1-20-2016

Antigen Specific Induced T Regulatory Cellular Therapy for Graft-Versus-Host Disease Following Allogeneic Bone Marrow Transplantation

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Allogeneic Bone Marrow Transplantation

by

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A dissertation submitted in partial fulfillment of
the requirements for the degree of
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Date of Approval:
January 15, 2016

Keywords: Alloreactive, Graft-versus-Leukemia, CD8 Induced Tregs, Minor-mismatched antigens, and Combination therapy

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Dedication

This dissertation is dedicated to my loving and supportive father and mother, Dennis and Shirley Heinrichs. Their constant encouragement and devotion got me through the most challenging parts of this process. A special dedication to my sister Erica Heinrichs; she motivated me to pursue my dreams even if the road to success was long and treacherous. Also I dedicate this work to my grandparents, Francis and Dolly Heinrichs and Lou Franklin who left this world too soon, but whose love lives on in me.

Finally, a special dedication to my future husband Dr. Kellen Voss; he stood by my side through the worst and best of times. He continues to encourage my dreams and turn them into our dreams. Life without him would truly not be worth living. I am so happy to end this chapter of my life and begin a new one with him by my side.

Acknowledgements

I am very thankful to my mentor Dr. Xue-Zhong Yu for his scientific guidance and support for me throughout my PhD career. His knowledge and expertise in transplantation immunology is unsurpassed. I appreciate that he encouraged me to move to the Medical University of South Carolina with him during my second year, which really enhanced my PhD experience and helped me grow as an individual. I also greatly appreciate my co-mentor Dr. Patricia Kruk for being a strong supporter of my scientific and personal goals throughout my PhD. She always reminded me to keep my eyes on the prize and pushed me to my highest potential. I would like to extend my great gratitude to my committee members, Dr. Claudio Anasetti, Dr. Sri Nagaraj, Dr. Amer Beg and Dr. Burt Anderson, for their kindness and helpful guidance these last four years.

I would like to especially thank all the past and current members of the Yu lab: Dr. Kelley Haarberg, Dr. Jianing Fu, Dr. Yongxia Wu, Dr. Hung Nguyen, Dr. Hanief Sofi, David Bastian, Kane Koassard, Steven Schutt, Dr. Anusara Daenthanasanmak, and Yuejun Liu. They have provided me with experimental assistance, editing manuscripts, and emotional support and encouragement these last four years.

A special thank you to my co-PhD students and best friends: Stephanie Blankenship, Adonis Mcqueen, Krishna Reddy, and Geofery Ciarolone for our study sessions and for being the coolest nerds around.

Finally, I would like to thank the technical support from the animal facilities and flow cytometry cores at both Moffitt Cancer Center and the Medical University of South Carolina for providing top of the line core that made my research a seamless enjoyable process.

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Abstract

Allogeneic hematopoietic stem cell transplantation (allo-HCT) has been a successful cellular therapy for patients suffering from hematological malignancies for many decades; however, the beneficial effects of graft-versus-leukemia (GVL) are classically offset by graft-versus-host disease (GVHD). GVHD occurs when major and/or minor human leukocyte antigen (HLA) mismatches between donor and recipient cause rapid expansion and activation of donor effector T cells (Teffs) resulting in end organ damage to the recipient's epithelial tissues. Given the lymphoproliferative nature of this disease, the standard treatment option is broad immunosuppression, which can result in primary disease relapse, steroid refractory GVHD, and/or opportunistic infection. A more targeted therapy that can selectively suppress GVH responses with maintained GVL responses would achieve the optimal goal of allo-HCT. Regulatory T cells (Tregs) both natural (nTregs) or induced (iTregs) could be potential cellular therapies for the treatment of GVHD, given their innate suppressive function. Initial clinical trials using nTregs have yielded positive results; however, nTreg cellular therapy has been cumbersome due to the necessity for   large scale *ex vivo* expansion given their low yield within an apheresis product and non-specific suppression. Conversely, iTregs can be generated from naïve T cells thus decreasing *ex vivo* culture times and can be educated with specific antigen thus providing targeted suppression, but a consensus on their efficacy for GVHD therapy has not been reached. Therefore, we investigated the efficacy of antigen specific iTreg therapy for the prevention of GVHD while maintaining GVL responses.

In Chapter 2, we evaluated the effectiveness of monoclonal HY-specific iTregs in GVHD attenuation. We chose HY as a target antigen because it is a naturally processed, ubiquitously expressed minor mismatch antigen carried by only male donors/recipients cited to increase GVHD prevalence when donor and recipient are sex-mismatched. Utilizing HY-transgenic mice in which all T

cells recognize HY antigen exclusively, we generated HY specific iTregs which effectively attenuating GVHD in male, but not female recipients in three murine bone marrow transplantation (BMT) models (major mismatch, parent to F1, and miHAg mismatch). We found HY specific iTregs lost stability in female recipients but remained stable and suppressive in male recipients suggesting expression of HY antigen was required for their suppressive function and stability. GVL responses were not compromised with the addition of HY specific iTregs in recipient mice using a pre-established tumor model. Thus, HY-specific iTregs can be generated and suppress GVHD in an antigen-dependent manner while sparing the GVL effect.

In Chapter 3, we extend our findings in Chapter 2, which provided proof of principle that antigen specific iTregs effectively control GVHD; however, this therapy has a limited translational potential. Therefore, we generated alloreactive CD4 and CD8 iTregs and evaluated GVHD attenuation and GVL preservation in either full or haplo-MHC mismatched BMT models. We found alloreactive CD4 iTregs significantly suppress lethal GVHD, but completely abrogated the GVL effect against aggressive tumors. Conversely, alloreactive CD8 iTregs moderately attenuated GVHD and possessed direct cytotoxicity against tumor cells. Therefore, to rescue the impaired GVL effect mediated by CD4 iTregs, we established a combinational therapy with CD8 iTregs. Indeed we found combination CD4 and CD8 iTreg therapy significantly suppressed GVHD while sparing GVL responses compared to either CD4 or CD8 singular therapy. Mechanistically, this was achieved by potent suppression of both CD4 and CD8 Teffs coupled with preserved cytolytic molecule expression by both CD8 iTregs and Teffs.

Taken together, we propose antigen specific iTreg therapy can effectively attenuate GVHD while preserving GVL responses. We further uncovered unique characteristics of CD4 and CD8 iTregs that can be exploited to achieve the optimal cellular therapy following allo-HCT.

Chapter 1: Background

Note to Reader

Portions of this chapter have been previously published (Heinrichs et al 2015, Journal of Immunology Research and Therapy) and are utilized with permissions from the publisher (see Appendix A)

1.1 Allogeneic HCT

Allogeneic hematopoietic cell transplantation (allo-HCT) provides a reconstituted, healthy immune system for patients suffering from bone marrow failure syndromes and hematological malignancies such as leukemias, lymphomas, and myelomas. Donors are identified by high-resolution typing of class I and II human leukocyte antigen (HLA), and typically selected by recipient matching at HLA-A, -B, -C, -DRB1, DQB1, and –DPB1 [1]. Disparity within the major HLA, or even minor histocompatibility antigens [2], may stimulate donor T cells to induce GVHD. However, this is offset by the anti-cancer graft-versus-leukemia (GVL) effect of the allograft.

1.2 GVHD Pathophysiology

The pathophysiology of GVHD is complex, involving many different T-helper cell types, which contribute to disease manifestation; we refer the readers to our extensive review discussing the characteristics of these cells [3]. In brief, following conditioning, damage to host tissues causes the release of pro-inflammatory cytokines and danger-associated molecular pattern molecules (DAMPs),

which in turn activate recipient antigen-presenting cells (APCs). These host APCs then present host antigens to the donor T cells, which rapidly expand and differentiate into effector T cells (Teffs). Following differentiation, Teffs migrate to the GVHD target organs (skin, liver, lung, and gut) and cause end organ damage (3). Despite extensive advancements in HLA matching, immunosuppressive drugs, and conditioning therapies, many patients receiving allo-HCT still succumb to primary disease (37%), GVHD (20%), or infection (17%), respectively (4). Clearly, there is room for improving the success of allo-HCT.

1.3 GVL Effect

Early evidence of the beneficial GVL responses was apparent when increased disparity between donor and recipient resulted in less primary disease relapse in allo-HCT patients, displayed by increased disease free survival compared to syngeneic recipients (5, 6). When recipients received T-cell depleted grafts in order to negate deleterious GVHD responses; they also displayed increased rates of leukemic relapse, pointing to the T-cell compartment as the driving force for GVL responses (7-9). Although NK cells can play a very important role in GVL function (10), the cytotoxic CD8 and CD4 T cell mediate the most potent killing of residual tumor cells. This is classically demonstrated with the use of donor lymphocyte infusion (DLI) in order to treat patients with relapse after allogeneic HCT, however DLI also often offsets by the induction of GVHD (11, 12). Recently, it was discovered that CD4 and CD8 cytotoxic lymphocytes (CTL) used distinct mechanisms to mediate tumor clearance with CD4 CTLs preferentially using Fas/Fas-L mediated apoptosis and CD8 CTLs using direct release of cytotoxic perforin (13, 14). To adequately harness the GVL effect without deleterious GVH responses deeper understanding of tumor specific antigens like HA-1 or HA-2 (15) and how to isolate and enrich T cells specific for those antigens will greatly enhance the outcome of allo-HCT.

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1.4 Regulatory T Cells

Many clinicians and scientists have begun to embrace the concept of harnessing our own suppressive immune cells, T regulatory cells (Tregs), to improve recipient survival and quality of life (16-18). A delicate balance exists between GVL and GVHD responses, with too much suppression leading to tumor relapse and too little suppression leading to alloreactivity and end organ damage (Figure 1.1). Alas, balancing these fine cellular mechanisms has yet to be realized. Nonetheless, Tregs, with their ability to acquire antigen specificity, may be the answer clinicians and scientists have been looking for.
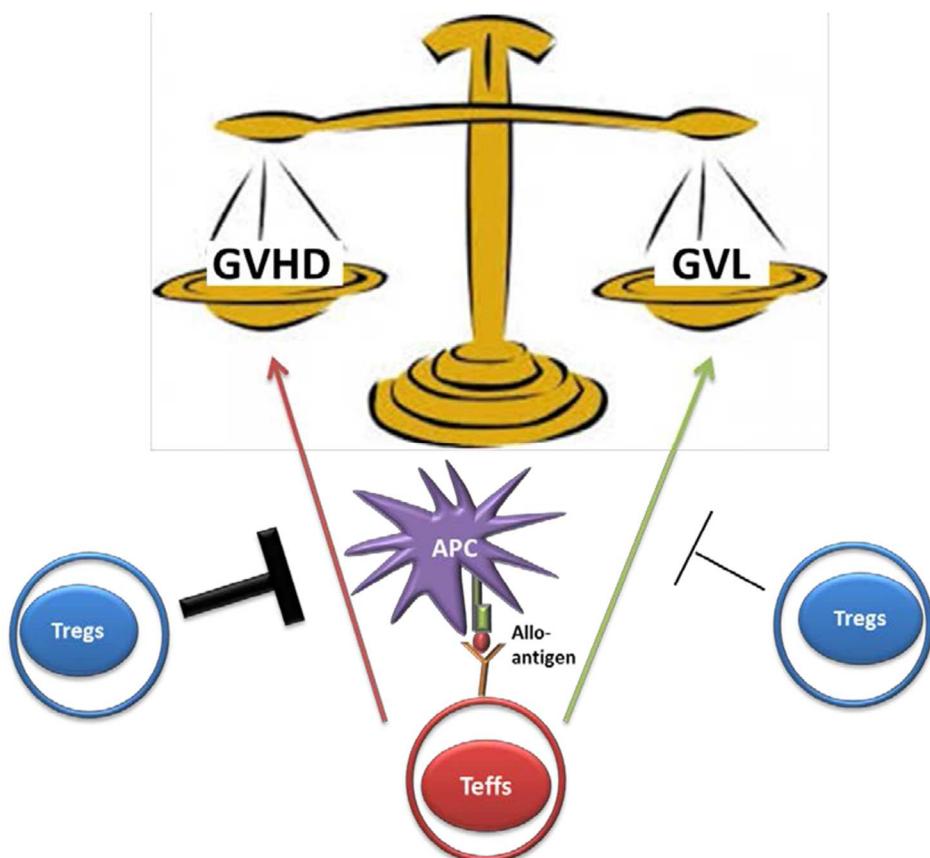
Figure 1.1 Delicate balance between GVH and GVL responses. Following allogeneic HSCT, effector T cells within the graft inoculum recognize non-hematopoietic and hematopoietic allo-antigens presented by host and/or donor APCs resulting in both graft-versus-host (GVH) and graft-versus-leukemia (GVL) responses. Treg therapy could improve outcomes in allo-HSCT by greatly inhibiting Teffs cells causing GVHD with little or partial inhibition of the GVL effect.

1.4.1 Initial Discovery

Tregs are relatively young, first being described as "suppressor T cells" in the 1970's by

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Gershon and Kondo, who conducted elegant experiments illustrating that induction of tolerance, was dependent on thymus-derived lymphocytes, and not B cells (19, 20). However, due to the inability to clearly characterize this suppressive lymphocyte population, controversial findings within the I-J region (21), and limitations in scientific techniques, the "suppressor T cells" fell off the scientific map for 12 years. In 1982, Sakaguchi and colleagues, while studying the effects of neonatal thymectomy on normal immune homeostasis, stumbled upon a very important discovery: within the CD4 T lymphocyte compartment were cells capable of causing autoimmune disease and those capable of preventing it (22).

1.4.2 CD25 and Foxp3

Thirteen years later, Sakaguchi was able to distinguish a reliable cell surface marker (CD25), which could differentiate between the protective CD4 T cells (CD25^{hi}) fraction from the pathologic CD4 cells (CD25^{low})(23). However, activated T cells can also express CD25, therefore negating the exclusivity of CD25 as marker for Tregs (24). Luckily, advances in intracellular staining techniques allowed for the discovery of Foxp3 (a member of the forkhead winged helix family), the master transcription factor for determining Treg fate and suppressive function (25). The finding that patients suffering from the autoimmune disease immunedysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) had inherited germline mutations within the *FOXP3* gene, which resulted in non-functional Tregs, solidified the specificity of Foxp3 to the Treg lineage (26). Scrufy mice, harboring a deletion of the Foxp3 gene, also display a lymphoproliferative disease characterized by multiorgan damage (27). The ability to definitively isolate and study Tregs (CD4^{+}CD25^{+}Foxp3^{+}) in autoimmune diseases clearly shows the major function of these cells is to maintain immune homeostasis (28, 29).

1.5 Natural and Induced T Regulatory Cells

With the identification of Foxp3, studies on Tregs increased exponentially and soon after we found

regulatory cells of the immune system were not just confined to expression of Foxp3 or even the T cell compartment. Over the years, multiple different flavors of regulatory cells have been discovered: Tr1 cells (30), CD8^{+}-Tregs (31, 32), myeloid derived suppressor cells (MDSC) (33), and B cells (B10 cells) (34). In this chapter, we will focus on CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells.

1.5.1 Development and Generation

As stated in the previous section, early neonatal thymectomy on day 3 versus day 7 of life pointed to the thymus as a major tissue associated with generation of Tregs (35). Experiments transferring the CD25^{+}CD4^{+} Tregs from the periphery and the resulting abolition of autoimmune disease in mice harboring genetic mutation within the Foxp3 gene (Scurfy) (25) hinted the Treg pool was actually comprised of two distinct subsets. Indeed, it is now widely accepted that Tregs can be either naturally derived from the thymus (nTregs) or converted from naïve CD4^{+}CD25^{-} T cells in the periphery termed as inducible Tregs (iTregs).

Both nTregs and iTregs have differential requirements for their generation, which helps characterize these two distinct subsets. nTregs are derived exclusively from the thymus. Upon recognition of self-antigen/self-MHC (major histocompatibility complex) with high affinity (36, 37), co-stimulation from CD28/B7 interactions (38), and IL-2 (although not required) (39), nTregs begin to increase expression of Foxp3 and acquire suppressive function (40) (41). iTregs, on the other hand, arise in the periphery from a population of naïve T cells, and therefore do not recognize self-antigens with high affinity (42). Instead, during chronic antigen exposure, including microbes in the gut and with suboptimal co-stimulation through CD28/B7, iTregs initiate the expression of Foxp3. In contrast to nTregs, iTregs require the presence of exogenous cytokines, IL-2 (39) and TGFβ (42), to fully differentiate into the commonly known suppressor T cells. Retinoic acid (RA) produced by CD103^{+} dendritic cells (DC) in the gut, has also been shown to further drive conventional T cells to express Foxp3 (43, 44) (Figure 1.2).
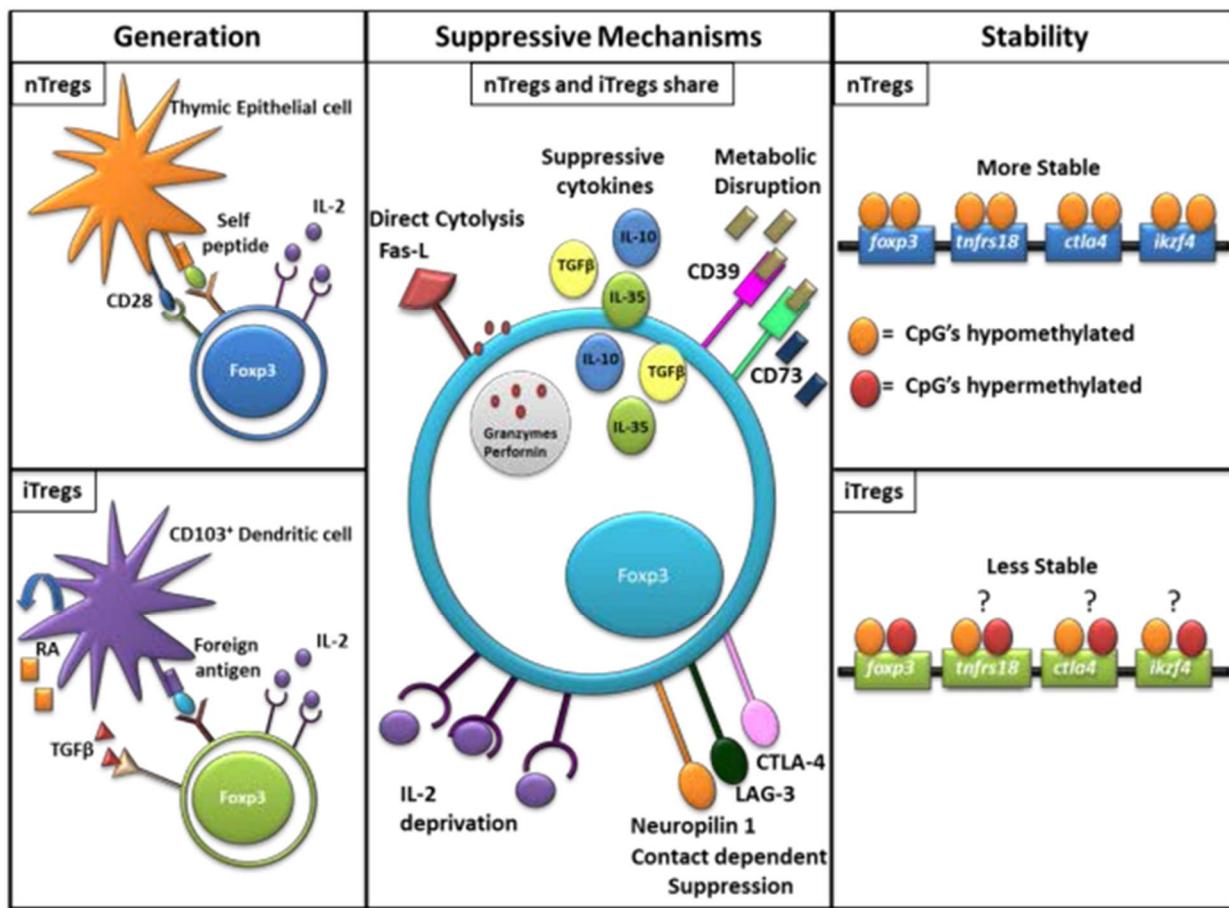
Figure 1.2 nTregs and iTregs: Generation, Suppressive Mechanisms, and Stability For generation nTregs and iTregs are distinct, with nTregs requiring recognition of self-antigen, costimulation, and IL-2; whereas iTregs recognize foreign antigen and require IL-2, TGFβ, and RA. nTregs and iTregs share suppressive mechanisms, broadly defined as direct cytolysis, suppressive cytokines, metabolic disruption, IL-2 deprivation, and contact dependent suppression. nTregs are more stable than iTregs with a fully demethylated CNS2 region with the foxp3 gene whereas iTregs sometimes display a partially methylated CNS2.

1.6 Suppressive Mechanisms

While nTregs and iTregs may differ in their requirements for generation, they utilize a multitude of similar mechanisms in order to maintain immune homeostasis (45, 46) (Figure 1.2). Tregs are activated via TCR engagement, which is absolutely necessary to mediate their suppressive function *in vivo*. In an elegant study using inducible genetic ablation of cell surface TCR complexes, Levine and colleagues found TCR stimulation was not required for Foxp3 expression, stability, or the ability of

Tregs to consume IL-2 (47). Instead, TCR activation is necessary for the expression of a limited number of genes, like IRF4, is required for activated Tregs to maintain suppressive function (47). The suppressive mechanisms of Tregs can be broadly classified into contact-dependent or contact-independent suppression.

1.6.1 Contact-Dependent Mechanisms

Contact-dependent suppression involves the expression of inhibitory molecules: CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), LAG-3 (lymphocyte activation gene 3), and Neuropilin-1. CTLA-4 inhibits expression of the costimulatory markers CD80/CD86 on the surface of APCs through trans-endocytosis (48), and thus results in decreased proliferation of T cells. Specific deletion of CTLA4 in Tregs resulted in decreased suppressive function (49). LAG-3 binds to MHC-Class II with a high affinity (31) on immature DCs and inhibits their maturation and co-stimulatory capacity (50). Neuropilin- 1, a recently discovered component of the Treg suppressive arsenal, was found to potentiate long-lasting interactions between Tregs and DCs. Neuropilin-1 ablation resulted in attenuated Treg suppressive function (51, 52).

