gal-9 transgenic recipients had a reduced GVHD lethality. Paradoxically, recipients of Tim-3 deficient and CD25 depleted donor T cells had a significantly reduced GVHD lethality with an associated reduced pathology in the colon. CD25-depleted Tim-$3^{-/-}$ donor T-cells underwent increased activation-induced cell death due to increased IFN-γ production. B7-H3 deficient recipients had accelerated GVHD lethality and had increased pathology in the colon. Interestingly, recipients of B7-H3 deficient donor T cells also had an accelerated GVHD lethality, with increased T-cell proliferation in the spleen as well as increased pathology in the colon. This work identifies B7-H3 as a negative regulator of acute GVHD.

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AICD	Activation induced cell death
APC	Antigen presenting cell
BMT	Bone Marrow Transplantation
CD40L	CD40 ligand
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CRD	Carbohydrate recognizing domain
CTL	Cytotoxic T lymphocyte
DLI	Donor lymphocyte infusion
DST	Donor specific transfusion
EAE	Experimental autoimmune encephalitis
Foxp3	Forkhead box P3
Gal-9	Galectin-9
GVHD	Graft versus host disease
GVT	Graft versus tumor
HLA	Human leukocyte antigens
HSCT	Hematopoeitic stem cell transplant
IFN	Interferon
IL	Interleukin
IgV	Immunoglobulin variable
mHAgs	Minor histocompatibility antigens
MHC	Major histocompatibility complex

MLR	Mixed lymphocyte reaction
MST	Median survival time
NK	Natural killer cell
PD1	Programmed death protein 1
PDL1	PD1 ligand 1
PDL2	PD1 ligand 2
qPCR	Quantitative polymerase chain reaction
TCR	T cell receptor
Tg	Trasngenic
Th	T helper
Tim-3	T cell immunoglobulin mucin
Treg	T regulatory cell

Chapter I: Introduction

Hematopoietic stem cell transplantation

Each year in the United States, there are over 30,000 new cases of leukemia and over 21,000 people will die of the disease.[1] For many of these patients, a bone marrow transplant (BMT) is their only chance for a cure. An allogeneic BMT, in which the patient receives major histocompatability (MHC) matched bone marrow, represents the best chance for disease free survival. Human leukocyte antigens (HLA) are the gene products of the MHC locus. HLA are expressed on the cell surfaces of all nucleated cells in the body and are important for allogeneic T-cell activation.[2] GVHD occurs in 19-66% of patients receiving HLA-identical sibling allogeneic-BMT and over 90% of patients receiving HLA-matched unrelated and HLA-unmatched related allogeneic-BMT depending on factors such as the age and sex of the donor and recipient.[3] GVHD limits the use and success of allogeneic HSCT and it is fatal to approximately 15% of recipients.[4] Allogeneic bone marrow transplants have advantages over autologous bone marrow transplants in that they provide a tumor free graft and a graft versus tumor (GVT) effect. Unfortunately, allogeneic BMTs are associated with an increased risk for development of graft vs host disease (GVHD). GVHD is a pathological condition in which mature T lymphocytes from the transplanted tissue of a donor initiate an immunologic attack on the cells and tissue of the recipient. Prevention of GVHD is currently achieved by either T-cell depletion of donor bone marrow or the use of global immune suppressive therapy. However, because of their inhibitory effects on donor T-cells, these approaches are

associated with an increased risk of leukemic relapse after BMT due to a decreased GVT effect.[5] Cellular strategies, such as donor lymphocyte infusions (DLI), are currently being pursued and are aimed at reducing GVHD while maintaining the GVT effect.[6] Specifically targeting the pathogenic T cells responsible for GVHD is critical for improving the outcome of BMT.

GVHD pathogenesis

It was 50 years ago that GVHD was initially reported using irradiated mice that were infused with allogeneic marrow and spleen cells.[7] Billingham defined GVHD as a syndrome in which donor immune-competent cells recognize and attack host tissues in immune-compromised allogeneic recipients.[8] Specifically, he had three requirements he defined to be necessary for GVHD. The first requirement was that the transplanted graft must contain immunologically competent cells. Second, the recipient must be immune compromised so they are incapable of rejecting the transplanted cells. Third, and last, the recipient and donor must have an antigen disparity.[9] T cells were identified to be the immune-competent cells.[10] Severity of GVHD following an allogeneic HSCT is correlated to the number of donor T cells that were transferred.[11] Prior to HSCT, recipients undergo an immune compromising conditioning regimen that can vary depending on age, sex, and disease. This prevents the transplanted donor cells from being rejected by the recipient. Antigen disparity between the donor and the recipient has two categories, major and minor histocompatibility antigens.

MHC mismatches generate immune responses when T cells directly recognize foreign MHC on the surface of host cells, with little or no contribution of the presented peptide. It is estimated that between 1-10% of T cells are directly alloreactive and lead to a massive activation of the immune system that induces cytokine release and tissue damage resulting in GVHD. Even when there is not a major MHC mismatch, GVHD can still occur through mismatches of minor histocompatibility antigens (mHAgs).[12] The frequency of mHAg-reactive T cells is thought to be lower, partially explaining the reduced risk of GVHD in fully MHC-matched transplants.

The pathogenesis of GVHD consists of three main phases. **First**, there is preconditioning-induced damage of the host that primes the immune system. Damage to the host epithelium from the conditioning regimen induces a "cytokine storm" that causes increased expression of molecules known to attract or activate donor T-cells. A correlation has been shown between increased doses of radiation with increased levels of pro-inflammatory cytokines such as TNF-α and IL-1.[13] Additionally, microbial products translocate into circulation upon epithelial cell damage in the lining of the gastrointestinal tract.[14] This serves to further amplify the activation state of host ACPs. During the **second** phase, donor T cells undergo activation, cytokine secretion, and trafficking to target tissues. Donor T cell activation occurs when T cells recognize foreign MHC molecules presented on host APCs (direct presentation) or peptides of host MHC molecules presented on donor APCs (indirect presentation). During direct

presentation, donor T cells recognize either the peptide bound to allogeneic MHC molecules or the foreign MHC molecules themselves.[15] Indirect allorecognition is entirely peptide dependent. Donor T cells can respond to peptides of host MHC molecules presented by donor APCs. Once the T cells are activated, they readily secrete inflammatory cytokines such as IFN-γ and IL-2 further amplifying the immune response. After priming and activation in the secondary lymphoid organs, donor T cells migrate to GVHD target tissues to cause tissue destruction. Expression of specific homing molecules (selectins, integrins, and chemokines) direct the migration to specific target organs.[16] This leads to the **third** phase: upon arrival at the GVHD target organs, donor T cells provoke considerable tissue destruction. The pathogenesis of acute GVHD culminates in the generation of multiple cytotoxic effectors that contribute to destruction of target tissues. There is a significant amount of data that suggests that soluble inflammatory mediators act in conjunction with direct cell-mediated cytolysis by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells to cause the wide range of deleterious effects seen during acute GVHD. Each of these three phases of pathogenesis amplifies the next step and provides for a feedback loop that continues the cycle and leads to the clinical features of acute GVHD.

Chronic GVHD was initially defined as occurring after 100 days after transplant and acute GVHD occurred before 100 days after transplant. However, it has been shown that the differences between acute and chronic GVHD involve distinct pathological processes, rather than exclusively time of onset. Acute

GVHD has strong inflammatory components and involves alloreactive donor T cell-mediated cytotoxic responses against the tissues of the recipient, mediated by cell-surface and secreted factors.[17] Once the tissue damage is initiated by the cytotoxic T cells, other effector cells are recruited and even more tissue damage is perpetuated. In contrast, chronic GVHD displays more of an autoimmune-like systemic syndrome that is associated with fibroproliferative features.

Graft versus tumor effect

GVHD has a beneficial effect on the incidence of leukemia relapse and the overall survival of patients with leukemia, known as graft-versus-tumor (GVT) effect.[18] It would be extremely beneficial to be able to separate out the GVT effect from the GVHD effect, but this possibility has yet to be firmly established. The role of T cells was established for both GVHD and GVT by the finding that depleting T cells from the transplant eliminated GVHD but resulted in an increased leukemia relapse rate.[19] Additionally, lymphocyte mediated anti-leukemia effects are shown in studies that use allogeneic DLI to treat relapse of myeloid leukemia after HSCT.[20-23]

Donor NK cells also exhibit GVT activity, when given early post transplant before T-cells become primed.[24] Studies have shown that adoptive transfer of Ly49-mismatched, MHC-matched NK cells reduced pulmonary tumor burden while presenting with reduced GVHD.[25] Current strategies to improve GVT effects are

based on selectively targeting tumor-specific killing and inhibiting immune escape mechanisms commonly used by tumors.

Methods of preventing GVHD

Reducing the initial damage from the conditioning regimen is one opportunity to reduce the severity of GVHD. Using non-myeloablative conditioning reduces the intensity of conditioning and reduces initial damage leading to a reduction in the incidence of acute GVHD.[26] However, reduced conditioning regimens then have to rely on the GVT effects to eliminate any residual malignant cells.

Another approach is to utilize a delayed lymphocyte infusion. This method relies on delaying the administration of donor T cells until after inflammation has subsided and could lead to a decrease in GVHD while preserving GVT. Although the donor T cells could mediate GVL effects without evidence of GVHD, the effect was much weaker than T cells that were freshly transferred.[27] It has also been noted that in murine models, DLI may lose its efficacy over time.[28]

Another promising cellular therapy is the transfer of T regulatory cells (Tregs) at the time of transplant. These cells are a subpopulation of CD4+ cells that are characterized by the expression of CD25 and the transcription factor Foxp3. They act to suppress autoreactive lymphocytes and control innate and adaptive immune responses.[29,30] Preclinical models have shown that adoptive transfer of

Tregs was highly effective at suppressing acute GVHD.[31-33] Additionally, it appears that Tregs preserve GVT in rodents.[34] Treg cell infusion is currently in phase I clinical trials.

Due to the crucial role that both donor $CD4^+$ and $CD8^+$ T cells have in GVHD pathogenesis, it stands to reason that the most effective approach to prevent GVHD is to focus on the depletion, tolerization, or functional incapacitation of donor T cells. T cell activation in response to an alloantigen requires at least two stimulatory signals.[35] The first signal occurs through the T cell receptor (TCR), which recognizes the antigen and is MHC restricted. While this signal is necessary to induce a full T cell response, it is not sufficient. The second signal is provided by co-stimulatory molecules and is needed to induce T cell proliferation, cytokine secretion, and effector function after TCR activation. The most extensively studied pathways involve interactions between CD28 and B7 molecules CD80 and CD86, and between CD40 and CD40 ligand (CD40L). Approaches to blocking the B7-CD28 pathway were limited by the adverse effects on Tregs and unwanted blockade of the CTLA-4 inhibitory pathway. Blockade of the CD40-CD40L interactions was found to be effective in reducing GVHD while augmenting Treg function in mice.[36,37] Co-inhibitory molecules act to counter-regulate T cell activity and include cytotoxic T lymphocyte antigen 4 (CTLA-4) that binds to CD80 and CD86 as well as programmed cell death protein 1 (PD-1), which binds to PD-1 ligand (PDL1) and PDL2. It may be possible to augment these negative regulatory pathways to inhibit GVHD. One strategy

utilizes a CTLA-4-Ig fusion protein and resulted in reduced GVHD.[38] However, inhibition of GVHD was not complete. PD-1 has been shown to play a role in the pathogenesis of acute GVHD. Blockade of PD-1 resulted in an IFN-γ dependent in increase in acute GVHD severity.[39] Agonistic antibodies to PD-1 may offer a novel strategy for preventing GVHD. Overall, reagents that selectively signal through these inhibitory pathways are not yet available for clinical GVHD prevention or therapy.

In summary, GVHD results from the accumulation of tissue damage mediated by T cell activation that promotes a feed-forward loop that is difficult to control once it is fully initiated. The pathogenesis of the process is regulated at many levels including, but not limited, inflammation from conditioning regimens, activation by APCs in secondary lymphoid organs, T cell effector mechanisms, cytokines, and target tissue environment. Systemic steroid therapy remains the standard primary therapy for GVHD, despite its negative broad immune suppression. Patients who have steroid-refractory GVHD have an extremely poor outlook, with long-term mortality rates that can reach 90%. New and improved therapies are greatly needed. By understanding the regulatory processes listed above, investigators hope to develop targeted treatments that restrain GVHD without resulting in the general immunosuppression.

Tim-3/Gal-9 Immune Regulation

T cell immunogloblulin and mucin (Tim) family consists of three identified proteins in both human and mouse (Tim-1, Tim-3, and Tim-4).[40] Tim-3 was the first identified family member and was originally identified as a molecule specifically expressed on IFN-γ producing $CD4^+$ T helper type (Th)1 and $CD8^+$ T cytotoxic type (Tc)1 cells in mice.[41] Later, Tim-3 was also found to be expressed on IFN-γ producing human T cells.[42] The human Tim-3 molecule shares approximately 70% homology with the murine Tim-3 molecule.[43]

Tim-3 protein structure

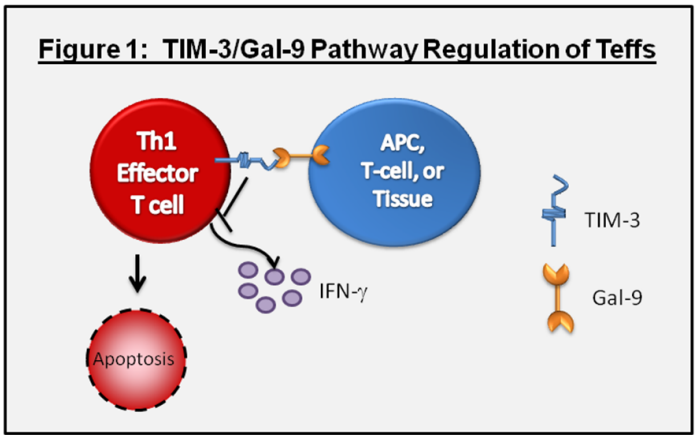
The murine Tim-3 gene encodes a 281-amino acid protein that is expressed on terminally differentiated Th1 cells and not on Th2 cells.[41] Studies looking at the crystal structures of the Tim-3-Ig-like domains determined they belong to the immunoglobulin variable (IgV) set and are composed of two, anti-parallel B-sheets that contain six conserved cysteine residues.[44-46] In the IgV domain of Tim-3, two N-linked sugars serve to facilitate interactions with carbohydrate recognizing proteins including galectin-9 (Gal-9). The Tim-3 protein is a type I cell-surface glycoproteins that share common structural features consisting of an N-terminal Ig-like domain followed by a mucin domain with O-linked and N-linked glycosylations, a single transmembrane domain and a cytoplasmic region that contains tyrosine phosphorylation motif. In addition to the full length molecule, there is also a soluble splice form (sTim-3) that lacks the mucin domain and the

transmembrane domain that has been identified.[47] The signaling pathway has not yet been conclusively established.

Gal-9 was demonstrated to be a ligand for Tim-3 by Kuchroo et al., by interacting with the N-linked sugars located in the IgV domain of Tim-3 via its carbohydrate recognizing domains (CRDs).[48] Gal-9 is a 40-kD S-type B-galactoside binding tandem-repeat-type lectin that contains two non-identical CRDs joined by a flexible linker peptide sequence of amino acids.[49,50] Gal-9 can localize to the plasma cell membrane or be secreted, the mechanism of which remains poorly understood.[49] Expression of Gal-9 is highly prevalent in tissues including bone marrow, lymph nodes, thymus, spleen, mast cells, T cells, B cells, macrophages, endothelial cells, and fibroblasts.[49,51] Accordingly, Gal-9 has been linked to the regulation of immune homeostasis and inflammation and the expression of Gal-9 has been shown to be regulated by IFN-γ.[51,52]

Tim-3 Immune Regulation

When TIM-3 binds with its ligand galectin-9, it inhibits Th1 responses and



induces peripheral tolerance [53-55]. The in vivo role of the TIM-3/Gal-9 pathway has been studied using blocking strategies relying on a monoclonal anti-TIM-3 antibody and a TIM-3 Ig fusion protein. Administration of these reagents resulted in an exacerbation of experimental autoimmune encephalomyelitis (EAE), colitis, collagen-induced arthritis (CIA), and autoimmune diabetes.[54] These reagents were also shown to eliminate transplant tolerance that was induced by donor specific transfusion (DST) and anti-CD154 treatment (costimulatory blockade).[54] These studies suggest that the normal TIM-3/Gal-9 signaling acts to dampen a Th1 immune response, and obstruction using the above reagents results in an amplified Th1 response and increased disease. Hence, a major function of the TIM-3/Gal-9 pathway is to limit adaptive immune responses, specifically Th1 immune responses. These results were solidified when Gal-9 was discovered to be the ligand for TIM-3 and caused cells to aggregate and undergo apoptosis in vitro as depicted in figure 1.[55] GVHD effects are mainly mediated by Th1 Teff cells. This makes the TIM-3/Gal-9 pathway an attractive target for attempting to dampen the response of Teff cells.

In addition to the direct effect that the TIM-3/Gal-9 pathway has on T cells, it may also play an indirect role by modulating Tregs. The possibility of creating or activating Tregs is of great interest, as work by our lab and others indicates that Tregs can ameliorate GVHD.[32-34] Tregs are also being pursued as cellular therapy for a variety of autoimmune conditions. These cells, classically defined as $CD4^+CD25^+Foxp3^+$ regulatory cells, suppress T cell responses by

mechanisms that are not completely clear. Various model systems have shown suppression by Tregs requires secretion of the suppressive cytokines IL-10 and/or TGF-β, or the killing of effector T cells by release of granzyme B.[56-58] It has been observed in vitro that TIM-3$^+$ Tregs are more resistant to Gal-9 induced apoptosis than Teffs. Mice treated with recombinant Gal-9 had an increased frequency of Tregs compared to Teffs, and naïve CD4+ T-cells were more readily converted into Foxp3$^+$ ex vivo.[59] Mechanisms responsible for this effect have not yet been reported in vivo.

Due to the ability of the TIM-3/Gal-9 pathway to suppress T cells, and because of its link to Treg biology, we propose to study this negative regulatory pathway in the context of GVHD. We hypothesize that Teffs expressing TIM-3 are negatively regulated by apoptosis and other mechanisms such as Tregs that in turn decrease GVHD lethality. T cells may proliferate less, become less activated, or undergo more apoptosis through this pathway. This effect may involve the activation or generation of Tregs. We further hypothesize that by inducing the TIM-3/Gal-9 pathway by administering rGal-9 or TIM-3 agonists we can augment the pathway for use as a possible therapy for treating or preventing GVHD.

B7-H3

B7-H3 is a member of the B7 family and is a type I transmembrane protein that shares approximately 25% amino acid identity with other B7 family members. It

is the most highly conserved between mice and humans.[60] The human B7-H3 molecule is approximately 90% homology with the murine B7-H3 molecule.[61] Although initial reports described B7-H3 as a positive co-stimulatory molecule, other reports have shown it to be inhibitory.

Protein Structure

While B7-H3 only shares approximately 25% amino acid homology with other B7 family members, it's secondary and tertiary structure (predicted from its primary sequence) are shown to be highly homologous to other B7 family molecules.[62] Murine B7-H3 has a single extracellular variable-type immunoglobulin (IgV)-IgC domain and an intracellular domain. However, human B7-H3 is found to have an additional isoform, known as 4Ig-B7-H3 that contains a nearly exact tandem duplication of the IgV-IgC domain.[61]

B7-H3 Immune Regulation

B7-H3 has been shown to be expressed on activated T cells as well as APCs (in humans and mice). Expression has also been shown on fibroblasts, synoviocytes, osteoblasts, and epithelial cells.[62] The molecular mechanisms regulating B7-H3 expression are still not clear.

B7-H3's function in immune regulation has yet to be clearly defined as both stimulatory[63-66] and inhibitory[67-70] properties have been described. Although TLT-2, a type I transmembrane glycoprotein was identified as a receptor for B7-H3[64],

others have shown no evidence for such an interaction in mouse or humans[69], further confounding elucidation of the nature of the biological response of the B7-H3 pathway.

Initial studies identified B7-H3 as a positive costimulatory molecule due to its capability to promote T cell proliferation and IFN-γ secretion in vitro.[63] Overexpression of B7-H3 by transfection of a mouse tumor cell line promoted an antitumor response leading to the regression of tumors and amplification of a CTL response.[65] When a B7-H3$^{-/-}$ mouse was used in an allograft rejection model, there was no difference in graft prolongation unless additional treatment with cyclosporine A or rapamycin was used leading to increased allograft survival.[66] Taken together, these studies would indicate that B7-H3 can act as a positive costimulatory molecule.

