Generation and Application of Antigen-Specific Induced Regulatory T cells in Allogeneic Bone Marrow Transplantation

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Generation and Application of Antigen-Specific Induced Regulatory T cells in
Allogeneic Bone Marrow Transplantation

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Transplantation, Graft-versus-Host Disease, Linked Suppression

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DEDICATION

This dissertation is dedicated to my wonderful and loving family. I honor the memory of my dad, the late John Maitland Semple, who departed this life June 24th, 2010. The greatest impact my dad has had on my life is his legacy of hard work and an honest life, a man unafraid to tell the truth, even when it hurts. The greatest gift he has given to me is almost 51 years of loving marriage to my mother, Kathleen Clover Semple. This dissertation is dedicated to my dear mom, who has always stressed the importance of an education throughout my life and is my strongest source of encouragement and support of my endeavors. To my wife Sheron, who has single-handedly taken care of our son during my tenure in graduate school, while at the same time attending nursing school. To my son Jaden, who brings me the greatest joy and is my constant source of determination and gives me the will to succeed. To my siblings Selwyn, Sherlyn, Hugh, Camille, Rhonda, Audrey, Conrad, Marcia and Leslyn who inspire me each day of my life. To my Aunt Yvonne and great-Aunt Darling for their love and constant encouragement and support over the years. To my extended family; brothers- and sisters-in-law, nephews, nieces, uncles, aunts and cousins, I thank you for your love, encouragement and support during these years. I honor the memory of my friend, the late John Nylander who passed away September 22, 2010. My deepest and sincerest gratitude to John, who has been a great buddy and co-worker who accompanied me to Florida when I began my studies in 2007. To all my many friends, thank you for all the love, support and encouragement. I love you all very much.
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LIST OF ABBREVIATIONS

APC Antigen presenting cell
Bcl-2 B-cell lymphoma 2
Bcl-X
Bcl-2-related gene, long isoform
BMDC Bone marrow-derived dendritic cells
BMT Bone marrow transplantation
CML Chronic myelogenous leukemia
CSA Cyclosporin
CTL Cytotoxic T leukocytes
CTLA-4 Cytotoxic T-leukocyte antigen 4 (also known as CD152)
CTLA-4-Ig Cytotoxic T-leukocyte antigen 4 - immunoglobulin
DC Dendritic cells
EAE Experimental autoimmune encephalomyelitis
EBV Epstein-Barr virus
Foxp3 Forkhead box P3
GVHD Graft-versus-Host Disease
GVL Graft-versus-Leukemia
GVT Graft-versus-Tumor
HCT Hematopoietic cell transplantation
IDO Indoleamine 2, 3 - dioxygenase
IPEX Immune dysregulation, polyendocrinopathy, enteropathy, x-linked
syndrome

IL-2 Interleukin-2
IL-7 Interleukin-7
IL-17 Interleukin-17
Itk IL-2 inducible T-cell kinase
iTreg Induced regulatory T cell
KI Knock-in
KO Knock-out
Lck Lymphocyte-specific protein tyrosine kinase
MBP Myelin basic protein
MHC Major histocompatibility complex
MiHA Minor histocompatibility antigen
MLR Mixed leukocyte reaction
MMF Mycophenolate mofetil acid
MTX Methotrexate
NFκB Nuclear factor κB
NK cell Natural killer cell
NKT cell Natural killer T-cell
NOD Non-obese diabetetic
nTreg Natural regulatory T cell
OVA Ovalbumin
PI3K Phosphatidylinositol 3-kinases
RAPA Rapamycin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SCT</td>
<td>Stem cell transplantation</td>
</tr>
<tr>
<td>TAC</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>TBI</td>
<td>Total body irradiation</td>
</tr>
<tr>
<td>TCD-BM</td>
<td>T cell-depleted bone marrow</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tconv</td>
<td>Conventional T cell</td>
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<tr>
<td>Teff</td>
<td>T effector cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β1</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell2</td>
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<tr>
<td>Th17</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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ABSTRACT