1.6.2 Contact-Independent Mechanisms

In conjunction with contact-dependent mechanisms, Tregs utilize contact-independent mechanisms that create an immunosuppressive milieu to counteract the inflammatory milieu. A brief list of such mechanism involves the secretion on anti-inflammatory cytokines (IL-10, TGFβ, and IL-35), IL-2 consumption, release of granzymes, and generation of adenosine through ectoenzymes CD39/CD73 on Treg cell surface. IL-10, the immunomodulatory cytokine, seems to be a tissue specific suppressive mechanism utilized by Treg cells at intestinal interfaces, as IL-10 deficient Tregs could not protect mice during transfer of CD45RB^{high}CD4^{+} T cells induced colitis (53). Supporting this tissue specificity, Rubtsov generated specific IL-10 ablation within Foxp3 expressing cells and found 40% of

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IL-10 deficient mice developed spontaneous colitis by 6 months of age, these same mice did not develop systemic autoimmunity (54). The major function of TGFβ mediated suppression by Tregs is surprisingly through contact-dependent, but APC-independent, induction of infectious tolerance, a process of conversion of naïve or effector T cells into suppressive CD4$^+$Foxp3$^+$ suppressor T cells (55). IL-35, much like TGFβ, has been implicated in conferring infectious tolerance by inducing iTr35 regulatory cell mediated suppression via IL-35 (56).

Interestingly, high expression of CD25 (IL-2 receptor alpha chain) not only aids in the identification of Treg cells but Tregs can also non-specifically sequester IL-2 from the inflammatory microenvironment. This was seen in experiments where addition of common-γ chain cytokines reversed Treg-mediated T cell apoptosis *in vitro* and *in vivo* (57). Since Tregs require activation through TCR signaling, it is no surprise they also express the ectoenzymes CD39/CD73, which convert extracellular adenosine triphosphate (ATP) into adenosine (58, 59). Tregs utilize adenosine by increasing its concentration within the inflammatory microenvironment which increases adenosine binding to A2A adenosine receptors expressed on DCs and T cells, subsequent increase of cyclic AMP and resulting inhibition of DC/T cells (60). Finally, Tregs can cause direct apoptosis of effector T cells (Teffs) through the release of granzymes (61).

1.6.3 Mechanisms Specific to GVH and GVL

With regards to GVL/GVHD responses the role of Treg generated granzymes is complex, Ley and colleague found granzyme B-expressing Tregs specifically accumulated in the tumor microenvironment and directly used granzyme-mediated apoptosis of NK and CD8 Teffs to inhibit tumor clearance (62). However, some years later Ley also noted Tregs do not use this granzyme-B mediated apoptosis to control Teffs during GVHD (63). More recently, Granzyme A has been shown to be critical for Tregs to control intestinal GVHD, where mice treated with Tregs deficient for granzyme A failed to rescue hosts from gastrointestinal GVHD (64). IL-10 was also found to be a key suppressive

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molecule nTregs use to suppress GVHD, shown by the inability of $CD4^+CD25^+$ Tregs from IL-10$^{-/-}$ mice to alleviate acute GVHD (65). Homing to the proper sites, lymph nodes and target organs, by expressing of CCR5 is also indispensable for Tregs to suppress GVHD as genetic ablation of CCR5 expression negates Tregs ability to attenuate GVHD (66). Therefore, it seems Tregs use their vast arsenal of suppressive mechanism to suppressive immune reactions in a context and tissue specific manner and further research is needed to exploit this aspect of Tregs for maximal therapeutic efficacy.

1.7 Stability

In order to realize the use of nTregs or iTregs for cellular therapy, whether for GVHD or autoimmune disorders, the safety of the therapy must be unequivocally harmless to the patients. To the advantage of cellular therapy is the fact that these cells arise naturally during immune homeostasis and off target side effects, like those seen with pharmacological therapy, should be reduced significantly. However, two different lineage-tracing studies revealed Foxp3 expression could be lost in a subset of Treg cells, name ex-Tregs. Whether these "ex-Tregs" displayed an activated effector phenotype or promiscuous transient Foxp3 expression differed based whether Foxp3 was tagged using the NOD BAC transgenic mice expressing GFP-Cre within the Foxp3 promoter crossed with ROSA-LSL-YFP mice (67) or using a tamoxifen-inducible GFP-Cre fusion with the estrogen receptor mutant (GFP-creERt2) crossed with ROSA-LSL-YFP (68), respectively. Hesitation among clinicians and scientists began with these initial lineage-tracing studies and was strengthened by demonstrating nTregs can lose expression of Foxp3 after repeated rounds of ex vivo stimulation (69, 70). Therefore, how can we ensure Treg cellular therapy remains suppressive and safe if the mater transcription factor and regulator of suppressive function, Foxp3, is lost following expansion? The environmental factors, external stimuli, and intrinsic mechanisms maintaining or negating the expression and stability of Foxp3 have exploded in the field of Tregs and still remain a hot topic of debate. Recently, multiple extensive reviews have

explored the notion of Treg stability versus Treg plasticity; with the general consensus Tregs poses the ability to display both of these characteristics depending on the microenvironmental signals (71, 72). Treg stability can be broadly separated into two subsets: the epigenetic control of Foxp3 (gene regulation) and the stability of Foxp3 (transcription factor).

1.7.1 Epigenetic Control of Foxp3

Classically, a stable Treg genetic signature consisted of highly demethylated CpG islands within the conserved non-coding sequence 2 (CNS2) of the Treg specific demethylation region (TSDR), with nTregs displaying fully demethylated CNS2 and iTregs displaying partially demethylated CNS2 regions (73). However, the field of Treg genetic stability has moved from a Foxp3 centric view to a multiple Treg signature gene view, termed "nTreg-Me" by Ohkura et al. (74). In these experiments, it was elegantly found CpG hypomethylation of four Treg signature genes *Foxp3, Tnfrs18 (GITR), Ctla4, and Ikzf4 (Eos)* was independent of Foxp3 expression and occurred following strong and/or chronic TCR signaling. Importantly, it was found cells can express Foxp3 but without a full nTreg-Me signature can lose stability and become plastic, secreting proinflammatory cytokines (74) (Figure 2). In line with this study was the establishment of the Treg-quintet, a complex of five redundant transcription factors that act in conjunction with Foxp3 to fully establish the Treg-signature (75). Any one of these factors, Eos, IRF4, GATA-1, Lef- 1, and Stab1 helps stabilize Foxp3 to its bind target site, either repressing (IL-2) or activating (CTLA-4) expression, and thus fully committing the cell to the Treg phenotype.

1.7.2 Foxp3 Protein Stability

Given expression of Foxp3 protein itself ensures inheritable maintenance of the Treg phenotype by directly binding to the CNS2 in a Cbfb-Runx1 demethylation dependent manner (73), many investigators have shifted focus as to what factors contribute to the stability of Foxp3 expression. Recently, some key negative (CDK2 and Stub1) and positive (PTEN and Ezh2) regulators have

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emerged. Cyclin-dependent kinase 2 (CDK2) was found to phosphorylate Foxp3, which then recruited the E3 ubiquitin ligase Scf/Fwb7, interesting when CDK2 was genetically deleted, the half-life of Foxp3 was dramatically increased resulting in a more potently suppressive Treg (76). Likewise, the E3 ubiquitin ligase, Stub1, was found to polyubiquinate Foxp3 in a heat shock protein 70 dependent fashions during inflammatory responses (77). Silencing of Stub1 decreased the degradation of Foxp3 and enhanced protection from T cell mediated colitis in mice (77). On the converse side, phosphatase and tensin homolog (PTEN) deficiency was found to lead to loss of CD25 expression and then eventual loss of Foxp3 expression and suppressive function, probably through overt signaling through PI3(K), a direct target of PTEN (78, 79). Finally the chromatin-modifying enzyme (Ezh2) was found to aid Foxp3 in binding to repression targets (IL-2 and IFNγ) genes to mediate their silencing, genetic ablation of Ezh2 lead to a decrease in Foxp3^{+} cells in non-lymphoid tissues and expression of genes resembling Teff cells at those sites (80) and failed to protect mice from autoimmune colitis (81). More specifically, Ezh2 may impact Tregs in tissue specific manner as Ezh2 deficient Tregs displayed reduced expansion on the spleen and lymph nodes, but not in the thymus and lamina propria (81). Furthermore, He et al. demonstrated Ezh2 plays an important role in Treg survival and expansion post BMT (82). Much more research is needed to understand exactly what can make and more importantly maintain a stable Treg cell if their use as a cellular therapy will be fully realized.

1.8 nTregs in GVHD

Given their natural presence, high stability, and important function to maintain homeostasis nTregs were the first subset of Tregs to be explored as a cellular therapy. The uncontrolled immune activation, high likelihood of disease (GVHD), limited therapeutic options, and steroid refraction surrounding allo-HCT made it the ideal candidate to test nTreg cellular therapy

1.8.1 Pre-Clinical Findings

Initial experiments in pre-clinical models found donor-type $CD25^+CD4^+$ regulatory T cells could suppress lethal acute GVHD in BALB/c recipients but only if a high ratio of 1:1 Treg to Teff cell was maintained (65). The knowledge nTregs only account for 5-10% of the total CD4 T cell population and high number needed to achieve GVHD attenuation; it became apparent nTregs would need to be expanded *ex vivo* in order to achieve a more efficient translatable therapy. A seminal study from Blazar's group in 2002, tested *ex vivo* polyclonal activated and expanded nTregs in three different models of lethal acute GVHD (83). Importantly, this study established nTregs could be expanded (67-fold) to sufficient numbers to attenuate GVHD thus solving the problem of low circulating nTregs. To further move to clinical reality, investigators strove to see if nTregs would suppress the beneficial GVL effect. Using two different tumor models, A20 and BCL1, freshly isolated $CD4^+CD25^+$ Tregs did not impair the ability of Teff cells to clear tumor at a 1:1 ratio, however, if the effector T cell dose was below a certain threshold the tumor relapsed (84).

1.8.2 Expansion of Human nTregs

With the strong preclinical findings indicating nTregs could functionally attenuate GVHD while maintaining GVL, the field moved quickly to translate murine findings to human nTregs. Levings isolated $CD4^+CD25^+$ human nTregs from peripheral blood and expanded them with IL-2 and allogeneic feeder cells. These expanded nTregs remained unresponsive to allogeneic DCs and anti-CD3 activation, while maintaining the ability to suppress autologous $CD25^-$ T cells *in vitro* (85). nTreg expansion of 100-fold was reached by Godfrey in 2004, using cell-sized dynabeads with anti-CD3 and anti-CD28 attached, CD4 feeder cells, and IL-2 (86). It was found these activated and expanded nTregs could potently suppress DC-driven allogeneic mixed lymphocyte reactions by 90%, and completely prevent the secretion of pro- inflammatory cytokines (86). Since cord blood transplants are often used in the clinic, researchers also tested whether nTreg isolation and expansion from this source could also be

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effective. Cord blood was found to contain a larger CD25^{bright} population compared to adult peripheral blood, in which the population was CD25^{dim} indicating a non-suppressive function. These nTregs displayed a comparable growth rate to peripheral nTregs, and were also potently suppressive against allogeneic CD4$^+$CD25$^-$ Teffs (87). Lastly, based on the finding human nTregs could be expanded more robustly using anti-CD3 loaded artificial APCs and could potently suppress xenogeneic GVHD (88), the first clinical trials were initiated for nTreg therapy for the treatment of GVHD.\

1.8.3 Antigen-Specific nTreg

Recently, a new concept has emerged regarding the expansion of nTreg cells for cellular therapy: selective expansion of the alloreactive nTregs within an apheresis product. This more personalized approach, using nTregs specific for both HLA-mismatched (18) and HLA-matched but minor antigen mismatched (miHAg) (89), yielded a high number of potently suppressive nTregs. Generation of these alloantigen specific nTregs differs from institutions with some using monocyte derived DCs (89, 90) or B cells (Leventhal and Wood personal communication); however each generates sufficient numbers for infusion into patients. These results have initiated the first clinical trial using personalized nTregs to prevent acute GVHD (17).

1.8.4 Clinical Trails

In 2009, the first patients were treated with ex vivo expanded CD4$^+$CD25$^+$CD127$^-$ nTregs from donor peripheral blood (91). In this initial trial only two patients were enrolled, nTreg therapy could only be initiated once standard immunosuppression failed. One patient developed acute GVHD and displayed transient alleviation of disease, however the Treg source was exhausted and the patient later succumb to multiorgan failure (91). The other patient developed chronic GVHD and once nTreg therapy was initiated significant reduction in symptoms was seen (91). Even though the sample size was very small, this study lead to the first dose escalation study for *ex vivo* expanded nTregs isolated from

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umbilical cord blood   (92). A dosing of 1, 3, 10, or 30 x 10^6 Tregs/kg was tested; of the 23 patients

enrolled 17 patients received their target dose and no dose-limited toxicities were observed. A modest

reduction in acute GVHD was observed in the 23 patients compared with historical controls (43% versus

61%) respectively (92). In a very bold clinical trial, freshly isolated nTregs from donor peripheral blood

were administered four days prior to transplant, followed by no post-transplant immunosuppression. Of

the 26 patients enrolled, only 2 developed GVHD, given no immunosuppression was administered; this

trial proved nTregs could be used as a prophylactic for GVHD (93). However, 13 of the 26 patients died

within 3 months post-transplant from other co-morbidities. These three clinical trials really opened the

door for Treg therapy; however, there are still improvements to be made. For instance, the expansion

potential of nTregs was still a major obstacle, with 5 patients not receiving sufficient cell doses (92) and

despite the high success of freshly isolated nTregs from the Di Ianni group; a high number 2:1 (Treg:

Teff) was still needed to prevent GVHD (93).

1.9 iTregs in GVHD

The study of iTregs in pre-clinical models of GVHD has been restricted to the *in vitro*

generated iTreg due to the fact an adequate marker to fully distinguish nTregs from iTregs has not been

established. Given conventional T cells constitute a larger percentage of peripheral blood or cord blood

products and have an increased activation capacity compared to nTreg cells, protocols to polarize these

cells into iTregs are currently being investigated. It is now well established that conventional CD4 T

cells isolated from peripheral lymphoid organs can begin to express Foxp3 upon polyclonal stimulation

with anti-CD3/anti- CD28 in the presence of TGFβ and IL-2 (42, 94, 95) and retinoic acid (RA) can

further enhance the expression of Foxp3 (43).

Table 1.1 Summation of Differences in iTreg Pre-Clinical Results. Differences in activation cue, polarizing cytokines, and infusion schedule have results in dramatically different results for iTreg therapy. Many more tests on GVL function are highly warranted.

Investigator	Activation Reagent	Polarizing Cytokines	Infusion Schedule	GVHD Outcome	GVL Outcome
Koenecke et al 2009	BM-derived mature DCs	IL-2 TGFβ RA	Tregs + Teff Day 0	No attenuation	Not tested
Beres et al 2011	Plate bound anti-CD3 Soluble anti-CD28	IL-2 TGFβ RA	Tregs + Teff Day 0	No attenuation	Not tested
Semple et al 2011	OVA peptide	IL-2 TGFβ RA	Tregs + Teff Day 0	Significant attenuation	Not tested
Sela et al 2011	CD11c⁺ DCs	IL-2 TGFβ RA	Tregs (multiple infusions) Teff Day 0	Significant attenuation	Not tested
Hippen et al 2011	KT64/86 With anti-CD3	IL-2 TGFβ Rapamycin	Tregs + Teff Day 0	Significant attenuation xenogeneic	Not tested
Zhang et al 2013	Plate bound anti-CD3 Soluble anti-CD28	IL-2 TGFβ Rapamycin	Tregs + Teff Day 0	Attenuation when stabilized by rapamycin/IL-2 complexes	Tregs impair CML clearance
Li et al 2015	HY peptide	IL-2 TGFβ RA	Tregs Day 0 Teff Day 3	Significant attenuation	Preserved GVL function
Heinrichs et al 2015	CD11c⁺ DCs	IL-2 TGFβ RA	Tregs Day 0 Teff Day 3	Significant attenuation	Tregs impair P815 clearance

1.9.1 Pre-Clinical Findings

Unlike, nTreg preclinical findings, which displayed similar results even across different expansion and GVHD models, there is still considerable controversy in the literature regarding iTreg therapy for the prevention or treatment of GVHD. This controversy seems to encompass differences in activation reagents, polarizing cytokines, and infusion schedules (Table 1.1).

1.9.2 Polyclonal versus Antigen Specific iTregs

iTregs generated using polyclonal activation (anti-CD3/anti-CD28) (96-98) are inferior to antigen-specific (99, 100) /alloantigen specific iTregs (101-103). Beres et al found a high percentage of iTreg conversion using polyclonal activation however even at a 1:1 (Treg: Teff) ratio these iTregs could not effectively attenuate acute GVHD (96); they claim the ineffectiveness of iTreg therapy directly stems from the loss of Foxp3 expression. This finding is directly in line with the subsequent study by Zhang et al. showing polyclonal activated iTregs failed to protect recipient mice and could even be

pathogenic if systemic rapamycin and IL-2 complexes were not co-administrated (97). Despite these two pre-clinical findings, Hippen et al was able to induce naïve T cells from human peripheral blood products to 240 x 10^9 iTregs when stimulated with KT64/86 cells (a K562 cell-based artificial APC expression CD86 and high affinity Fc receptor loaded with anti-CD3) and these iTregs potently suppressed xenogeneic GVHD (98). In contrast to polyclonal activation, we have shown, using OT-II and HY-transgenic naïve T cells stimulated with either OVA or HY peptide, OVA (99) and HY-specific (100) CD4 iTregs potently suppress acute GVHD, even at low Treg: Teff ratios. This higher potency correlates with the ability of antigen-specific iTregs to recognize antigen, as antigen-specific iTregs failed to protect recipient mice when the cognate antigen was not expressed. Emphasizing continuous activation of Tregs through TCR engagement is essential for their suppressive function.

Naïve B6 T cells generated with either BALB/c BM-derived mature DCs (101) or CD11c$^+$ splenic DCs(102) to induce alloreactive iTregs yield conflicting results, with the former being ineffective at protecting mice from GVHD due to loss of Foxp3 expression, whereas the latter significantly attenuates GVHD in a non-irradiation BMT model and iTregs persist for 6 months in recipient mice. We have generated alloreactive CD4 iTregs using the same generation conditions as Sela et al (CD11c DCs) and have found these iTregs to be potently suppressive and effective attenuate GVHD in a fully MHC-mismatched irradiated BMT (103). It is no surprise antigen-specific iTregs are more potent and suppressive than polyclonal iTregs as a recently study found the two different activation signals impart different   phenotypic profiles to each iTreg (104). Physiologically activated iTregs displayed better control of Th1 responses and a broader range of chemokine and chemokine receptor expression than anti-CD3/CD28 activated iTregs (104); this could explain the differences seen between investigators with regards to   iTregs ability to attenuate GVHD.

1.9.3 HY Antigen

The diversity of minor histocompatibility antigens (miHAg) among the population is quite

extensive and represents a hindrance following allo-BMT in HLA-matched donor and recipient pairs (105). miHAg, like HY, are fragments or peptides of endogenous host proteins that are bound to the clefts of HLA molecules (106). Whereas, in HLA- mismatched transplants the T cells recognize the HLA molecule as foreign, in a HLA-matched transplant, the T cells recognize the miHAg present within the HLA binding groove and mediate GVHD. HY is the most well studied miHAg due to the high incidence of sex-disparity allo-BMTs (107) (108-110). Given its high polymorphism HY antigen has been associated acute GVHD risk between female donors and male recipients, which makes it an ideal target antigen for iTreg therapy (107).

1.9.4 Polarizing Cytokines

Differences in the polarizing conditions also accounts for the discrepancy seen in iTreg therapy for GVHD. IL-2 and TGFβ are present throughout all experiments however some investigators use rapamycin (97, 98) and others using RA (96, 99-103). Since Rapamycin has been shown to preferentially suppress Teff cells while allowing for the growth/conversion of iTregs, the addition of this compound to generation conditions should yield a more pure population of iTreg cells (111). However, we and others have proven RA greatly increases conversion of naïve T cells into iTregs displaying potent suppressive function. RA was shown to increase the histone acetylation and methylation within the CNS elements of the Foxp3 promoter region thus increasing accessibility of binding partners to the Foxp3 promoter (112).

1.9.5 Infusion Schedule

Finally the infusion schedule seems to play a major part in the outcome of GVHD attenuation with iTreg therapy. Almost all studies use iTregs as a prophylactic therapy; iTregs have yet to be shown to be beneficial as a treatment modality. Most investigators infused iTregs with T-cell depleted bone marrow and CD25-depleted Teff cells all within 24 hours of irradiation (96, 97, 101, 102). With the

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finding that initial infusion of nTregs two days prior to Teff infusion resulted in robust expansion of nTregs with 10-fold less needed to attenuate GVHD (113), we strove to apply this infusion schedule to iTreg cell therapy. Indeed we found infusion of iTregs prior to Teff cells greatly increased the potency of iTregs to attenuate GVHD (100, 103). Despite all these conflicting results the first dose escalation clinical trial using iTregs induced as described in (98) will be tested in adults receiving non-myeloablative HLA- identical sibling donor transplantation (114). The outcome of this trial will greatly increase of understanding of iTreg cellular therapy and results are eagerly awaited.

1.10 CD8 iTregs in GVHD

A less understood population of suppressor T cells is derived from the CD8 T cell lineage (31, 32). Surprisingly after allogeneic BMT in murine models, a significant population of $CD8^+CD25^+Foxp3^+$ iTregs emerges early after transplant (115, 116) and not after syngeneic transplant. These CD8 iTregs were found to express similar suppressive molecules as compared with CD4 iTregs (GITR, CD44, CTLA-4, and CD25) and could compensate for the CD4 iTregs to attenuate GVHD (115). However, CD8 iTregs did express increased levels of $\alpha 4\beta 7$ as compared to CD4 iTregs (116). Importantly, when these CD8 iTregs were isolated from recipient mice and used as a prophylactic in secondary recipients they could significantly attenuate GVHD (116). To correlate these findings to human cells, patients' peripheral blood was tested 6 months post-transplant and surprisingly no $CD8^+CD25^+Foxp3^+$ iTregs were found. Authors later found all patients had received cyclosporine as a prophylaxis and thus concluded CD8 iTregs were acutely sensitive to cyclosporine inhibition (116). Future experiments are needed to see if this population arises in patients receiving different prophylactic therapy like rapamycin.