Several contrasting studies show that B7-H3 can act as a negative costimulatory molecule. B7-H3 has been shown to inhibit T cell proliferation and impair T helper 1 (Th1) responses.[67] Others also showed that B7-H3 inhibited T cell activation and effector cytokine production and when B7-H3 was blocked there was an exacerbation of experimental autoimmune encephalomyelitis (EAE).[68] Furthermore, B7-H3 blockade resulted in accelerated cardiac graft rejection associated with enhanced IFN-γ production.[70] B7-H3 expression has also been shown to contribute to the evasion of cancer immune surveillance.[71-73]

Chapter II: Contrasting acute graft-versus-host disease effects of Tim-3/galectin-9 pathway blockade dependent upon the presence of donor regulatory T-cells

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T-cell immunoglobulin mucin-3 (Tim-3) is expressed on pathogenic T-cells and its ligand, galectin-9 (gal-9), is up-regulated in inflamed tissues. When Tim-3^{+} T-cells encounter high gal-9 levels, they are deleted. Tim-3 is up-regulated on activated T-cells during GVHD. Inhibition of Tim-3/gal-9 binding by infusion of a Tim-3-Ig fusion protein or Tim-$3^{-/-}$ donor T-cells increased T-cell proliferation and GVHD lethality. When the Tim-3/gal-9 pathway engagement was augmented by using gal-9 transgenic recipients, GVHD lethality was slowed. Together, these data indicate a potential for modulating this pathway to reduce disease by increasing Tim-3 or gal-9 engagement. Paradoxically, when Tim-3/gal-9 was inhibited in the absence of donor T-regulatory cells (Tregs), GVHD was inhibited. GVHD reduction was associated with decreased colonic inflammatory cytokines as well as epithelial barrier destruction. CD25-depleted Tim-$3^{-/-}$ donor T-cells underwent increased activation-induced cell death due to increased IFN-γ production. To our knowledge, these studies are the first to show that whereas the absence of Tim-3/gal-9 pathway interactions augments systemic GVHD, concurrent donor Treg depletion paradoxically and surprisingly inhibits GVHD. Thus, while donor Tregs typically inhibit GVHD, under some conditions, such Tregs actually may contribute to GVHD by reducing activation-induced T-cell death.

Introduction

GVHD remains the leading cause of morbidity and mortality post-BMT. Patients are given immune suppressive therapy to prevent or diminish the severity of GVHD following allogeneic BMT, which in turn increases the risk of infection and disease recurrence. Novel GVHD strategies remain a high priority.

The T-cell immunoglobulin mucin (TIM) family consists of 3 proteins (TIM 1, 3, 4), homologous in mouse and human.[40] Tim-3 was the first described member[41] and has been the most well-studied. Differentiated T-effector cells (Teffs) express Tim-3 with the highest density on Th1, lower density on Th17, and no expression on Th2 cells.[74,75] The expression of galectin-9 (gal-9), identified as a ligand for Tim-3, is up-regulated in inflamed tissues.[48,76-78] When Tim-3^{+} Teffs encounter high gal-9 levels, they are deleted.[48,49,79,80] A major function of the Tim-3/gal-9 pathway is to limit immune responses under conditions of tissue inflammation and injury. In vivo blockade of Tim-3/gal-9 interaction or the use of Tim-3 knockout (-/-) mice increases Th1 cells within inflamed tissues.[41,47,81]

When Tim-3 binds with gal-9, Th1 responses are inhibited and peripheral tolerance induced.[47,48,81] In vivo blocking strategies relying on monoclonal anti-Tim-3 antibody and Tim-3-Ig fusion protein showed exacerbation of experimental autoimmune encephalomyelitis (EAE) and autoimmune diabetes.[41,54] Transplant tolerance induced by donor-specific transfusion and anti-CD154 treatment was impaired.[47] Thus, Tim-3/gal-9 signaling acts to dampen a Th1 immune response,

whereas signaling blockade results in an amplified Th1 response and increased disease. These results were solidified when gal-9 was discovered to be the ligand for Tim-3 and caused cells to aggregate and undergo apoptosis in vitro.[48] Hence, a major function of the Tim-3/gal-9 pathway is to limit adaptive Th1 responses. GVHD effects are largely mediated by Th1 Teffs, making the Tim-3/gal-9 pathway an attractive target for regulating GVHD lethality.

Although there is evidence for a negative regulatory function of the Tim-3/gal-9 pathway in autoimmunity, its role in acute GVHD is unclear. We show that during acute GVHD, donor T-cells rapidly up-regulate Tim-3 and non-hematopoietic cells up-regulate gal-9. Allogeneic T-cell proliferation was increased upon inhibition of Tim-3. Tim-3 inhibition with Tim-3-Ig or use of Tim-$3^{-/-}$ donor T-cells accelerated GVHD lethality. Conversely, gal-9 transgenic (Tg) recipients had a significantly reduced rate of GVHD. These results suggest that Tim-3/gal-9 signaling negatively regulates T-cells during GVHD and inhibiting Tim-3/gal-9 increases Teffs and GVHD lethality. Paradoxically and surprisingly, when Tim-3 was inhibited in the absence of donor Tregs, GVHD lethality was significantly reduced. This result was explained by an increased level of IFN-γ secretion that leads to increased activation-induced cell death (AICD). Recipients of Treg depleted Tim-$3^{-/-}$ donor T-cells had less damage to the epithelial layer of the colon as well as a reduced percentage of inflammatory cytokine secretion. These results suggest that increased levels of IFN-γ can lead to protection of the colon from GVHD and reduce the lethality rate.

Methods

Mice

C57BL/6 ($H2^b$) and BALB/c ($H2^d$) mice were purchased from the National Institutes of Health. B6D2F1 ($H2^{b/d}$) mice were purchased from The Jackson Laboratories. Mice expressing gal-9 under the β-actin promoter and TIM-$3^{-/-}$ mice are on the BALB/c background and were previously described.[82,81] B6-L2G85 (luc^+) express luciferin under the β-actin promoter were kindly provided by Dr. Robert Negrin (Palo Alto, CA).[83] TEa $CD4^+$ Tg T-cells express a TCR that recognizes the peptide ASFEAQGALANIAVDKA in the context of I-A^b and were previous described.[84] TEa Tg mice (kindly provided by Dr. Alexander Rudensky (New York, NY) were crossed with B6-L2G85 mice to produce cells that were TEa^+luc^+. Mice were bred and housed in a specific pathogen-free facility in microisolator cages and used at 6-16 weeks of age. All experiments were approved by the institutional animal care and use committee of the University of Minnesota.

Bone marrow transplantation

Mice were lethally irradiated by an x-ray source on d-1. A total of $1x10^7$ bone marrow (BM) cells with or without purified T cells were infused on d0. For GVHD induction, T-cells were isolated from lymph nodes and purified by incubation with phycoerythrin-labeled antibodies to CD19, γδ-TCR, and DX5 or NK1.1 (eBioscience), incubation with anti-phycoerythrin beads and depletion on

magnetic column (Miltenyi Biotec). Flow cytometric phenotyping demonstrated >95% purity. Mice were monitored daily for survival, weighed twice weekly, and examined for clinical GVHD. Where indicated, mice were clinically scored for GVHD as described.[85]

Frozen tissue preparation

Tissues including colon, small intestine, liver, lung, and spleen were taken at indicated days after transplantation, embedded in Optimal Cutting Temperature (OCT) compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80°C.

Immunofluorescence

Six μm cryosections were fixed in acetone and incubated with monoclonal rat anti-gal-9 (Biolegend, San Diego, CA) overnight at 4°C, and anti-CD45-FITC (eBioscience) for 30min at room temperature. Fluorochrome-labeled secondary antibodies to rat (Jackson Immunoresearch) were incubated for 30min at room temperature. Slides were mounted with DAPI slow fade gold (Invitrogen, Carlsbad, CA). Confocal images were acquired on an Olympus FluoView 500 Confocal Laser Scanning Microscope under 40x/0.9 oil-immersion objective lens using Fluoview 3.2 software (Olympus) and then processed with Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

CFSE experiments

T-cells purified from lymph nodes were labeled for 15min with 5μM CFSE (carboxyfluorescein diacetate succinimidyl diester; Invitrogen) at room temperature followed by quenching with fetal bovine serum. $1x10^7$ labeled purified T-cells were infused into lethally irradiated recipients. Spleens were harvested on indicated days, and single cell suspensions were made. Cells were surface stained and acquired on a FACS LSRII Fortessa (BD Biosciences). Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

Flow Cytometry

Single-cell suspensions were stained with the following monoclonal antibodies (mAbs): Tim-3, CD8a, CD4, CD45.1, $H2K^d$, IFN-γ, IL-2, IL-17, Granzyme-B, Ki-67, Annexin V, Activated Caspase-3. For cytokine detection, cells were stimulated in vitro with cell stimulation cocktail and protein transport inhibitor cocktail (eBioscience) for 5 hours. Cells were then surface stained with appropriate surface antibodies followed by fixing and permeabilizing with the Fix/Perm permeabilization kit (Invitrogen), and labeled with the appropriate intracellular antibodies. Cell apoptosis and death was measured by staining cells with Annexin-V staining kit and 7-AAD (eBioscience). Phenotypic acquisition of cells was performed on the LSRII Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

FITC-Dextran Assay

FITC-dextran assay was used to evaluate mucosal integrity. Mice were given 400µl of FITC-dextran orally (Sigma #FD4-1G) at a concentration of 40mg/ml in PBS. Plasma was collected from peripheral blood, mixed 1:1 with PBS and analyzed on a plate reader at excitation/emission wavelength of 485/535nm.

Antibodies and Reagents

Hybridoma clone 5D12[52] was used to produce αTIM-3 mAb. Protein was purified over a protein G column and brought to a concentration of 2mg/mL in PBS. In some studies, Tim-3-Ig (100µg/dose) or control human IgG was injected intra-peritoneally every day from d-1 to 5 and three times per week until d28. Where indicated, transplanted mice received αIFN-γ (100µg/dose; NCI) or rat IgG intraperitoneally twice per week from d-1 to 28.

T-cell isolation

Spleens were processed into a single-cell suspension followed by red cell lysis. Colons were harvested, cut longitudinally and then into 5mm pieces. Gut pieces were incubated with 15.4mg/ml DTE in CMF with 10% serum (30min at 37°C) followed by treatment with 5mM EDTA in RPMI with 10% serum (15min at 37°C). Tissue was incubated three-times with 1 mg/ml collagenaseD (Roche, Indianapolis, IN) in RPMI with 5% serum (45min/37°C). Lymphocytes were purified on a 44/67% percoll gradient (800*g* at 20°C for 20min).

Treg suppression assay

Tregs were isolated from spleens and of BALB/c or Tim-3$^{-/-}$ mice. T-cells were labeled with 1μM CFSE. T/NK cell-depleted splenocytes from BALB/c mice were used as APCs. 4×10^5 CFSE-labeled T-responder cells were stimulated with 4×10^5 APCs in RPMI-c and 0.25μg/mL purified α-CD3. Four days later, cells were harvested and proliferation determined by CFSE dilution.

BLI studies

Xenogen IVIS imaging system was used. Firefly luciferin substrate (0.1 mL; 30 mg/mL) was injected intraperitoneally, and imaging performed immediately after substrate injection. Data were analyzed and presented as photon counts per area.

Statistics

The Kaplan-Meier product-limit method was used to calculate survival. Differences between groups were determined using log-rank statistics. Group comparisons were analyzed by Student *t* test or one-way ANOVA with a tukey multiple comparison test. *P* values ≤ 0.05 were considered to be significant.

Results

Tim-3 expression is up-regulated on donor T-cells during acute GVHD

Tim-3 can be expressed on subsets of differentiated T-cells that have undergone 2-3 rounds of cell division[81]. To determine whether Tim-3$^+$ Teffs are induced during acute GVHD generated in fully conditioned recipients, C57BL/6 (B6; H2^b) recipients were lethally irradiated followed by an infusion of BALB/c (H2^d) BM with or without purified donor T-cells from BALB/c mice to induce GVHD. Whereas Tim-3 was expressed on an average of 41% of CD4$^+$ and 76% of CD8$^+$ donor T-cells on d7 of the GVHD response, few naïve T-cells were Tim-3$^+$. To examine the in vivo kinetics of Tim-3 up-regulation on donor T-cells, we performed an in vivo mixed leukocyte reaction (MLR). B6 recipients were lethally irradiated and given 10^7 BALB/c purified CFSE-labeled donor T-cells. On days 3, 4, and 5 post transfer, splenocytes were analyzed for CFSE dilution and Tim-3 expression. Donor T-cells began up-regulating Tim-3 on d3 and by d5 over 50% of donor CD4$^+$ and over 80% of donor CD8$^+$ T-cells express Tim-3 after several rounds of division (Figure 1B). Although gal-9 expression was not observed on CD4$^+$ or CD8$^+$ T-cells by flow cytometry (not shown), gal-9 expression was up-regulated in the spleen on d7. Figure 1C shows immunofluorescent staining of spleen from the BM control and GVHD mice on d7 post-BMT. Gal-9$^+$ (shown in red) cells were CD45$^-$ but surrounded CD45$^+$ cells (shown in green) in the spleen, indicating that non-hematopoietic, stromal cells strongly expressed gal-9 in GVHD recipients.

Tim-3/gal-9 pathway inhibition leads to increased allogeneic proliferation in vitro and in vivo and accelerated GVHD lethality

Previously, it has been shown that alloreactive T-cells have increased expansion following αTim-3 blockade.[81,86] After we determined that Tim-3 expression was highly up-regulated during acute GVHD, we sought to determine if allogeneic T-cells would increase their proliferation when the Tim-3 pathway was blocked. Initially, we performed an in vitro MLR by mixing B6-purified T cells with irradiated BALB/c stimulators (1:1) and adding monoclonal αTim-3 blocking antibody in three different concentrations. Figure 2A shows that αTim-3 mAb at 5 μg/mL resulted in significantly increased proliferation on d5 through d8 compared the irrelevant IgG control. The increased proliferation in vitro was dose dependent.

An in vivo assay was used to establish the average number of divisions of divided cells (proliferative capacity) of Tim-$3^{-/-}$ vs WT T-cells. B6 mice were transplanted with 10^7 allogeneic T-cells from BALB/c donors labeled with CFSE. On days 3 and 4, splenocytes were analyzed for CFSE dilution. Donor T-cells had very low division on d3 and there was no significant difference between the groups. By d4 Tim-$3^{-/-}$ vs. WT donor T-cells had significantly increased proliferative capacity (Figure 2A). These results indicate that inhibition of Tim-3 increase allogeneic T-cells proliferation as compared to controls.

Due to the increased expression of Tim-3 during acute GVHD and increased proliferative capacity of allogeneic donor T-cells upon Tim-3 blockade, we reasoned that GVHD would be augmented upon Tim-3/gal-9 pathway inhibition. Soluble Tim-3-Ig fusion protein (Tim-3-Ig) binds gal-9, precluding Tim-3 engagement. To test its effect on GVHD, B6 recipients were lethally irradiated, given BALB/c BM with or without BALB/c splenocytes ($15x10^6$) and Tim-3-Ig or IgG at doses of 100μg IP every day from d-1 to 5 and then 3 times/week until d21. Figure 3A shows recipients of Tim-3-Ig had a significant increase in GVHD lethality with a median survival time (MST) of only 9 days vs. 33 days for recipients of IgG (p=0.012).

To determine whether Tim-3 expression on donor T-cells would impact GVHD lethality, lethally irradiated WT B6 mice were given BALB/c BM with or without purified T-cells from BALB/c or Tim-$3^{-/-}$ mice to induce GVHD. Despite the fact that Tim-$3^{-/-}$ T-cells had increased proliferative capacity, T-cell mediated GVHD lethality was augmented, albeit more modestly than with using blocking Tim-3-Ig and WT cells. Figure 3B shows pooled survival data with Tim-$3^{-/-}$ donor T-cells having a trend (p=0.083) toward accelerated lethality (MST, 59 days) compared to WT (MST, 82 days). To strengthen these data, clinical scoring was performed. B6 mice were lethally irradiated and given BALB/c BM and $3x10^6$ BALB/c or Tim-$3^{-/-}$purified T-cells. Consistent with the survival data, figure 3C shows that mice that received Tim-$3^{-/-}$ donor T-cells had significantly increased clinical scores

(d10-d31, $p<0.05$). In summary, both using a blocking reagent and using Tim-$3^{-/-}$ donor cells resulted in augmented GVHD lethality.

Decreased lethality in Gal-9 Tg recipients

It has been shown that Gal-9 Tg mice have a reduced ability to prime a Th1 immune response and generate Teffs.[87] Based on this premise, we sought to determine if Gal-9 Tg recipient mice could inhibit GVHD lethality. In these mice, Gal-9 expression is driven by a ubiquitous promotor (β-actin), which leads to high gal-9 especially in the small intestine, liver and lung (Supplemental Figure 1A). Gal-9 Tg mice co-express Gal-9 on $B220^+$, $CD11b^+$, and $CD11c^+$ APCs present in the spleen (Supplemental Figure 1B). BALB/c or Gal-9 Tg mice were lethally irradiated and given B6 BM and $2x10^6$ B6 purified T-cells. Figure 3D shows that Gal-9 Tg recipients had a significant decrease in lethality rate (MST, 35.5 days) compared to WT recipients (MST, 26.5 days). To determine if the decreased lethality rate was dependent upon Tim-3 expression by donor T-cells, Gal-9 Tg mice were lethally irradiated and given BALB/c BM and $3x10^6$ BALB/c or Tim-$3^{-/-}$ purified T-cells. Gal-9 Tg recipients that received Tim-$3^{-/-}$ donor T-cells did not have the survival advantage seen with Tim-3 expressing BALB/c T-cells (Figure 3E). This demonstrates that Tim-3 expression on donor T-cells was necessary for the survival advantage in Gal-9 Tg recipients.

Donor Treg depletion increases survival rates when the Tim-3/gal-9 pathway is blocked

We have shown that depletion of Tregs from donor T-cell grafts worsens GVHD, while donor Treg add-back diminishes GVHD lethality.[33] To determine if donor Treg mediated GVHD suppression was diminished by using Tim-3$^{-/-}$ versus WT T-cells, we depleted the graft of Tregs. B6 recipients were lethally irradiated and given BALB/c BM ± Treg (CD25)-depleted donor purified T-cells from WT BALB/c or Tim-3$^{-/-}$ mice. Remarkably, and contrary to our predictions, mice that received Tim-3$^{-/-}$ purified Treg-depleted T-cells were protected against GVHD lethality compared to recipients of WT Treg depleted T-cells ($p<0.001$), resulting in a MST of 104 days vs. a MST of 32 days respectively (Figure 4A). Clinical scoring showed significantly decreased scores for recipients of Tim-3$^{-/-}$ Treg-depleted T-cells (not shown). To determine whether these surprising findings were unique to the mode of Tim-3/Gal-9 pathway blockade, we used Tim-3-Ig instead of Tim-3$^{-/-}$ donor T-cells. Lethally irradiated B6 recipients were given BALB/c BM ± CD25-depleted donor purified T-cells and Tim-3-Ig or IgG (100µg d0 to 5 then qod until d28 post-BMT). Recipients of WT Treg-depleted T-cells and irrelevant IgG treatment had a rapid GVHD lethality course with a MST of 32 days. Consistent with findings using Tim-3$^{-/-}$ Treg-depleted T-cells, Figure 4B shows that recipients of Treg-depleted BALB/c T-cells and Tim-3-Ig treatment were protected against GVHD lethality compared to recipients of WT purified Treg depleted T-cells ($p<0.01$), resulting in 75% vs 0% long-term survival, respectively. Collectively, these data from two distinct but complementary approaches support the notion that blockade of the Tim-3/gal-9 pathway in the absence of donor Tregs inhibits, rather than augments, GVHD lethality.

To investigate if the Tim-3 pathway had differential effects on Teffs vs. Tregs, we performed in vitro studies to address expression and suppressive capacity. Naïve Tregs were isolated from BALB/c and Tim-3$^{-/-}$ mice and activated and expanded in vitro. Neither the naïve or activated Tregs expressed Tim-3 (Supplemental Figure 2A). A Treg suppression assay was performed using naïve Tregs isolated from the spleen of BALB/c and Tim-3$^{-/-}$ mice. Tregs were combined with CFSE labeled CD25-depleted purified T-cells and irradiated APCs in vitro and stimulated with αCD3. The cells were then harvested on d4 and analyzed for CFSE dilution. There was no significant difference in the ability of WT Tregs to suppression Teff division compared to Tim-3$^{-/-}$ Tregs (Supplemental Figure 2B).