CD28 co-stimulation is required for the generation of naturally occurring regulatory T cells (nTregs) in the thymus through Lck-signaling. However, high level of CD28 suppresses the generation of induced Tregs (iTregs) from naïve CD4 T cells, although underlying mechanism(s) has not been defined. Here we investigated the role of CD28-mediated signaling pathways in the suppression of Treg generation. We used a series of transgenic (Tg) mice on CD28-deficient background that bears WT CD28 or mutated CD28 in its cytosolic tail incapable of binding to Lck, PI3K or Itk. Regardless of exogenous IL-2, strong CD28 costimulation suppressed iTreg generation through Lck signaling. Using a GVHD model to test the role of CD28-mediated iTreg suppression in T cell pathogenicity in vivo, we found that CD28-Lck T cells induced significantly less GVHD than T cells from CD28-WT mice. Furthermore, we found that the recipients of T cells from CD28-Lck mice generated significantly more iTregs than those with T cells from CD28-WT, which contribute to reduced graft-versus-host disease (GVHD) development in recipients of CD28-Lck T cells. These results indicate that CD28 costimulation can negatively regulate Treg generation and may provide an avenue for control of T-cell immunity or tolerance by regulating Tregs using the CD28 signal as a target. We went a step forward and investigated the therapeutic potential of antigen-specific iTregs in the prevention of GVHD. Donor hematopoietic stem cells and mature T cells are transplanted into a lymphopenic host to potentially cure many cancers and hematopoietic diseases like leukemia in bone marrow transplantation (BMT) or
hematopoietic stem cell transplantation (HCT), but the frequent development of GVHD is the main drawback of this treatment. nTregs suppress the development of GVHD and may spare the graft-versus-tumor activity. However, nTregs are a minor (~5%) subpopulation of CD4 helper T cells in healthy individuals, and using *in vitro* expanded nTregs is a common strategy to test their therapeutic potential in BMT. The concern of *in vitro* expanded nTregs may include their stability of Foxp3 (master regulatory gene for the development and function of regulatory T cell) expression and suppressive function, survival *in vivo*, and the non-selective suppression of the pre-activated nTregs. Antigen-specific activation of the regulatory T cells is important for optimal function. In this study, we used an alternative strategy to generate antigen-specific, iTregs and assessed their suppressive potential by comparing their effectiveness in preventing GVHD with polyclonal iTregs. We found that antigen-specific iTregs prevented GVHD lethality in recipients that expressed the target antigen, but were not protective of recipients who did not express the target antigen. Furthermore, antigen-specific iTregs were significantly more efficient than those polyclonal Tregs in the prevention of GVHD. These results reveal the therapeutic potential of antigen-specific iTregs to prevent GVHD efficiently and selectively, and provide the rationale to use antigen-specific iTregs in clinical HCT.
CHAPTER 1.

INTRODUCTION

Regulatory T cells.