Currently only two groups have published pre-clinical experiments on *in vitro* generated CD8 iTregs testing the ability of these cells to attenuate GVHD, each with opposite findings. While testing polyclonal CD4 iTregs, Zhang et al simultaneously, generated polyclonal CD8 iTregs and found them to

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be equally pathogenic due to loss of Foxp3 expression 3 weeks post-transplant (97). They also found CD8 iTregs to be less responsive to Foxp3 stabilization, using rapamycin and IL-2 complex treatments, as compared to CD4 iTregs (97). Due to the inability to attenuate GVHD, the GVL function was not assessed in the study. Further work on CD8 iTreg therapy by Zheng and colleagues was to isolate naïve human CD8+CD25-CD45RA+CD45RO- and generate alloreactive CD8 iTregs (termed CD8hi) by stimulating solely with hCD40-B cells (117). These CD8hi iTregs potently suppressed GVHD induced by hPBMC injected into Rag2-/-γc-/- mice (humanized mouse model of GVHD). Authors then used lymphoblastoid cell line (LCL) to assess CD8hi ability to maintain the GVL effect; infusion of CD8hi iTregs did not impair the GVL effect as LCL tumor was cleared within the blood of recipient mice as compared to PBS treated controls (117). Interestingly, it was found CD8hi iTregs had direct cytotoxicity against LCL tumors through Fas- FasL, perforin, and granzyme B pathways as inhibition of either negated the lysis of LCL tumors *in vitro*. The direct cytotoxic effect of CD8 iTregs is in direct correlation with our own findings using murine alloreactive CD8 iTregs in GVHD (103). We find CD8 iTregs possess some direct toxicity against P815 mastocytoma, however not enough to fully eradicate tumor without Teff cell infusion (103). Interesting, these cytolytic CD8 iTregs were moderately effective at GVHD attenuation (103).

1.11 Tregs Effect on GVL Preservation

Although attenuation of GVHD is the main focus of investigators in assessing the potential of Treg therapy, suppression of Teffs can only reach a certain threshold before these cells are unable to clear recipients of residual tumor cells (the GVL effect). In fact, the increase in Treg numbers in the peripheral blood and/ or tumor microenvironment positively correlates with tumor relapse or growth in mice and humans (118, 119). With regards to nTreg therapy, pre-clinical models show contrasting results   depending on the type of tumor tested. In models using A20 (84, 120) and BCL1 (84), Tregs did not inhibit the GVL effect. However, the GVL effect was inhibited in a model using P815 mastocytoma

(120). Be that as it may, initial nTreg clinical trials indicated no increased incidence of tumor relapse compared to historical controls (92). iTregs, on the other hand, seem to be more complex. Zhang et al. found polyclonal activated CD4 iTregs, despite being unable to attenuate GVHD without the addition of rapamycin, also impaired the capacity of Teffs to clear primary myeloid blast crisis CML. This impairment was not due to rapamycin administration, as mice treated only with rapamycin did not succumb to tumor mortality (97). In our lab, we found HY-specific iTregs could attenuate GVHD and still maintain the GVL effect, even against pre-established P815 mastocytoma tumors (100). However, our recent data shows alloreactive CD4 iTregs, when infused three days prior to Teffs, significantly impairs the GVL effect (103).

1.12 Modifying Tregs

Tregs are the master regulators of balance in our immune systems. Given their natural function, we have tried to exploit them to control immune disorders characterized by unbridled inflammation (namely autoimmunity/GVHD). However, isolation, expansion, and reinfusion of Tregs did not result in an adequate therapy. Thus, investigators are eagerly testing new strategies to increase the specificity, stability, and activity of Tregs. Chimeric antigen receptor (CAR) modified T cells have shown great promise for increasing the antitumor effects in acute and chronic B cell malignancies (121, 122) as well as some solid tumors (123). Since Tregs are themselves derived from the same lymphocyte progenitors, it is tempting to envision the use of CARs to increase Treg specificity and stability. In this regard, specificity was easily achieved, illustrated by two studies using hapten-specific CAR Tregs were more potent in alleviating experimental colitis than unmodified Tregs (124, 125). Given the increased potency of antigen-specific iTregs compared to polyclonal iTregs, it would be ideal to find a way to engineer iTregs that could specifically suppress responses against tissue damage (GVHD), while ignoring responses to tumor antigens (GVL). To increase stability, silencing of Stub1, a molecule that ubquinates Foxp3, was tested by infecting Tregs with lentivirus containing sh-Stub1 (silencing RNA).

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It was found sh-Stub1 Tregs were more stable during experimental colitis induction (77). Likewise, Restifo and colleagues established BACH2 as a key partner for Foxp3 stability, as genetic deletion resulted in Tregs inability to suppress lethal inflammation in RAG KO mice (126). Therefore, retroviral induced expression of BACH2 in iTregs could potentially increase their stability and should be further investigated.

1.13 Combinational Therapy

The dichotomy we have seen between CD4 and CD8 iTregs, especially with regards to GVL and GVHD responses, raises the question as to whether these cells can work together to optimize the outcome of allo-HCT? As our knowledge about cellular and biological processes continues to expand, clinicians and scientists have moved from a singular approach in order to incorporate a combinational therapeutic approach in a vast majority of disease models. Alloreactive CD4 iTregs are able to potently attenuate GVHD, yet severely compromise GVL function. Alloreactive CD8 iTregs only modestly attenuate GVHD, but possess GVL capability (103). We hypothesized combining these two cellular therapies   would result in attenuation of GVHD while preserving the GVL effect. Indeed, we found in allogeneic BMTs, a combination of CD4 iTregs and CD8 iTregs was effectively able to decrease GVHD while maintaining GVL (103). With regards to combinational therapy, two investigators have found the addition of Rapamycin and IL-2 complexes in conjunction with iTreg infusion creates optimal attenuation of GVHD (97, 116). We believe even more beneficial combinational therapies will emerge for GVHD in years to come.

Chapter 2: HY-Specific Induced Regulatory T Cells Display High Specificity and Efficacy in the Prevention of Acute Graft-versus-Host Disease

Note to Reader

This chapter has been previously published (Li & Heinrichs et al. J Immunol. 2015, Jul 15; 195(2): 717-25) and are utilized with permissions from the publisher (see Appendix B)

2.1 Abstract

Naturally derived regulatory T cells (nTregs) may prevent graft-versus-host disease (GVHD) while preserving graft-versus-leukemia (GVL) activity. However, clinical application of nTregs has been severely hampered by their scarce availability and non-selectivity. To overcome these limitations, we took alternative approaches to generate Ag-specific induced Tregs (iTregs) and tested their efficacy and selectivity in the prevention of GVHD in pre-clinical models of bone marrow transplantation (BMT). We selected HY as a target antigen because it is a naturally processed, ubiquitously expressed minor histocompatibility antigen (miHAg) with a proven role in GVHD and GVL effect. We generated HY- specific iTregs (HY-iTregs) from resting CD4 T cells derived from TCR transgenic mice, in which CD4 cells specifically recognize HY peptide. We found HY-iTregs were highly effective in preventing GVHD in male (HY$^+$) but not female (HY$^-$) recipients using MHC II-mismatched, parent → F1 and miHAg- mismatched murine BMT models. Interestingly, the expression of target Ag (HY) on the hematopoietic or non- hematopoietic compartment alone was sufficient for iTregs to prevent GVHD. Furthermore, treatment with HY-iTregs still preserved the GVL effect even against pre-established leukemia. We found HY-iTregs were more stable in male than in female recipients. Furthermore, HY-

iTregs expanded extensively in male but not female recipients, which in turn significantly reduced donor effector T-cell (Teff) expansion, activation, and migration into GVHD target organs resulting in effective prevention of GVHD. This study demonstrates iTregs specific for HY miHAg are highly effective in controlling GVHD in an Ag-dependent manner while sparing the GVL effect.

2.2 Introduction

Allogeneic bone marrow transplantation (BMT), as a treatment for leukemias, lymphomas, and myelomas, has historically been hampered by the detrimental effects of graft-versus-host disease (GVHD). Allogeneic T cells within the graft inoculum recognize both major and minor mismatch antigens on leukemic and host tissues, resulting in either beneficial graft versus leukemic (GVL) or deleterious graft-versus host (GVH) effect. Clinicians and scientists still struggle to separate the GVL and GVH responses; among other strategies, the use of naturally derived regulatory T cells (nTregs) has been shown to be a promising approach to effectively control GVHD in animal studies and initial clinical trials. However, isolation and expansion of nTregs still remains a significant obstacle to establishing nTreg therapy as a standard for GVHD treatment. This is due to the low frequency and high number of nTregs needed to effectively control GVHD. Another concern regarding nTreg therapy centers on the loss of the GVL effect. Given nTregs are non-selective suppressors; this therapy could result in suppression of allogeneic T cells responding to leukemic cells and therefore increased relapse in patients. Establishing Ag-specific inducible T regulatory (iTreg) cell therapy for the treatment of GVHD may solve the previously stated disadvantages of nTreg therapy. First, iTregs can be generated from naïve T cells, under specific polarizing conditions, offering a greater number of primary cells for initial expansion. Secondly, we propose, by conferring antigen specificity or antigen education during iTreg generation, we can overcome the high number needed for efficiency as compared to non-specific nTreg cell therapy. Finally, we propose drawing the fine line between GVL and GVH responses can be obtained by conferring Ag-specificity.

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In experimental autoimmune disease models, Ag-specific Tregs are highly effective in controlling autoimmune diabetes, gastritis, and encephalomyelitis (127-129). Others and we have initiated studies to evaluate the effects of Ag-specific iTregs in the prevention of GVHD and in the maintenance of GVL activity. We previously generated OVA-specific iTregs by *foxp3* transduction or TGFβ-induction, and demonstrated they persist long-term *in vivo* and suppress GVHD in non-myeloablative and myeloablative BMT models when activated by the cognate Ag; either constitutively expressed or introduced via immunization (130, 131). However, we used a nominal Ag to activate Ag-specific iTregs in our preliminary studies, which may not represent clinical settings. Therefore, it is crucial to extend these studies by testing iTregs specific for naturally processed alloantigens, in this case, HY Ag. HY is a minor histocompatibility Ag (miHAg) expressed solely by male recipients. Clinical data shows MHC-matched BMT between female donors and male recipients increased the risk for acute GVHD development (132) and HY-specific alloresponses (109, 133-135). Therefore, due to its clinical relevance, we generated HY specific iTregs and tested their efficiency, stability, and selectivity in suppressing acute murine GVHD.

2.3 Materials and Methods

2.3.1 Mice

C57BL/6 (B6, H-2^b, CD45.2$^+$, BALB/c (H-2^d) and (B6 x DBA2) F1 (BDF1, H-2$^{b/d}$) mice were purchased from the National Cancer Institute. B6 Ly5.1 (H-2^b, CD45.1$^+$), B6 bm12 (H-2^b), BALB.b (H-2^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Foxp3^{gfp} knock-in (KI) strain was obtained from A. Rudensky's laboratory (136, 137). Luciferase-transgenic (*Luc*-Tg) strain on B6 background was kindly provided by R. Negrin (Stanford Univ., CA) (138). Anti-HY TCR Tg Marilyn mice (CD4$^+$Tg, H-2^b, I-A^b restricted) was kindly provided by C.R. Mainhart (NIAID, Bethesda, MD).

Marilynn Foxp3^{gfp} knock-in (KI) and (B6 x bm12) F1 strains were produced by crossbreeding. All the mice were housed in a pathogen-free condition at H. Lee Moffitt Cancer Center and Drug Discovery Building at MUSC. All experimental procedures were approved by the IACUC.

2.3.2 T-cell purification and iTreg generation

Total T cells or CD4$^+$CD25$^-$ T cells were purified through negative selection using magnetic beads as described in our previous work (131, 139). The purity of CD4$^+$CD25$^-$ cells ranged from 85 to 95%, but CD4$^+$CD25$^+$ cells was always less than 1% among total CD4$^+$ cells. To generate HY-specific iTregs, CD4$^+$CD25$^-$ T cells from TCR Tg (Marilynn) Foxp3^{gfp} KI mice were seeded at 2.5 x 10^5/ml and stimulated with 0.5 μg/ml HY peptide in the presence of 1.25 x 10^6/ml irradiated syngeneic T-cell depleted (TCD)-splenocytes as APCs with 5 ng/ml TGF-β1, 5 ng/ml IL-2 and 10 nM retinoic acid for 6 days.

2.3.3 Immuno-fluorescence analysis.

Multiple-color flow cytometry was performed to measure the expression of surface molecules according to standard techniques. Intracellular Foxp3 expression was measured with a Foxp3 detection kit from eBioscience (San Diego, CA), according to manufacturer's instruction. Intracellular cytokines were measured according to standard techniques, as described in our previous work (140).

2.3.4 BMT and bioluminescent imaging (BLI).

The procedures for induction of acute GVHD were described in previous publications (131, 140). BALB.b mice were exposed to total body irradiation (TBI) at 850-900 cGy (2 split doses) at day - 1. (B6 x bm12) F1 or BDF1 mice were exposed to 1200 - 1300 cGy TBI (2 split doses). TCD-BM cells alone or in combination with purified T cells from B6 donors were injected via the tail vein into recipients within 24 hrs after irradiation. Recipient mice were monitored every other day for clinical

signs of GVHD, such as ruffled fur, hunched back, lethargy or diarrhea, and mortality. Animals judged

to be moribund were euthanized and counted as GVHD lethality. To generate BM chimeras, female or

male recipients were lethally irradiated and transplanted with TCD-BM from male or female syngeneic

donors, and thus HY antigens were expressed only on epithelial tissues (F → M) or hematopoietic cells

(M → F). *In vivo* BLI of the recipients transplanted with allogeneic T cells from *Luc*-Tg B6 donors and

BM from non-Tg B6 donors was performed using an IVIS200 charge-coupled device imaging system

(Xenogen). For GVL induction, p815-luc mastocytoma cells were injected either on day of transplant

with iTregs or three days prior to irradiation (pre-established tumor model).

2.4 Results

2.4.1 HY-specific iTregs suppress polyclonal T-cell response to alloantigens in vitro

iTregs can be generated from conventional CD4 T cells upon TCR stimulation in the presence

of TGFβ, and addition of retinoic acid (RA) further increases the generation of iTregs (42, 139, 141). In

this study we selected HY as target antigen, because it is a naturally processed and ubiquitously

expressed miHAg with a proven role in GVHD and GVL responses (6-10). HY-specific iTregs were

generated from $CD4^+CD25^-$ T cells from Marilyn Foxp3^{gfp} KI mice by stimulating with HY^{Ab}*Dby*, in

the presence of IL-2, TGFβ and RA (Figure 2.1A, upper panels) and purified (purity 94%± 3%) by

FACS sorting   (Figure 2.1A, lower panels). Foxp3^{gfp} reporter gene allows us to obtain purified Foxp3$^+$

iTregs, but this strategy cannot be applied in humans and GFP KI may affect the function of Tregs

(142, 143). Therefore, to exclude any confounding effect, we generated HY-specific iTregs from

$CD4^+CD25^-$ T cells of Marilyn mice (Figure 2. 1B, upper panels). $CD4^+CD25^{hi}$ cells (purity 92% ± 3%)

were purified through positive selection for CD25 using magnetic beads (Figure 2. 1B, lower panels).

Thus, iTregs were routinely generated from non-Foxp3^{gfp} $CD4^+CD25^-$ T cells and isolated for

$CD4^+CD25^{hi}$ using magnetic beads. We then tested the suppressive function of HY-specific iTregs *in*

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vitro, and found these iTregs suppressed ~50% proliferation of polyclonal T cells in response to allogeneic APCs at 1:16 ratio of Treg: Teff in the presence of HY-peptide, but the same iTregs had little suppressive activity in the presence of nominal OVA peptide (Figure 2.1C), confirming the activation of iTregs is required for their suppressive function *in vitro*.
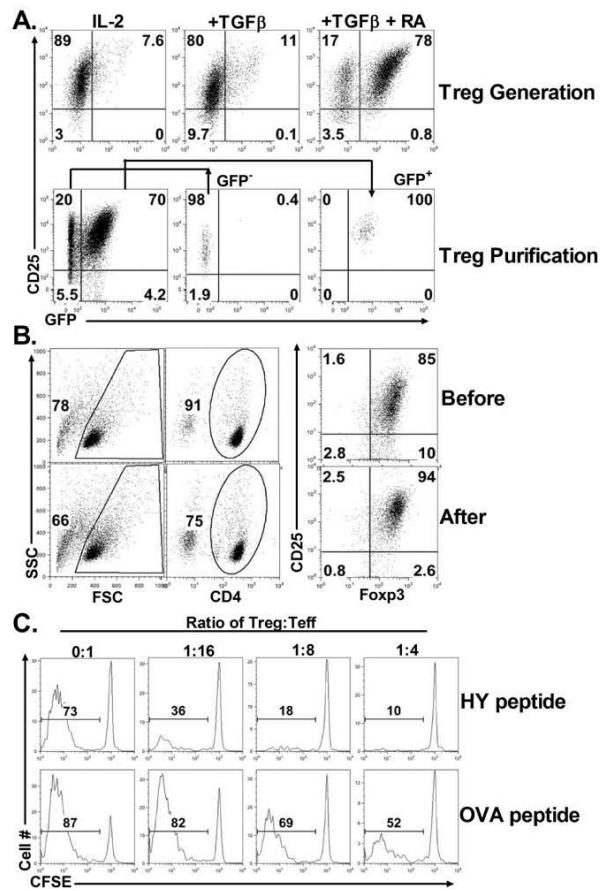
Figure 2.1 Generation and isolation of HY-specific iTregs. (A) CD4+CD25- cells were purified from spleen and lymph nodes of TCR Marilynn Foxp3gfp KI mice, and stimulated with HY-peptide (0.5 µM) in the presence of irradiated TCD-splenocytes plus IL-2 (5 ng/ml). To generate iTregs, media was supplied with either TGFβ (5 ng/ml) alone or TGFβ and RA (10 nM). Five to six days after culture, cells were harvested and tested for expression of CD4, CD25, and GFP by flow cytometry. The phenotype of cultured cells under the different condition is shown on gated live CD4+ cells (upper panels). CD4+ CD25+GFP+ (iTregs) and CD25+GFP- cells (controls) were purified by FACS sorting (lower panels). (B) CD4+CD25- cells were purified from spleen and lymph node of TCR Marilyn mice, and iTregs were generated in the presence of TGFβ and RA as described in A. Six days after culture, CD4+CD25hi cells (iTregs) were isolated by enriching CD4+ cells through negative selection and then purifying CD25hi cells through positive selection using magnetic beads. The phenotype of cultured cells is shown on gated live CD4+ cells before (upper panels) and after (lower panels) iTreg isolation. These results represent accumulative data obtained from more than 10 experiments. (C) CD4+CD25- purified T cells from B6 Ly5.1+ mice were labeled with CFSE and stimulated at 2 x 10^5/well with irradiated TCD-splenocytes from female (B6 x bm12) F1 mice at 6 x 10^5/well in 96-well plates. Various numbers of HY-specific iTregs were added into culture to achieve indicated

Treg: Teff ratios in the presence of HY (upper panels) or control OVA (lower panels) peptide at 0.5 μg/mL. Six days after cell stimulation, cultured cells were harvested and stained for the expression of Ly5.1 and CD4. CSFE profiles were shown on gated Ly5.1$^+$CD4$^+$ Teffs. These data represents 1 of 3 replicate experiments. ND: not done