Tim-3/gal-9 pathway blockade results in decreased gut injury in recipients of Treg-depleted donor grafts

To investigate the mechanism of increased survival, we sought to evaluate the gastrointestinal tract, which is a major GVHD target organ. B6 recipients were lethally irradiated and given BALB/c BM ± CD25-depleted donor purified T-cells from BALB/c or Tim-3$^{-/-}$ mice. To measure epithelial integrity of the GI tract, we used a FITC-dextran assay in which the loss of epithelial cell integrity results in leakage of FITC-dextran from the intestine into the peripheral blood. FITC-dextran was administered orally to mice on d14 and d21 and serum levels were measured 4 hours later. On d14, mice that received Tim-3$^{-/-}$ CD25-depleted

donor T-cells had a significantly reduced level of FITC-dextran in the serum (1.629 μg/mL) compared to recipients of WT CD25-depleted T-cells (2.762 μg/mL) indicating increased epithelial integrity (Figure 5A; p=0.046). On d21, there was also a trend (p=0.078) toward reduced levels in the serum of recipients of CD25-depleted Tim-$3^{-/-}$ T-cells. On both days, there was no significant difference between the group that received BM only and the group that received CD25-depleted Tim-$3^{-/-}$ T-cells. Histopathology scores of the gut on d21 showed a trend ($p<0.07$) towards reduced pathology in the colon of recipients of CD25-depleted Tim-$3^{-/-}$ T-cells (not shown).

The colon was examined for the presence of T-cells expressing effector cytokines that cause tissue damage. B6 hosts were infused with BALB/c BM ± BALB/c CD25-depleted purified T-cells from BALB/c or Tim-$3^{-/-}$ mice. On d21, lamina propria lymphocytes (LPLs) were isolated, re-stimulated, and stained for the expression of IL-2, IFN-γ, and IL-17, known effector molecules involved in GVHD. Expression of IL-2, IFN-γ, and IL-17 in $CD4^+$ and $CD8^+$ T-cells was significantly decreased in recipients of CD25-depleted Tim-$3^{-/-}$ vs WT T-cells (Figure 5B). These data suggest that in our model where Tim-3 is inhibited in the absence of donor Tregs, the GI tract is better protected, which is associated with increased overall survival.

Elevated IFN-γ leads to activation-induced cell death

One explanation for the paradoxical results seen with the Tim-3/gal-9 blockade in recipients of unmanipulated vs Treg-depleted donor T-cells could be the over exuberant production of IFN-γ resulting in donor T-cell activation induced cell death (AICD) in the latter case. To examine this possibility, B6 recipients were lethally irradiated and given BALB/c BM and WT or Tim-$3^{-/-}$ purified T-cells that were either unmanipulated or CD25-depleted. On d7 post-BMT, splenocytes were, re-stimulated in vitro in the presence of brefeldin A, and T-cells were analyzed for IFN-γ expression by ICC staining. Figure 6 shows T-cells obtained from recipients of Tim-$3^{-/-}$ compared to WT donor T-cells had significantly increased IFN-γ expression in both donor $CD4^+$ ($2.4x10^5$ vs. $1.2x10^5$) and $CD8^+$ ($1.8x10^5$ vs. $1.1x10^5$) T-cells. Along with IFN-γ, we also found that T-cells isolated from recipients of Tim-$3^{-/-}$ had increased levels of the effector molecule Granzyme B ($CD4^+$, $3.0x10^5$ vs. $1.2\ x10^5$; $CD8^+$, $5.4x10^5$ vs. $2.1x10^5$). Ki-67 staining was used to determine that Tim-$3^{-/-}$ donor T-cells were also proliferating more than WT T-cells ($CD4^+$, $1.1x10^5$ vs. $3.0x10^4$; $CD8^+$, $1.3x10^5$ vs. $4.1x10^4$). These results indicate that simultaneously inhibiting Tim-3/gal-9 and depleting donor Tregs leads to increased donor T-cell effector molecule secretion and increased proliferation.

The increased activation state that is observed in Tim-$3^{-/-}$ donor T-cells compared to WT donor T-cells supports the possibility that these cells are undergoing AICD, reducing the number of donor alloreactive T-cells, and ultimately leading to an increased survival rate. To examine AICD of donor T-cells, B6 mice were

lethally irradiated and infused with BALB/c BM and BALB/c or Tim-3$^{-/-}$ purified CD25^{-} donor T-cells. Splenocytes were analyzed on d7 post transplant. First, we analyzed the number of cells that were expressing Annexin V/7-AAD (indicating dead cells). There were significantly increased numbers of Tim-3$^{-/-}$ donor T-cells that were AnnexinV^{+}/7AAD^{+} compared to WT donor T-cells (Figure 6B). There were significantly increased numbers of Tim-3$^{-/-}$ compared to WT donor T-cells that were active-caspase 3^{+}, a more specific cell-death marker. Cumulatively, these data suggest that recipients of Tim-3$^{-/-}$ Treg-depleted donor T-cells are more activated leading to increased AICD, which was associated with decreased GVHD lethality.

To further analyze AICD, we sought to track the T-cell expansion/contraction in vivo using a monoclonal tracer system.[88] B6D2F1 mice were lethally irradiated and infused with B6 NTCD BM, 5x10^{5} B6 purified T-cells, and 2.5x10^{4} TEa monoclonal luciferase enriched T-cells (Tim-3-Ig vs. IgG treatment). Initially, mice that received Tim-3-Ig had a significant increase in T-cell expansion followed by a contraction that resulted in a significantly smaller T-cell pool at later time points in the assay (d20-24).

Blocking IFN-γ eliminates the promotion of AICD

To further analyze the role of IFN-γ in promoting AICD, we sought to inhibit IFN-γ to determine whether this cytokine was responsible for the survival differences between the recipients of CD25-depleted WT or Tim-3$^{-/-}$ donor T-cells. B6

recipients received BALB/c BM ± CD25-depleted donor purified T-cells from WT BALB/c or Tim-3$^{-/-}$ mice and were treated with either IgG or α-IFN-γ antibody (400µg twice per week). Whereas recipients of CD25-depleted Tim-3$^{-/-}$ T-cells had a MST of 74 days, the lethality rate was significantly higher (p=0.046) in those receiving CD25-depleted WT T-cells with a MST of 55 days. The groups that received anti-IFN-γ mAb treatment had no statistically significant difference between the survival curves with recipients of either CD25-depleted WT or Tim-3$^{-/-}$ experiencing 100% lethality by 2 weeks post-BMT. In summary, IFN-γ is necessary for the increased survival that is observed when Tim-3/gal-9 is inhibited in the absence of donor Tregs.

Further studies were done to analyze the effect of IFN-γ blockade. B6 recipients were infused with BALB/c BM ± CD25-depleted donor purified T-cells from WT BALB/c or Tim-3$^{-/-}$ mice and were treated with either IgG or αIFN-γ antibody. Contrary to Figure 6, blocking IFN-γ resulted in a significantly decreased number of Tim-3$^{-/-}$ donor T-cells that were AnnexinV^{+}/7AAD^{+} compared to WT T-cells and eliminated any difference in Ki-67^{+} at d7 post transplant. LPL's were isolated on d21 post-transplant. Significantly fewer cells were found in recipients of Tim-3$^{-/-}$ vs WT donor T-cells. Blocking with αIFN-γ resulted in a trend (p<0.06) towards increased cells in recipients of Tim-3$^{-/-}$ T-cells compared to WT. When we analyzed the number of proliferating LPLs, Ki-67^{+} cell number in recipients of Tim-3$^{-/-}$ vs WT T-cells was decreased. Blocking IFN-γ resulted in a significant

increase in the number of Ki-67^{+} cells in recipients of Tim-3$^{-/-}$ T-cells compared to WT.

Discussion

Here, we demonstrate the important role for the Tim-3/gal-9 pathway in GVHD in MHC-disparate recipients. Tim-3 was found to be highly up-regulated on infused donor T-cells suggesting a role in GVHD regulation. Inhibition of Tim-3/gal-9 binding led to an increase in proliferation of donor T-cells both in vitro and in vivo. Blocking the Tim-3/gal-9 pathway with Tim-3-Ig resulted in significant augmentation of GVHD lethality. Importantly, to our knowledge, we have shown for the first time that depletion of Tregs in the absence of Tim-3 resulted in the amelioration of GVHD lethality. Tim-3$^{-/-}$ donor T-cells that were CD25-depleted resulted in decreased gut GVHD as evidenced by increased mucosal integrity (decreased FITC-dextran serum levels) as well as decreased effector cytokine production by donor T-cells. We found that this paradoxical result was caused by the increased levels of IFN-γ causing AICD of the donor T-cells. Blocking IFN-γ eliminated lethality differences between WT and Tim-3$^{-/-}$ CD25-depleted donor T-cell infusion suggesting that IFN-γ levels are critical for regulating GVHD.

Previously, we showed that negative regulatory pathways play an important role in regulating GVHD.[89-91] This study examined the role of a novel negative regulator Tim-3 and its expression and role in regulating acute GVHD. Infused donor T cells rapidly up-regulated Tim-3 and caused striking up-regulation of the

ligand gal-9 on non-hematopoietic, stromal cells. Gal-9 is up-regulated by IFN-γ, which is increased during acute GVHD.[78,92] Tim-3 ligand is detected on $CD4^+$ and to a much lesser extent $CD11c^+$ cells by Tim-3-Ig fusion protein staining.[47,81] However, a recent finding showed that a monoclonal α-Gal9 antibody did not stain human resting $CD4^+$ cells.[93] It is possible that the Tim-3L protein used in earlier studies was binding to alternative Tim-3 ligands other than Gal-9 as was discussed by Cao et al.[44,94]

In the absence of Tim-3, donor T-cells were able to proliferate more rapidly. These data are consistent with other studies involving a Th1 response and showed that effector Th1 cells express Tim-3 and proliferation was increased when the Tim-3 pathway was blocked with Tim-3-Ig.[47,81] The Tim-3-Ig fusion protein was used to show that mice were more susceptible to autoimmune diseases such as EAE and diabetes. To evaluate the effect of Tim-3 on GVHD, we first used the Tim-3-Ig to block the pathway. As expected, Tim-3 pathway blockade with Tim-3-Ig resulted in significantly increased lethality compared to controls that received human IgG1. These data confirm and extend those of Oikawa et. al. who showed in a non-irradiated parent-into-F1 model of a graft-versus-host response that Tim-3 expression was up-regulated on $CD8^+$ T-cells, albeit not $CD4^+$ T-cells, and a blocking anti-Tim-3 mAb caused weight loss and increased the frequency of both $CD4^+$ and $CD8^+$ T-cells expressing IFN-γ.[76] As further evidence for the fact that the Tim-3-Ig fusion protein was blocking the Tim-3/gal-9 pathway, we also used $Tim\text{-}3^{-/-}$ donor T-cells to inhibit the pathway.

Compared to the Tim-3-Ig, the GVHD lethality observed in recipients of Tim-3$^{-/-}$ donor T-cells were more modestly augmented. This could be due to the binding of Tim-3-Ig to receptors other than gal-9[44,94] or compensatory suppressive mechanisms that have overlapping functions with Tim-3. With respect to the latter, Tim-3$^{-/-}$ mice do not develop spontaneous autoimmunity, even though it has been shown that Tim-3$^{-/-}$ mice are more susceptible to autoimmune disease induction.[81]

Our data are consistent with reduced Tim-3^{+} Teff deletion by gal-9^{+} cells due to Tim-3-Ig blockade and provide in vivo evidence that the Tim-3/gal-9 pathway is an important negative regulator of GVHD. As predicted from this hypothesis, Gal-9 Tg vs WT recipients had significantly decreased GVHD lethality, which should help eliminate harmful Tim-3^{+} Teff. Systemic administration of recombinant galectin-9 (rGal-9) has been shown to improve allogeneic cardiac as well as skin grafts.[95,96] Increased allograft survival was consistent with deletion of CD4^{+}Tim-3^{+} Th1 cells. Due to these promising findings, it may be possible to develop the use of reagents, such as rGal-9, for use in the clinic.

Our most surprising finding was the amelioration of GVHD lethality when Tim-3/gal-9 binding was inhibited in the absence of Tregs. Tregs have a known inhibitory role in GVHD and Treg deletion of donor T-cell grafts typically leads to a more aggressive GVHD lethality response.[33] Initially, we sought to determine whether inadequate Treg-mediated suppression in the context of Tim-3/gal-9

pathway blockade contributed to augmented GVHD lethality. Since Tregs can express gal-9, blockade of Tim-3/gal-9 binding may directly reduce the suppression of Tim-3^+ T-cells by gal-9^+ Tregs, thereby augmenting alloreactive Teff responses.[86,97] Contradictory to our expectations, inhibiting Tim-3/gal-9 binding by using Tim-$3^{-/-}$ donor T-cells or Tim-3-Ig in the absence of donor Tregs did not augment but rather actually reduced GVHD lethality.

The gut is the site of the largest immune compartment in the body and has a very complex immunologic environment. A significant amount of morbidity and mortality from GVHD is a consequence of damage to the gut leading to increased intestinal permeability. This allows for endotoxin to cross the epithelial barrier invoking a cytokine storm leading to even more tissue damage. Studies have shown that an increased level of cytokine production in the gut, mainly by donor infiltrating T-cells, can mediate acute GVHD.[98,99] Conversely, it has been shown that inhibiting cytokines (IFN-γ or IL-12) can inhibit acute GVHD in murine models.[100,101] A reduced inflammatory response may be a mechanism responsible for the reduced epithelial damage and slowed GVHD lethality that we observed in recipients of Treg depleted Tim-$3^{-/-}$ donor T-cells. Our results show a lower percentage of both $CD4^+$ and $CD8^+$ donor T-cells were secreting inflammatory cytokines (IFN-γ, IL-2, and IL-17) in the lamina propria of the colon. This reduction in inflammatory cytokines could mean a reduction of gut epithelial damage as supported by our finding of reduced serum concentration of orally administered FITC-dextran. We concluded that recipients of Treg-depleted Tim-

$3^{-/-}$ donor T-cells had a decreased GVHD lethality rate due to a reduction of inflammatory cytokine secretion in the gut and increased integrity of the epithelial barrier.

Previously, we have reported that high IFN-γ production results in AICD in $CD4^+$ and to a lesser extent, $CD8^+$ T cells.[102] IFN-γ has anti-proliferative properties and has been shown to be a potent inducer of negative regulatory pathways that can limit GVHD injury. Consistent with this hypothesis, it has been shown that the loss of IFNγ via the use of IFN-$\gamma^{-/-}$ donor T cells or neutralizing anti-IFN-γ mAb markedly accelerated GVHD lethality.[103] We revealed increased IFN-γ levels in the spleen during the early phases of GVHD (d7) in recipients of Treg-depleted Tim-$3^{-/-}$ vs WT donor T-cells. IFN-γ was shown to be required for AICD of T-lymphocytes.[104] This early burst of IFN-γ could be responsible for an increased level of AICD in donor T-cells. Granzyme-B is capable of mediating AICD of T-cells.[105] Recipients of Treg depleted Tim-$3^{-/-}$ donor T-cells had significantly increased granzyme-B secretion. Increased cell death was detected by measuring levels of Annexin V/7-AAD expression as well as levels of activated caspase-3. This provides a mechanism for the gut protection we observed that led to a reduced GVHD lethality rate.

In summary, the Tim-3/gal-9 pathway can act as a suppressor of acute GVHD. Tim-3 is up-regulated in the early stages of GVHD on Teffs where it can regulate T-cell proliferation and affect GVHD lethality. We linked the inhibition of the Tim-

3/gal-9 pathway in the absence of donor Tregs to critical IFN-γ levels capable of ameliorating GVHD. It may be possible to translate our findings that enhanced signaling of the Tim-3/gal-9 pathway early post-transplantation to dampen the Th1 response and reduce GVHD in the presence of a T-cell replete graft by using reagents such as rGal-9 protein.

Authorship

R.G.V. designed and performed research, analyzed the data, and wrote the paper. P.A.T. performed experiments, provided advice, and edited the paper. Q.Z. performed experiments. A.P-M. and M.H. provided data. M.H., D.L., T.B.S., A.C.A. and V.K.K. provided essential reagents or mice, advice, and edited the paper. B.R.B. designed, organized, and supervised research and edited the paper. The authors thank Beverly Norris for purification of the αTim-3 monoclonal antibody and Nick Bade and Kazutoshi Aoyama for technical assistance.

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Conflicts of Interest

The authors declare no competing financial interests.

Figure Legends

Figure 1: Tim-3/Gal-9 expression is upregulated during acute GVHD. A. B6 mice were lethally irradiated (11.0Gy) and infused with 10^7 MHC-mismatched BALB/c NTCD BM and $3x10^6$ BALB/c purified T cells. Recipient mice were sacrificed on d7, along with 3 naïve BALB/c mice for control, and the spleens were examined by flow cytometry for Tim-3 expression. Cells were gated on CD4 or CD8 positive, H-$2K^d$ positive events. B. 10^7 CFSE-labeled BALB/c purified T cells were transferred into lethally irradiated B6 recipients. Spleens were harvested and analyzed by flow cytometry for CFSE dilution and Tim-3 expression on days 3, 4, and 5. Data shown are representative of 4 mice/group/day. Cells were gated on CD4 or CD8 positive, $H2K^d$ positive events. Numbers indicate percentage of Tim-3^+ cells. C. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c purified T cells and sacrificed on d7. Cryosections from the spleen of BM only recipients (top picture) and T-cell recipients (bottom picture) were stained for CD45 in FITC (shown in green) and Gal-9 (shown in red). Original magnification 20x. Pictures are representative of two experiments, 3-4 mice/group.

Figure 2: Inhibiting Tim-3 increases allogeneic T-cell proliferation. A. MLR was performed by mixing B6-purified T cells with irradiated BALB/c stimulators (1:1) and αTim-3. These cultures were pulsed with 3H-thymidine on the indicated days and harvested 16 hours later. Proliferation was determined as a measure of radioactive uptake. One Way ANOVA with tukey multiple comparison

test P<0.001 n=5 per group. ■, Control IgG; ○, αTim-3 5D12 mAb B. 10^7 CFSE-labeled WT or Tim-$3^{-/-}$ BALB/c purified T cells were transferred into lethally irradiated CD45.1^+ congenic B6 recipients. Spleens were harvested and analyzed by flow cytometry for CFSE dilution and on days 3 and 4. Representative histograms shown are representative of 4 mice/group/day. Filled histogram, WT donor; Open histogram, Tim-$3^{-/-}$ donor. Cells were gated on CD45.1^+, $H2K^d$ positive, CD4 or CD8 positive events.

Fig. 3. Inhibiting Tim-3 results in accelerated lethality while increasing Gal-9 expression results in decreased lethality. A. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $15x10^6$ BALB/c splenocytes. Survival plot of IgG control (closed circle) vs. Tim-3-Ig treatment (open circle) is shown p=0.012. n=21-24 per group. B. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $1x10^6$ BALB/c or $1x10^6$ Tim-$3^{-/-}$ purified T-cells. Survival plot of BALB/c (closed circle) vs. Tim-$3^{-/-}$ (open circle) donor is shown. p=0.083. n=16 per group. C. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $1x10^6$ BALB/c or $1x10^6$ Tim-$3^{-/-}$ purified T-cells. Mice were analyzed for clinical scores. n= 8 per group. D. BALB/c or Gal-9 Tg mice were lethally irradiated and given 10^7 B6 NTCD BM and $2x10^6$ B6 purified T-cells. Survival plot of BALB/c vs. Gal-9 Tg recipients is shown. p<0.01. n=24 per group E. Gal-9 Tg mice were lethally irradiated and given 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or $3x10^6$ Tim-$3^{-/-}$ purified T-cells. Survival plot of BALB/c vs. Tim-$3^{-/-}$ donors is shown. p=0.217. n=12 per group

Fig. 4. Inhibiting Tim-3 in the absence of Tregs leads to decreased lethality. A. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-$3^{-/-}$ purified $CD25^-$ T-cells. Survival plot of BALB/c (closed circle) vs. Tim-$3^{-/-}$ (open circle) donor is shown $p<0.001$. n=15 per group. B. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and 10^6 BALB/c $CD4^+CD25^-$ T-cells. Survival plot of IgG control (closed circle) vs. Tim-3-Ig (open circle) treatment is shown. $p<0.01$. n=8 per group.

Fig. 5. Inhibiting Tim-3 in the absence of T-regs reduces gut pathology. A. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-$3^{-/-}$ purified $CD25^-$ T-cells. 16mg of FITC-dextran was administered orally to mice on d14 (n=4, p=0.046) and d21(n=4, p=0.0778). Serum levels were measured 4 hours later. B. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-$3^{-/-}$ purified $CD25^-$ T-cells. Mice were sacrificed on day 21 and lamina propria lymphocytes were analyzed for effector cytokines.

Fig. 6. Elevated IFN-γ levels lead to AICD. A. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-$3^{-/-}$ purified $CD25^-$ T-cells. Mice were sacrificed on day 7 and splenocytes were analyzed for IFN-γ (n=4), Granzyme-B (n=8), and Ki-67 (n=8). *$p<0.05$, ***$p<0.001$ B. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$

BALB/c or Tim-3$^{-/-}$ purified CD25^{-} T-cells. Mice were sacrificed on day 7 and splenocytes were analyzed for Annexin V/7-AAD (n=4) and activated caspase-3 (n=8, $p<0.05$). C. B6D2F1 mice were lethally irradiated and infused with 10^7 B6 NTCD BM, $5x10^5$ B6 purified T-cells, and $2.5x10^4$ TEa monoclonal Luciferase enriched T-cells.