Manipulating the suppressive population of T cells is an exciting possibility in the field of immunology, especially in autoimmunity and graft rejection. Lymphocytes with the ability to induce tolerance were first described in the early 1970s [1, 2]. During the 1980’s, appearance of naturally occurring suppressor T cells in spleens of neonatal or irradiated mice were temporarily related to the ease of induction of tolerance [3]. These naturally occurring suppressor cells inhibited the antigen-specific cytolytic arm of alloreactive immune responses, but left intact the antigen-specific suppressive arm of the immune response [3]. However, the concept of a specialized population of suppressor cells was not well understood and it was not until the 1990’s that research on suppressor T cells was revisited. Subsets of syngeneic CD4 T cells were depleted and the remaining CD4 T cells were transferred into lymphopenic hosts and evaluated for the development of autoimmunity [4-6]. The transfer of CD25-depleted CD4 T cells into athymic nude mice resulted in systemic multiorgan autoimmune syndromes. Sakaguchi et al also reported that CD25, the interleukin-2 (IL-2) receptor α-chain found on CD5^hi^ and CD45RB^lo^ subsets of CD4 T cells is an important surface marker for suppressor cells [5]. However, on a per-cell basis, CD4^+^CD25^+^ T cells were more potent than CD5^hi^ or CD45RB^lo^ T cells in preventing the incidence and severity of autoimmunity [5].
The suppressor T cells were renamed regulatory T cells (Tregs), even though regulatory T cells might both enhance or suppress immune responses [7]. The neonatal thymectomy experiments that were conducted with the discovery of Tregs revealed that Tregs are developed in the thymus. Almost undetectable levels of peripheral CD4+CD25+ T cells were present one week after thymectomy done on day 3, and much less splenic CD4+CD25+ T cells were detected in the thymectomized adult mice when compared with unmanipulated controls [8]. It should be noted that these results determined that the CD4+CD25+ regulatory T cells prevented autoimmunity not only in the neonatally thymectomized mice, but also in the lymphopenic animals [5, 9]. After the identification of Tregs, characterizing the phenotype of these regulatory cells was the focus of many researchers. CD4+CD25+ T cells were still the accepted phenotype of Tregs. However, this phenotype was not reliable since CD25; the high-affinity subunit of the IL-2 receptor was a T cell activation marker. Rudensky’s group and others reported that Foxp3, a member of the forkhead winged-helix family of transcription factor was required for the control of regulatory T cell development and also for its function [10, 11]. The role of Foxp3 in humans is not as clear-cut as in mice. Foxp3 is the most specific marker for Tregs and when overexpressed in conventional effector T cells (Teffs), these Teffs can be converted into suppressive T cells which function like Treg cells [12]. Mutation in the Foxp3 transcription factor results in scurfy mouse and an autoimmune syndrome in humans known as immunedysregulation, polyendocrinopathy, and enteropathy, x-linked (IPEX) [13].
Naturally-derived regulatory T cells.

Two types of CD4 T regulatory cells are described based on their origin; naturally derived regulatory T cells and regulatory cells induced in response to antigenic challenge [14]. Natural T regulatory cells are generated in the thymus and migrate to the periphery as regulatory cells [15]. Tregs account for approximately 5-10% of peripheral CD4+ in mice and a slightly smaller fraction in humans [16]. It is believed that thymic stromal cells, including cortical and medullary thymic epithelial cells and dendritic cells (DCs) contribute to the differentiation of nTregs, but there is controversy regarding to what extent the stromal cell components are required for this process [17, 18]. Relatively little is known about the developmental requirements of regulatory T cells [7]. However, thymic development of nTreg cells requires high-affinity interactions between their T cell receptor (TCR) and self-peptide-major histocompatibility complex (MHC) presented by thymic stromal cells [19]. Conventional CD4+ T cells (Tconv) also require MHC class II expression and interaction with TCR for their development, but they are low-affinity peptide-MHC class II interactions that result in positive selection [20-22]. There is also a requirement of Foxp3 for nTreg development in the thymus [10, 11], but the need for costimulation in the thymus of Treg development is not well understood [23] because costimulation in the thymus pushes conventional CD4+ cells to undergo negative selection [24-26]. Sakaguchi et al found that costimulatory signals are necessary for nTreg development because the number of nTregs in the thymus decrease after the loss of CD40 or CD28 expression [27]. However, how CD28 costimulation promote generation and maintenance of Tregs is not fully understood, but it is possible that CD28 costimulation increases the avidity of intrathymic TCR engagement for stimulating Treg
generation or that Tregs require CD28 costimulation for survival and expansion since CD28 costimulation promotes IL-2 secretion [23]. One study reported that both IL-2 and IL-7 in the thymic microenvironment are required for the development of nTregs in mice [28]. However, in a definitive study, Tai et al reported that costimulatory signals through the Lck-binding motif in the CD28 cytosolic tail initiate Treg differentiation in thymocytes [23].