2.4.2 HY-specific iTregs prevent GVHD in activation-dependent manner

Next, we examined whether HY-specific iTregs were able to prevent GVHD induced by polyclonal T cells in a B6 → (B6 x bm12) F1 BMT model, in which donor effector CD4$^+$ T cells (Teff) recognize mismatched recipient MHC II alloantigen (H2^{bm12}). In this model, Teffs at indicated dose induced ~60% GVHD lethality while addition of iTregs at the same time of BMT significantly reduced GVHD lethality in male ($p < 0.01$) but not in female recipients ($p = 0.7$) (Figure 2.2 A ,B), indicating recognition of HY antigen by HY-specific iTregs was indispensable for their suppressive function of allogeneic responses *in vivo*. To assess whether donor reconstitution was impaired by iTreg therapy, 80 days post BMT, we observed male recipients who received Teffs plus HY-specific iTregs had comparable numbers of total spleen, B and T cells to those of BM alone (controls without GVHD), whereas the recipients of BM + Teff (GVHD controls) had significantly reduced numbers of spleen, B and T cells (vs. BM alone, $p<0.05$, Figure 2.3). These results indicate HY-specific iTregs promoted long-term immune reconstitution and did not cause chronic GVHD in male recipients. We next determined whether infusion of HY-specific iTregs prior to Teffs promotes Treg expansion and increases therapeutic potential of Tregs (113). To this end, we utilized the same model and infused HY-specific iTregs 3 days prior to Teffs and found these iTregs completely prevented GVHD lethality in male recipients ($p<0.01$) (Figure 2.2 C, D).
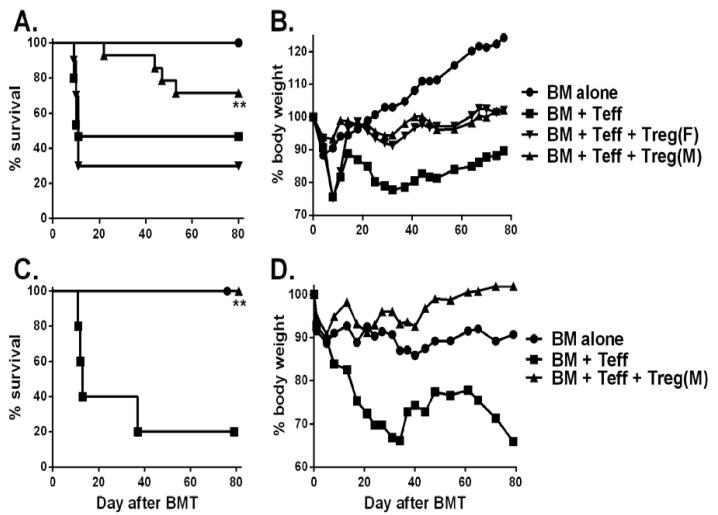
Figure 2.2 HY-specific iTreg attenuation of GVHD is antigen dependent. Male or female (B6 x bm12) F1 mice were lethally irradiated and transferred with 5×10^6 TCD-BM alone or plus $1\text{-}1.5 \times 10^6$/mouse CD25-depleted CD4^{+}T cells (Teffs) from B6 donors. HY-specific iTregs were generated and isolated as described in Figure 1, were added at $0.5\text{-}0.75 \times 10^6$/mouse into donor graft at the same time of BMT. Overall survival (A) and body weight changes are shown. The data are pooled from 3 replicate experiments with 10-15 mice in each group. In a separate experiment (n = 5-6), male (B6 x bm12) F1 mice were lethally irradiated and transferred with TCD-BM alone or plus 1×10^6/mouse HY-specific iTregs on day 0. CD25-depleted CD4^{+} T cells (Teffs) from B6 donors were injected at 2×10^6/mouse on day 3 after BMT. Recipient survival (C) and body weight changes (D) are shown. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.
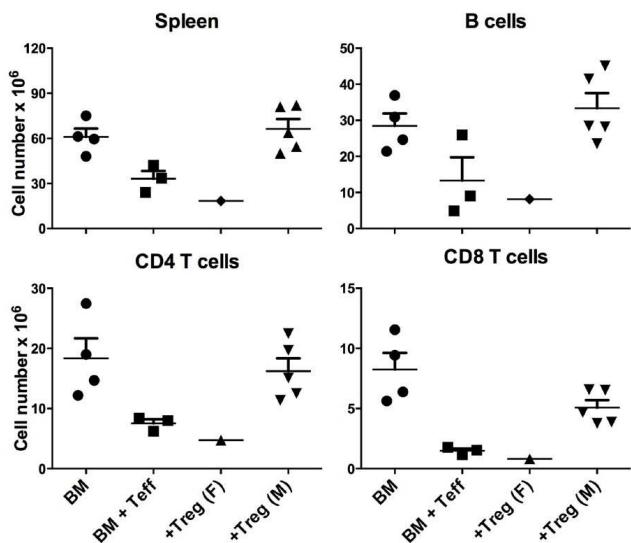
Figure 2.3 Effects of HY-specific iTregs on long-term donor immune reconstitution. Lethally irradiated (B6 x bm12) F1 mice were transplanted with TCD-BM alone or TCD-BM plus CD4^{+}CD25^{-} Teffs with or without HY-specific iTregs in male or female recipients as described in Figure 1. Eighty days after BMT, survival recipients were euthanatized and their splenocytes were counted and stained for expression of H2K[b] (donor type), CD4, CD8 and B220. The numbers of total spleen cells, donor B cells (H2K[b+]B220^{+}), CD4 (H2K[b+]CD4^{+}) T cells, and CD8 T cells (H2K[b+]CD8^{+}) were shown. These data represent 1 of 2 replicate experiments.

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In clinical BMT, most patients receive grafts from MHC-matched and multiple miHAg-mismatched donors. In an effort to mimic a clinical scenario, we used the B6 → BALB.b (both H2^b) model, in which donor and recipient mice differ by at least 29 different miHAg loci (144). HY-specific iTregs were highly effective in preventing GVHD in male (p<0.05) but not female BALB.B recipients (Figure 2.4 A, B). Likewise, haploidentical transplantation is extensively used in clinic. Utilizing B6 →DF1 model, we further confirmed HY-specific iTregs were highly effective in preventing GVHD in male (p<0.05) but not female BDF1 recipients (Figure 2.4 C, D). To further support our long-term data in the B6 → BDF1 model, we analyzed pathology scores and found male recipients who received iTregs had significantly reduced pathologic damage within the liver, small intestine, large intestine, and lung compared to all other groups (Figure 2.4E; p < 0.01). In agreement with survival data, female recipients receiving iTregs had comparable pathologic injury in target organs to Teff alone groups, further supporting necessity for iTregs to recognize specific Ag to exert their suppressive function. Taken together, these findings support the use of HY-specific iTregs in clinically relevant BMT models.
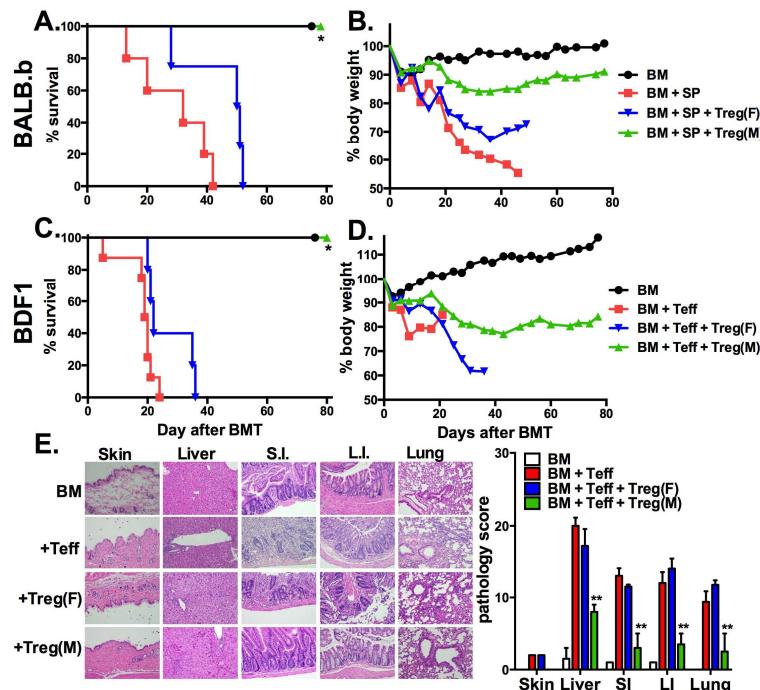
Figure 2.4 Effect of HY-specific iTregs in the prevention of GVHD in miHAg- or haplo-mismatched BMT model. Male or female BALB.b mice were lethally irradiated and transferred with TCD-BM alone or plus 4 x 10^6/mouse HY-specific iTregs. On day 3, 25 x 10^6/mouse total splenocytes from normal B6 donors were injected into the recipients previously transferred with BM alone or BM plus iTregs. Recipient survival (A) and body weight changes (B) are shown. The data are pooled from 2 replicate experiments with 9-10 mice in each group.

Male or female BDF1 mice were lethally irradiated and transferred with TCD-BM alone or plus at 4×10^6/mouse CD25- depleted total T cells from normal B6 donors. HY-specific iTregs were also included at 2×10^6/mouse into donor graft 3 days after BMT for some recipients. Recipient survival (C) and body weight changes (D) are shown. The experiments were done 2-3 times for each BMT model, but the data presented are from one experiment with 5-8 mice in each group using cell doses indicated. Seven days post Teff injection, BDF1 recipients as described in the legend were sacrificed and pathology samples of the skin, lung, liver, small and large intestine were collected (E) represents the pathology score of the various organs with 4 mice per group. Asterisk indicates statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$

2.4.3 HY-specific iTregs suppress the expansion and activation of donor T cells

We next assessed the cellular mechanism by which HY-specific iTregs suppress alloreactive Teffs *in vivo*. Taking advantage of *Luc*-Tg mice, the expansion and infiltration of *Luc*-Tg Teffs can be measured *in vivo* over time using BLI assay (145). To use this method, we titrated the dose of T cells required for mediating GVHD and found at least 4-fold lower numbers of *Luc*-Tg T cells were required to cause GVHD lethality comparable to normal B6 T cells (Figure 2.5).
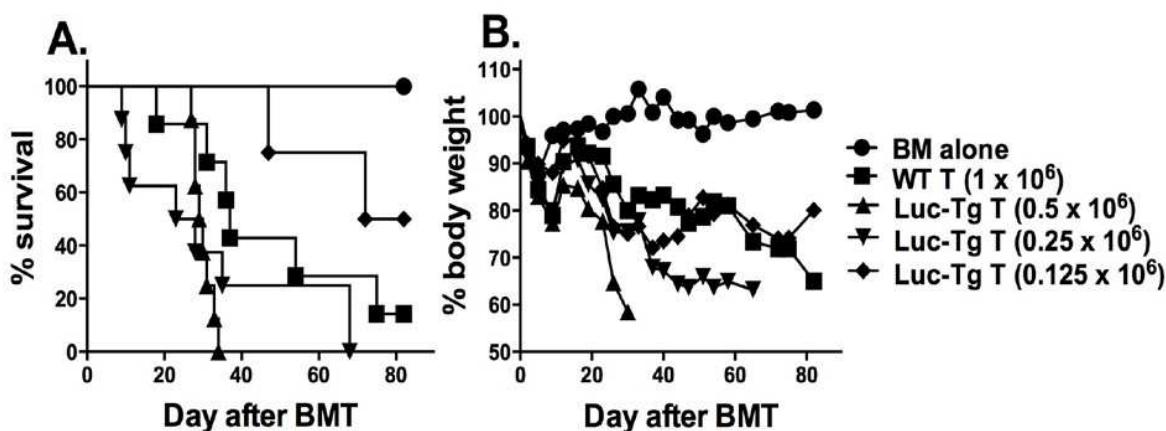
Figure 2.5 Pathogenicity of T cells from B6 Luc-Tg mice in GVHD. CD25-depleted total T cells (Teffs) were purified from spleens and lymph nodes of normal B6 or B6 Luc-Tg mice through negative selection. Lethally irradiated BALB/c mice were transplanted with TCD-BM or plus Teffs from normal or Luc-Tg B6 donor at the doses indicated. Recipient survival (A) and body weight changes (B) are shown. The data are pooled from 2 replicate experiments with 8-10 mice per group.

This data suggested *Luc*-Tg T cells might be significantly more pathogenic in the induction of acute GVHD. Using 0.25×10^6 *Luc*-Tg CD4$^+$, although there were relatively low signal intensities and no significant difference among groups on day 6 following Teff injection (Figure 2.6 A), throughout later observation periods, the BLI intensity was significantly reduced in male recipients receiving HY-specific iTregs compared to recipients with Teff alone ($p< 0.001$) or in female recipients transplanted

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HY-specific iTregs (p = 0.02) (Figure 2.6 A, B). Furthermore, male recipients receiving HY-specific iTreg showed less dispersed BLI signal, mainly confined to the spleen, compared to other recipients (Figure 2.6 B). Similar results were observed in miHAg- or haplo-mismatched BMT models (Figure 2.6 C-F). These data suggest HY specific iTregs regulate allogeneic Teff expansion and infiltration into GVHD target organs, such as the gut and liver.
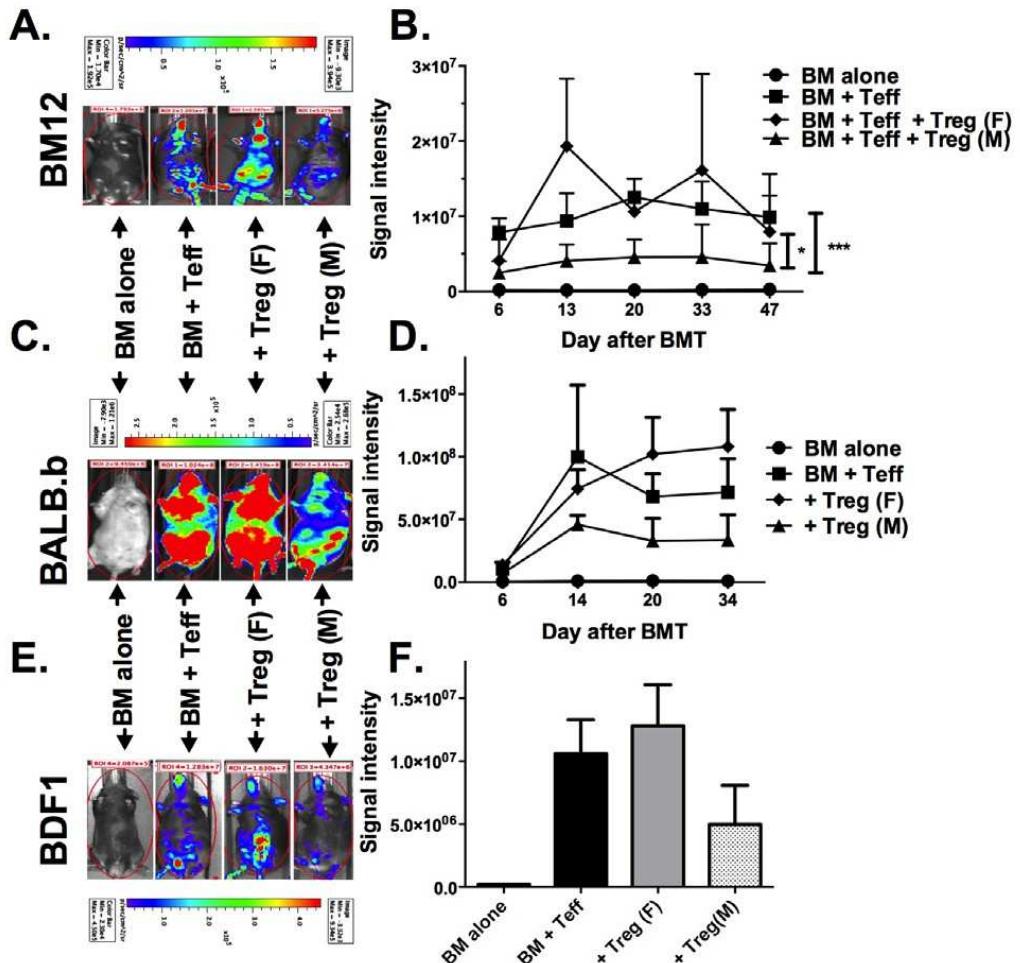
Figure 2.6 Effects of HY-specific iTregs on Teff expansion in vivo. The results from experiments using B6→Bm12 as described in Figure 2. The BLI signal strength was shown on one representative mouse from each group (A). Average signal intensity from each group of recipients throughout the experimental time points (B). The results were from the experiments using B6 → BALB.b BMT model as described in Figure 3 A and B. The BLI signal strength was shown on one representative mouse from each group at day 14 after BMT (C). Average signal intensity from each group of recipients was shown at the time points indicated (D). The results were from the experiments using B6 → BDF1 BMT model as described in Figure 3 C and D. The BLI signal strength was shown on one representative mouse from each group (E), and average signal intensity from each group of recipients was shown (F) on day 13 after BMT. The image is shown in one representative mouse in each group. The data represent one of 2 replicate experiments with 5-6 mice per group in each experiment.

To further evaluate the effect of iTregs on the expansion and migration of Teffs, we transferred

Teffs isolated from B6 Ly5.1[+] mice and HY-specific iTregs along with TCD-BM isolated from normal

B6 donors into (B6 x bm12) F1 recipients. Seven days after BMT, we measured Teffs (CD4$^+$Ly5.1$^+$)

and iTregs (CD4$^+$TCRVb6$^+$Ly5.1$^-$) in recipient spleen (Figure 2.7 A, C) and liver (Figure 2.7 B, D). We

found iTregs expanded extensively in the spleen and migrated substantially to the liver of male but not

female recipients, ($p < 0.01$, in spleen and liver), and the number of Teffs in the liver of male recipients

was dramatically reduced (Figure 2.7 D).
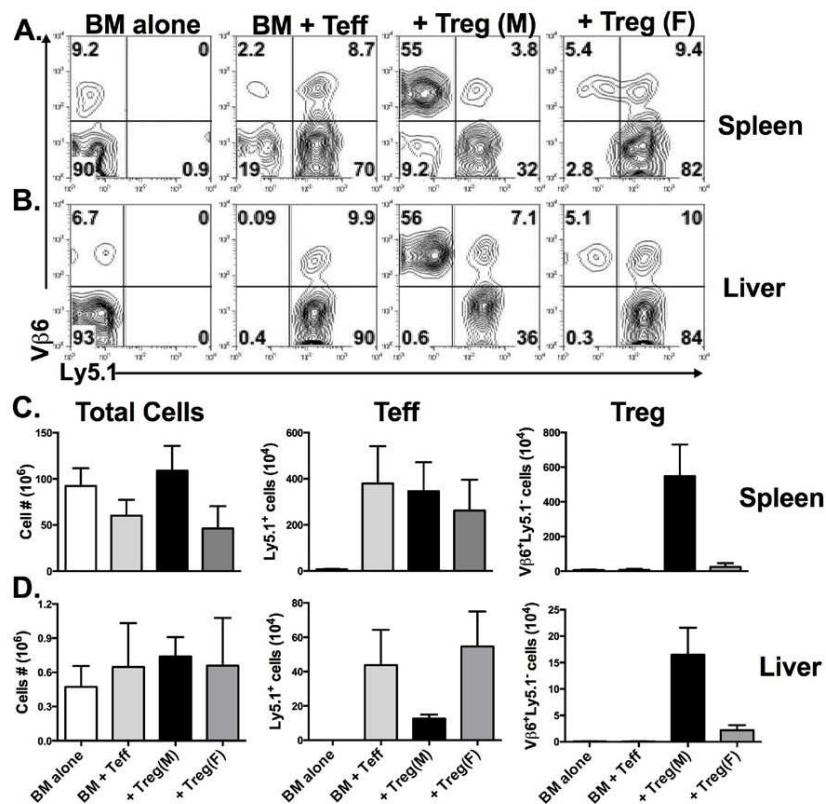
Figure 2.7 Stability and Efficiency of HY-specific iTregs. Lethally irradiated male or female (B6 x bm12) F1 mice were transplanted with B6 TCD-BM plus 1.5 x 10^6/mouse Teffs (CD4$^+$CD25$^-$) isolated from B6 Ly5.1$^+$ mice. At the same time, 0.75 x 10^6/mouse HY-specific iTregs were also included into the donor graft to some recipients. Seven days after BMT, recipient spleen and liver were harvested and measured for expansion and infiltration of iTregs and Teffs. Mononuclear cells were isolated from recipient spleen (A) and liver (B), and expression of TCRVb6 and Ly5.1 was shown in gated CD4$^+$ live cells. The average numbers of total cells, Teffs (CD4$^+$Ly5.1$^+$) and iTregs (CD4$^+$Vβ6$^+$Ly5.1$^-$) per mouse were shown in recipient spleen (C) and liver (D). Each group includes 3-4 mice, and the data represents 1 of 3 replicate experiments. Asterisk indicates statistical significance: *p<0.05,
**p<0.01, ***p<0.001

To extend these findings to the haploidentical BMT model, we transferred BM and Teffs from B6 Ly5.1[+] mice and HY-specific iTregs (Ly5.1[-]) into irradiated B6D2F1 recipients. Fourteen days after BMT, analysis of IFNγ and TNFα secretion within the spleen of effector CD4 and CD8 T cells, showed male recipients had significantly decreased secretion of pro-inflammatory cytokines (p<0.01) whereas there was no significant difference between Teff alone and female recipients (Figure 2.8 A, B). In correlation with our flow data, analysis of the serum cytokine levels, 14 days post BMT, revealed male recipients had significantly reduced levels of IFNγ and TNFα (p<0.01) whereas there was no difference between female recipients and Teff alone groups (Figure 2.8 C). These cellular findings are consistent with our long-term survival data, proving the recognition of cognate antigen is necessary for iTregs to control Teffs in order to attenuate GVHD.
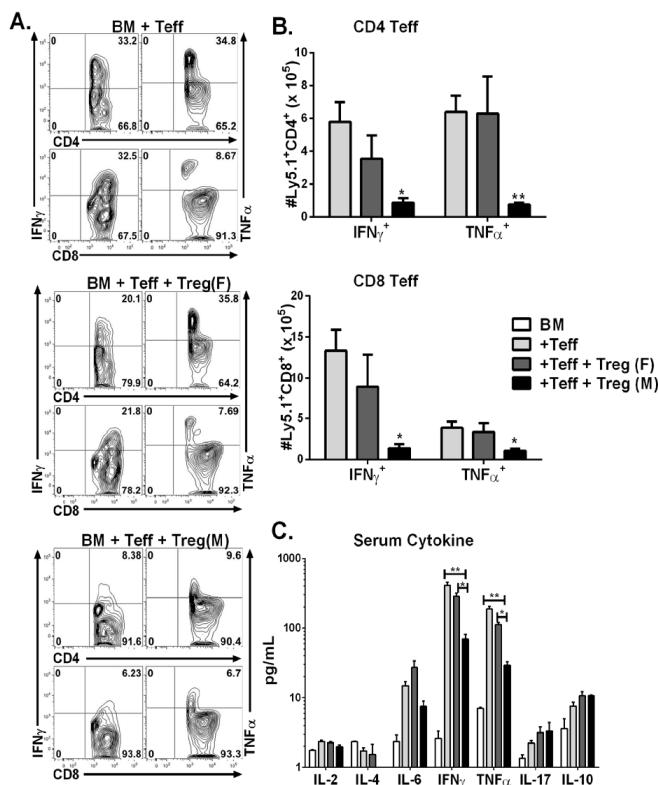
Figure 2.8 HY-specific iTregs suppress activation and expansion of Teffs. Male or Female BDF1 mice were lethally irradiated and transferred with TCD-BM alone or plus 4 x 10^{6}/mouse CD25-depleted total T cells from normal B6 Ly5.1[+] donors. HY-specific iTregs were also transplanted at 2 x 10^{6}/mouse on day 0 of BMT. Fourteen days after Teff injection, recipient spleen and liver were harvested and total T cells isolated 4 mice per group. (A)

Representative flow analysis of IFNγ and TNFα with effector CD4 and CD8 T cells. B) Represents the absolute number of CD4 and CD8 Teffs secreting IFNγ and TNFα within the recipient spleen. (C) Serum was collected on day 14 and cytokine levels were assessed by cytometric bead analysis, one representative analysis of two independent experiments. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