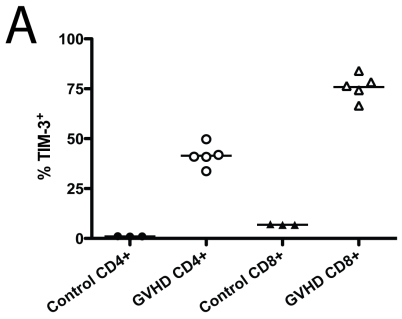
Fig. 7. Blocking IFN-γ eliminates the promotion of AICD A. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-3$^{-/-}$ purified CD25^{-} T-cells. Recipients of Tim-3$^{-/-}$ CD25-depleted T-cells had a significantly ($p = 0.046$) higher survival than those given WT CD25-depleted T-cells. Survival plot of IgG control vs. αIFN-γ treatment is shown. n=8 per group. B. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-3$^{-/-}$ purified CD25^{-} T-cells. Mice were sacrificed on day 7 and splenocytes were analyzed by flow cytometry for Annexin V (n=4) and Ki-67 (n=5-8). *$p<0.05$ C. B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c or Tim-3$^{-/-}$ purified CD25^{-} T-cells for IgG control treatment and $1x10^6$ BALB/c or Tim-3$^{-/-}$ purified CD25^{-} T-cells for αIFN-γ treatment. Mice were sacrificed on day 21 and lamina propria lymphocytes were analyzed for total CD4^{+} and CD8^{+} cell numbers (*$p<0.05$) and total Ki-67 (*$p<0.05$, **<0.01).

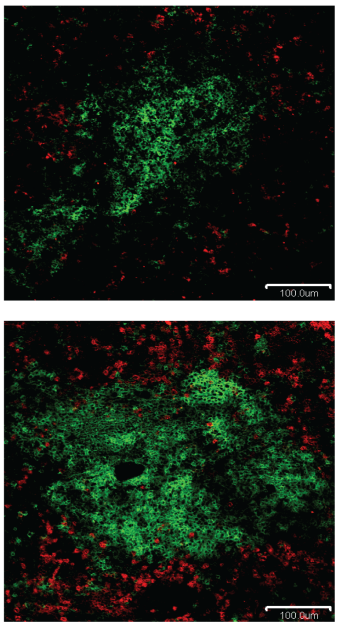
SF 1. Gal-9 staining pattern in GVHD organs. A. GVHD target organs (lung, jejunum, ileum, and liver) from WT B6 and Gal-9 Tg B6 mice were sectioned. Slides were stained for Gal-9 and counterstained with hematoxylin. B. Gal-9 Tg

B6 mice were sectioned and stained with Gal-9 (shown in red) and either B220, CD11b, or CD11c (shown in green).

SF 2. Tim-3 is not expressed on Tregs, does not contribute to a difference in Treg suppressive capacity, and does not alter Treg viability. A. Tregs ($CD4^+CD25^+$) were isolated from the spleen of BALB/c and Tim-$3^{-/-}$ mice. Naïve Tregs had no Tim-3 expression by flow cytometry. Tregs were activated in vitro with plate bound αCD3 in the presence of IL-2 (100U/mL) for 4 days. They were further expanded in the presence of IL-2 for 3 more days (day 7 in culture). Neither the activated or expanded Tregs expressed Tim-3. (Filled histogram, WT Tregs; Open histogram, Tim-$3^{-/-}$ Tregs. Cells were gated on $CD4^+CD25^+Foxp3^+$ events. B. Treg suppression assay was performed as described. Proliferating T-cells were quantified by the dilution of CFSE resulting in a $CFSE^{low}$ population. There was no difference in the suppressive capacity of WT vs Tim-$3^{-/-}$ Tregs. C. 10^6 WT purified T cells and 10^6 WT or Tim-$3^{-/-}$ CFSE-labeled Tregs ($CD4^+CD25^+$) were transferred into lethally irradiated $CD45.1^+$ congenic B6 recipients. Spleens were harvested and analyzed by flow cytometry for Treg viability on days 10. Cells were gated on $CD45.1^-$, $H2K^d$ positive, $CD4^+$, $Foxp3^+$, viability dye positive events.

Figure 1


A
100
75
50
25
0
% TIM-3+
Control CD4+
GVHD CD4+
Control CD8+
GVHD CD8+


C
100.0um
100.0um

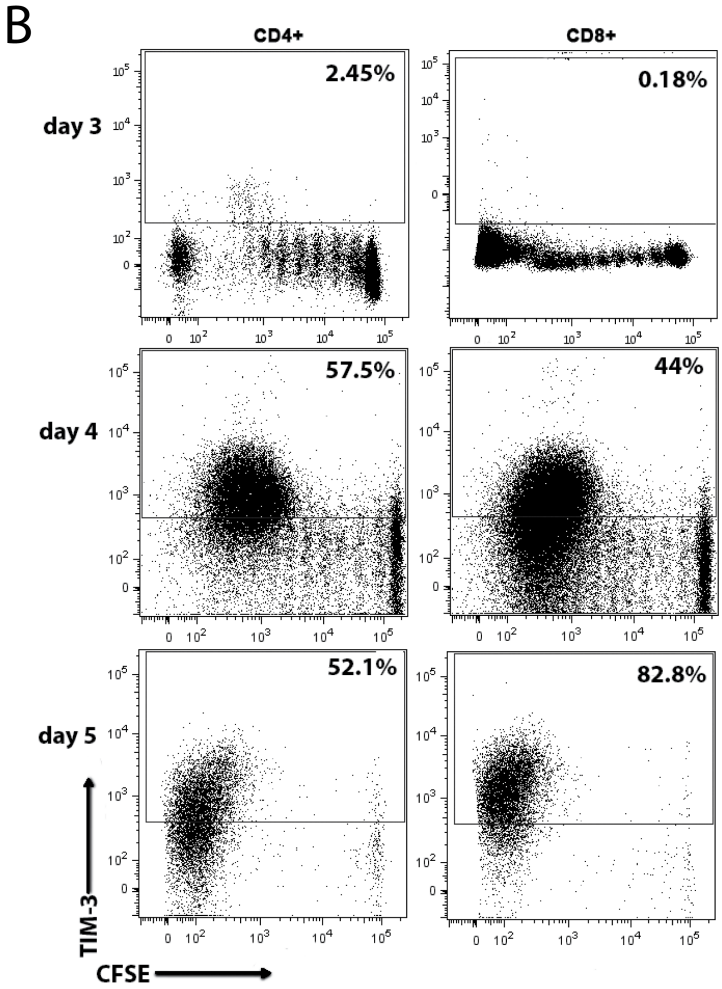

B
CD4+
CD8+
day 3
2.45%
0.18%
day 4
57.5%
44%
day 5
52.1%
82.8%
TIM-3
CFSE

Figure 2

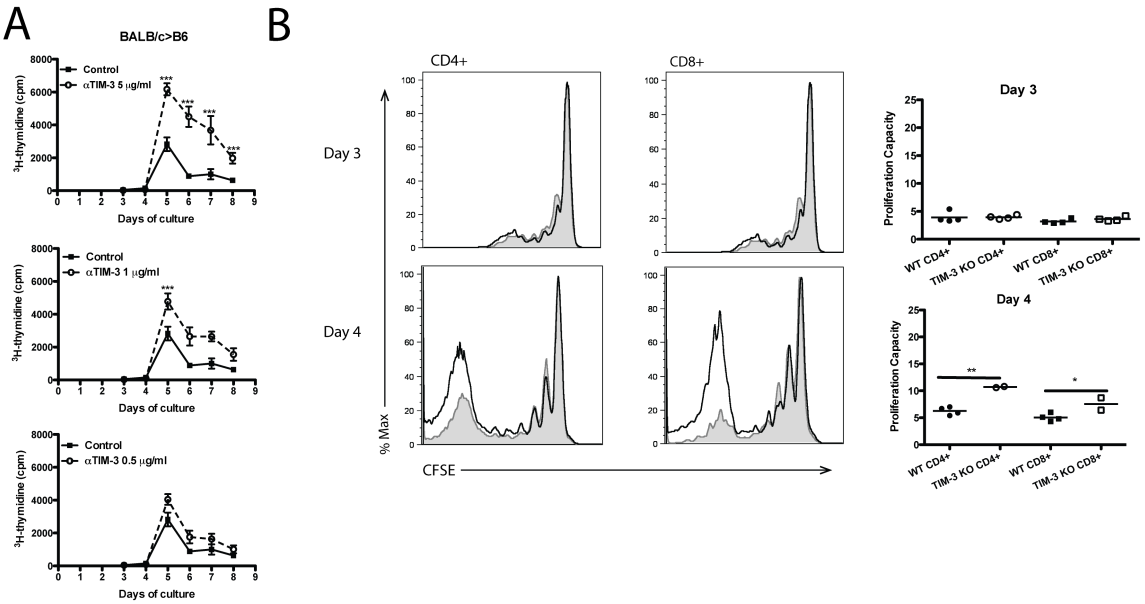


Figure 3

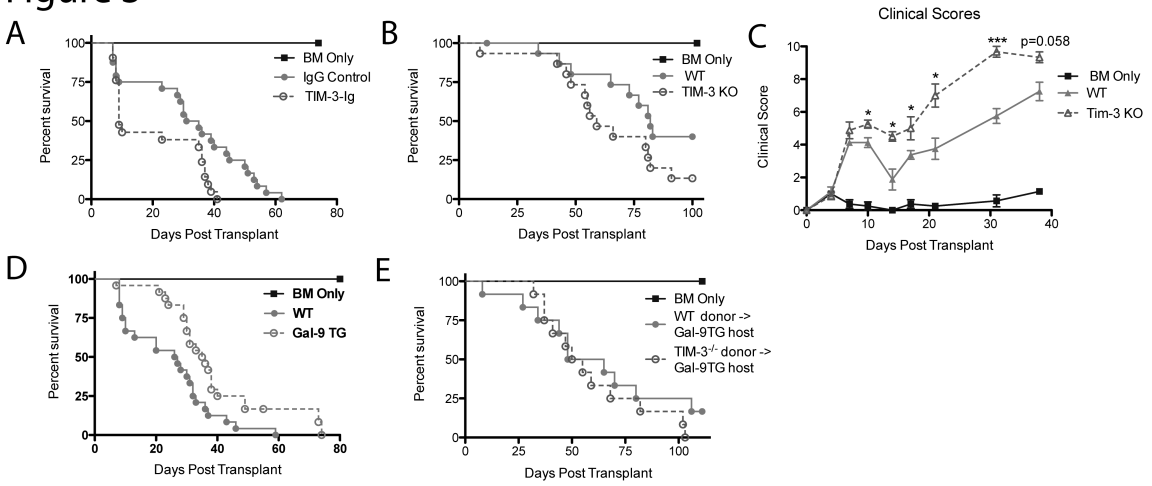
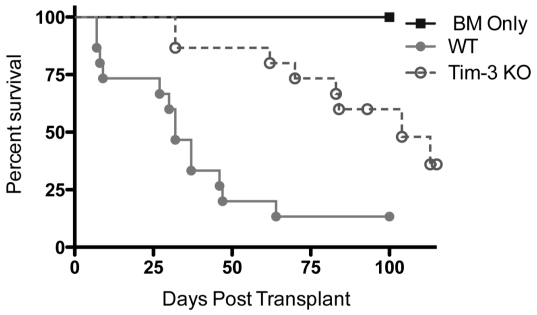


Figure 4

A


100
75
50
25
0
Percent survival
0
25
50
75
100
Days Post Transplant
BM Only
WT
Tim-3 KO

B

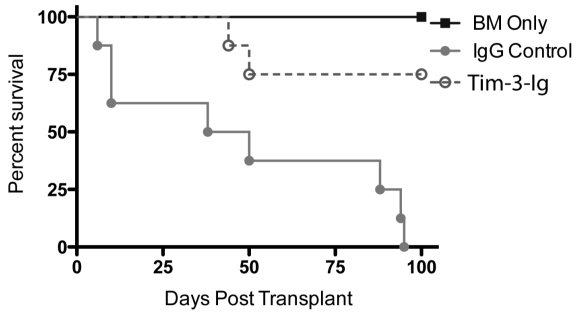

100
75
50
25
0
Percent survival
0
25
50
75
100
Days Post Transplant
BM Only
IgG Control
Tim-3-Ig

Figure 5

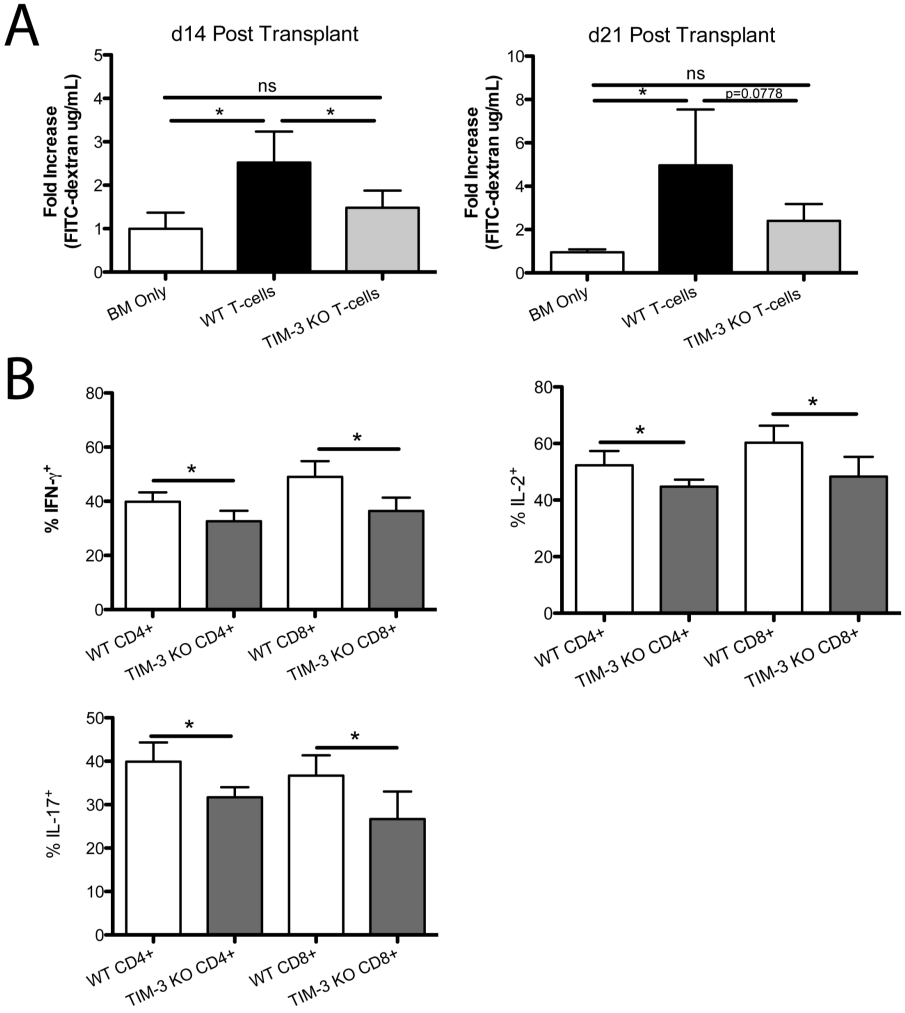

A
d14 Post Transplant
Fold Increase (FITC-dextran ug/mL)
ns
*
*
BM Only
WT T-cells
TIM-3 KO T-cells
d21 Post Transplant
Fold Increase (FITC-dextran ug/mL)
ns
*
p=0.0778
BM Only
WT T-cells
TIM-3 KO T-cells
B
% IFN-γ+
% IL-2+
% IL-17+
WT CD4+
TIM-3 KO CD4+
WT CD8+
TIM-3 KO CD8+