**Functions of regulatory T cells.**

Tregs are indispensable for maintaining immunological self-tolerance and immune homeostasis and are critical for preventing activation of auto reactive T cells [29, 30]. These cells suppress the activation, proliferation and effector functions which includes cytokine production of immune cells like CD4\(^+\) and CD8\(^+\) T cells, natural killer (NK) and NKT cells, B cells and other antigen presenting cells (APCs) *in vitro* and *in vivo* [27]. Tregs inhibit the differentiation of naive T cells into Th1 and Th2 [31] and suppress their immune responses in the prevention of autoimmunity [32]. The ability to suppress immune responses allows Tregs to prevent the development of autoimmune disease, immunopathology and allergy, and also function in the maintenance of allograft tolerance and fetal-maternal tolerance during pregnancy [33]. Recent evidence indicates that the use of Tregs (CD4\(^+\)Foxp3\(^+\)) is one of the promising approaches to control Graft-versus-Host-Disease (GVHD) in numerous mouse models [34-39] in addition to early clinical trials [40, 41]. Tregs have a promising potential as immunotherapy for the induction of tolerance in autoimmunity and organ and bone marrow transplantation [42].
With the recent identification of a new lineage of differentiated naïve T cells, Th17, studies have focused on the role of Tregs in controlling the Th17 cells. A recent study found that Tregs have an inhibitory role on Th17 differentiation and the IL-17 production, much like the effect on Th1 and Th2 differentiation [43]. However, some reports indicate that Th17 cells are not inhibited but rather enhanced by Tregs [44, 45]. Li et al investigated the role of Tregs on IL-17 production in the absence of exogenous polarizing cytokines and found that simultaneous activation of naive Tconv and Tregs in the presence of APC induced differentiation of Tregs but not Tconv into IL-17 producers, and IL-1β was mandatory for this function [46].

**Mechanisms of suppression.**

There are many unresolved issues pertaining to the suppressive functions of Tregs, but several fundamental conclusions are widely accepted. It is generally accepted that Tregs are activated by either antigen-specific TCR ligation or the presence of polyclonal stimuli [47, 48]. However, after the Tregs are activated, they can suppress in a non-antigen specific manner [47, 49]. Even though cell contact between the suppressor and responder cells is required for nTreg suppression [50, 51], the type of cells that the Tregs target whether the CD25+ or the APCs is still controversial [7]. However, Tregs have been shown to inhibit the expansion of Teffs in vivo [35]. A recent study by Tawara et al found that alloantigen expression by the host APCs is necessary and sufficient for induction of GVHD protection by donor Tregs. [52]. The main mechanism of suppression seemed to be inhibition of IL-2 transcription in the responder population [7]. Because of
the indiscriminate nature of Treg suppression, the important antitumor immune responses may also be suppressed, which may allow the progression of tumor in the body. Therefore, even though immunologists have been continually working to define Treg cells at the molecular level [53, 54]; more studies are still needed to understand the immunobiology of Tregs.

**Induced regulatory T cells.**

Induced regulatory T cells (iTregs) have identical phenotype as nTregs, but are differentiated from conventional CD4$^+$ T cells when exposed to specific stimuli in the periphery which includes altered activation signals or modulating cytokines [15]. *In vitro*, iTregs can be generated from naïve CD4$^+$CD25$^-$Foxp$^-$ T cells after TCR-stimulation with additional TGFβ [55]. TGFβ also inhibits T-cell proliferation, the production of pro-inflammatory cytokines, and is involved in cell cytolysis [56]. It was reported that immune regulation of iTregs occurs preferentially through the release of soluble factors such as the suppressive cytokines IL-10 and TGFβ [48, 57, 58]. TGFβ and IL-2 induce naïve CD4$^+$ T cells to become Foxp3$^+$ iTregs [59, 60], while the combination of TGFβ and IL-6 induces the production of IL-17-producing cells (Th17) [44, 61-63]. It is believed that there is plasticity between the Treg and Th17 lineages because of the role of the common suppressive cytokine TGFβ [64]. Xu *et al* reported that nTregs can be induced by IL-6 to become Th17 cells in the absence of TGFβ [65]. In contrast, Zheng *et al* found that iTregs failed to become Th17 when stimulated with IL-6 [66]. The apparent resistance of iTregs to Th17 conversion suggests a more effective role than nTregs in an
inflammatory milieu and may suggest a role for IL-2 and TGFβ in maintaining immunological homeostasis [66].