To address the stability of Ag-specific iTregs after injection, we performed a time course analysis of HY- specific iTregs within male and female recipient's spleen and liver 7, 14 and 21 days after our previously described B6→BDF1 model. The male recipients retained the high number of CD4$^+$Vβ6$^+$Foxp3$^+$ iTregs in their spleens on day 7, 14, and 21 post BMT (p< 0.001), whereas the number of HY-specific iTregs declined over time in the female recipients (Figure 2.9 A, B). The male recipients also had higher   numbers of CD4$^+$Vβ6$^+$ iTregs than female recipients on day 7 in their livers, although Foxp3 expression was retained similarly at this time point (Figure 2.9 C, D). In the liver, the numbers of iTregs were more strikingly different on days 14 or 21 between male and female recipients. Furthermore, iTregs were   highly stable in the male recipients reflected by their Foxp3 expression, whereas iTregs rapidly lost their Foxp3 expression in female recipients (p<0.001) (Figure 2.9 D). Taken together, iTregs were highly   stable and expanded extensively after Ag-stimulation, and in turn effectively suppress Teff expansion and activation and migration into GVHD target organs regardless of BMT models
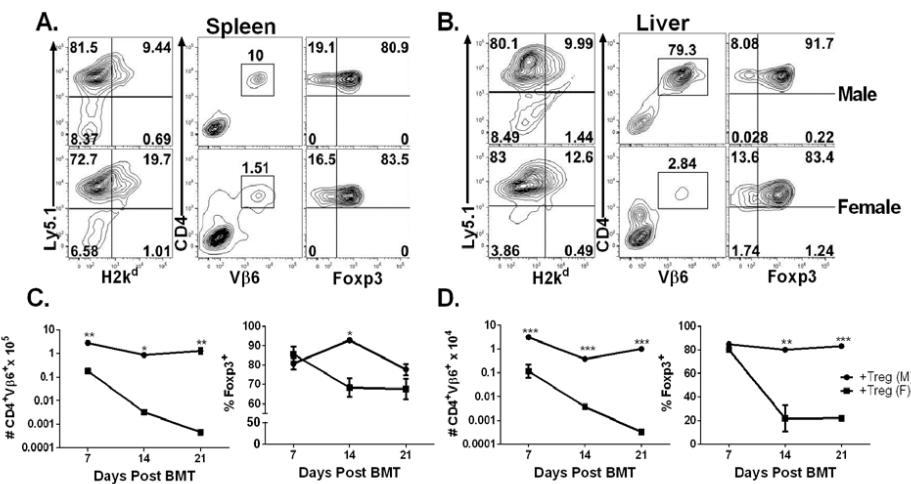
Figure 2.9 HY-specific iTregs are highly stable under inflammatory conditions. Male or Female BDF1 mice were lethally irradiated and transferred with TCD-BM alone or plus 4 x 10^6/mouse CD25-depleted total T cells from normal B6 Ly5.1$^+$ donors. HY-specific iTregs were also transplanted at 2 x 10^6/mouse on day 0 of BMT. On days 7, 14, and 21 post BMT spleen (A, C) and liver (B, D) were collected from recipient mice for analysis. Phenotypes of cells isolated from recipient spleen (A) or liver (B) are displayed. Absolute numbers of originally

infused iTregs ($H2K^{b+}CD4^+V\beta6^+$) in spleen (C) or liver (D) are depicted on the left panels. Percentages of Foxp3 expression on gated iTregs in spleen (C) or liver (D) were shown on the right panels. Asterisk indicates statistical significance:
*$p<0.05$, **$p<0.01$, ***$p<0.001$.

2.4.4 Expression of target antigen on epithelial tissues is not required for iTregs to prevent GVHD

It has been widely accepted donor T cells have to recognize alloantigens expressed on epithelial tissues in order to cause GVHD in myeloablative BMT models (146). However, it is not clear whether Tregs require expression of target antigen on epithelial tissues in order to suppress GVHD. To address this question, we created 2 types of BM chimeras by transplanting donor (male or female) BM into lethally irradiated syngeneic recipients (female or male), so the HY antigen was only expressed either on hematopoietic cells (M → F chimeras) or on epithelial tissues (F → M chimeras). We then transplanted TCD-BM plus Teffs from B6 donors with or without additional HY-specific iTregs into these lethally irradiated chimeric recipients. In B6 → BALB.b (miHAg-mismatched) and B6 → BDF1 (haplo- mismatched) BMT models, we found HY-specific iTregs were highly capable in preventing GVHD, and the efficacy was comparable in either type of chimeric recipients (Figure 2.10 A, B), indicating target antigen expressed on either compartment is sufficient for iTregs to exert their suppression in GVHD.
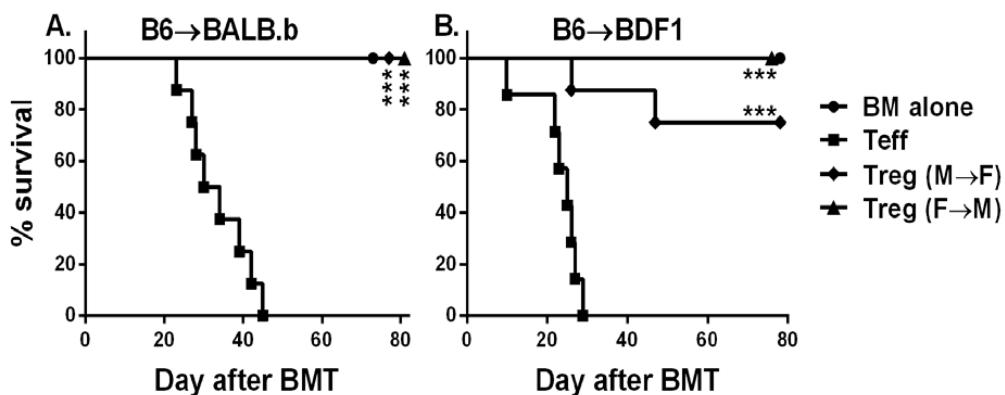
Figure 2.10 Effect of HY-antigen distribution on HY-specific iTreg-medicated protection. (A) Male → female or female → male BM chimeras were generated using BALB.b mice as described in "Material and Method". These BM chimeras were lethally irradiated again and divided into 2 cohorts, each of which were

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transferred with TCD- BM alone or plus 25 x 10^6/mouse total splenocytes from normal B6 donors. HY-specific iTregs were also included at 4 x 10^6/mouse into donor graft at the day of BMT for some recipients. Recipient survival is shown, and the data represent 5-8 mice in each group. (B) Male → female or female → male BM chimeras were generated using BDF1 mice as described in "Material and Method". These BM chimeras were lethally irradiated again and divided into 2 cohorts, each of which were transferred with TCD-BM alone or plus 4 x 10^6/mouse CD25-depleted total T cells from normal B6 donors. HY-specific iTregs were also included at 2 x 10^6/mouse into donor graft at the day of BMT for some recipients. Recipient survival is shown, and the data represent 7-8 mice in each group. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001

2.4.5 HY-specific iTregs essentially preserve the GVL effect

To determine the effect of HY- specific iTregs on the GVL activity, we utilized the clinically relevant B6→BDF1 (haplo-mismatched) BMT model with the injection of p815-luc$^+$ mastocytoma cell line. One day after lethal irradiation, we injected TCD-BM from B6 donors and HY-specific iTregs into male recipients, three days later we then injected B6 Teff cells and p815-luc$^+$ cells. We observed mice receiving BM + p815 alone all succumbed to tumor mortality within 20 days post BMT, as seen by high BLI signal with little weight loss, however mice received an addition of Teff cells died from GVHD indicated by decreased weight loss with little to no BLI signal (Figure 2.11 A-D). The addition of HY- specific iTregs significantly increased survival (p<0.001) and significantly delayed tumor mortality (p<0.001) as seen by maintained body weight and low BLI signal (Figure 2.11 A-D).
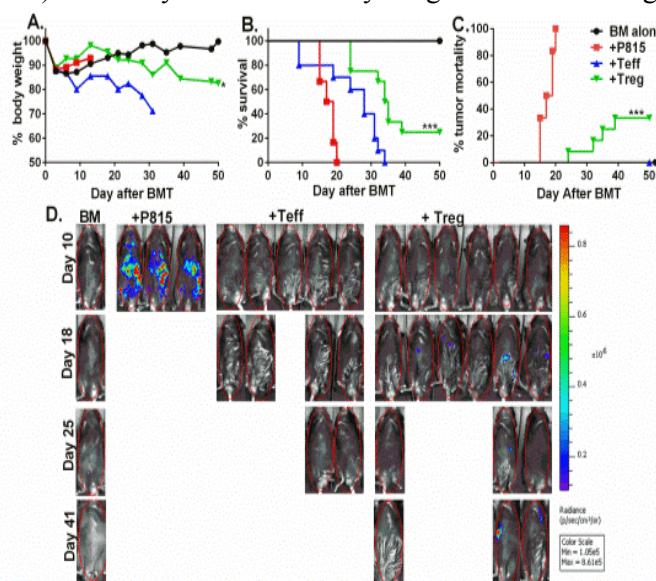
Figure 2.11 HY-Specific iTregs spare the GVL effect. B6D2F1 male recipient mice were lethally irradiated and injected with B6 BM with or without HY-specific iTregs, three days later CD25 depleted Teffs plus 5000 p815-luc mastocytoma cells were injected. Mice were monitored for body weight loss (A), survival (B), and tumor morality using the IVIS 200 imager throughout the course of study. The data depicted in A and B is

pooled from 2 replicate experiments, but the imaging shown (D) is from one of these 2 replicate experiments. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001

In order to better mimic clinical circumstance where patients have already established tumor, we generated a pre-established tumor model by injecting p815-luc$^+$ cells to the recipients 3 days prior to irradiation and 7 days prior to Teff infusion. One day after irradiation, male recipients were transplanted with BM plus HY-specific iTregs and three days later Teffs were infused. As shown in Figure 2.12, 50% of the recipients of BM plus p815 tumor died within 50 days of BMT without body weight loss and strong BLI signal indicating tumor relapse (Figure 2.12, A-C). The recipients of BM plus Teffs also died within 62 days with body weight loss and no detectable BLI signal, indicating GVHD mortality. HY- specific iTreg infusion significantly attenuated GVHD (p<0.05), reflected by higher percentage of survival and no tumor relapse reflected by no BLI signal (Figure 2.12 A-C, p < 0.05). Taken together, these data indicate the HY- specific iTregs largely preserved the GVL activity mediated by Teffs.
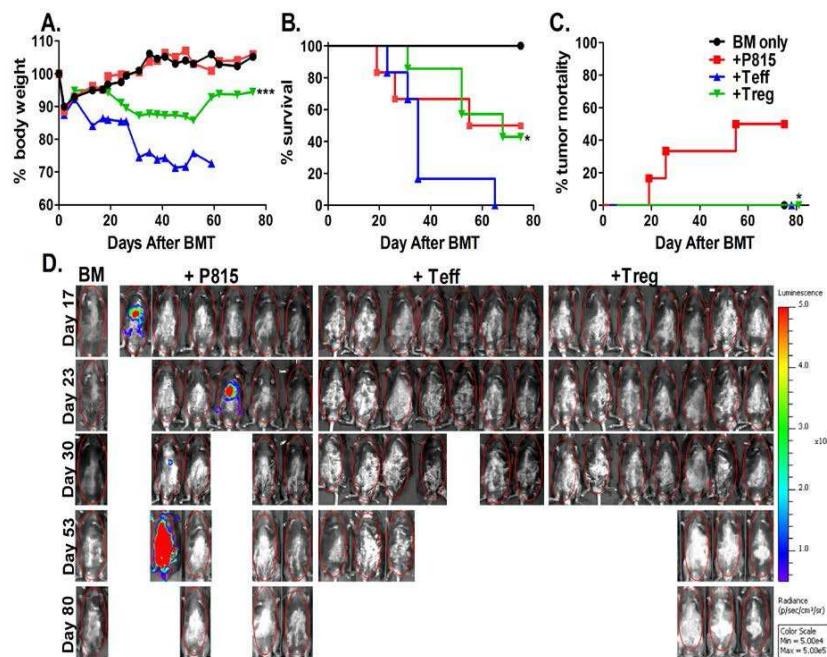
Figure 2.12 HY-specific iTregs largely preserve GVL effect in pre-established tumor model. Male BDF1 mice injected with 5000 P815-luc cells three days prior to irradiation. Tumor bearing mice were lethally irradiated and transplanted with 5 x 10^6 BM and HY-specific iTregs 2 x 10^6/mouse from B6 donors. Three days later, the recipient mice received 4 x 10^6 CD25-depleted total T cells. Recipient body weight (A) and survival (B) are shown. Data are from one experiment of two replicates with similar but not the same settings. Mice were imaged once weekly and then once every two-three weeks to monitor tumor growth (C and D) using an IVIS 200 imager. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.\

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2.6 Discussion

Aiming to increase the potency and selectivity of Treg therapy, by using TGFβ-induced Ag-specific iTregs, our previous studies have demonstrated Ag-specific iTregs, once activated in the recipient, are significantly more effective than expanded polyclonal nTregs in the prevention of GVHD (130, 131). The current study substantially extended our previous work by generating and testing iTregs specific for naturally processed alloantigens. Given the knowledge female to male transplants occur frequently in the clinic and these patients are at a greater risk of developing GVHD due to miHAg mismatched antigens, like HY, we strove to provide clinical relevance by generating HY-specific iTregs. We found monoclonal iTregs specific to HY miHAg were highly effective in preventing GVHD in activation-dependent manner. Furthermore, we observed HY-specific iTregs largely preserve the GVL effect (Figure 2.11 and 2.12). Given p815 used in our study is a mastocytoma cell line originally derived from male DBA2 mice (147), therefore is susceptible to antigen-specific T cell- rather than NK cell- mediated killing. Our results indicate miHAg-specific iTregs still permit the GVL activity against the tumor that expresses such a miHAg. This observation is important and clinically relevant, because many miHAg, such as HY, are ubiquitously expressed.

Unlike freshly isolated nTregs, iTregs are generated from naïve CD4 T cells and thus the number of iTregs is essentially unlimited (Figure 2.1). In current nTreg cell expansion protocols for clinical application, long culture period and multiple rounds of expansion are required to reach an optimal number of cells (88, 148) still with potential loss of Foxp3 expression (69). Given iTregs rapid expansion potential (98), this will decrease culture times and in turn resolve the fear of Foxp3 loss *in vitro*, however there is still concern regarding iTregs stability *in vivo*. Given our results showing iTregs remain highly stable even under extreme inflammatory conditions (Figure 2.9), this current work gives strong rationale to move iTreg therapy into the clinic. A potential concern was raised by some studies showing *in vitro* generated iTregs were less suppressive than nTregs (149, 150) and failed to prevent GVHD (96, 151). On the other hand, there is also substantial evidence in the literature supporting

iTregs as or more effective than nTregs in suppressing immune responses *in vivo* (42, 127, 152-158). Consistent with our previous studies using OVA-specific iTregs[4,5], the current work demonstrated HY-specific iTregs were highly effective in preventing GVHD in clinically relevant murine models of allogeneic BMT in an Ag- dependent manner (Figure 2.2 and 2.4).

The stability and efficacy of iTregs still appears to be controversial with regards to controlling GVHD. However, our results are also supported by the reports from Steinman's group (102), who demonstrated iTregs generated with allogeneic DCs in the presence of TGF-β and RA maintained Foxp3 expression and exhibited higher levels of CNS2 demethylation in the Foxp3 gene, a marker for stability. Stability of iTregs generated by others and us may be partially attributed to the presence of RA, which was shown to promote iTregs through increasing histone methylation and acetylation within the promoter and conserved non-coding DNA sequence elements at the Foxp3 gene (112)(38). More strikingly, we interpret the efficacy of iTregs in the attenuation of GVHD is directly related to TCR-driven activation and expansion *in vivo* (Figure 2.7 and 2.9). The iTregs used in our studies were ~ 100% Ag-specific and able to expand when recognizing cognate Ag, whereas the iTregs used in studies by others were polyclonal and only a small fraction (e.g. < 1%) of them were able to expand when recognizing alloAgs. Our data clearly shows that iTregs not activated by cognate Ag were unable to expand and were ineffective in the prevention of GVHD. Along this line, Sela et al. showed DC-induced, alloAg-specific iTregs are capable of preventing GVHD (36). However, the therapeutic efficacy of their iTregs was lower than HY-specific iTregs used in our current study but higher than polyclonal iTregs used in other studies (27-29), indicating the efficacy directly correlates with the frequency of alloreactive cells among different types of iTregs. Taken together, these data provide direct evidence TCR-driven activation and expansion of iTregs after infusion is essential for their therapeutic efficacy in the control of GVHD.

Since it is commonly accepted GVHD development requires donor T cells that recognize alloantigens expressed on epithelial tissues, we hypothesized Tregs must also recognize antigens

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expressed on epithelia in order to prevent GVHD. A recent study by Tawara et al. proposed the hosts

APCs are necessary and sufficient for GVHD protection by donor Tregs (159). By creating BM

chimeras as recipients in which the alloantigens to be recognized by Tregs are expressed on either

hematopoietic cells or parenchymal tissues, we observed HY-specific iTregs were highly capable in

preventing GVHD in either type of chimeric recipients (Figure 2.10). These results indicate target

antigen expressed on either compartment is sufficient for iTregs to exert their suppression in GVHD.
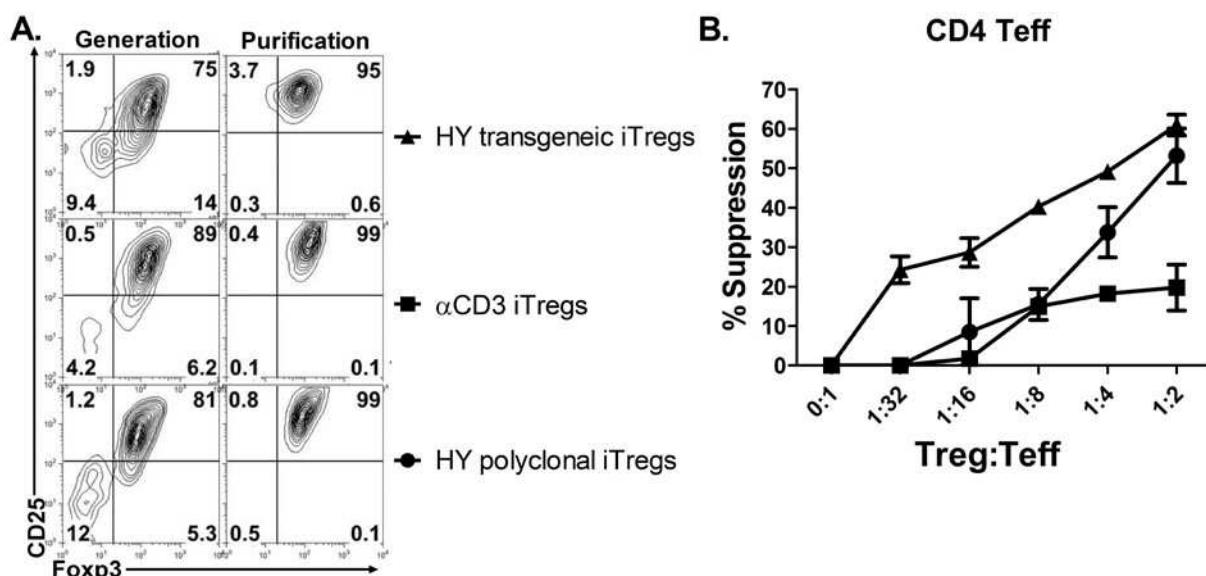
Figure 2.13 Education with HY antigen enhances suppressive function of iTregs. HY- TCR transgenic iTregs were generated as described in Figure 1. Polyclonal HY iTregs were generated by isolating WT B6 resting CD4+CD25- T cells and stimulated with syngeneic DCs with HY peptide, IL-2 (5ng/mL), TGFβ (5ng/mL), and RA (10nM) for 5 days. After 5 days, cells were harvested and restimulated with syngeneic DC's with HY peptide for an additional 5 days. Anti-CD3 polyclonal iTregs were generated by stimulating resting CD4+CD25- T cells with syngeneic APCs and α-CD3 with IL-2 (5ng/mL), TGFβ (5ng/mL), and RA (10nM) for 3 days. Different iTregs were harvested and purified through CD25-magnetic bead positive selection (A). iTreg suppressive function was tested by CFSE labeling CD4$^+$CD25$^-$ B6 T cells and stimulating them with BDF1 male APCs. HY transgenic, HY polyclonal, or αCD3 iTregs were then titrated at the indicated ratios. After 5 days culture, cells were harvested and CFSE dilution was assessed through flow cytometry. Percentage of suppression was calculated by using the control (without iTregs) as the baseline for uninhibited T cell proliferation (B).

The current work using TCR transgenic T cells clearly provides the evidence miHAg-specific

iTregs were effective in the prevention of acute GVHD. To translate the finding into clinical

application, one could generate Ag-specific human iTregs by transducing TCR-gene into CD4 T cells

and then induce them into iTregs *in vitro*. Alternatively and also more practically, miHAg-reactive

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iTregs could be generated from polyclonal CD4 T cells. In fact, we were able to generate HY-reactive polyclonal iTregs by 2-rounds of stimulation of CD4 T cells with dendritic cells from normal female B6 mice in the presence of HY-peptide (Figure 2.13A). These iTregs enriched for HY-specificity exhibited significantly higher efficiency in suppressing B6 CD4 T cells in response to APCs from BDF1 male mice as compared to polyclonal iTregs generated after anti-CD3 stimulation (Figure 2.13B). Furthermore, we recently have shown human nTregs specific for HY miHAg (89) can be extensively expanded *ex vivo*, which demonstrates the feasibility to acquire sufficient human HY-specific iTregs for clinic trials. In   conclusion, the current pre-clinical study provides strong rationale to apply human miHAg-specific iTregs in the clinic for the prevention of GVHD in patients after allogeneic HCT.