Figure 6

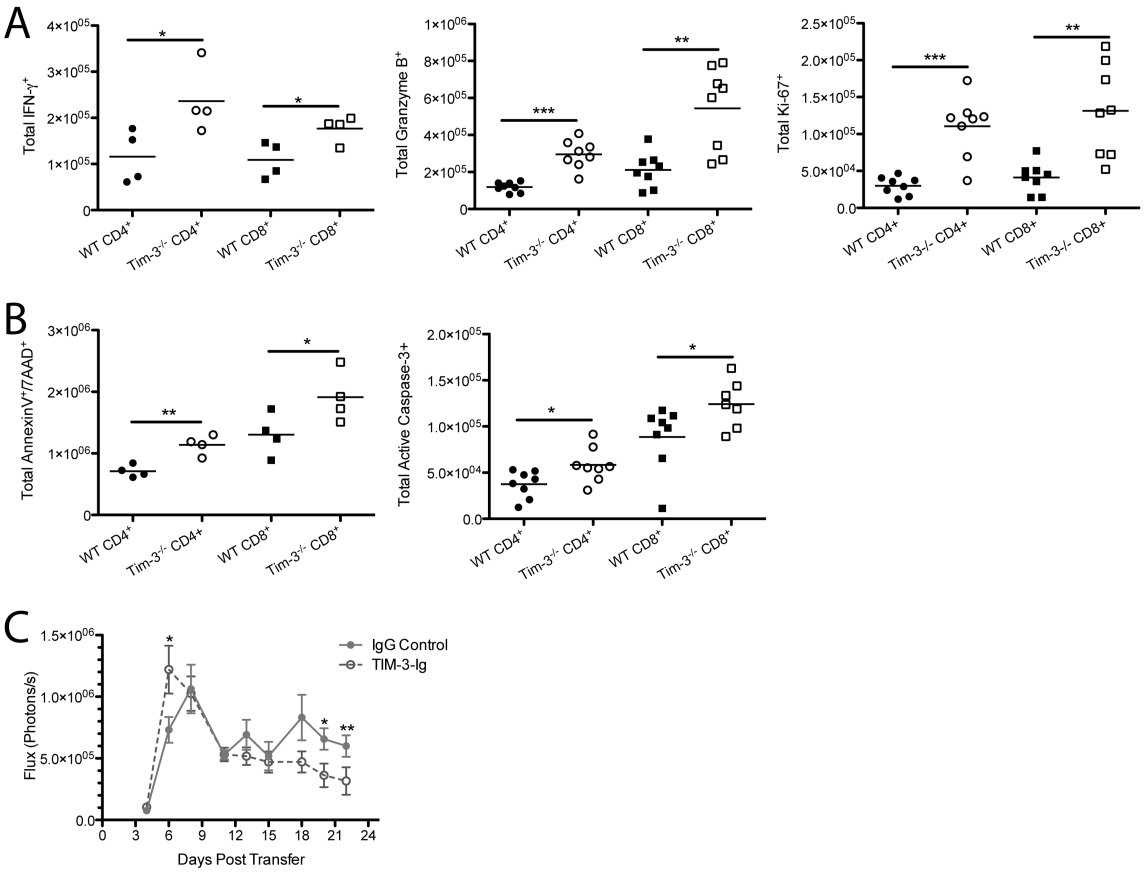
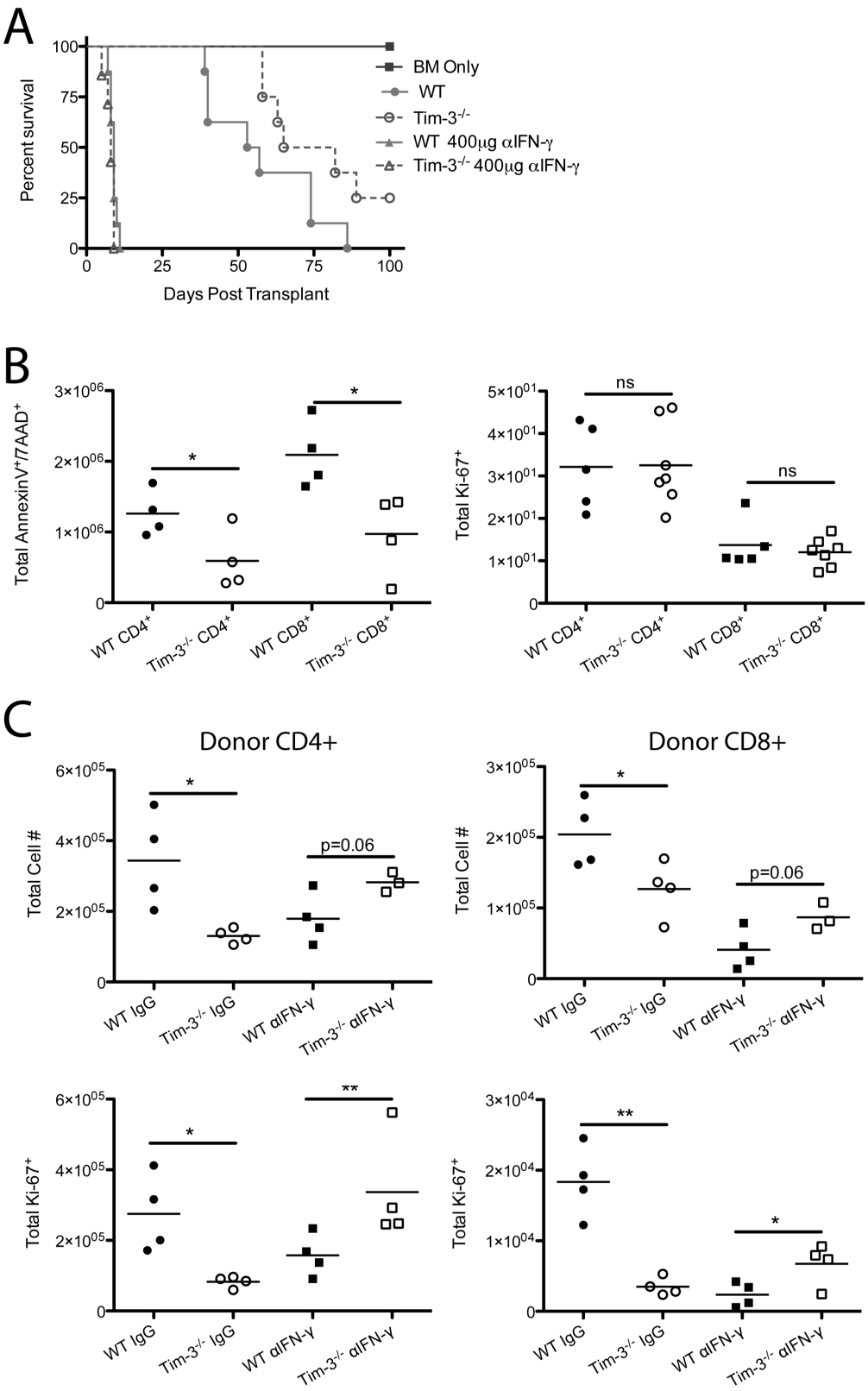
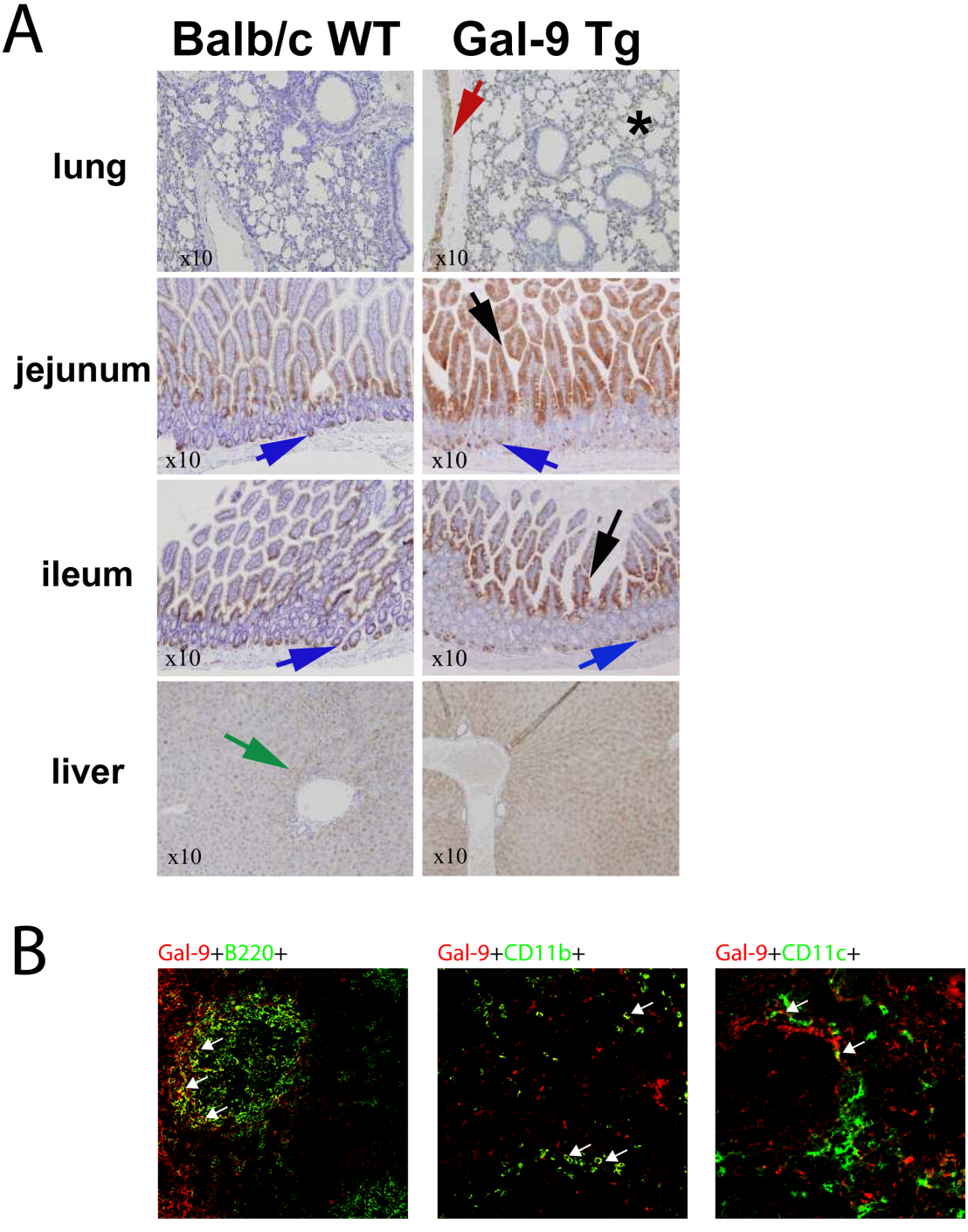

A
Total IFN-γ+
Total Granzyme B+
Total Ki-67+
WT CD4+
Tim-3-/- CD4+
WT CD8+
Tim-3-/- CD8+
B
Total AnnexinV+/7AAD+
Total Active Caspase-3+
C
IgG Control
TIM-3-Ig
Flux (Photons/s)
Days Post Transfer

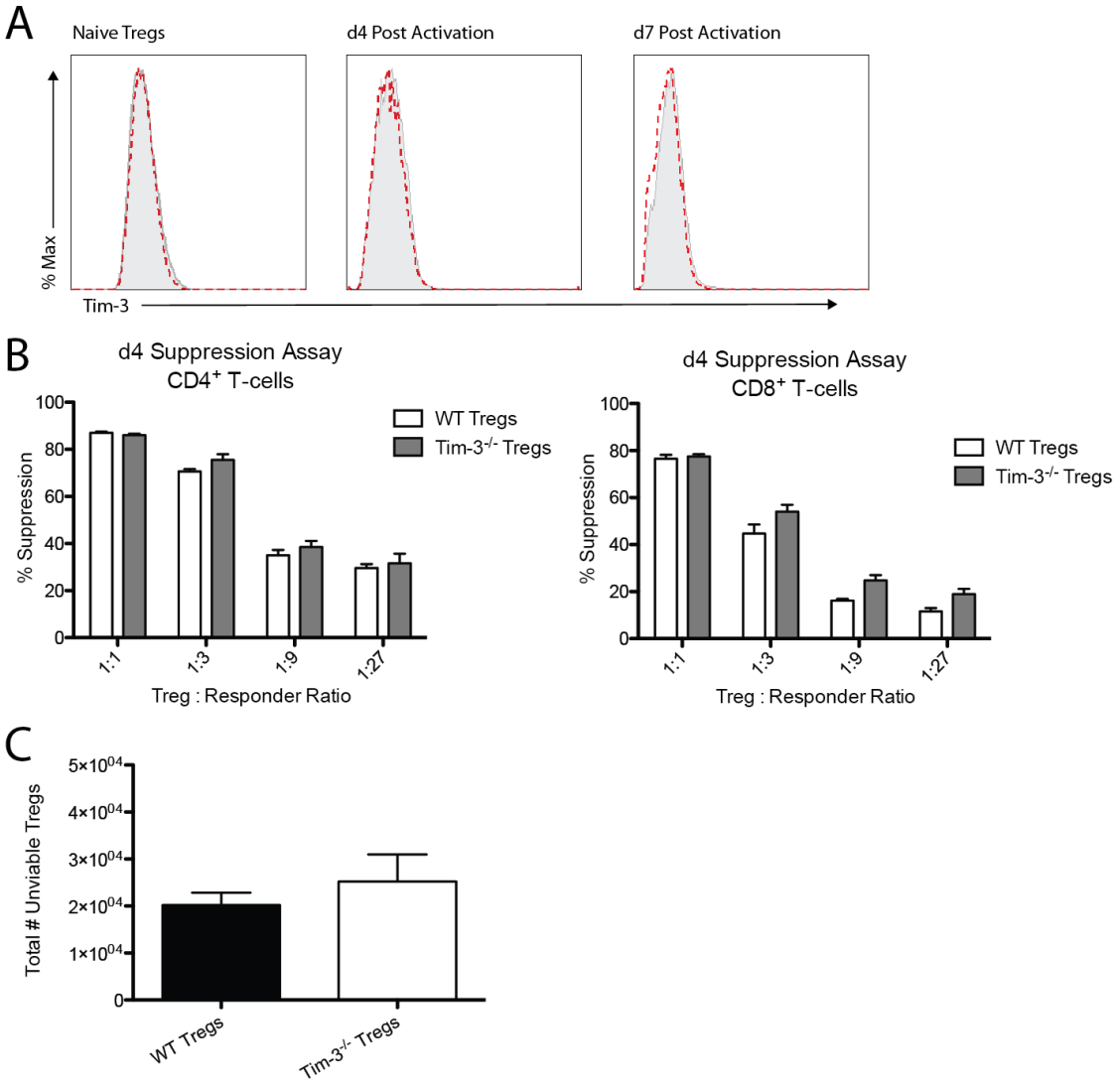
Figure 7


A
Percent survival
Days Post Transplant
BM Only
WT
Tim-3-/-
WT 400μg αIFN-γ
Tim-3-/- 400μg αIFN-γ
B
Total AnnexinV+7AAD+
Total Ki-67+
WT CD4+
Tim-3-/- CD4+
WT CD8+
Tim-3-/- CD8+
ns
C
Donor CD4+
Donor CD8+
Total Cell #
Total Ki-67+
WT IgG
Tim-3-/- IgG
WT αIFN-γ
Tim-3-/- αIFN-γ
p=0.06

Supplemental Figure 1



Supplemental Figure 2



Chapter III: B7-H3 expression in donor T cells and host non-hematopoietic cells negatively regulates graft-versus-host disease lethality.

Veenstra RG, Flynn R, Kreymbok K, Taylor PA, Panoskaltsis-Mortari A, Allison JP, Mak TW, van den Brink MR, Blazar BR.

Members of the B7 family have been shown to be important for regulating immune responses by providing either positive or negative costimulatory signals. The function of B7-H3 has been controversial. We show that B7-H3 is up-regulated in GVHD target organs including the colon, liver, and lung. Infusion of allogeneic donor T-cells into B7-H3$^{-/-}$ vs wildtype (WT) recipients also resulted in increased GVHD lethality, associated with increased T-cell proliferation, colonic inflammatory cytokines as well as epithelial barrier destruction. Allogeneic B7-H3$^{-/-}$ versus WT donor T-cells also had increased T-cell proliferation and GVHD lethality, associated with increased proliferation and cytokine secretion in the spleen, intraepithelial lymphocyte inflammatory cytokines and intestinal permeability. Although B7-H3$^{-/-}$ Tregs were less suppressive than WT Tregs, GVHD also was augmented in recipients of B7-H3$^{-/-}$ Treg-depleted grafts. We also show that T-cells lacking B7-H3 are still capable of providing graft-versus-leukemia effects without increased GVHD in a delayed lymphocyte infusion model. We conclude under these conditions that B7-H3 is responsible for providing a negative costimulatory signal, providing a basis for developing a treatment that increases signaling through the B7-H3 pathway to decrease the allogeneic immune responses such as those seen in GVHD.

Introduction

GVHD remains the leading cause of morbidity and mortality after BMT. Patients are given immune suppressive therapy to prevent or diminish the severity of GVHD following allogeneic BMT, which in turn increases the risk of infection and disease recurrence. Novel GVHD strategies remain a high priority.

B7-H3 is a member of the B7 family whose function in immune regulation has yet to be clearly defined. B7-H3 is a type I transmembrane protein and the most highly conserved B7 family member between mice and humans.[63] A wide range of cells express B7-H3 including activated T-cells, NK-cells, DCs, and macrophages.[63,67,106] B7-H3 is also expressed on non-hematopoietic cells including fibroblasts, synoviocytes, osteoblasts, and epithelial cells.[107-109] Although TLT-2, a type I transmembrane glycoprotein was identified as a receptor for B7-H3[64], others have shown no evidence for such an interaction in mouse or humans[69], further confounding elucidation of the nature of the biological response of the B7-H3 pathway.

Initial studies identified B7-H3 as a positive costimulatory molecule due to its capability to promote T-cell proliferation and IFN-γ secretion.[63] Overexpression of B7-H3 by transfection of a mouse tumor cell line promoted an antitumor response leading to the regression of tumors and amplification of a CTL response.[65] When a B7-H3$^{-/-}$ mouse was used in an allograft rejection model,

there was no difference in graft prolongation unless additional treatment with cyclosporine A or rapamycin was used, leading to increased allograft survival.[66] Taken together, these studies would indicate that B7-H3 can act as a positive costimulatory molecule. However,the role of B7-H3 remains unclear as both stimulatory[63-66] and inhibitory[67-70] properties have been described.

Due to the controversy in the field, we sought to further define the role B7-H3 plays in the context of acute GVHD. We show that during acute GVHD, B7-H3 is upregulated in GVHD target organs. B7-H3$^{-/-}$ recipients had accelerated GVHD lethality, more damage to the epithelial layer of the colon as well as an increased percentage of inflammatory cytokine secretion from intraepithelial lymphocytes, consistent with the role of B7-H3 as a negative costimulatory pathway member. Recipients of B7-H3$^{-/-}$ donor T-cells also had accelerated GVHD lethality, along with increased damage to the epithelial layer of the colon. Lamina propria and intraepithelial lymphocytes showed increased inflammatory cytokine secretion. Together, these results suggest that B7-H3 signaling negatively regulates T-cells both directly and indirectly during GVHD and inhibiting B7-H3 increases Teffs and GVHD lethality.

Methods

Methods

Mice

C57BL/6 ($H2^b$) and BALB/c ($H2^d$) mice were purchased from the National Institutes of Health. $B7H3^{-/-}$ mice are on the B6 background and were made using B6 ES cells as previously described.[67] Mice were bred and housed in a specific pathogen-free facility in microisolator cages and used at 6-16 weeks of age. All experiments were approved by the institutional animal care and use committee of the University of Minnesota.

Bone marrow transplantation

Mice were lethally irradiated by an x-ray source on d-1. A total of $1x10^7$ bone marrow (BM) cells with or without purified T-cells were infused on d0. For GVHD induction, T-cells were isolated from lymph nodes and purified by incubation with phycoerythrin-labeled antibodies to CD19, γδ-TCR, and DX5 or NK1.1 (eBioscience), incubation with anti-phycoerythrin beads and depletion on magnetic column (Miltenyi Biotec). Flow cytometric phenotyping demonstrated >95% purity. Mice were monitored daily for survival, weighed twice weekly, and examined for clinical GVHD. Where indicated, mice were clinically scored for GVHD as described.[85]

Quantitative PCR (qPCR)

cDNA was synthesized from TRIzol-isolated total RNA using the SuperScript® III First Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For quantitative real-time reverse transcription–PCR experiments, reactions containing the TaqMan Universal PCR Master Mix and probes for B7-H3 (Mm00506020_m1) or glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_g1) as the control gene were run on the Applied Biosystems 7300 Real Time PCR System and analyzed with ABI Relative Quantification Study software (Applied Biosystems, Foster City, CA). The $2^{(-\Delta\Delta C_t)}$ method was used to generate RQ for quantifying B7-H3 expression in samples in comparison with unmanipulated wild-type (wt) mice. The formula used was RQ = 9i.

Frozen tissue preparation

Tissues including colon, small intestine, liver, lung, and spleen were taken at indicated days after transplantation, embedded in Optimal Cutting Temperature (OCT) compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80°C.

CFSE experiments

T-cells purified from lymph nodes were labeled for 15min with 5μM CFSE (carboxyfluorescein diacetate succinimidyl diester; Invitrogen) at room temperature followed by quenching with fetal bovine serum. $1x10^7$ labeled purified T-cells were infused into lethally irradiated recipients. Spleens were

harvested on indicated days, and single cell suspensions were made. Cells were surface stained and acquired on a FACS LSRII Fortessa (BD Biosciences). Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

Flow Cytometry

Single-cell suspensions were stained with the following monoclonal antibodies (mAbs): Tim-3, CD8a, CD4, CD45.1, $H2K^d$, IFN-γ, IL-2, IL-17, Granzyme-B, Ki-67, Annexin V, Activated Caspase-3. For cytokine detection, cells were stimulated in vitro with cell stimulation cocktail and protein transport inhibitor cocktail (eBioscience) for 5 hours. Cells were then surface stained with appropriate surface antibodies followed by fixing and permeabilizing with the Fix/Perm permeabilization kit (Invitrogen), and labeled with the appropriate intracellular antibodies. Cell apoptosis and death was measured by staining cells with Annexin-V staining kit and 7-AAD (eBioscience). Phenotypic acquisition of cells was performed on the LSRII Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

FITC-Dextran Assay

FITC-dextran assay was used to evaluate mucosal integrity. Mice were given 400µl of FITC-dextran orally (Sigma #FD4-1G) at a concentration of 40mg/ml in PBS. Plasma was collected from peripheral blood, mixed 1:1 with PBS and analyzed on a plate reader at excitation/emission wavelength of 485/535nm.

T-cell isolation

Spleens were processed into a single-cell suspension followed by red cell lysis. Colons were harvested, cut longitudinally and then into 5mm pieces. Gut pieces were incubated with 15.4mg/ml DTE in CMF with 10% serum (30min at 37°C) followed by treatment with 5mM EDTA in RPMI with 10% serum (15min at 37°C). Tissue was incubated three-times with 1 mg/ml collagenase D (Roche, Indianapolis, IN) in RPMI with 5% serum (45min/37°C). Lymphocytes were purified on a 44/67% percoll gradient (800*g* at 20°C for 20min).

Treg suppression assay

Tregs were isolated from spleens and of B6 or B7-H3$^{-/-}$ mice. T-cells were labeled with 1μM CFSE. T/NK cell-depleted splenocytes from B6 mice were used as APCs. 4×10^5 CFSE-labeled T-responder cells were stimulated with 4×10^5 APCs in RPMI-c and 0.25μg/mL purified α-CD3. Five days later, cells were harvested and proliferation determined by CFSE dilution.

Mixed Lymphocyte Reaction (MLR)

BM cells were isolated from WT or B7-H3-/- mice and were cultured with GM-CSF (10ng/mL) and IL-4 (10ng/mL) for 5 days. BM derived dendritic cells (DC) were then irradiated (1000 rad). T-cells were purified from the lymph nodes of BALB/c mice and labeled with CFSE. T-cell responders were mixed at a 10:1 ratio with the BM derived DC stimulators and plated in a 96 well plate. Nine days later, cells were harvested and proliferation determined by CFSE dilution.

Cell Lines

A20 is a BALB/c-derived lymphoma cell line that was modified to express luciferase as previously described.[110] To induce lymphoma, a lethal dose ($1x10^6$) of A20 cells were injected into mice by lateral tail vein injection on d53 after transplant.

Statistical analysis

The Kaplan-Meier product-limit method was used to calculate survival and differences between groups were determined using log-rank test. For all other data, Student's *t*-test was used to analyze differences between groups. $P<0.05$ was considered as significant.

Results

B7-H3 expression is up-regulated in target organs during acute GVHD

B7-H3 has been shown to be expressed on activated T-cells, epithelial cells, in addition to APCs including DCs, B-cells, and macrophages.[62] Inflammatory cytokines, like those present in GVHD, have been shown to increase B7-H3 expression on DCs and monocytes.[63] To determine whether B7-H3 is induced during acute GVHD, C57BL/6 (B6; $H2^b$) recipients were lethally irradiated followed by an infusion of BALB/c ($H2^d$) BM with or without purified donor T-cells from BALB/c mice to induce GVHD. GVHD target organs (colon, ileum, liver, lung, and spleen) were harvested d7,14, and 21 after transplant and B7-H3 expression was assessed. None of six commercially available anti-B7-H3 antibodies provided a sufficient signal-to-noise ratio (not shown). Therefore, qPCR was performed. We observed that mice receiving allogeneic T-cells had highly up-regulated B7-H3 mRNA by day 21 after transplant in the colon, liver, and lung (Figure 1).