Because of the infrequency of the nTreg population in peripheral blood, they will have to be expanded with anti-CD3/anti-CD28 coated microbeads or allogeneic APCs to attain adequate numbers for therapeutic use. Even though the expansion of nTregs is feasible, there are a number of issues that prevent the application of expanded nTregs in the clinic. These include the stability of their Foxp3 expression, their suppressive function and survival *in vivo* and the non-selective suppression of pre-activated polyclonally-expanded nTreg. Because of these issues, there has been effort towards the use of iTregs instead of expanded nTregs, which is a major portion of research in this dissertation. iTregs were previously used in the treatment of autoimmune diseases [67]. Also, it was recently reported that iTregs are significantly more potent at suppressing T-cell activation *in vitro* and are equally effective as freshly isolated nTregs at attenuating chronic colitis *in vivo* [68].

**Other T cells with regulatory functions.**

In addition to CD4⁺CD25⁺FOXP3⁺ Treg cells, other T cells also possess regulatory activity in mice. The CD4⁺ T cell subsets are induced in the periphery and include the CD4⁺IL-10⁺Foxp3⁻ (T₉₁) cells, which secrete IL-10 and the CD4⁺TGFβ⁺ (T₉₁) cells which secrete TGFβ and have been reported in *in vivo*-induced oral tolerance in mice [69]. Other subsets of regulatory cells include CD8⁺ T cells which have
phenotypic and functional features similar to CD4$^+$ Tregs [70-72]. However, most of these subsets of cells like the IL-10-secreting T$_R$1 cells, the TGFβ-secreting T helper 3 (T$_{H3}$) cells, the CD4$^+$CD8$^-$ T cells and the CD8$^+$CD28$^+$ T cells can acquire their regulatory functions after antigenic stimulation in the periphery, which is in contrast to nTregs, which are developed in the thymus with specialized suppressive function [70-72]. Our study focuses on Foxp3$^+$ nTregs and iTregs.

**CD28 Costimulation**

**T cell activation.**

T cell activation requires the recognition of specific peptide-MHC on the surface of APCs. Foreign protein antigens compete with self-antigens for binding to MHC and thus T cells have evolved to recognize low numbers of specific peptide-MHC complexes. The low numbers of peptide-MHC interactions and the low affinity of TCR for peptide-MHC complexes are not sufficient for effective T cell activation. Thus, interactions between costimulatory molecules present on the T cells and APCs provide the second signal that is required for effective T cell activation. The costimulatory molecules are important to the immune system and manipulating these molecules may provide pertinent information for control of the immune response. CD28 is the major costimulatory molecule present on T cells and provides the second signal for effective T cell activation. There is therapeutic interest in CD28 costimulation because manipulating these costimulatory signals might provide a means either to enhance or to terminate the
immune response [73]. CD28 is a useful target for immune-regulation because of its
costimulatory function. The ligands of the CD28 receptor, B7-1 (CD80) and B7-2
(CD86) are expressed mainly on B cells, DCs, and thymic medullary epithelial cells,
although other cells may have a low expression [73]. CD28 amplifies TCR signals and
positively regulates T cell activation [73]. CD28 costimulation reduces the number of
TCRs that must be triggered to achieve T-cell activation, and enhance T cell survival and
proliferation, as well as the production of cytokines and cytokine receptors.