Chapter 3: CD8 Tregs Promote GVHD Attenuation and Overcome the Impaired GVL Effect Mediated by CD4 Tregs in Mice

Note to reader

This chapter has been previously published (Heinrichs et al, Annual ASH meeting, Orland FL 2015) and are utilized with permissions from the publisher (see Appendix C)

3.1 Abstract

Adoptive natural regulatory T cell (nTreg) therapy has improved the outcome for patients suffering from graft-versus-host disease (GVHD) following allogeneic hematopoietic cell transplantation (allo-HCT). However, fear of broad immune suppression and subsequent dampening of beneficial graft- versus-leukemic (GVL) responses remains a challenge. To address this concern, we generated alloreactive induced Tregs (iTregs) from resting CD4 or CD8 T cells and tested their ability to suppress GVH and maintain GVL responses. We utilized major mismatched and haploidentical murine models of HCT with host-derived lymphoma or leukemia cell lines to evaluate GVH and GVL responses simultaneously. Alloreactive CD4 iTregs were effective in preventing GVHD, but abrogated the GVL effect against aggressive leukemia. Alloreactive CD8 iTregs moderately attenuated GVHD while sparing the GVL effect. Hence, we hypothesized a combination of CD4 and CD8 iTregs could achieve the optimal goal of allo-HCT. Indeed, the combinational therapy was superior to CD4 or CD8 iTreg singular therapy in GVHD control; importantly, the combinational therapy maintained GVL responses. Cellular analysis uncovered potent suppression of both CD4 and CD8 effector T cells by the combinational therapy resulting in effective prevention of GVHD, which could not be achieved by

either singular therapy. Gene expression profiles revealed alloreactive CD8 iTregs possess elevated expression of multiple cytolytic molecules compared to CD4 iTregs, which likely contributes to GVL preservation. Our study uncovers unique differences between alloreactive CD4 and CD8 iTregs can be harnessed to create an optimal iTreg therapy for GVHD prevention with maintained GVL responses.

3.2 Introduction

Allogeneic hematopoietic cell transplantation (allo-HCT) was established to treat patients suffering from hematological malignancies encompassing leukemias and lymphomas. Allo-HCT replaces a patient's diseased hematopoietic system with a healthy donor stem cell source. The degree of human leukocyte antigen (HLA) disparity dictates the beneficial graft-versus-leukemia (GVL) response, where donor T cells recognize and kill residual leukemic cells, and the deleterious graft-versus-host disease (GVHD), where donor T cells recognize and damage host epithelial tissues (3). Unfortunately, a therapy separating these two responses within allo-HCT recipients has yet to be established. Presently, the primary therapy for GVHD is broad immune suppression, which can lead to relapse of the underlying malignancy or life-threatening infections (160).

Natural T regulatory cells (nTregs) and induced T regulatory cells (iTregs) play key roles in suppressing autoimmunity (26) and in maintaining immune homeostasis (41). nTregs arise from the thymus, recognize self-antigen with high affinity, and represent a stable cell lineage(36). iTregs arise in the periphery (42), recognize non-self-antigens, and are less stable due to their propensity to lose Foxp3 expression, the master transcription factor (40) associated with Treg suppressive function.

Harnessing Treg's inherent suppressive nature to control GVHD has been an ongoing area of investigation. Initial clinical trials using nTregs have yielded positive results (91-93). However, nTregs constitute a small percentage of circulating T cells and require expansion due to the high number needed for GVHD attenuation (93); thus, reaching optimal cell doses for patients (92) remains an

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obstacle. Likewise, nTregs are non-selective suppressors, which could have significant impairment of the beneficial GVL response. Conversely, iTregs can be generated in large numbers (98) and enriched for antigen specificity (99, 161), which could result in targeted separation of GVH and GVL responses. However, iTreg therapy has been surrounded by controversy, with some investigators citing beneficial effects (88, 102, 115, 117) and others a complete lack of efficacy (96, 97, 101). The lack of a consensus on iTreg therapy seems to stem from differences in activation agents (polyclonal vs. antigen-specific), polarizing cytokines, and infusion schedules employed across laboratories. Yet, it is clear that antigen specificity enhances iTregs potency in GVHD attenuation, but these findings used monoclonal antigen specific   iTregs that cannot be readily translated to the clinic (99, 161). Therefore, to extend antigen-specific iTreg therapy into clinical settings, we tested the efficacy of alloreactive iTregs in attenuating GVHD and preserving GVL responses.

Utilizing well-established murine models of allo-HCT, we demonstrated alloreactive CD4 iTregs potently suppress GVHD; yet GVL is completely lost against aggressive leukemia. Alloreactive CD8 iTregs are less stable than CD4 iTregs, but still moderately attenuated GVHD while preserving the GVL effect. To overcome the obstacles of each singular therapy, we established a combination therapy using both CD4 and CD8 iTregs, which was superior in GVHD attenuation and preservation of GVL responses. Mechanistic studies demonstrated, even under the same polarization conditions, CD4 and CD8 iTregs represent two distinct regulatory populations that suppress GVH or maintain GVL responses. Finally, we uncovered unique gene expression patterns between alloreactive CD4 and CD8 iTregs, which provide further understanding of these cells types and rationale to apply combinational iTregs therapy after allo- HCT.

3.3 Materials and Methods

3.3.1 Mice

C57BL/6 (B6; H-2^b), BALB/c (H-2^d), B6-Ly5.2 (H-2^b), B6DF1 (B6 x DBA2) F1 (H-2$^{b/d}$) were purchased from NCI. B6-Foxp3-GFP (H-2^b) was kindly provided by A. Rudensky. All animals were housed in specific pathogen-free conditions in the America Association for Laboratory Animal Care-accredited Animal Resource Center at the Medical University of South Carolina. The Institutional Animal Care and Use Committee of the Medical University of South Carolina approved all work.

3.3.2 iTreg Generation

CD4$^+$CD25$^-$ and CD8$^+$CD25$^-$ resting T cells were purified from C57BL6 spleen and lymph nodes as previously described (161). CD11c$^+$ dendritic cells were purified from BALB/c mice using CD11c microbeads (Miltenyi) positive selection. CD4 or CD8 T cells were stimulated with CD11c DCs at a 1:10 (T cell: DC) ratio in the presence of IL-2 (5ng/mL), TGFβ (5μg/mL), and retinoic acid (RA) (40nM) for 5 or 4 days respectively.

3.3.3 GVH/GVL Models

Major histocompatibility complex (MHC)-mismatched (B6→BALB/c) (162) and haploidentical (B6→BDF1) (163) BMT models were used as previously established. Recipients were monitored for survival and body weight loss twice/week. In GVL models, tumor doses ranged from 5,000-10,000 per mouse. Tumor mortality and GVHD mortality were distinguished as previously described (162). Representative samples of GVHD target organs were excised from recipient's 14 days post-BMT and subjected to pathology scoring as previously described (164).

3.3.4 Flow Cytometry and Intracellular Cytokine Staining

Mononuclear cells were isolated from recipient spleen and liver as previously described (165) and stained for surface molecules using standard flow cytometry protocols. Foxp3 was detected using established manufacturer's instructions Foxp3 fixation/permeablization kit (eBioscience). Intracellular cytokines and granzyme expression were detected from spleen and liver lymphocytes following in vitro phorbol-myristate-acetate/ionomycin stimulation as previously described (163). Cells were analyzed using the LSRII Diva software (BD Biosciences) and FlowJo (Treestar).

3.3.5 Microarray

$CD8^+Foxp3^+$ and $CD4^+Foxp3^+$ cells were generated three independent times from Foxp3-GFP reporter mice and sorted purified (BD FACSAria II cell sorter), $CD8^+Foxp3^-$ and $CD4^+Foxp3^-$ were used as controls. RNA was extracted using Trizol and isolated using Qaigen RNAeasy Mini Kit and quality assessed using 2100 Bioanalyzer (Agilent). Total RNA (250ng) was converted using the WT Plus Kit (Affymetrix) and hybridized to Mouse 430 2.0T Genechips (Affymetrix) according to manufacturer's instructions. Raw data (CEL files) were normalized by Robust Multi-array average (RMA) 70 using the gene level summarization method in Expression Console software (Affymetrix). Comparisons were performed using dChip software 71 using strict criteria of absolute fold change> 2 and p<0.05. Calculations based on 50 iterations estimated a false discovery rate (FDR) of < 1 % for all comparisons.

3.3.6 Statistics

For comparison of recipient survival among groups in GVHD experiments and tumor mortality, the log-rank test was used to determine statistical significance. To compare pathology scores, cytokine levels, and effector T cells, a Student *t* test was performed.

3.4 Results

3.4.1 Alloreactive CD4 iTregs suppression of GVHD is antigen specific

Our previous findings illustrate antigen specificity increases iTregs suppressive potential (161). However, HY-specific monoclonal iTregs have limited potential to be translated to the clinic. Therefore, we generated alloreactive CD4 iTregs and tested their ability to alleviate GVHD while maintaining GVL activity. We utilized generation conditions previously described (102); as a control, polyclonal CD4 iTregs were generated (99). iTregs generated with $H2^{d+}$ DCs (Figure 3.1 A) were highly suppressive toward CD4 and CD8 T-cell responses to $H2^{d+}$ alloantigens, and more effective than polyclonal iTregs *in vitro* (Figure 3.1B). These alloreactive iTregs were also capable of suppressing allogeneic responses *in vivo*, reflected by a significant reduction of T-cell proliferation and IFNγ secretion (Figure 3.1C, D). Using an MHC-mismatched B6 to BALB/c HCT model, we injected enriched $H2^{d}$-alloreactive iTregs three days prior to effector T cells (Teffs), and found these iTregs significantly attenuated GVHD, displayed by improved survival and body weight maintenance (Figure 3.2A, B) and decreased pathological damage in all tissues (Figure 3.2C).
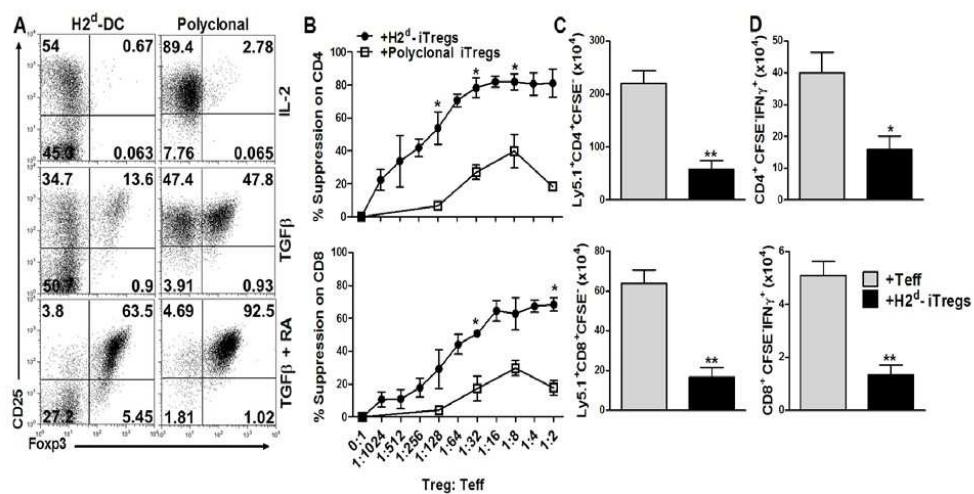
Figure 3.1 Alloreactive CD4 iTregs display superior suppressive function than polyclonal iTregs. Naïve CD4^{+}CD25^{-} T cells were isolated from B6 mice and plated with either BALB/c DCs (alloreactive) or with α-CD3 + B6 APCs (polyclonal) with IL-2, TGFβ, and retinoic acid. Foxp3 expression was assess through Flow cytometry, Activity of iTregs was assessed by suppressing allo-response of T effector cells in vitro (B) and in vivo (C,D).* p < 0.05, ** p < 0.01, *** p < 0.001.
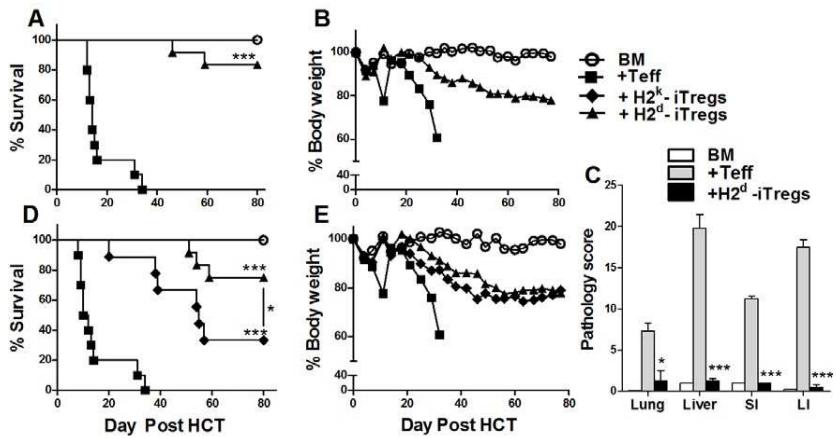
Figure 3.2 Alloreactive CD4 iTregs attenuation of GVHD is antigen-specific. BALB/c mice were lethally irradiated (700cGy) and transplanted with 5 x 10^6 TCD-BM from B6 donors and 1 x 10^6 CD25^{hi} H2^d specific iTregs. Three days later 1.0 x 10^6 CD25 depleted B6 Teff cells were injected alone or with a second dose of H2^d iTregs (1-0.5 x 10^6). Recipients were monitored for survival (A) and body weight loss (B) for 80 days. During on replicate experiment GVHD target organs were excised, subjected to H&E staining, and sent to a collaborator for pathological scoring (C). Recipient BALB/c mice were lethally irradiated (700cGy) and transplanted with 5 x 10^6 TCD-BM from B6 donors and 1 x 10^6 CD25^{hi} H2^d or H2^k specific iTregs. Three days later 1.0 x 10^6 CD25-depleted B6 Teff cells were injected alone or with a second dose of H2^d or H2^k specific iTregs (1-0.5 x 10^6). Recipients were monitored for survival (D) and body weight loss (E) for 80 days. * P<.05; ** P<.01; *** P<.001. All error bars indicate standard error of the mean (SEM)

To evaluate the impact of iTreg allo-reactivity in the prevention of GVHD, we generated third-party (H2^k)-reactive iTregs and compared the ability of H2^d- vs. H2^k-alloreactive iTregs to suppress GVHD. As expected, host (H2^d)-reactive iTregs were superior to third-party (H2^k)-reactive iTregs in suppressing GVHD (Figure3.2D, E). To understand the mechanism of how iTreg allo-reactivity affects iTreg function, we followed the fate of CD4 iTregs to observe their effect on Teffs. Host (H2^d)-reactive iTregs displayed greater persistence as compared to H2^k-iTregs (Figure 3.3A, B). Furthermore, host (H2^d)-reactive iTregs potently suppressed the expansion of CD4 Teffs and reduced the expansion of CD8 Teffs (Figure 3.3A, B). Conversely, the third-party (H2^k)-reactive iTregs reduced the expansion of CD4 Teffs but had no effect of CD8 Teffs, which may account for their ability to moderately attenuate GVHD lethality. Taken together, alloreactive iTregs effectively attenuate GVHD and host- reactive iTregs have greater stability and potency in GVHD prevention
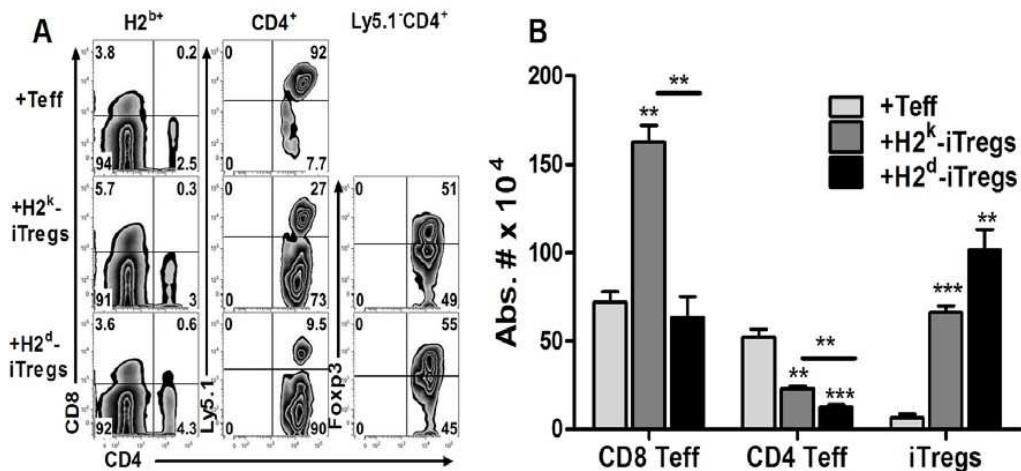
Figure 3.3 Alloantigen specificity is essential for iTreg function and stability. BALB/c mice were lethally irradiated and transplanted with TCD-BM and either H2^d or H2^k iTregs as described in Figure 3.1. Three days later Ly5.1$^+$ CD25-depleted Teff cells were injected. Fourteen days later recipients spleen excised, mononuclear cells isolated, and stained for indicated surface molecules, representative flow gating strategy is shown (A). Based on flow percentages absolute numbers of Teff and iTregs were calculated for the spleen (B) * P<.05; ** P<.01; *** P<.001. All error bars indicate standard error of the mean (SEM)

3.4.2 Alloreactive CD4 iTregs impair GVL activity against aggressive leukemia

Since GVHD is intimately linked to GVL activity, we tested whether alloreactive CD4 iTregs could suppress GVHD without impairing GVL responses. To further increase iTreg yield and translational potential, we tested non-enriched iTregs (bulk after culture) pathogenicity following allo-HCT and found non-enriched alloreactive iTregs (CD4, CD8, or in combination) were not pathogenic (Figure 3.4A and B). Consequently, we used non-enriched iTregs in all experiment thereafter. Initially, we found CD4 iTregs significantly attenuated GVHD (Figure 3.5A) while largely preserving GVL activity against A20 B cell lymphoma (Figure 3.5B, C). To ensure the maintenance of GVL function was not tumor specific, we used a haploidentical B6 to BDF1 HCT model with host-original mastocytoma (P815). Surprisingly, the addition of CD4 iTregs completely abrogated the ability of Teffs to mediate GVL function, reflected by all recipients with CD4 iTregs succumbing to tumor mortality (Figure 3.5D, E) with heavy tumor load (Figure 3.5F) similar to those with BM plus tumor alone. To understand the strikingly different outcomes we encountered between A20 and P815 models, we found A20 cells express significantly higher levels of MHC-II and CD86 compared to P815 (data

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not shown), suggesting A20 lymphoma is more immunogenic than P815, possibly contributing to the

differential sensitivities we observed. Taken together, while CD4 alloreactive iTregs are effective in

GVHD control, they may impair the GVL activity against aggressive and low immunogenic tumors.
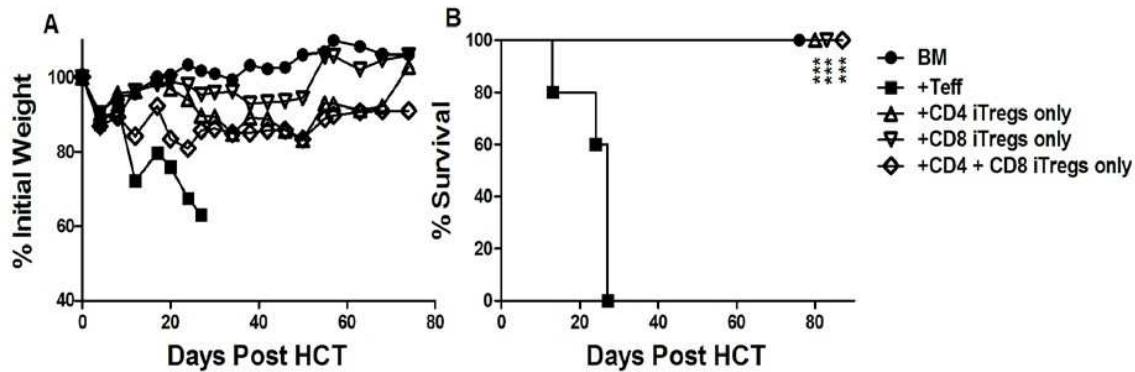
Figure 3.4 Non-enriched alloreactive iTregs are not pathogenic. BALB/c mice were lethally irradiated and transplanted with 5×10^6 TCD-BM, 1×10^6 CD4 iTregs, 1×10^6 CD8 iTregs, or 0.5×10^6 CD4 $+ 0.5 \times 10^6$ CD8 iTregs. Three days later 0.5×10^6 CD25-depleted Teff cells were injected. Recipients were monitored for survival and body weight loss for 80 days, n=8. ***P<0.005
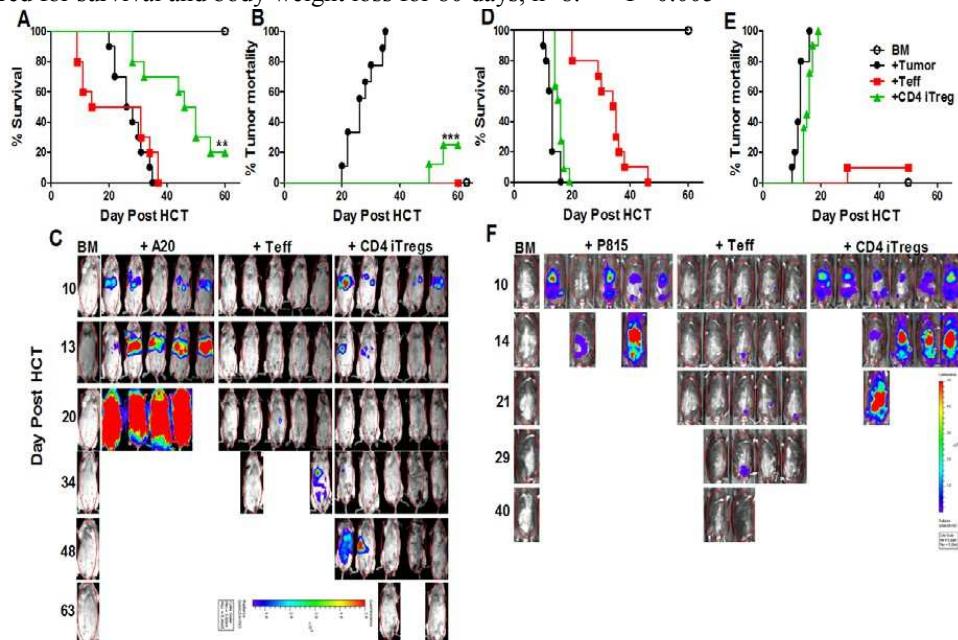
Figure 3.5 Alloreactive CD4 iTregs impair GVL activity against aggressive leukemia. BALB/c mice were lethally irradiated and transplanted with 5×10^6 TCD-BM, 1×10^6 CD4 iTregs, and 1×10^4 luc-A20 cells. Three days later 0.5×10^6 CD25-depleted Teff cells were injected. Recipients were monitored for survival (A) tumor mortality (B) and tumor load (C) for 80 days n=10/group. BDF1 recipient mice were lethally irradiated (1200cGy) split dose 3 hours apart and transplanted with 5×10^6 TCD-BM, 2×10^6 CD4 iTregs, and 5×10^3 luc-P815 cells. Three days later 2×10^6 CD25-depleted Teff cells were injected. Recipients were monitor for survival (D) tumor mortality (E) and tumor load (F) for 60 days, n=15/group. * P<.05; ** P<.01; *** P<.001. All error bars indicate standard error of the mean (SEM).