Absence of B7-H3 expression on host cells increases allogeneic proliferation in vitro and GVHD lethality in vivo

After we determined that B7-H3 expression was highly up-regulated during acute GVHD, we sought to determine if allogeneic T-cells would increase their proliferation when B7-H3 was blocked. We performed an in vitro MLR by co-culturing BALB/c-purified T-cells with irradiated B6 or B7-$H3^{-/-}$ BM derived DC stimulators (10:1). Figure 2A shows that T-cells stimulated with B7-$H3^{-/-}$ DC

stimulators had significantly increased proliferation compared to wildtype (WT) DC stimulators.

To determine whether host B7-H3 expression would regulate GVHD lethality, lethally irradiated WT B6 or B7-H3$^{-/-}$ mice were given BALB/c BM with or without purified T-cells from BALB/c mice to induce GVHD. Figure 2B shows survival data with B7-H3$^{-/-}$ recipients having significantly accelerated lethality compared to WT [median survival time (MST), 11 days vs. 57 days, $p<0.0001$]. The acceleration of GVHD lethality in B7-H3$^{-/-}$ recipients was not dependent on donor Tregs as evidenced by the marked acceleration in GVHD following purified CD25-depleted T-cells. Clinical and histological assessment revealed changes consistent with the survival data. WT B6 or B7-H3$^{-/-}$ mice were lethally irradiated and given BALB/c BM and 1x10^6 BALB/c purified T-cells. Figure 2B shows that B7-H3$^{-/-}$ recipient mice had significantly increased clinical scores (d7-d30; $p<0.05$). Histological analysis in Figure 2C shows that the colon of B7-H3$^{-/-}$ recipients had significantly increased pathology. Thus, lack of B7-H3 expression in the recipient leads to augmented GVHD.

To investigate the mechanism of reduced survival, we evaluated donor T-cells localized to the spleen early post-BMT.. WT B6 or B7-H3$^{-/-}$ mice were lethally irradiated and given BALB/c BM and 1x10^6 BALB/c purified T-cells. On day 7, splenocytes were isolated. The number of $\alpha 4\beta 7$ integrin expressing cells were significantly increased in B7-H3$^{-/-}$ recipients compared to WT (Figure 2D: CD4+,

$2.6x10^5$ vs. $3.8x10^5$; CD8+, $1.2x10^5$ vs. $2.1x10^5$), correlating to the increased histopathology scores of the colon. Splenocytes were re-stimulated for 5 hours with PMA and ionomycin in the presence of brefeldin A and monensin, and T-cells analyzed for IL-2 expression by intracellular cytokine (ICC) staining. There was a significant increase in the number of IL-2 expressing T-cells obtained from B7-H3$^{-/-}$ recipients compared to WT recipients for both donor CD4$^+$ ($9.4x10^5$ vs. $11.7x10^5$) and CD8$^+$ ($1.7x10^6$ vs. $2.8x10^6$) T-cells. Ki-67 staining was used to determine that the number of proliferating T-cells obtained from B7-H3$^{-/-}$ recipients were also significantly greater than WT T-cells (CD4$^+$, $1.1x10^5$ vs. $1.6x10^5$; CD8$^+$, $8.4x10^4$ vs. $12.5x10^4$). Compared to WT recipients, T-cells from B7-H3$^{-/-}$ recipients had increased proliferation and effector cytokine secretion as well as increased expression of α_4B_7 integrin.

Absence of B7-H3 expression in recipients results in increased gut injury in recipients

To further investigate the mechanism of decreased survival, we evaluated the gastrointestinal tract, a major GVHD target organ and that had an increased histopathology score. B6 or B7-H3$^{-/-}$ recipients were lethally irradiated and given BALB/c BM and purified T-cells from BALB/c mice. To measure epithelial integrity of the GI tract, we used a FITC-dextran assay in which the loss of epithelial cell integrity results in leakage of FITC-dextran from the intestine into the peripheral blood. FITC-dextran was administered orally to mice on d21 and serum levels were measured 4 hours later. B7-H3$^{-/-}$ recipients had a significantly

increased level of FITC-dextran in the serum (1.47 μg/mL) compared to recipients of WT CD25-depleted T-cells (0.65 μg/mL) indicating decreased epithelial integrity (Figure 3A; $p<0.05$).

To determine how B7-H3 deficiency affected T-cells within the intestine itself, intraepithelial lymphocytes (IELs) were isolated from the colon on d21 post-transplant. Significantly more infiltrating donor IELs were found in B7-H3$^{-/-}$ vs WT recipients (Figure 3B). When we analyzed the number of proliferating IELs, both CD4^{+} and CD8^{+} donor T-cells had an increased percent of Ki-67^{+} cells in B7-H3$^{-/-}$ vs WT recipients (Figure 3B). On d21 after transplant, IELs were isolated, re-stimulated as above, and stained for the expression of IFN-γ, IL-17, and TNF-α, known effector molecules involved in GVHD. The percent expression of IFN-γ, IL-17, and TNF-α in CD4^{+} and CD8^{+} T-cells was significantly increased in B7-H3$^{-/-}$ vs WT recipients (Figure 3B). These data suggest that in our model where B7-H3 is absent in recipient tissues, the GI tract is less protected, which is associated with decreased overall survival.

Absence of B7-H3 expression on donor T-cells leads to accelerated GVHD lethality

T-cells that were activated with PMA/ionomycin show an increased expression of B7-H3.[63] To determine whether B7-H3 expression on donor T-cells would impact GVHD lethality by inhibiting T-cell responses analogous to its role on host tissues, lethally irradiated WT BALB/c mice were given B6 BM with or without

purified T-cells from B6 or B7-H3$^{-/-}$ mice to induce GVHD. Figure 4A shows survival data with B7-H3$^{-/-}$ donor T-cells having a significantly accelerated lethality compared to WT (MST, 32 days vs. 67.5 days). Consistent with the survival data, Figure 4B shows that mice that received B7-H3$^{-/-}$ donor T-cells had significantly increased clinical scores (d8; d21-d34, $p<0.05$). While the histopathology scores showed the mice had severe GVHD (Supplemental Figure 1), there was not a significant difference between the WT and B7-H3$^{-/-}$ groups that was seen during clinical scoring.

B7-H3 deficient recipients were hypersusceptible to GVHD caused by Treg-replete or Treg-depleted grafts. However, such data do not provide insight as to whether B7-H3 expression on Tregs might alter their suppressor function. Therefore, studies were performed using B7-H3$^{-/-}$ than WT Tregs and purified T-cells stimulated with αCD-3 mAb. Such T-cells were suppressed less well by B7-H3$^{-/-}$ than WT Tregs at each of 3 different Treg:T-cell ratios in vitro, with an apparently more profound loss in CD8 versus CD4 T-cell suppression (Figure 4C). Therefore, we sought to determine whether the reduced in vitro suppressor activity of B7-H3-/- vs WT Tregs would translate into a decrement in GVHD acceleration by B7-H3$^{-/-}$ T-cells compared to WT T-cells if donor Tregs were depleted prior to transplant. Despite diminution of Treg mediated suppression of T-cell responses in vitro by B7-H3$^{-/-}$ vs WT Tregs, and as was seen in Figure 4D, the B7-H3$^{-/-}$ recipients, the acceleration of GVHD lethality of B7-H3$^{-/-}$ donor T-cell recipients was not dependent on the presence of donor Tregs. Taken together,

these data confirm that the absence of B7-H3 expression on donor T-cells results in augmented GVHD lethality.

Absence of B7-H3 expression on donor T-cells results in increased donor T-cell proliferation and activation

We sought to determine whether allogeneic T-cells would increase their in vitro and in vivo proliferation when B7-H3 was absent. First, we performed an in vitro MLR by co-culturing B6 or B7-H3$^{-/-}$ purified T-cells with irradiated B6 BM derived DC stimulators (10:1). Figure 5A shows that B7-H3$^{-/-}$ vs WT CD4+ and CD8+ T-cells stimulated with BALB/c DC stimulators had significantly increased proliferation on day 5. Next, splenocytes were isolated on day 7 from recipients of B7-H3$^{-/-}$ vs WT donor T-cells. In contrast to WT recipients, mice receiving B7-H3$^{-/-}$ donor T-cells had significantly more total donor T-cells, donor CD4^{+} T-cells, as well as a trend ($p=0.0861$) of donor CD8^{+} T-cells in the spleen (Figure 5B). In addition, there were significantly more B7-H3$^{-/-}$ donor T-cells that were proliferating compared to WT T-cells (CD4^{+}, $2.2x10^5$ vs. $0.98x10^5$; CD8^{+}, $1.2x10^6$ vs. $0.68x10^6$). Splenic T-cells were stimulated as above and analyzed for IFN-γ and IL-2 expression by ICC staining. Figure 5C shows recipients of B7-H3$^{-/-}$ compared to WT donor T-cells had significantly higher numbers of IFN-γ expressing CD4^{+} ($1.9x10^5$ vs. $0.81x10^5$) and CD8^{+} ($1.6x10^6$ vs. $1.1x10^6$) T-cells as well as significantly increased numbers of IL-2 expressing T-cells (CD4^{+}, $9.0x10^5$ vs. $11.7x10^5$; CD8^{+}, $1.7x10^6$ vs. $2.8x10^6$). In addition to having a higher number of cells proliferating, the proportion of cells undergoing apoptosis was

significantly reduced in B7-H3$^{-/-}$ donor T-cells (CD4^{+}, 72.6% vs. 52.9%; CD8^{+}, 68.7% vs. 47.7%). The number of α_4B_7 integrin expressing cells were significantly increased in B7-H3$^{-/-}$ recipients compared to WT (CD4+, 2.5x10^{5} vs. 3.8x10$^{5;}$ CD8+, 1.2x10^{6} vs. 2.2x10^{6}). Compared to WT, B7-H3$^{-/-}$ donor T-cells had increased proliferation, effector cytokine secretion, α_4B_7 integrin expression, as well as being less susceptible to apoptosis.

Absence of B7-H3 expression on donor T-cells results in increased gut injury in recipients

To further investigate the mechanism of decreased survival, we used the FITC-dextran assay described above. FITC-dextran was administered orally to mice on d21 and serum levels were measured 4 hours later. On d21, mice that received B7-H3$^{-/-}$ donor T-cells had a modestly increased level of FITC-dextran in the serum (1.477 μg/mL) compared to recipients of WT (0.6497μg/mL) indicating decreased epithelial integrity (Figure 6A; p=0.0773). IELs were isolated on d21 after transplant. When we analyzed the number of proliferating IELs, Ki-67^{+} cell numbers in recipients of B7-H3$^{-/-}$ vs WT T-cells was increased approximately 3 fold for both CD4^{+} and CD8^{+} (Figure 6B). Additionally, the proportion of cells undergoing apoptosis (Annexin V^{+}) was significantly reduced. This suggests that B7-H3$^{-/-}$ donor T-cells are proliferating more while dying less and leading to increased gut injury. On d21 after transplant, IELs and LPLs were isolated, re-stimulated, and stained for the expression of IFN-γ, IL-2, IL-17, and TNF-α. Significantly more total donor IELs, CD4^{+} and a trend of CD8^{+} cells were found in

recipients of B7-H3$^{-/-}$ vs WT T-cells in addition to significantly increased CD4^{+} and CD8^{+} LPLs (Figure 6C). IEL expression of IFN-γ and TNF-α in CD4^{+} and CD8^{+} T-cells, IL-2 in CD8^{+} T-cells, and IL-17 in CD4^{+} T-cells was significantly increased in recipients of B7-H3$^{-/-}$ vs WT donor T-cells. LPL expression of IFN-γ, TNF-α, IL-2, and IL-17 was significantly increased in recipients of B7-H3$^{-/-}$ vs WT donor T-cells (Figure 6C). These data suggest that in our model where B7-H3 is absent on donor T-cells, lymphocytes accumulate in the GI tract by increased proliferation and increased survival. These lymphocytes are also more inflammatory as evidenced by increased cytokine production leading to increased damage to the GI tract, which is associated with decreased overall survival.

B7-H3$^{-/-}$ T-cells can mediate GVL without inducing GVHD when given later after BMT

We have shown that utilizing a delayed lymphocyte infusion (DLI) while blocking a negative regulatory pathway (PD1/PD-L1 pathway) can provide potent graft-versus-leukemia (GVL) effects without GVHD effects.[111] Since B7-H3 appears to negatively regulate T-cells in our acute GVHD model, we sought to determine if they could be used to facilitate GVL without inducing GVHD. BALB/c mice were lethally irradiated and given 10^7 BALB/c BM. Mice were infused with $30x10^6$ B6 or B7-H3$^{-/-}$ splenocytes, or saline injected without supplemental cells as a control, day 50 after transplant. Three days later, the mice were infused with $1x10^6$ A20^{luc} tumor cells. Mice were monitored for survival as well as GVHD clinical scores. Mice that received no DLI (saline control) had over 50% tumor-related

lethality. Mice that received WT or B7-H3$^{-/-}$ splenocytes had only 8% and 0% lethality respectively. Additionally, mice did not have significant GVHD scores in any of the groups. These data suggest that B7-H3$^{-/-}$ T-cells can provide a potent GVL effect without GVHD when DLI is performed under these conditions.

Discussion

Here, we demonstrate the important role for the B7-H3 pathway in acute GVHD in MHC-disparate recipients. B7-H3 was found to be up-regulated in GVHD organs including the colon, liver, and lung 21 days after transplant. B7-H3 inhibition using B7-H3$^{-/-}$ recipients accelerated GVHD lethality. Interestingly, using B7-H3$^{-/-}$ donor T-cells also accelerated GVHD lethality. Both recipients of B7-H3$^{-/-}$ donor T-cells as well as B7-H3$^{-/-}$ recipients had more damage to the epithelial layer of the colon as well as an increased percentage of inflammatory cytokine secretion. Our results indicate that B7-H3 acts as a negative costimulatory molecule in the context of a GVHD response.

This work examined the role of a novel immune regulator B7-H3 and its expression and role in regulating acute GVHD. Acute GVHD induced by T-cell infusion in MHC-disparate recipients lead to an up-regulation of B7-H3$^{-/-}$ mRNA in GVHD target organs. B7-H3 is up-regulated by IFN-γ, which is increased during acute GVHD.[63,67] The B7-H3 ligand has not been firmly established. Hashiguchi et al described a direct interaction between B7-H3 and TLT-2,[64] however, others did not confirm this finding.[69] The identification of ligands would greatly advance the understanding of B7-H3-cell interactions.

Other negative regulatory pathways have been shown to play an important role in regulating acute GVHD.[39,91,112,113] To evaluate the effect of B7-H3 on GVHD, we first used B7-H3$^{-/-}$ recipient mice to inhibit the pathway. This resulted in

significantly increased lethality and clinical scores compared to WT recipient mice. These data support previous studies where inhibiting B7-H3 in a Th1 mediated model lead to earlier onset of EAE[67] and an increased rate of cardiac graft rejection.[70] Tregs have a known inhibitory role in GVHD[33] and we sought to determine whether impaired Treg-mediated suppression in the context of B7-H3 pathway inhibition contributed to augmented GVHD lethality. We did observe that B7-H3$^{-/-}$ Tregs were not able to suppress αCD3 mediated T-cell proliferation as well as WT Tregs. However, depletion of CD25^{+} cells from the donor graft still resulted in increased GVHD lethality in B7-H3$^{-/-}$ recipients, indicating that B7-H3 expression on donor T-cells down-regulates GVHD through a thymus-derived Treg-independent mechanism.

The gut is a major target organ for acute GVHD and results in a significant amount of morbidity and mortality. Studies have shown that an increased level of cytokine production in the gut, mainly by donor infiltrating T-cells, can mediate acute GVHD.[99,114] Increased histopathology scores were found in the colon of B7-H3$^{-/-}$ recipients at d7 and d21 after transplant. Additionally, our data shows that a higher percentage of both CD4+ and CD8+ donor T-cells were secreting inflammatory cytokines (IFN-γ, TNF-α, and IL-17) and had increased Teff proliferation in the intraepithelial layer of the colon in B7-H3$^{-/-}$ recipients 21 days after transplant. These data are consistent with other studies that showed inhibiting B7-H3 lead to increased IFN-γ secretion as well as increased proliferation.[67,68,70] The increased Teff proliferation and inflammatory cytokine

secretion could mean increased gut epithelial damage as supported by our finding of increased serum concentration of orally administered FITC-dextran. We conclude that B7-H3$^{-/-}$ recipients have an increased GVHD lethality rate due to an increase of inflammatory cytokine secretion in the gut and decreased integrity of the epithelial barrier.

Activated T-cells have been shown to express B7-H3 and expression can be up-regulated by IFN-γ.[63,67] To determine the possible contribution of B7-H3 expression on donor T-cell, we used B7-H3$^{-/-}$ donor T-cells. Again, we sought to determine if the augmented GVHD lethality was related to any impairment in Treg-mediated suppression in the context of B7-H3 pathway inhibition. As before, depletion of CD25^{+} cells from the donor graft still resulted in increased GVHD lethality. Analysis of the spleen at day 7 after transplant showed that there was an increased number of proliferating donor T-cells (Ki-67^{+}) as well as a reduced percentage of donor cells undergoing apoptosis (Annexin V^{+}). We also observed more IFN-γ production in recipients of B7-H3$^{-/-}$ donor T-cells. These data are consistent with other studies that showed inhibiting B7-H3 lead to increased IFN-γ secretion as well as increased proliferation.[67,68,70] Increased gut epithelial damage could result from the increased Teff proliferation and inflammatory cytokine secretion. As expected, we noted increased epithelial damaged that was demonstrated by an increased serum concentration of orally administered FITC-dextran. We conclude that B7-H3$^{-/-}$ recipients have an

increased GVHD lethality rate due to an increase of inflammatory cytokine secretion in the gut and decreased integrity of the epithelial barrier.

After observing that B7-H3 negatively regulated T-cells in our acute GVHD model, we hypothesized that B7-H3$^{-/-}$ donor T-cells could be used to facilitate GVL without inducing GVHD by utilizing a delayed DLI model. Our results show that when mice were challenged at d50 with tumor and given DLI three days later, the mice that received B7-H3$^{-/-}$ donor T-cells had a GVL response comparable to WT donor T-cells. Despite the high level of acute GVHD seen upon transfer of B7-H3$^{-/-}$ donor T-cells at the time of transplant, performing a DLI at d53 after BM transfer circumvents GVHD. We conclude that B7-H3$^{-/-}$ T-cells can provide a potent GVL effect without GVHD in this model of delayed DLI.

The role of B7-H3 remains to be controversial with reports of both positive[63-66] and negative[67-70] costimulatory roles in models of autoimmunity and tumor immunobiology. A possible explanation for such conflicting data may be that B7-H3 is capable of binding to more than one receptor. Members of the B7/CD28 family are known to be capable of being both activators and negative regulators of the T-cell response. It is therefore possible that B7-H3 may have different ligands that can provide positive or negative signals. Currently, only one potential ligand has been suggested. Hashiguchi et al found that triggering receptor expressed on myeloid cells (TREM) like transcript (TLT-2) interacted with B7-H3 that enhanced CD8 T-cell activation. Blockade of this pathway using

a monoclonal antibody inhibited contact hypersensitivity responses.[64] These data would suggest that TLT-2 may function as a ligand for B7-H3. However, others have found no evidence for such an interaction in either mice or humans.[69] In order to understand the mechanisms of the role B7-H3 has on in the immune system, all potential ligands will need to be identified.

Another possible explanation for the contrasting data is the physiological differences between the models used to study B7-H3. Suh et al demonstrated that B7-H3 is a negative regulator that preferentially affects Th1 responses. B7-H3 expression on DCs was upregulated in the presence of IFN-γ, but was suppressed in the presence of IL-4. B7-H3$^{-/-}$ mice developed more severe airway inflammation than did WT mice in conditions in which T helper cells were differentiated toward Th1 rather than Th2.[67] Under Th1 conditions, B7-H3$^{-/-}$ mice developed more severe airway inflammation than WT mice with more activated T-cells in the bronchoalveolar lavage and had enhanced IFN-γ production. These results were not seen under a Th2-mediated airway hypersensitivity that had no apparent differences between the B7-H3$^{-/-}$ and WT group. Additionally, in a model of cardiac transplantation where Th1 responses mediate rejection, using a B7-H3 blocking antibody or B7-H3$^{-/-}$ recipients lead to accelerated rejection.[70] Acute GVHD effects are largely mediated by Th1 responses and we demonstrated that inhibiting B7-H3 by using B7-H3$^{-/-}$ recipients as well as B7-H3$^{-/-}$ donor T-cells lead to accelerated GVHD lethality. Additionally, we also

observed more IFN-γ production and increased Teff proliferation that is in agreement with the above studies.

In summary, these studies demonstrate that the B7-H3 pathway acts as a suppressor of acute GVHD and by inhibiting B7-H3 GVHD disease severity is augmented. Reagents that enhance signaling of the B7-H3 pathway early post-transplantation may be used to dampen the Th1 response and reduce GVHD.