Treg generation.

Another important function of CD28 costimulation apart from T cell activation
involves the generation of nTregs in the thymus and their survival and homeostasis in the
periphery. It was noted previously that prevention of CD28 ligation with CTLA4-Ig
exacerbated autoimmune disease in non-obese diabetic (NOD) mice [74]. Likewise, NOD
mice develop more rapid and severe autoimmune diabetes in CD28 KO background as
compared with WT mice, which suggest a role for CD28 in autoimmunity. Furthermore,
mice deficient in CD28 or its ligands have reduced numbers of nTregs [74], which further
linked CD28 with a role in nTreg generation. CD28 KO mice lack potent costimulation
for T effector cells and also for nTregs, the most effective mediators of self-tolerance and
result in a balanced deficit that results in the preservation of Ag-mediated activation [75].
However, IL-2 is dispensable for the induction of Foxp3 expression and nTreg
development in the thymus, but it is essential for the survival and homeostasis of nTregs
in the periphery [76]. CD28 costimulation was shown to be important for nTreg survival
and homeostasis in the periphery [76]. In a recent study it was demonstrated conclusively that CD28 costimulation is essential for nTreg development in the thymus independently of IL-2, even when TCRs are engaged in high-affinity interactions with their agonist antigen [23]. Our group subsequently reported that CD28 costimulation is critical for iTreg generation in the periphery and the requirement of CD28 in iTreg generation is also dependent on IL-2 production [77]. Efficient development of nTregs in the thymus relies on CD28 costimulation through its cytosolic Lck-binding motif and c-Rel leading to activation of NF-κB [78-80]. Recently, Vang et al reported that Cd28(-/-) mice lack Treg progenitors [81]. It is necessary to understand the developmental requirements of Tregs in order to effectively utilize these cells in immune therapy.

**CD28-mediated signaling in Treg generation**

The CD28 receptor lacks catalytic activity and therefore relies on associated kinases to execute its functions [82]. CD28 cytoplasmic domain has multiple motifs that recruit and activate Lck, PI3K and Itk kinases, which in turn mediate distinct CD28 functions. Upon T cell stimulation, the cytoplasmic tail of CD28 is phosphorylated at Tyr170 (in the context of YMNM) and creates a docking site for Src homology (SH)2 domain-containing proteins. A point mutation of this motif (F170MNM) compromises T cell survival due to failure of Bcl-XL up-regulation, but does not affect Treg generation in the thymus [23]. The PI3K-regulated pathway is one of the major signaling pathways and is activated by TCR, IL-2R, and CD28 stimulation, leading to T-cell activation, proliferation, and cell survival [83]. Akt, a serine-threonine kinase, is a key downstream
effector of the PI3K signaling pathway, which in response to PI3K activation, phosphorylates and regulates the activity of various targets including kinases, transcription factors and other regulatory molecules. Akt functions as a key regulator of various critical cell functions including glucose metabolism, cell proliferation and survival.

Hematopoietic Stem Cell Transplantation

Allogeneic hematopoietic stem cell transplantation (HCT) is a curative therapy for many hematologic, some epithelial malignancies, and a variety of non-malignant diseases [84]. HCT represents the most effective treatment for patients with high risk and relapsed hematologic malignancies [12]. However, donor T cells included in the graft react with recipient alloantigens present on APCs and produce a syndrome consisting of diarrhea, weight loss, skin changes, and liver abnormalities called GVHD. Despite the enormous potential of HCT, the risks associated with GVHD limit its extensive application [85]. Billingham, an early pioneer in the field of BMT, described three requirements for the development of GVHD [86]. First, the donor graft must contain immunologically competent cells (mature T cells). It was seen in both experimental and clinical allogeneic BMT that the severity of GVHD correlates with the number of donor T cells transfused [87]. Second, the recipient must be immune-compromised and incapable of rejecting the transplanted cells. And, finally the recipient must express tissue antigens that are not present in the transplant donor [87].
**Direct and indirect presentation.**