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3.4.3 CD8 iTregs moderately attenuate GVHD and fully preserve GVL function

Recent studies showed CD8 iTregs arise after allogeneic HCT, which play a significant role in GVHD attenuation (116). Interestingly, a recent report also suggested human CD8 iTregs possess direct cytotoxicity against LCL-tumor cell lines (117). We therefore generated alloreactive CD8 iTregs and evaluated their ability to control GVHD and preserve the GVL response. Using similar polarizing conditions, we were able to generate 30-40% Foxp3$^+$ CD8 iTregs (Figure 3.6A). These CD8 iTregs displayed significantly higher levels of CD39$^+$CD73$^+$, CTLA-4, and granzyme B compared to nTregs and CD4 iTregs (Figure 3.6B), and were equally suppressive of allogeneic responses as CD4 iTregs (Figure 3.6C). Despite potent suppressive function *in vitro*, alloreactive CD8 iTregs only moderately attenuated GVHD (Figure 3.7A), but preserved the GVL effect against P815 tumor (Figure 3.7B, C). Interestingly, CD8 iTregs themselves could significantly delay tumor mortality (Figure 3.7A-C). *In vitro* cytotoxic assays confirmed CD8 iTregs possess direct cytotoxicity against tumor cells (Figure 3.7SD). These results provide evidence alloreactive CD4 and CD8 iTregs are fundamentally different with regards to GVH and GVL responses, with CD4 iTregs potently suppressing Teffs and CD8 iTregs contributing to GVL maintenance.
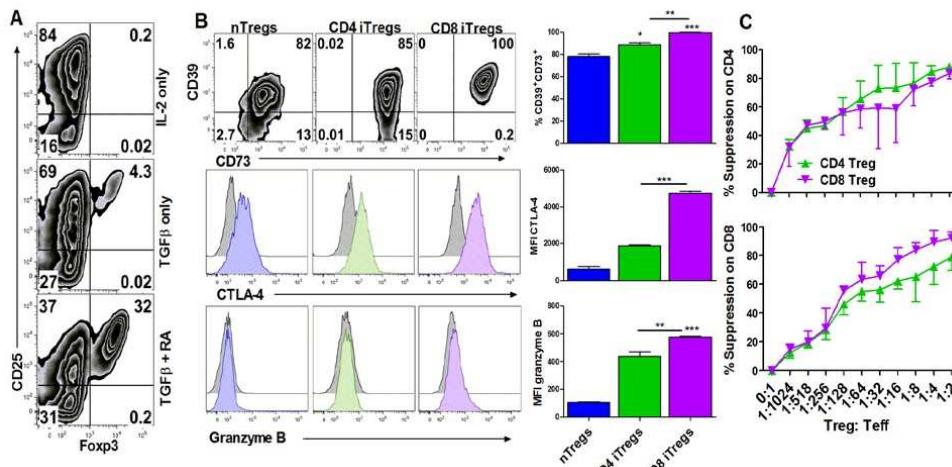
Figure 3.6 Generation and Function of Alloreactive CD8 iTregs. Naïve CD8$^+$CD25$^-$B6 T cells were stimulated with BALB/c DCs with IL-2 (5ng/mL), TGFβ (5ng/mL) and retinoic acid (40nM) for 4 days (A). Expression of CD39, CD73, CTLA4, and granzyme B were assessed between nTregs, alloreactive CD4 iTregs, and alloreactive CD8 iTregs post generation (B) and mean fluorescence intensity quantified (B). In vitro suppressive function between alloreactive CD4 and alloreactive CD8 iTregs was assessed through suppressive of CFSE labeled alloreactive B6 T cells (C). Error bars indicate the mean of standard error.
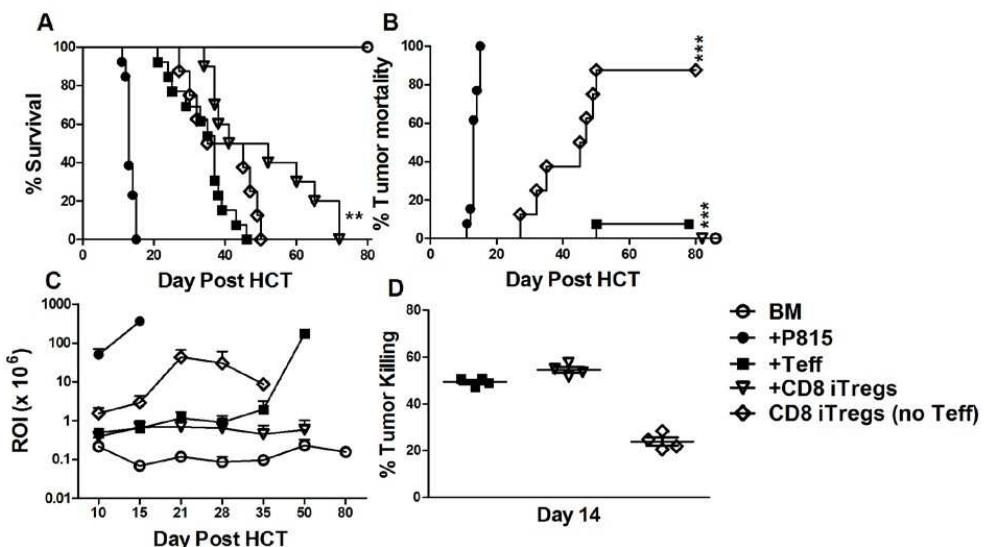
Figure 3.7 CD8 iTregs moderately attenuate GVHD and maintain GVL responses. BDF1 recipient mice were lethally irradiated (1200cGY,split doses) and transplanted with 5 x 106 B6 TCD-BM, 4 x 106 CD8 iTregs, and 5 x 103 luc-P815 cells. Three days later 2 x 106 CD25-depleted Teff cells were injected. Recipients were monitored for survival (A) and tumor mortality (B). Every week to every other week mice were subjected to whole body imaging, region of signal intensity was quantified using IVIS software for each group (C). BDF1 mice were transplanted as described in (A), fourteen days post HCT splenocytes were plated with either CFSEhi P815 or CFSElow EL4 tumor cells for 16 hours. Percentage tumor killing was calculated as ratio EL4:P815 (no splenocytes control) / EL4: P815 (each group), tumor killing was normalized to total donor CD8 T cells in splenocytes (D). n=15. * P<.05; ** P<.01; *** P<.001. Error bars indicate the mean of standard error.

3.4.4 Combination of CD4 and CD8 iTregs is superior to singular iTregs therapy in GVHD attenuation

We further reasoned these two types of iTregs could compensate for each other's weaknesses and provide an optimized cellular therapy. We first assessed the potency of a combination therapy (CD4 + CD8 iTregs) to suppress GVHD using B6 to BALB/c HCT model. To negate an additive effect within the combinational therapy, the absolute iTreg product injected was equivalent to CD4 or CD8 iTregs singular therapy. We observed the inclusion of CD8 iTregs did not abrogate the ability of CD4 iTregs to alleviate GVHD, and in fact attenuated GVHD more potently than CD4 iTreg singular therapy (Figure 3.8A). By using β-actin luciferase transgenic B6 donors to monitor the expansion of Teffs *in vivo*, we found the combinational therapy significantly reduced the expansion of Teffs compared to either CD4 or CD8 iTreg singular therapy reflected by significantly decreased bioluminescent signal over time (Figure 3.8B).

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To understand the cellular mechanism of iTreg therapy on GVHD, we performed a time course study to monitor both Teff and iTregs after allo-HCT. Recipient's BALB/c spleen, liver, and mesenteric lymph nodes (mLN) were harvested on day 5, 8, and 11 post HCT. On day 5, Thy1.1$^+$ Teffs were not readily detectable in any of the recipients. On day 8, the expansion of Teffs was significantly suppressed by all iTreg therapies (Figure 3.8C). On day 11, all the recipients with Teffs alone succumbed to GVHD. Combinational therapy with CD4 and CD8 iTregs controlled Teff expansion significantly better than either singular therapy (Figure 3.8C), which correlated with a less intense bioluminescence signal observed in the combinational therapy (Figure 3.8B). Interestingly, CD4 iTregs potently controlled the expansion of CD4, but not CD8 Teffs, whereas CD8 iTregs potently controlled the expansion of CD8, but not CD4 Teffs (Figure 3.8D). Furthermore, the combination of CD4 and CD8 iTregs potently controlled the expansion of both CD4 and CD8 Teffs (Figure 3.8D). To evaluate the fate of infused iTregs, we tabulated absolute numbers of residual Foxp3$^+$ infused individual iTregs (Thy1.2$^+$), which reflects iTregs expansion and stability. Overall, equivalent or greater expansion/stability of iTregs was observed when infused in combination as compared to each alone in recipient spleens and mLNs (Figure 3.8E). While CD8 iTregs accumulated in mLN, there were significantly fewer in the liver when infused alone versus together with CD4 iTregs (Figure 3.8E). To track the maintenance and stability of infused iTregs, we generated iTregs from Ly5.1$^+$ B6 mice, performed HCT as described in Figure 3.8, and evaluated Foxp3 expression 17 days post-transplant. Consistently, we found CD8 iTregs expanded and/or survived longer than CD4 iTregs (Figure 3.9A, C), albeit at the loss of Foxp3 expression. Given the injected iTregs were non-enriched (~60% Foxp3 expression) at the day of transplant, we found CD4 iTregs had significant retention of Foxp3 expression (~40%) and were more stable than CD8 iTregs (Figure 3.9A, B). Taken together, the combinational therapy was more effective in preventing GVHD than either singular therapy, likely because the former was able to control both CD4 and CD8 Teffs simultaneous
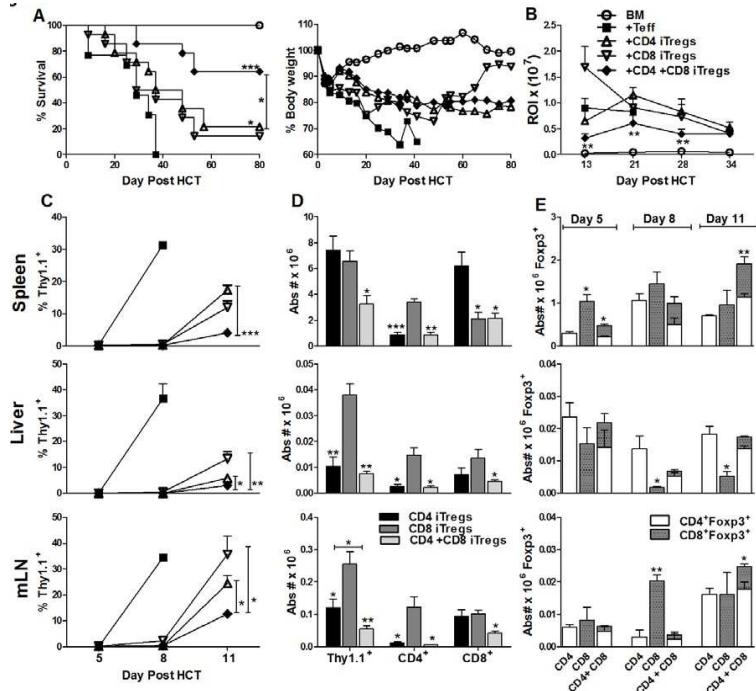
Figure 3.8 Combination of CD4 and CD8 iTregs superior to singular iTregs therapy in GVHD attenuation. BALB/c mice were lethally irradiated and transplanted with 5×10^6 TCD-BM, 1×10^6 CD4 iTregs, 1×10^6 CD8 iTregs, or 0.5×10^6 CD4 + 0.5×10^6 CD8 iTregs. Three days later 0.5×10^6 CD25-depleted Teff cells were injected. Recipients were monitored for survival and body weight loss for 80 days (A), n=20/group. During one replicate experiment, 0.25×10^6 luc-Teff were injected and expansion monitored over the first 40 days post BMT (B). BALB/c mice were lethally irradiated and transplanted as described in A, however Teff cells were injected from Thy1.1$^+$ reporter mice. On days 5, 8, and 11 recipient spleens, liver, and mesenteric lymph nodes harvested and Teff expansion analyzed over time (C) as well as characteristics (D). iTreg absolute number was also quantified over time in all three organs (E). * P<.05; ** P<.01; *** P<.001. All error bars indicate standard error of the mean (SEM).
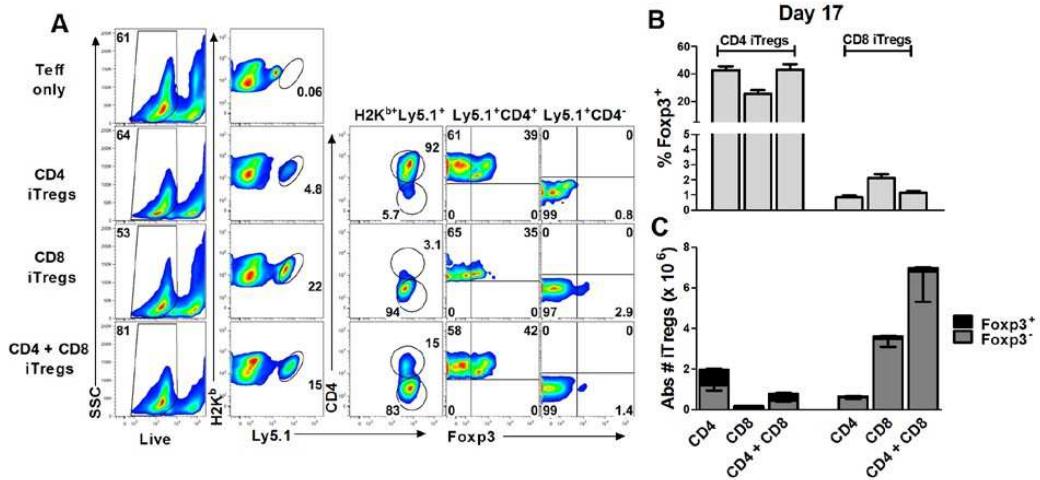
Figure 3.9 CD4 iTregs display greater stability than CD8 iTregs. BALB/c mice were lethally irradiated and transplanted with 5×10^6 B6 TCD-BM, 1×10^6 CD4, 1×10^6 CD8, or 0.5 CD4 + 0.5×10^6 CD8 iTregs generated from Ly5.1$^+$ mice. Three days later, CD25-depleted 0.5×10^6 Teff cells were injected. Seventeen days post- transplant, spleens were collected and iTregs analyzed as described in the representative flow

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diagram (A). Quantification of percentage of Foxp3 (B) and absolute number (C) per mouse n=4. Error bars indicate the mean of standard error

3.4.5 Combinational therapy rescues inhibited GVL response of CD4 singular therapy

To exclude potential model specific phenomena, we further evaluated the efficacy of combinational therapy in B6 to BDF1 HCT model. We identified an appropriate combined dose of CD4 and CD8 iTregs potently attenuated GVHD significantly better than either iTreg therapy alone. This was supported by pathological analysis of GVHD target organs; including the liver, lung, small intestine and colon (Figure 3.10A, B). Taken together with the data presented in Figure 3.8, we conclude the combinational therapy is superior to single iTreg therapy across different HCT models.

We next addressed the most pertinent question as to whether the combinational therapy can spare the GVL effect against P815. Fourteen days post HCT, we observed the recipients given CD4 iTregs alone had significantly increased tumor burden within the liver, lung, spleen, and gut as compared to those receiving Teffs alone (Figure 3.10C, D). However, the recipients given either CD8 iTregs alone or combinational therapy had significantly reduced tumor burden as compared to those receiving CD4 iTregs alone within all organs examined (Figure 3.10C, D). These results suggest CD8 iTregs could overcome the impaired GVL effect mediated by CD4 iTregs. To extend this observation to long-term survival studies, we simultaneously evaluated the effect of combinational therapy on GVHD and GVL responses against P815. Indeed, the recipients with the combinational therapy had significantly improved long-term survival, resulting from reduced GVHD lethality and tumor mortality (Figure 3.11 A-C). Taken together with data presented in Figure 3.5 and Figure 3.7, we conclude the combinational therapy has distinct advantages over either CD4 or CD8 iTreg singular therapy resulting in GVHD prevention and GVL preservation.
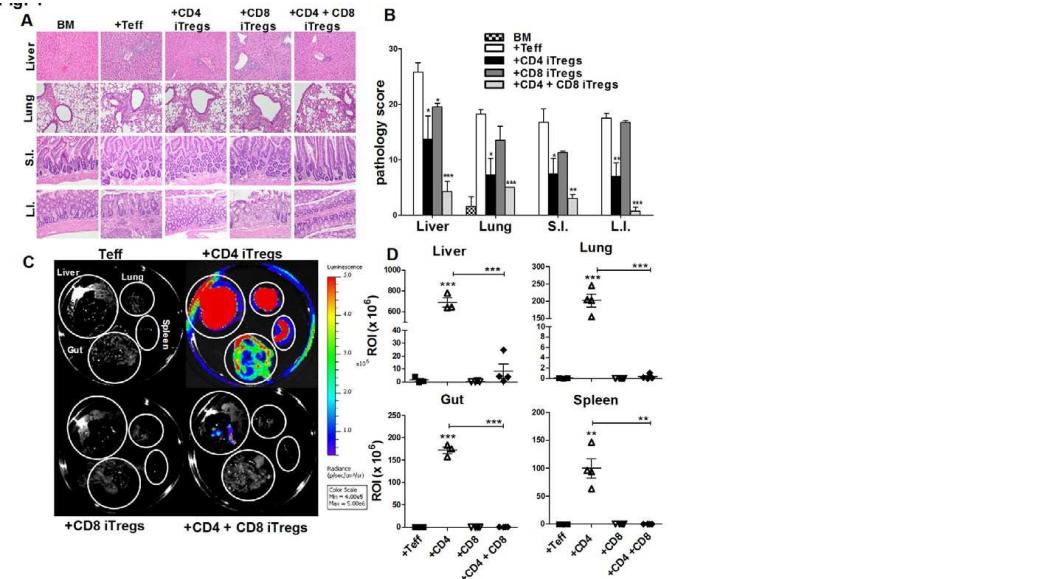
Figure 3.10 Combinational therapy reduce GVHD pathology and tumor burden. BDF1 recipient mice were lethally irradiated (1200cGy) and transplanted with 5×10^6 B6 TCD-BM, and either 2×10^6 CD4 iTregs, 4×10^6 CD8 iTregs, or a combination of both CD4 and CD8 iTregs, and 5×10^3 luc-P815 cells. Three days later 2×10^6 Ly5.1$^+$ CD25-depleted Teffs were injected. Fourteen days post BM injection; GVHD target organs were excised for pathological analysis. A representative photomicrograph of each group depicting the average disease score morphology (A) and quantification of pathological damages to organs (B) are shown. Before mice were euthanized, luciferin was injected, and organs were excised followed by imaging using IVIS 200 imager (C), a representative mice from each group n=4. Organ bioluminescent was quantified using Living Imager software (D). * P<.05; ** P<.01; *** P<.001. Error bars indicate the mean of standard error.
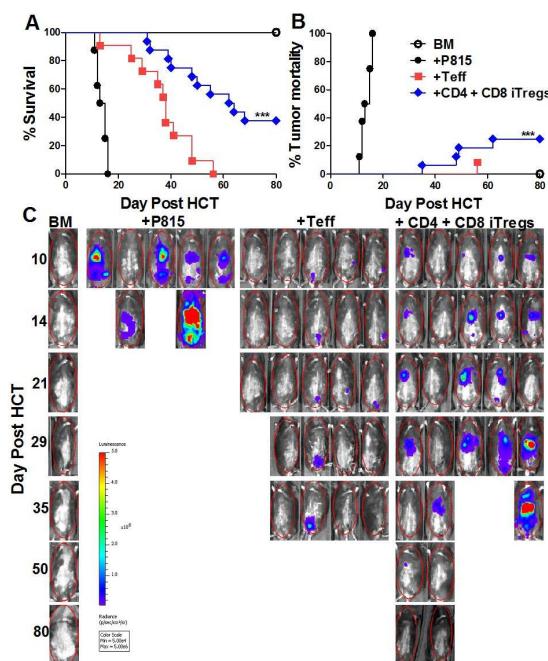
Figure 3.11 Combinational therapy preserves the GVL response. BDF1 recipient mice were lethally irradiated (1200cGY) and transplanted with 5×10^6 TCD-BM, 2×10^6 CD4 iTregs, 4×10^6 CD8 iTregs, and 5×10^3 luc-P815 cells. Three days later 2×10^6 B6 CD25-depleted Teffs were injected. Recipients were monitored for survival (A), tumor mortality (B), and tumor load (C) for 80 days, n=15. * P<.05; ** P<.01; *** P<.001.