Authorship

R.G.V. designed and performed research, analyzed the data, and wrote the paper. R. F. performed experiments and edited the paper. K.K. performed experiments and edited the paper. C.M.H. performed experiments and edited the paper. P.A.T. performed experiments, provided advice, and edited the paper. A.P-M. provided data. B.R.B. designed, organized, and supervised research and edited the paper.

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Conflicts of Interest

The authors declare no competing financial interests.

Figure Legends

Figure 1. B7-H3 Expression is upregulated in target organs during acute GVHD

B6 mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM with or without $2x10^6$ BALB/c purified T-cells. Mice were sacrificed on day 21 after transplantation and the colon, liver, and lung were examined for B7-H3 mRNA by qPCR.

Figure 2. B7-H3 pathway inhibition on recipients leads to accelerated GVHD lethality

A. MLR was performed by co-culturing BALB/c-purified T-cells that were CFSE labeled with irradiated B6 or B7-H3$^{-/-}$ DC stimulators (10:1). Cells were analyzed by flow cytometry on d9. Cells were gated on $H2K^b$ positive, viability dye negative, CD4 or CD8 positive events and analyzed for dilution of CFSE. (n=5, *p<0.05, **p<0.01) B. B6 or B7-H3$^{-/-}$ mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $2x10^6$ BALB/c purified T-cells. Survival plot of WT (closed circle) vs. B7-H3$^{-/-}$ (open circle) is shown p<0.0001. n=16 per group. B6 or B7-H3$^{-/-}$ mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $1x10^6$ CD25-depleted BALB/c purified T-cells. Survival plot of WT (closed circle) vs. B7-H3$^{-/-}$ (open circle) is shown p<0.0001. n=16 per group. C. B6 or B7-H3$^{-/-}$ mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $2x10^6$ BALB/c purified T-cells. Mice were analyzed for weights and clinical scores. (n=8, *p<0.05, **p<0.01) D. Mice were transplanted as in C. 21

days after transplant, colons were harvested, sectioned and stained by H&E. Sections were scored for pathology. (n=4, *p<0.05, **p<0.01) E. B6 or B7-H3$^{-/-}$ mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $1x10^6$ BALB/c purified T-cells. Mice were sacrificed on day 7 and splenocytes were analyzed for total cell numbers of α_4B_7, IL-2, and Ki-67 expression (n=5, *p<0.05, **p<0.01)

Figure 3. B7-H3 pathway inhibition results in increased gut injury in recipients

A. B6 or B7-H3$^{-/-}$ mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $3x10^6$ BALB/c purified T-cells. 16mg of FITC-dextran was administered orally to mice on d21 and serum levels were measured 4 hours later (n=4, *p<0.05, **p<0.01) B. B6 or B7-H3$^{-/-}$ mice were lethally irradiated and infused with 10^7 BALB/c NTCD BM and $2x10^6$ BALB/c purified T-cells. Mice were sacrificed on day 21 and intraepithelial lymphocytes were analyzed for total cell numbers (n=4, p<0.05). Intraepithelial lymphocytes were analyzed for Ki-67 as well as effector cytokines IFN-γ, IL-17, and TNF-α (n-4, p<0.05)

Figure 4. B7-H3 inhibition on donor T-cells augments GVHD

A. BALB/c mice were lethally irradiated and infused with 10^7 B6 NTCD BM and $1x10^6$ B6 or B7-H3$^{-/-}$ purified T-cells. Survival plot of WT (closed circle) vs. B7-H3$^{-/-}$ (open circle) is shown (p<0.0001, n=16 per group). BALB/c mice were

lethally irradiated and infused with 10^7 B6 NTCD BM and $1x10^6$ B6 or B7-H3$^{-/-}$ CD25-depleted purified T-cells. Survival plot of WT (closed circle) vs. B7-H3$^{-/-}$ (open circle) is shown ($p<0.05$, n=8 per group) B. BALB/c mice were lethally irradiated and infused with 10^7 B6 NTCD BM and $1x10^6$ B6 or B7-H3$^{-/-}$ purified T-cells. Mice were analyzed for weights and clinical scores. (n= 8 per group, *$p<0.05$, **$p<0.01$, ***$p<0.001$)

A. MLR was performed by co-culturing B6 or B7-H3$^{-/-}$ purified T-cells that were CFSE labeled with irradiated BALB/c DC stimulators (10:1). Cells were analyzed by flow cytometry on d5. Cells were gated on H2K^b positive, viability dye negative, CD4 or CD8 positive events and analyzed for dilution of CFSE. (n=5, *$p<0.05$) B. BALB/c mice were lethally irradiated and infused with 10^7 B6 NTCD BM and $1x10^6$ B6 or B7-H3$^{-/-}$ purified T-cells. Mice were sacrificed on day 7 and splenocytes were analyzed for total cell numbers (n=4) C. Additionally, splenocytes were analyzed for Ki-67, IFN-γ, IL-2, and Annexin V, α_4B_7 expression (n=4). *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 6. B7-H3 inhibition on donor T-cells leads to increased gut injury

A. BALB/c mice were lethally irradiated and infused with 10^7 B6 BM and $1x10^6$ B6 or B7-H3$^{-/-}$ purified T-cells. 16mg of FITC-dextran was administered orally to mice on d21 and serum levels were measured 4 hours later (n=4). B. BALB/c mice were lethally irradiated and infused with 10^7 B6 BM and $1x10^6$ B6 or B7-H3$^{-/-}$

purified T-cells. Mice were sacrificed on d21 after transplant and intraepithelial lymphocytes were analyzed for Ki-67 and Annexin V expression. (n=4) C. BALB/c mice were lethally irradiated and infused with 10^7 B6 BM and $1x10^6$ B6 or B7-H3$^{-/-}$ purified T-cells. Mice were sacrificed on d21 after transplant and intraepithelial lymphocytes and lamina propria lymphocytes were analyzed for IFN-γ, TNF-α, IL-2, and IL-17 expression. (n=4)

*p<0.05, **p<0.01, ***p<0.001

Figure 7. B7-H3 inhibition maintains tumor rejection following DLI

A. BALB/c mice were lethally irradiated and infused with 10^7 BALB/c TCD BM. Mice were infused with $30x10^6$ B6 or B7-H3$^{-/-}$ splenocytes, or PBS as a control, day 50 after transplant. On day 53 after transplant, the mice were infused with $1x10^6$ A20^{luc} tumor cells. (n=10) B. Mice were monitored for clinical scores and not significant on d30 or d75. (n=10)

Supplemental Figure 1. B7-H3-/- Tregs are less suppressive than WT Tregs

Treg suppression assay was performed as described. Proliferating T-cells were quantified by the dilution of CFSE resulting in a CFSE^{low} population. There was significant decrease in the suppressive capacity of B7-H3$^{-/-}$ Tregs compared to WT.

Supplemental Figure 2. Histopathology scores for recipients of B7-H3$^{-/-}$ donor T-cells. BALB/c mice were lethally irradiated and infused with 10^7 B6

NTCD BM and $2x10^6$ B6 or B7-H3$^{-/-}$ purified T-cells. GVHD organs were harvested d7, d14, and d21 after transplant, sectioned and stained by H&E. Sections were scored for pathology. (n=4)

Figure 1

A

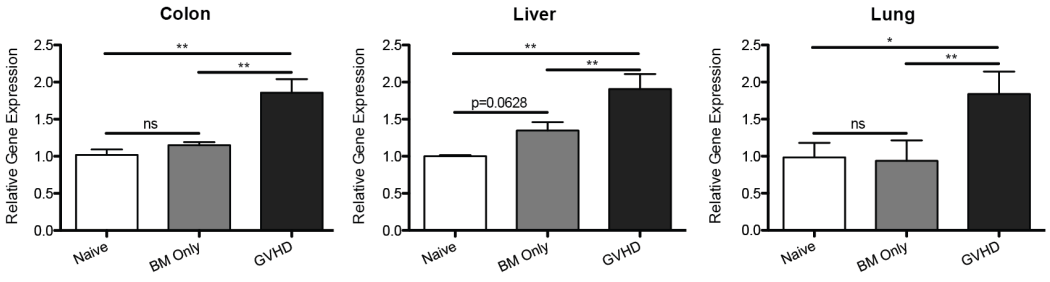

Colon
Liver
Lung
Relative Gene Expression
2.5
2.0
1.5
1.0
0.5
0.0
**
**
ns
**
**
p=0.0628
*
**
ns
Naive
BM Only
GVHD

Figure 2

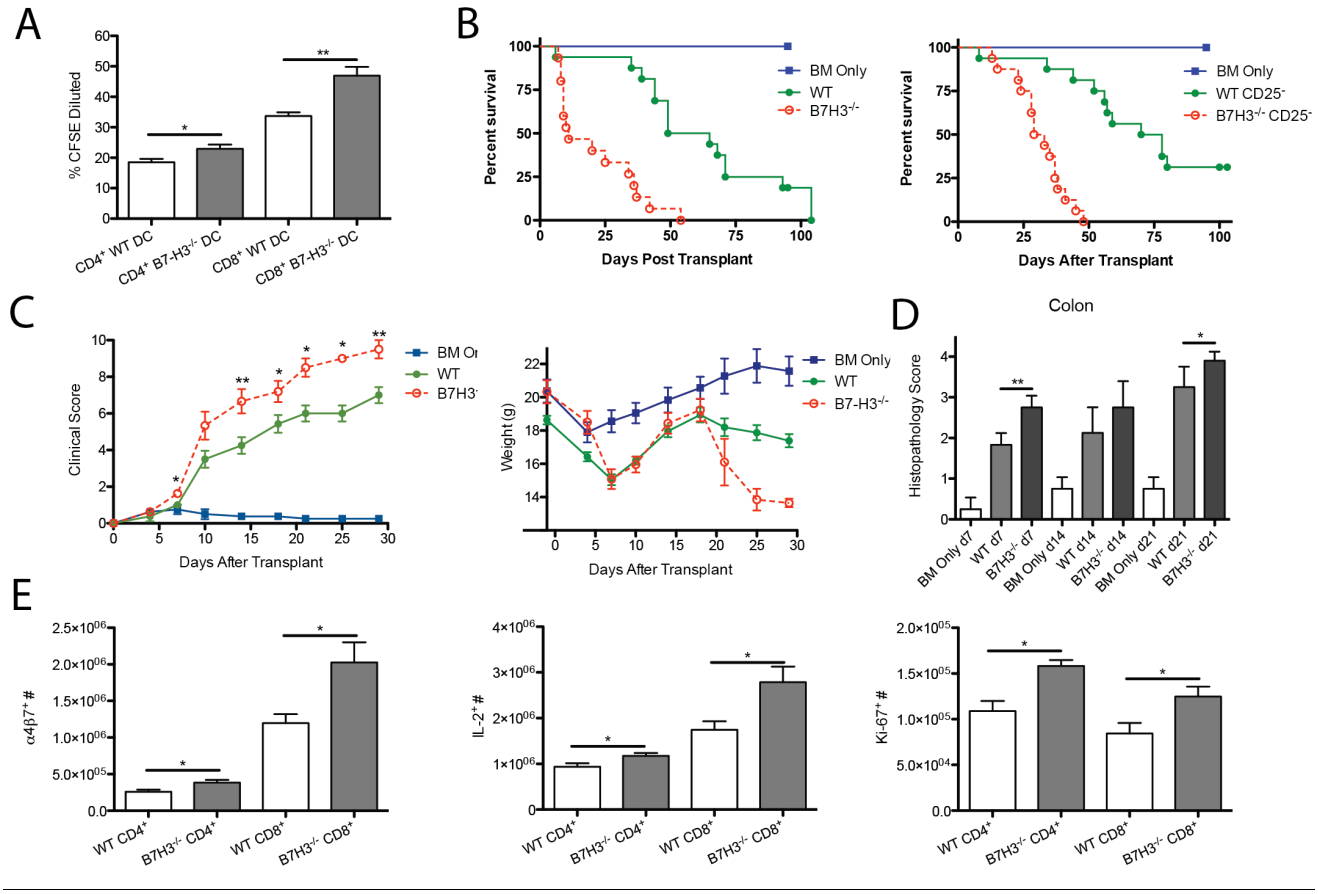

A
% CFSE Diluted
CD4+ WT DC
CD4+ B7-H3-/- DC
CD8+ WT DC
CD8+ B7-H3-/- DC
B
Percent survival
Days Post Transplant
BM Only
WT
B7H3-/-
Days After Transplant
WT CD25-
B7H3-/- CD25-
C
Clinical Score
Days After Transplant
Weight (g)
D
Colon
Histopathology Score
BM Only d7
WT d7
B7H3-/- d7
BM Only d14
WT d14
B7H3-/- d14
BM Only d21
WT d21
B7H3-/- d21
E
α4β7+ #
IL-2+ #
Ki-67+ #
WT CD4+
B7H3-/- CD4+
WT CD8+
B7H3-/- CD8+

Figure 3

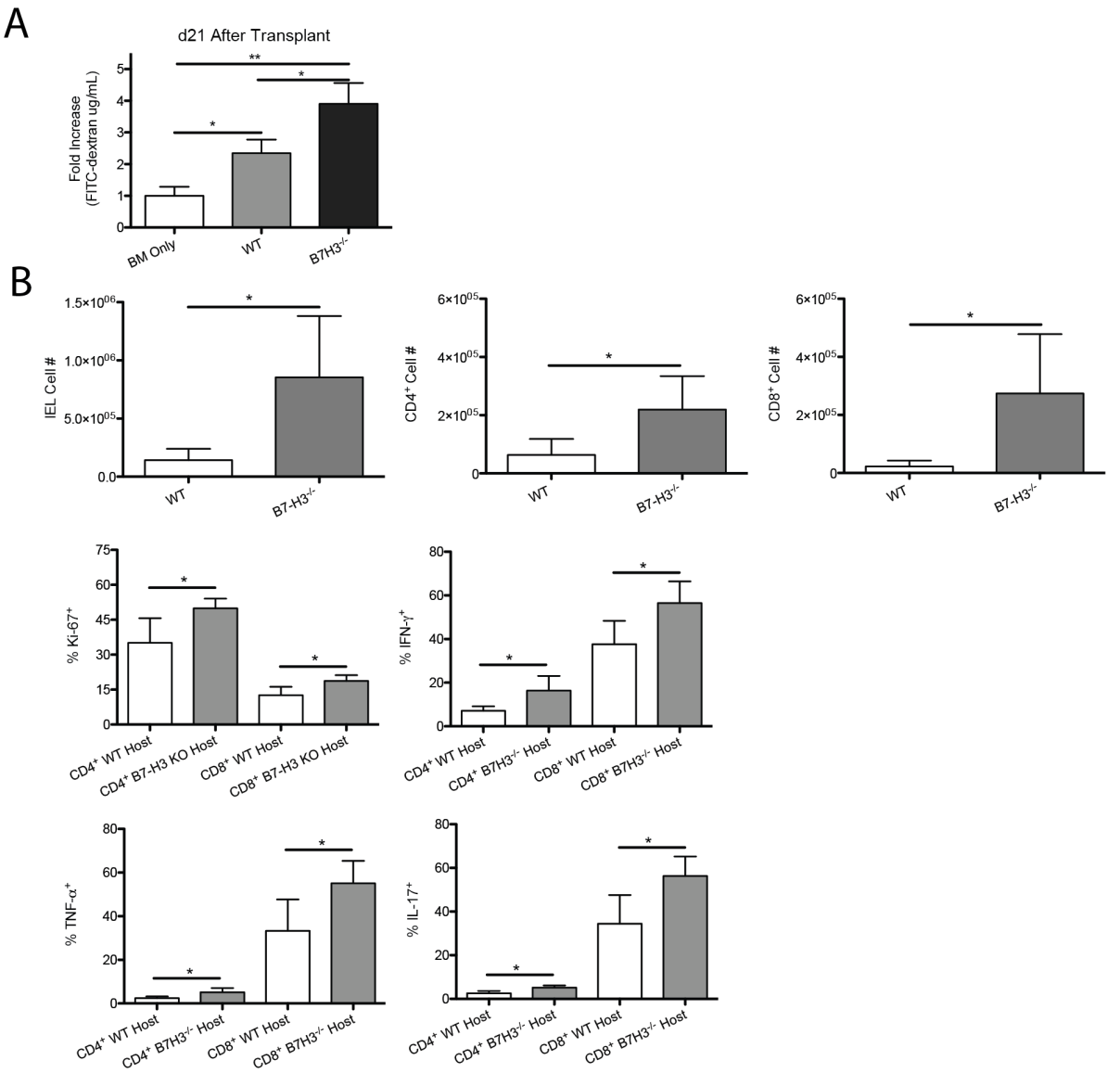

A
d21 After Transplant
Fold Increase (FITC-dextran ug/mL)
BM Only
WT
B7H3-/-
B
IEL Cell #
CD4+ Cell #
CD8+ Cell #
% Ki-67+
% IFN-γ+
% TNF-α+
% IL-17+
CD4+ WT Host
CD4+ B7-H3 KO Host
CD8+ WT Host
CD8+ B7-H3 KO Host
CD4+ B7H3-/- Host
CD8+ B7H3-/- Host

Figure 4

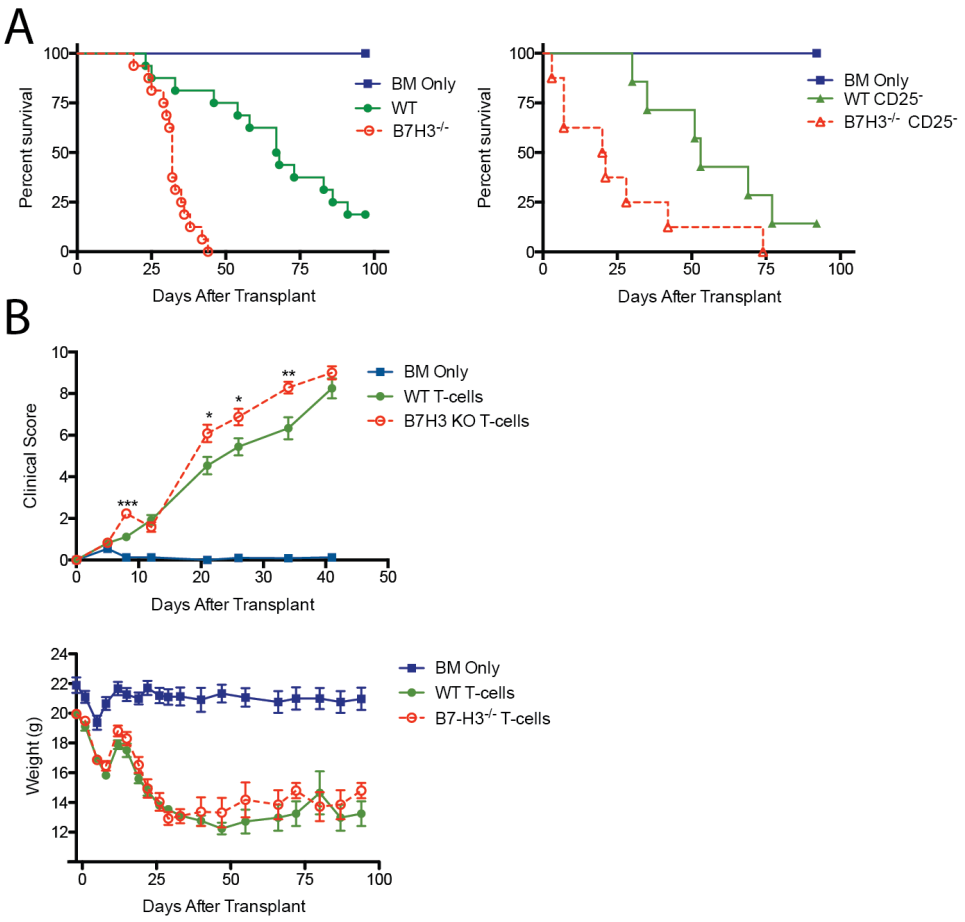

A
Percent survival
Days After Transplant
BM Only
WT
B7H3-/-
WT CD25-
B7H3-/- CD25-
B
Clinical Score
Days After Transplant
BM Only
WT T-cells
B7H3 KO T-cells
***
*
*
**
Weight (g)
Days After Transplant
BM Only
WT T-cells
B7-H3-/- T-cells