After allogeneic HCT transplants, both host- and donor-derived APCs are present in secondary lymphoid organs [88-90]. The donor T cells that are included in the graft recognize host alloantigens that are presented by either host APCs (direct presentation) or donor APCs (indirect presentation) [91, 92]. In the case of direct presentation, the donor T cells recognize either peptide bound to allogeneic MHC molecules or allogeneic MHC molecules without peptide [91, 93], whereas in indirect presentation, T cells respond to the peptide generated by degradation of the allogeneic MHC molecules which are presented on self-MHC [93]. It was previously reported that host APCs, rather than donor APCs, are important for GVHD induction in MiHA mismatch [92]. Studies indicate that presentation of distinct target antigens by the host and donor type APCs might play a differential role in mediating damage to target organs [92, 94, 95]. Additionally, recent findings indicate that alloreactive Tregs specific for both directly and indirectly presented alloantigens are required for the induction of tolerance in organ transplantation [96, 97].

**Effect of immunosuppressive agents.**

Immunosuppressive agents are included as an essential component of the allogeneic HCT regimen to reduce the risks of developing GVHD [16]. These immunosuppressive drugs impair T cell function and may protect from GVHD. Tacrolimus (TAC) and methotrexate (MTX) used in combination constitute the current standard of care for GVHD prevention after allogeneic HCT [98]. Randomized clinical trials have shown that TAC/MTX is superior to Cyclosporin (CSA)/MTX in the prevention of acute GVHD. It was reported that Grade II-IV acute GVHD was
significantly lower with TAC/MTX compared to CSA/MTX in both sibling donor (32% vs. 44%; p=0.01), and unrelated donor (56% vs. 74%; p= 0.0002) transplant trials [99, 100]. TAC and CSA are calcineurin inhibitors, while rapamycin (RAPA) or serolimus and mycophenolate mofetil acid (MMF) are immunosuppressants which affect the activation and function of T cells [101]. RAPA inhibits the mTOR pathway activity which is located downstream of IL-2/PI3K signaling. The mTOR pathway is a downstream target of Akt phosphorylation and has been linked to the PI3K signaling pathway, but the exact mechanism for connection is unknown. The mTOR signaling pathway is one of the targets of B7:CD28 co-stimulation and plays a critical role in the control of cap-dependent mRNA translation, cell growth, and proliferation [102]. RAPA inhibits protein kinase activity of the mTOR/raptor complex 1 and is currently used to prevent allograft rejection as well as an antineoplastic agent, because of its anti-proliferative property [103].

Tregs appear to be relatively resistant to effects of RAPA, and the drug may even promote generation of Tregs in vitro or allow their selective outgrowth in culture [104-106]. It was recently reported that RAPA has potent antiproliferative effects on antigen-stimulated CD4+ T cells and can promote the conversion of the naïve CD4+ T cells into Tregs in vivo and even promote their persistence in vivo [103]. RAPA reduces the expansion of Tconv in vivo, but does not affect the phenotype of Tregs or the expression of homing molecules, which suggests that RAPA uses different means to inhibit mTOR. However, it was also shown that RAPA did not affect the polyclonal expansion of donor-type Tregs after transplantation [107]. Additionally, there is a synergistic protective effect of Treg and RAPA against acute GVHD-related morbidity and mortality based on
reduced expansion of conventional T cells after BMT [107]. Immunosuppression greatly impairs immune reconstitution and increases the risk of infection while at the same time it diminishes and can even completely abrogate the important T cell-mediated graft-versus-leukemia (GVL) effect [108]. For these reasons, it is absolutely necessary to provide an alternative treatment option to immunosuppressive agents following HCT.