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3.4.6 CD8 iTregs contribute to the preserved GVL effect by maintaining cytolytic functions

To understand why the GVL effect was preserved upon addition of CD8 iTregs (as opposed to CD4 iTregs alone) we analyzed the levels of effector molecules on all donor cells injected (Teffs + iTregs) to accurately reflect the total cytolytic potential within recipient mice. Importantly, this analysis will not exclude the cytolytic molecules, such as IFNγ, granzyme B, and fas-L, which are secreted or expressed by CD8 iTregs themselves (Figure 3.6). Fourteen days post HCT; CD4 iTregs alone significantly reduced the percentage of IFNγ$^+$ cells among total donor T cells (H2^{d-}, Teff + iTregs) in recipient spleens and livers (Figure 3.12A). The percentage of IFNγ$^+$ cells among donor T cells was partially restored with addition of CD8 iTregs (Figure 3.12B). To a lesser extent, the absolute number of H2^{d-}IFNγ$^+$ cells was also significantly increased in the recipients given combinational therapy compared to those of CD4 iTregs alone (Figure 3.12C). Combinational therapy was also able to restore the expression of granzyme B and fas-L on H2^{d-}CD8$^+$ cells, which were impaired by CD4 iTregs (Figure 3.12D). These results indicate the combinational therapy achieves GVL preservation by maintaining sufficient levels of IFNγ secretion and cytolytic molecule expression.
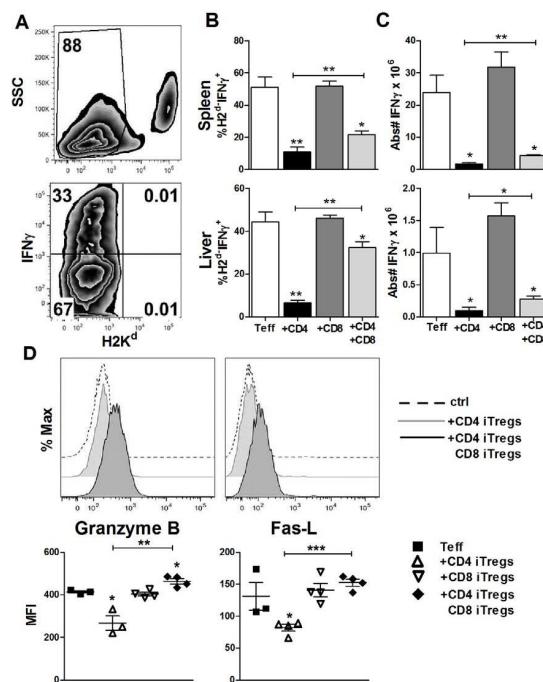
Figure 3.12 CD8 iTregs contribute to the preserved GVL effect mediated by combinational therapy.
BDF1 mice were transplanted as stated in Fig 6. Fourteen days post BMT spleen and liver mononuclear cells

were isolated. Representative flow gating strategy (A) displays IFNγ secretion from Teffs and injected iTregs (H2K^{d-}). Percentages of indicated cell populations, IFNγ production (B), and absolute number are quantified (C). Cells were also stained for granzyme B and Fas-L, with a corresponding isotype control (D) representative histogram analysis of the isotype control, CD4 singular or combination therapy. Graphical quantification of the mean fluorescence intensity for each molecule is displayed in (D), n=4. * P<.05; ** P<.01; *** P<.001. Error bars indicate the mean of standard error.

3.4.7 Alloreactive CD4 and CD8 iTregs display differential gene expression profiles

Given CD4 and CD8 iTregs had distinct effects on GVH and GVL responses; we strove to understand molecular differences between these two types of iTregs and if different molecular signatures could confirm our long-term studies. By using T cells from Foxp3-GFP reporter mice, we generated alloreactive iTregs and sorted pure CD4$^+$Foxp3$^+$ and CD8$^+$Foxp3$^+$ iTregs. The gene expression of alloreactive iTregs (Foxp3$^+$) and Teffs (Foxp3$^-$) was compared at the genomic level. Among a total of 34,356 genes analyzed, there were 235 genes differentially expressed between CD4 and CD8 Foxp3$^+$ iTregs and 688 genes differentially expressed between CD4 and CD8 Foxp3$^-$ Teffs. By excluding genes that differ between CD4 and CD8 T cells independent of Foxp3 expression, we further identified 85 of the 235 genes uniquely upregulated and 71 of the 235 genes uniquely downregulated in CD8$^+$Foxp3$^+$ vs. CD4$^+$Foxp3$^+$ iTregs; as the expression of those 156 (85 + 71) genes were not significantly different in CD8$^+$Foxp3$^-$ vs. CD4$^+$Foxp3$^-$ Teffs (Figure 3.13A, B). To identify the genes or molecules that may contribute to the distinct activity of CD4 and CD8 iTregs on GVH/GVL responses, we focused on those that regulate cytolytic function, Foxp3 stability, Treg suppressive mechanisms, and plasticity. 32 representative genes among those were displayed in a heat map (Figure 3.13A). Consistent with increased cytolytic activity of CD8 iTregs and hence GVL preservation (Figure 3.6 and 3.7), we found granzyme B, granzyme C, fas-L, perforin, and DAPK2 (death associated protein kinase 2) were significantly and uniquely upregulated in CD8$^+$Foxp3$^+$ vs. CD4$^+$Foxp3$^+$ iTregs (Figure 3.13C). Among five transcription factors associated with Foxp3 stability, also known as the "Treg quintet"(75), 3 of the 5 genes were significantly downregulated in CD8$^+$Foxp3$^+$ iTregs; reflecting their instability as compared to CD4 counterparts (Figure 3.13D).

Among the effector molecules Tregs utilize to mediate suppression, we found LAG3, CD39, and FGL2 (fibrinogen-like protein 2) were upregulated, whereas CD28 and Neuropilin-1 were downregulated in CD8$^+$Foxp3$^+$ vs. CD4$^+$Foxp3$^+$ iTregs (Figure 3.13E), suggesting CD8 and CD4 iTregs may utilize different mechanisms to control the immune response. In addition, CD4$^+$Foxp3$^+$ cells upregulated genes associated with Treg plasticity, such as RORγt, IL-17R, IL-6R and CCR6, as compared with CD8$^+$Foxp3$^+$ cells, suggesting CD8 iTregs, although less stable, represent a more terminally differentiated population (Figure 3.13F). Overall, CD8 iTregs and CD4 iTregs are quite distinct on the molecular level, with CD8 iTregs expressing more cytolytic molecules and less plasticity than CD4 iTregs.
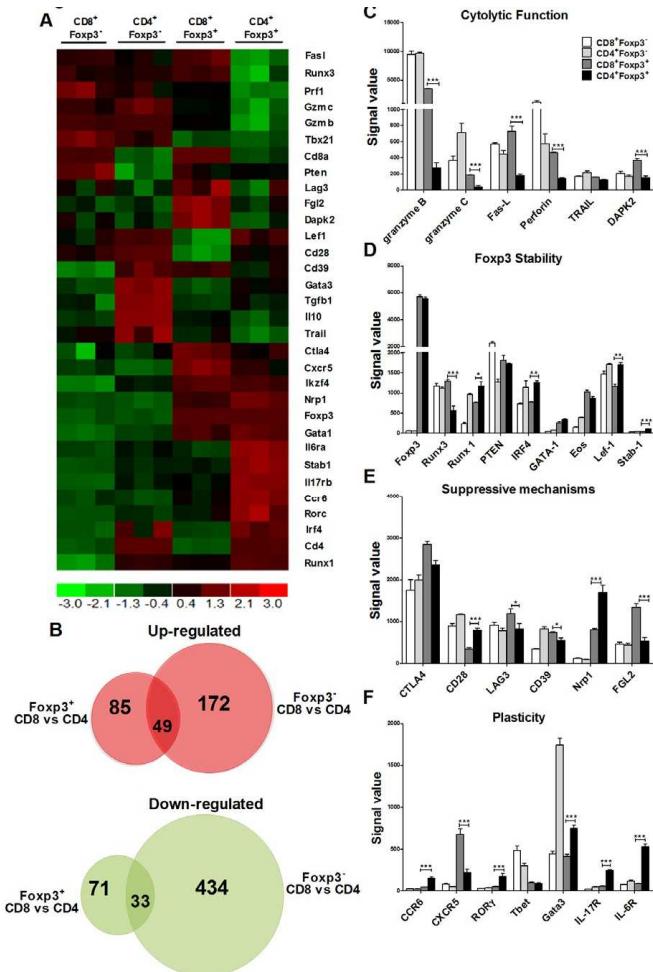
Figure 3.13 Alloreactive CD4 and CD8 iTregs display differential gene expression profiles. Naïve CD4 or CD8 B6-Foxp3-GFP T cells were stimulated with BALB/c CD11c^{hi} DC with IL-2 only, or with TGFβ and RA three separate times. GFP/Foxp3$^-$ CD4 or CD8 cells were FACS sorted from the culture with IL-2 alone, and GFP/Foxp3$^+$ CD4 or CD8 cells were sorted from the culture with IL-2 plus TGFβ and RA. Total RNA was isolated from 2 x 10^6 cells, and then labeled and hybridized to microarray chips for gene profiling. 32

representative genes display differential gene expression were shown (A). dChip software using FC>2 and p<0.05 identified unique genes that were exclusive to either Foxp3 expression or independent of Foxp3 expression (B). Signal values for genes relevant to cytotoxic function (C) Treg stability (D) suppressive mechanisms (E) or Treg plasticity were displayed (F). * P<. 05; ** P<. 01; *** P<. 001. Error bars indicate the mean of standard error.

3.5 Discussion

In the current study, we demonstrate host alloreactive iTregs are more potent in GVHD prevention than third-party alloreactive iTregs following allogeneic HCT. This is in accordance with our previous findings (161) that antigen recognition increases iTreg therapeutic potential. To date, an effective therapy distinguishing between GVH and GVL responses is highly desirable for patients with hematologic malignancies. Here, we established the first combinational alloreactive iTreg therapy, using both CD4 and CD8 iTregs, to provide distinct suppression of GVHD with maintained GVL responses. We uncovered unique roles for alloreactive CD4 and CD8 iTregs following allo-HCT. CD4 iTreg singular therapy potently suppressed GVHD but significantly impaired GVL responses. Conversely, CD8 iTreg singular therapy had a lesser impact on GVHD but fully preserved GVL activity. Although the current study utilizes iTregs reactive to major (MHC) antigen, we have validated the generation protocols can be applied to minor antigen (miHAg) settings (data not shown); suggesting miHAg-reactive iTregs could be utilized to prevent GVHD induction from miHAg disparity. Together, the combinational therapy using these two types of iTregs had complementary roles: CD4 iTregs reduced the expansion of Teffs to attenuate GVHD, and CD8 iTregs maintained optimal activation and cytolytic potential of Teffs, thus preserving GVL function.

Although controversy surrounds iTreg therapy for GVHD attenuation, a critical look into the variability among generation conditions clearly demonstrates why differential results exist. First, the activating agent (polyclonal vs. physiological) results in noticeably different outcomes for GVHD, as polyclonal activated iTregs are unable to mitigate disease (96, 97) while physiological activated iTregs significantly attenuate GVHD (99, 102, 161). It has been found physiologic

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activation imparts iTregs with more homing characteristics, like chemokine receptors, than polyclonal activated iTregs (104), which may provide advantages for physiologic iTregs on GVHD attenuation. Second, the difference between the third polarizing cytokine (rapamycin vs. RA) has a significant effect on the iTreg product. Interestingly, the use of rapamycin resulted in an unstable iTreg therapy (97), which could only be effective if rapamycin was continuously administered post-transplant, whereas RA has been shown to yield a stable iTreg product (99, 102, 161). The mechanism of action of these two compounds is quite different, with rapamycin indirectly suppressing expansion of contaminating Teffs through mTOR inhibition (166); whereas RA directly enhances the accessibility of the conserved non-coding sequence within the Foxp3 promoter, thus specifically enhancing Foxp3 stability. In this study, we established the optimal generation conditions for iTreg therapy on GVHD, physiological activation (allogeneic DCs) and RA polarization.

Of biological importance, we uncovered unique roles for CD4 and CD8 iTregs following allo-HCT. To our knowledge, this is the first study to show preferential suppression of CD4 Teff by CD4 iTregs and CD8 Teff by CD8 iTregs. Our cellular and molecular analysis uncovered increased expression of multiple cytolytic molecules on CD8, but not on CD4 iTregs, which likely contributes to the ability of CD8 iTregs to delay tumor relapse on their own. Of clinical importance, human alloreactive CD8 iTregs were also found to possess direct cytotoxicity against LCL tumor cell lines (117), providing a correlation between our findings and human specimens. Given their innate cytotoxic nature, these findings could be further expanded to use CD8 iTregs as a novel cell source for donor lymphocyte infusion (DLI) given to patients suffering from primary disease relapse (167-169). The stability of Foxp3 expression was another unique difference harbored by CD4 and CD8 iTregs, with CD4 iTregs displaying increased expression of multiple Treg stabilizing molecules, like the "Treg quintet", which was lacking in CD8 iTregs. These molecular findings mirror our cellular time course study of alloreactive iTregs and other's observations (97) that CD4 iTregs represent a stable cell subset

following allo-HCT; whereas CD8 iTregs are less stable, especially in GVHD target organs. Importantly, we observed that non-enriched CD4, CD8, or combinational therapy, even with loss of Foxp3 expression, was not pathogenic following allo-HCT, suggesting those iTregs are safe in cellular therapy. Together, our study proves CD4 and CD8 iTregs, even when generated under similar conditions, possess distinctly different characteristics. More work is needed to optimize the dose ratio of CD4 and CD8 iTregs and to manipulate either CD4 or CD8 iTreg characteristics to create a superior singular iTreg for GVH prevention and GVL maintenance. Similarly, multiple infusions of either CD4 or CD8 iTregs may be necessary to achieve sufficient efficacy and superior outcome in clinical settings.

Primary disease relapse is the leading cause of mortality, occurring in 37% of recipients (4), following allo-HCT. Therefore, when establishing a new therapeutic option for GVHD attenuation, maintaining GVL function is essential. A consensus on whether Tregs may preserve the GVL effect has not been reached, due to the limited tumor models tested. Notably, our studies show CD4 iTregs abrogate GVL activity against evasive P815 mastocytoma, which correlates with impaired CML clearance found   by others (97). Mirroring our observations, alloreactive nTreg (120) therapy resulted in maintained GVL activity against A20 but not against P815. The authors hypothesize clearance of P815 is strongly dependent on alloreactive T cells, which are sharply reduced by infused Tregs; which we also observed in our CD4 iTreg therapy. In the current study, we demonstrate a striking reduction in cytolytic molecules, such as granzyme B and fas-L on Teffs by CD4 iTregs, which likely contributes to the compromised GVL effect. This finding also provides a rationale to combine CD4 iTregs with cytolytic CD8 iTregs for GVL preservation.

We consider the most exciting finding in this work to be the combinational therapy's ability to rescue the impaired GVL effect mediated by CD4 singular therapy. Two main factors contribute to the unhindered GVL effect observed within the combinational therapy: 1) CD8 iTregs expression of cytolytic molecules that can directly lyse tumor cells and, 2) although the expansion of Teffs is dramatically inhibited, the residual cells are poly-functional, with sustained expression of IFNγ,

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granzyme B, and fas-L. While underlying mechanisms of GVL maintenance may still contribute, we identify these two factors, which provide a significant therapeutic effect. An effective therapy following allo-HCT requires conflicting immune responses, both suppression and cytolytic potential, and rarely can one immune cell type simultaneously possess each of these functions without complicated cellular manipulation. Uniquely, our combinational therapy utilizes the innate strengths of two immune cell types to achieve both suppression (CD4) and cytolytic potential (CD8) following allo-HCT. In summary, these findings provide the rationale for a targeted dual cellular therapy to reduce GVHD propensity and primary disease relapse, the two major hindrances obstructing allo-HCT success.

Chapter 4: Conclusions and Future Directions

The field of regulatory T cell therapy has come a long way since their discovery in 1970. However, there is still a long way to go. Although nTregs are an effective source for therapy, their low proliferative potential remains a major issue. Additionally, the expansion of GMP-grade nTreg for use in allo-HCT recipients requires significant clinical infrastructure and coordination. This work has shown both biologically (100) and translationally (103) iTregs can be generated rapidly and potently suppress GVHD. However GVL can be impaired using CD4 singular iTreg therapy. With the field trending towards investigating the differential abilities between CD4 versus CD8 iTregs with regards to GVHD and GVL responses, the convergence of these two therapies seems inevitable. The upcoming clinical trials involving both alloreactive nTregs and polyclonal iTregs will give us detailed insight into the next steps for improving iTreg cellular therapy for the treatment of GVHD.

The most compelling finding during this work was the therapeutic potential of alloreactive CD8 iTregs, which has the greatest potential for future experimental investigations. The molecular and cellular findings in Chapter 3 show alloreactive CD8 iTregs to be significantly less stable compared to alloreactive CD4 iTregs, a future project could focus on stabilizing Foxp3 expression within CD8 iTregs to provide a superior singular iTreg therapy. For example, Foxp3 stabilization can be achieved via inhibition of DNA methyltransferase by utilizing azacytidine thus resulting in a more demethylated Foxp3 promoter and stable Foxp3 expression. Azacytidine has been shown to generate induced Tregs in vivo following allogeneic transplantation giving this compound strong promise as an agent to stabilize/ increase iTreg conversion *ex vivo* (170). With new discoveries of molecules or proteins affecting Foxp3 stability, the list of agents is endless, for example, Stub1 (77), complement receptor inhibition (171), and Sirtuin1 (172) all represent potential targets to stabilize Foxp3 expression with alloreactive CD8 iTregs.

Stub1 is an E3-ubiquitin ligase that was shown to ubiquitate Foxp3 that lead to it being targeted for degradation, inhibiting Stub1 activity could greatly increase the half-life of the Foxp3 protein which is essential for lineage stability. Recently, binding of pro-inflammatory C3a and C5a (complement) to their cognate receptors C3aR and C5aR on T cells was shown to increase differentiation of T cells into Th1 or Th17 phenotype, whereas blocking these receptors lead to increase in Treg generation. Finally, Sirtuin1, a histone deacetylase which facilitates condensation of chromatin, was recently found to deacetylate Foxp3, by inhibiting Sirtuin1 activity acetylation of Foxp3 was increase and subsequently increasing their suppressive function.

On the other hand given alloreactive CD8 iTregs possess direct cytotoxicity against tumor cell lines however do not induce GVHD on their own characterizes these cells as a potential new cell source for DLI infusion. The anti-tumor effects of DLI infusion have substantially helped patients suffering from primary disease relapse however 50% of those patients end up with GVHD (167, 168). It would be an interesting approach to utilize alloreactive CD8 iTregs for DLI infusion given their inability to cause   acute GVHD but can mediate beneficial GVL clearance.

Finally, all the data presented in the current studies utilized either monoclonal or MHC-major mismatch settings in order to generate iTregs. This firmly establishes that antigen-specific Tregs potent efficiency to control GVHD, however, MHC-major mismatch transplant are never performed clinically due to high resolution typing of donor and recipient prior to transplant. Therefore, extension of these findings into minor-antigen mismatch setting would be even more clinically relevant and directly translational. There are multiple minor antigen preclinical models that could be utilized to generate alloreactive minor antigen specific iTregs (B10D2 to DBA2) (173). Consequently, these pre-clinical studies provide the rationale to support the first clinical trial in testing host-alloantigen reactive Tregs in GVHD (NCT01795573 led by Dr. Anasetti). Overall, our studies have significantly enhanced the knowledge and the translational potential of antigen-specific iTreg therapy for allo-HCT

Chapter 5: List of References

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Principal Investigator: YU, XUE-ZHONG
Department: Microbiology & Immunology

AR#: 3221
Title: Prevention of GVHD While Preserving GVL Effects

Initial Approval Date: February 28, 2013

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Statement of Investigator:

The Principal Investigator assures the following:

1. Alternatives to the use of live vertebrate laboratory animals were considered.

2. This experiment does not unnecessarily duplicate work performed by the PI.

3. The PI has consulted with MUSC staff veterinarians for any procedure involving pain or distress concerning appropriate use of anesthetics and analgesics, or PI is using accepted veterinary procedures.

4. Any facilities where survival surgical procedures or postoperative recovery is to be performed have been inspected and approved by the IACUC.

5. Personnel have been adequately trained for the procedures which they will be performing.

6. The number of animals to be used has been carefully considered and the minimum number required to obtain valid results will be used.
===
Statement of the IACUC:

This proposal has been reviewed and approved by the Committee. The IACUC gives assurance that it complies with the Public Health Policy on Humane Care and Use of Laboratory Animals and all applicable provisions of the Animal Welfare Act.

Signature of Approval: _____

Rupak Mukherjee, Ph.D., Interim Chair
Institutional Animal Care and Use Committee

About the Author

Jessica Heinrichs was born in Tampa, Florida in 1986 to Shirley and Dennis Heinrichs. She received her Bachelors of Science degree in 2008 from Florida State University. During her undergraduate works she published one coauthor manuscript on the morphology of the spade foot tadpole in Dr. Bryant Chase's lab. After graduating she worked as a lab manager for 2 years in Dr. Kimberly Hughes lab, where her work was focused on increasing the longevity of Drosophila melanogaster by controlling age of first reproduction. She then entered the Ph.D. program in biomedical sciences at the University of South Florida's College of Medicine in the fall of 2011. During her first two years she worked at Moffitt Cancer Center in the laboratory of Dr. Xue-Zhong Yu studying the use of iTreg cellular therapy to attenuate GVHD in pre-clinical animal models. In 2013, she moved with Dr. Yu to the Medical University of South Carolina where he had accepted a new position to complete her dissertation. At the Medical University of South Carolina she published two first author manuscripts and one review paper on the use of regulatory T cells following allogeneic bone marrow transplantation. Her work received two abstract achievement awards from the American Society of Hematology for the annual 2014 and 2015 meetings in San Francisco and Orlando, respectively. Following completion of her degree, she has accepted a post- doctoral position at Northwestern University in Chicago, Illinois in Dr. Joseph Leventhal's lab working on antigen-specific nTregs therapy to establish tolerance in kidney recipients.