Figure 5

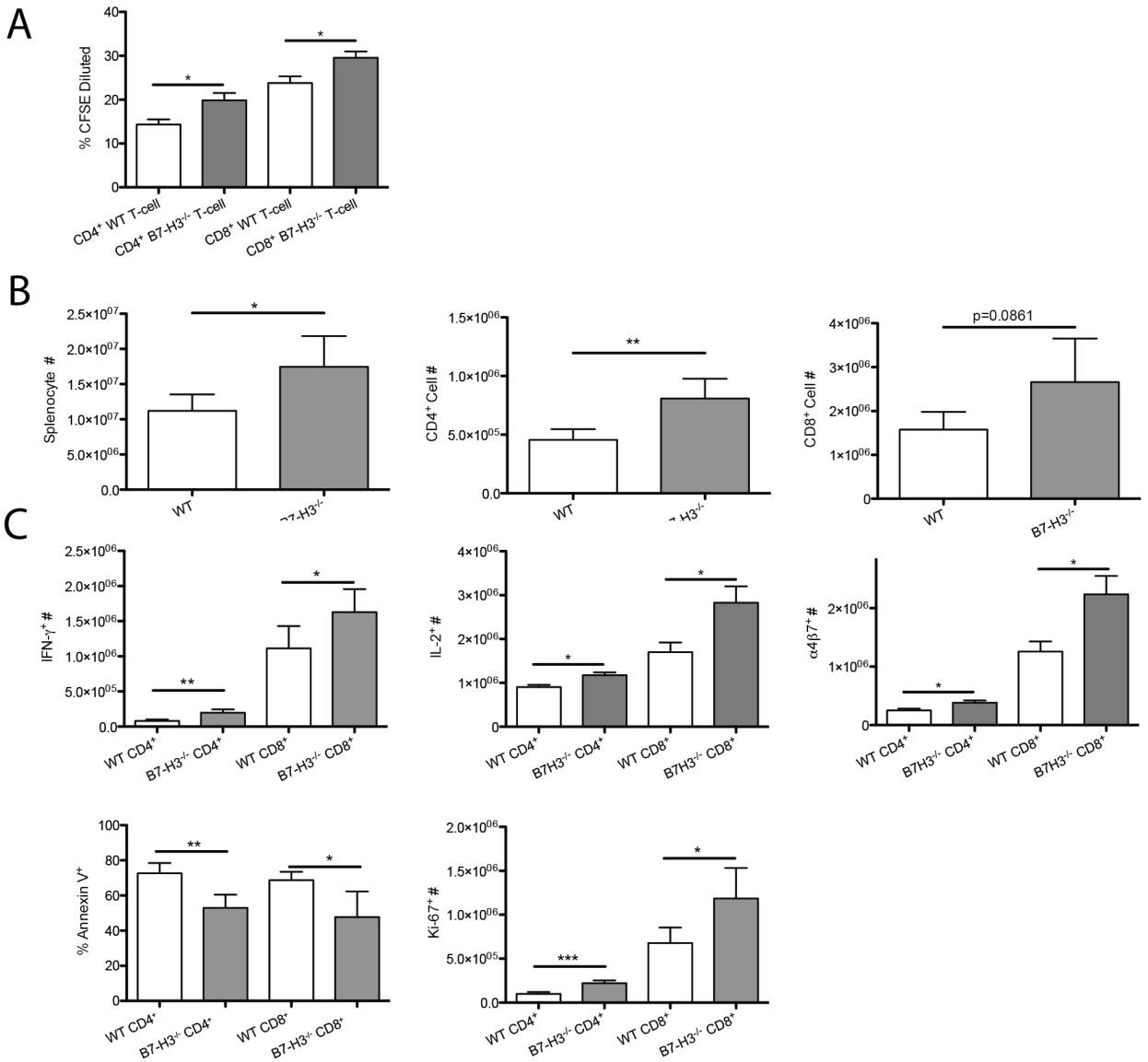

A
% CFSE Diluted
CD4+ WT T-cell
CD4+ B7-H3-/- T-cell
CD8+ WT T-cell
CD8+ B7-H3-/- T-cell
B
Splenocyte #
CD4+ Cell #
CD8+ Cell #
p=0.0861
WT
B7-H3-/-
C
IFN-γ+ #
IL-2+ #
α4β7+ #
WT CD4+
B7-H3-/- CD4+
WT CD8+
B7-H3-/- CD8+
% Annexin V+
Ki-67+ #

Figure 6

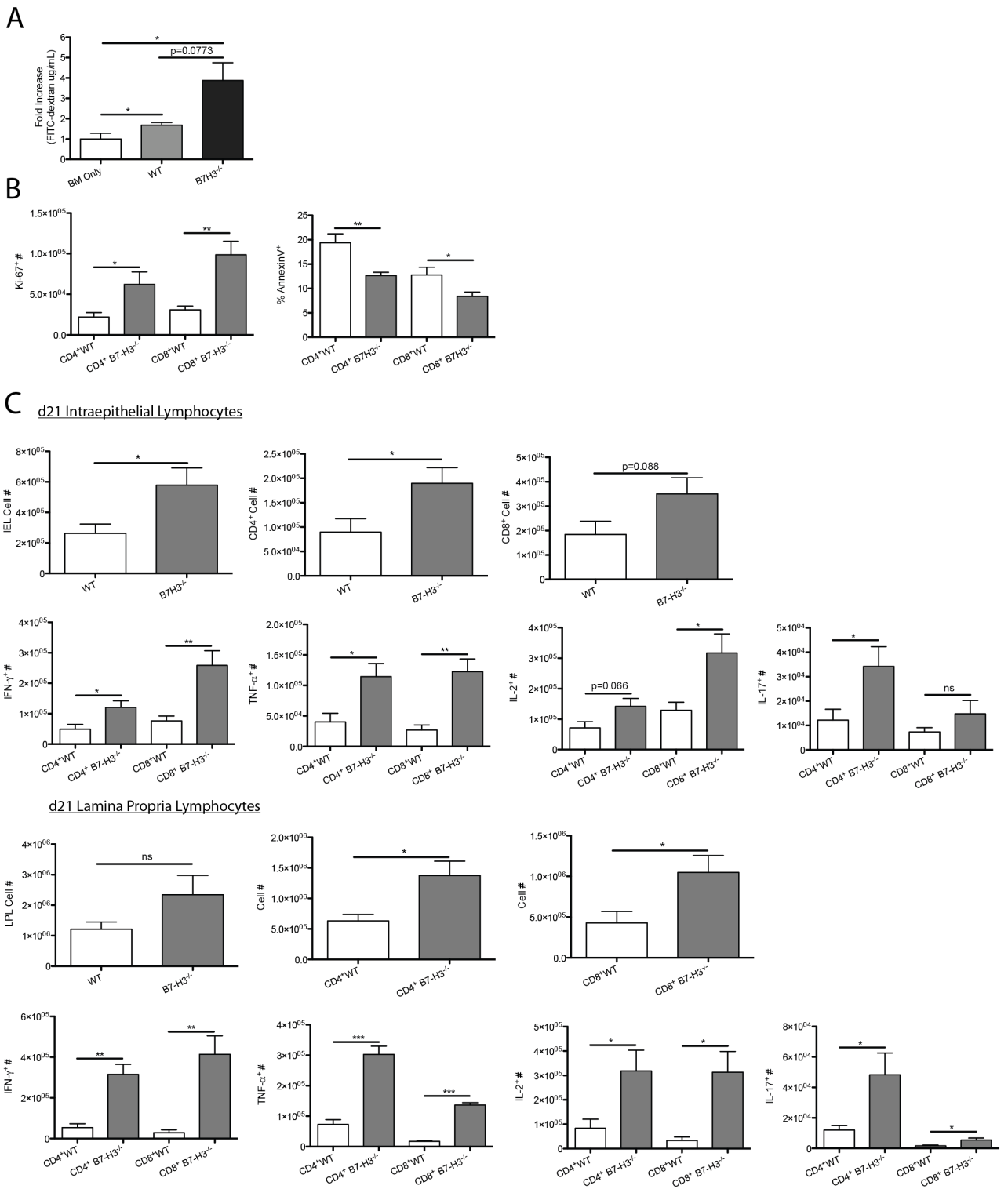
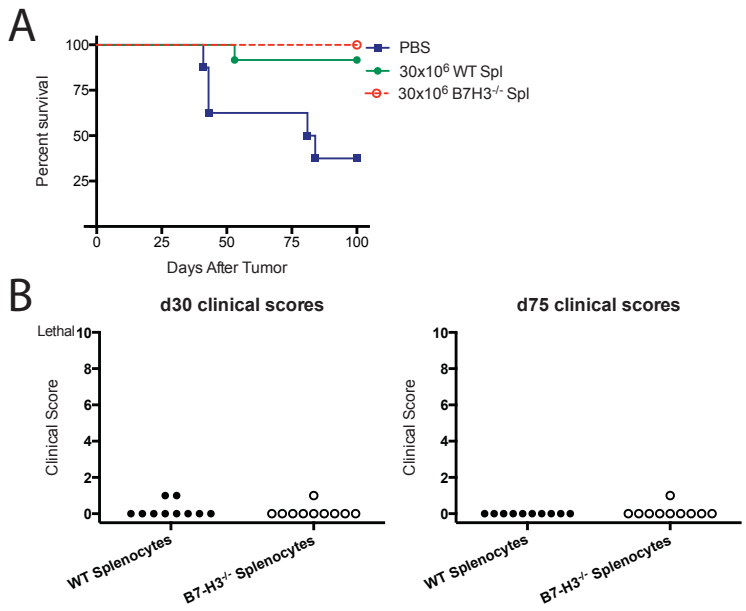
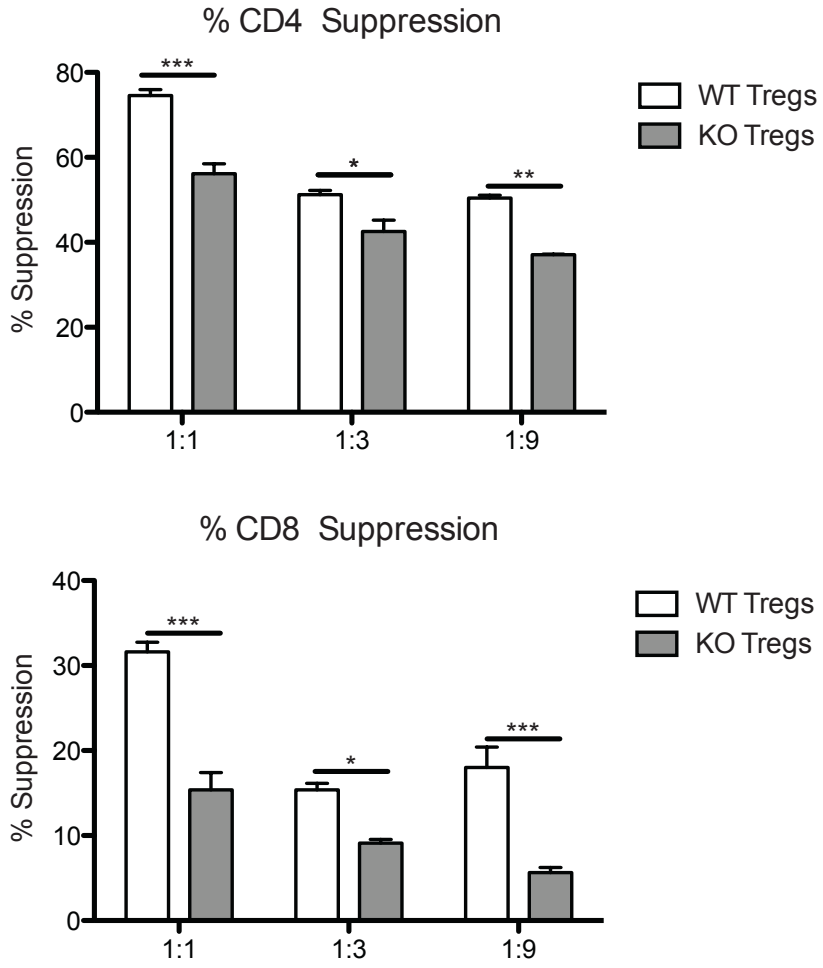

A
Fold Increase (FITC-dextran ug/mL)
p=0.0773
BM Only
WT
B7H3-/-
B
Ki-67+ #
% AnnexinV+
CD4+WT
CD4+ B7-H3-/-
CD8+WT
CD8+ B7-H3-/-
C
d21 Intraepithelial Lymphocytes
IEL Cell #
CD4+ Cell #
CD8+ Cell #
p=0.088
IFN-γ+ #
TNF-α+ #
IL-2+ #
p=0.066
IL-17+ #
ns
d21 Lamina Propria Lymphocytes
LPL Cell #
Cell #

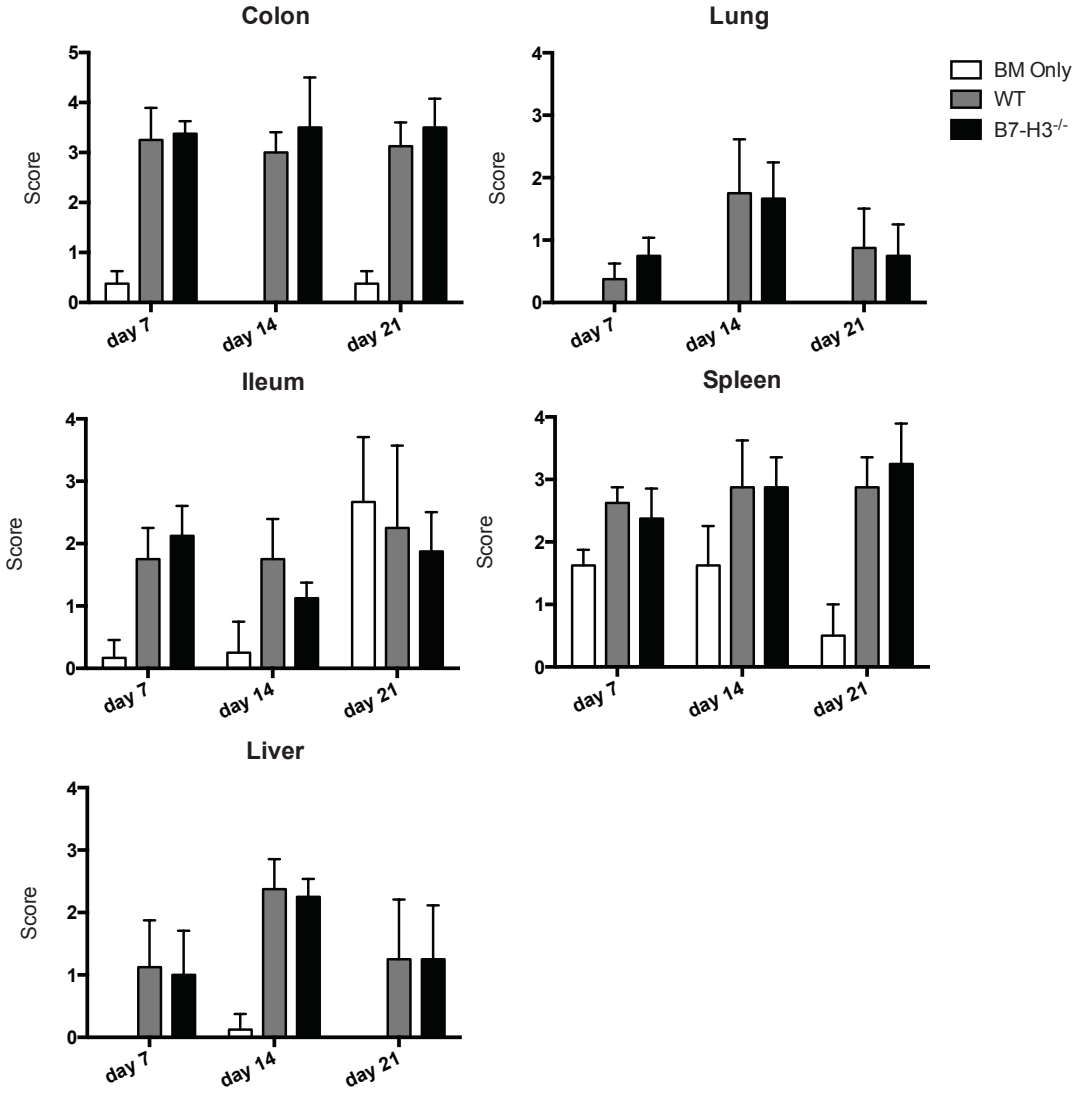
Figure 7


A
100
75
50
25
Percent survival
0
25
50
75
100
Days After Tumor
PBS
30x10^6 WT Spl
30x10^6 B7H3^-/- Spl
B
d30 clinical scores
d75 clinical scores
Lethal
10
8
6
4
2
0
Clinical Score
WT Splenocytes
B7-H3^-/- Splenocytes

Supplemental Figure 1



Supplemental Figure 2



Chapter IV: Concluding Statements

GVHD is a serious complication following HSCT that results in significant morbidity and mortality. This high morbidity and mortality prevents the use of HSCT to treat a wider range of diseases. By developing better GVHD treatments and prevention, patients will have increased survival and a better quality of life. By preventing or reducing GVHD, HSCT treatment could be extended to other conditions that are currently difficult to treat. There needs to be a more comprehensive understanding of immune regulators of GVHD in order to develop potential therapeutic targets and strategies.

Results that were outlined above show that negative immune regulators have a significant impact on GVHD biology. Tim-3/gal-9 pathway inhibition had a contrasting role in our model of acute GVHD depending on the presence of donor Tregs, as outlined in chapter 2. Tim-3 inhibition lead to a modestly augmented GVHD lethality. Teff cells underwent accelerated allo-proliferation by using $Tim^{-/-}$ T cells or blocking with a Tim-3-Ig fusion protein. This supported previous studies that showed Th1 Teffs had increased proliferation when blocked with Tim-3-Ig.[47,81] Furthermore, we demonstrated that gal-9 Tg recipients had reduced GVHD lethality and the effect was not observed when $Tim\text{-}3^{-/-}$ donor T cells were given to the gal-9 Tg recipients. Our data are consistent with the literature that describes $Tim\text{-}3^{+}$ Teff deletion by $gal\text{-}9^{+}$ cells and provides in vivo evidence that the Tim-3/gal-9 pathway is an important negative regulator of GVHD. Our most astonishing finding was the amelioration of GVHD lethality when Tim-3/gal-9 binding was inhibited in the absence of Tregs. This was

completely paradoxical to what would have been predicted knowing that eliminating Tregs leads to augmented GVHD. We went on to discover that inhibiting the Tim-3/gal-9 pathway in the absence of Tregs lead to a marked increase in IFN-γ secretion leading to activation induced cell death (AICD) of Teffs and therefore reduced GVHD lethality. Typically, IFN-g is thought to be inflammatory and damaging in the context of GVHD. However, this data suggests that over a certain threshold, IFN-γ can trigger AICD and prevent further inflammation and damage. This data demonstrates that there are usually multiple mechanisms of negative regulation that act to restrain immune hyper-activation. This reduced lethality was the result of having reduced gut damage demonstrated by fewer inflammatory cytokines and a more intact epithelial barrier. Together, this evidence shows that the Tim-3/gal-9 pathway can act as a suppressor of acute GVHD. It may be possible to translate our findings that enhanced signaling of the Tim-3/gal-9 pathway early post-transplantation to reduce the Th1 response and reduce GVHD in the presence of a T-cell replete graft by using reagents such as rGal-9 protein.

In studies looking at B7-H3, we found it to be a negative regulator of acute GVHD lethality as outlined in chapter 3. B7-H3 inhibition on recipients lead to augmented GVHD lethality along with increased gut injury. Teffs in the intraepithelial layer of the colon showed increased proliferation, inflammatory cytokine secretion as well as reduced epithelial layer integrity. Additionally, when B7-H3 is inhibited on donor T cells, there is augmented GVHD lethality. Early

after transplant (d7) the spleen has an increase in Teff proliferation and IFN-γ secretion as well as decreased apoptosis. Later after transplant (d21), the IELs of the colon had increased proliferation and decreased apoptosis and consequently a more damaged epithelial barrier. Both the intraepithelial and lamina propria layers had increased inflammatory cytokine secretion. Taken together, these data demonstrate B7-H3 expression in donor T cells and host non-hematopoietic cells negatively regulates GVHD lethality. Additional investigation will be necessary to elucidate the potential binding partners of B7-H3 so that the biology of the pathway can be better understood. These studies demonstrate that the B7-H3 pathway acts as a suppressor of acute GVHD and by inhibiting B7-H3 GVHD disease severity is augmented. Reagents that enhance signaling of the B7-H3 pathway early post-transplantation may be used to dampen the Th1 response and reduce GVHD.

It is clear that multiple regulatory mechanisms are involved in the biology of GVHD. Two negative regulatory pathways were described just in this thesis, and still more exist that have already been published,[89-91] and there will undoubtedly be more discovered in the near future. These pathways have the potential to have additive or even synergistic immune regulation. It would be ideal to be able to dampen the immune system to avoid lethal GVHD but maintain enough of a response to enable GVL. It will be important to demonstrate how these pathways work together so that better treatment plans and strategies can be developed.

In summary, this thesis presents research detailing the previously unidentified role for the Tim-3/gal-9 pathway as well as the B7-H3 pathway in acute GVHD. This data has increased our understanding of two crucial GVHD regulatory pathways and provides future strategies for developing new therapies that may help to decrease the morbidity and mortality of GVHD.

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