**Graft-versus-Host Disease**

GVHD, one of the major complications of allogeneic HCT is caused by donor T cells reacting against host alloantigens. These same alloreactive donor T cells can provide the beneficial GVL effect as well resulting in reduction in leukemia relapse [12]. These T cells also promote hematopoietic engraftment and reconstitute T cell immunity especially in adults with reduced thymic function [108]. The first step in the development of GVHD results in activation of the APCs [85]. BMT conditioning regimens administered before the infusion of donor cells along with the underlying disease and infections result in tissue damage and secretion of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 to create a ‘cytokine storm’ [109, 110]. These changes increase the expression of adhesion molecules, costimulatory molecules, MHC antigens and chemokine gradients, which alert the residual host and the infused donor immune cells [109]. The donor T cells infused with the graft then interact with the primed APCs and the end result is activation, proliferation, differentiation and migration of the alloreactive donor T cells. These alloreactive donor T cells cause extensive damage to the GVHD target organs (skin, gut, lung and liver) by cellular effectors like cytotoxic T lymphocytes (CTLs) and inflammatory effectors like cytokines. It is believed that the onset and course of GVHD
depend on the degree of major and minor MHC disparity as well as the T cell dose [16]. It was also reported that residual host APCs play an important role in the induction of acute GVHD by CD8\(^+\) T cells in the minor MHC-mismatched model [111]. However, Teshima et al. found that the alloantigens located on the host epithelial tissues, which are GVHD target tissues, are not required to trigger acute GVHD [112]. Tawara et al. investigated the role of APCs in the suppression of GVHD by donor Tregs and reported that alloantigen expression by host APCs is necessary and sufficient for the induction of GVHD protection by donor Tregs [52]. They also found that this requirement is independent of their effect on the maintenance of Treg number and the production of IL-10 or Indoleamine 2, 3-dioxygenase (IDO) by the host APCs [52].

Clinical GVHD can either be classified as acute or chronic [108]. Acute GVHD is characterized by damage to the skin, liver and the gastrointestinal tract, while chronic GVHD is characterized by more diverse manifestations which resemble autoimmune syndromes with eosinophilic fasciitis, scleroderma-like skin disease and salivary and lacrimal gland involvement [108]. For epidemiological studies, GVHD can be classified as either acute or chronic based on the time at which it occurs after HCT [108, 113, 114]. With such classification, acute GVHD occurs within the first 100 days post transplantation, while chronic GVHD occurs 100 days post transplantation [108]. However, many investigators prefer the pathological classification because the histology associated with acute GVHD can still persists 100 days post transplantation, especially when immunosuppressants are withdrawn from the patient [108]. Acute GVHD is responsible for 15% to 40% of mortality and is the major cause of morbidity after allogeneic HCT, while chronic GVHD occurs in up to 50% of patients who survive three
months after HCT [84, 113]. The incidence of chronic GVHD can be as high as 70% in human transplant recipients [108].

Apart from prophylaxis with immunosuppressive drugs, several strategies are being investigated to reduce the risk of developing GVHD. Some of these strategies include the selective depletion of alloreactive T cells in the donor graft, the use of umbilical cord blood as the source of the donor graft and the use of monoclonal antibodies and other novel drugs. Since GVHD is caused by the alloreactive T cells in the donor graft reacting against the recipient tissues, eliminating the offending T cells before graft infusion can effectively remove the risk of GVHD. However, patients who have had T cell-depleted BMT are more likely to have tumor or malignancy relapse and are at an increased risk for graft failure and infection. However, newer techniques to effectively deplete the alloreactive donor T cells from the graft are being investigated. In one study, CD62L(+) T cells (naïve and a subset of memory T cells) were selectively depleted and the CD62L(-) T cells (a subset of memory T cells) failed to proliferate in response to alloantigen and prevented the development of GVHD [115].

Bortezomib is a proteasome inhibitor that blocks the activation of nuclear factor κB (NFκB) [116] and is FDA approved for the treatment of multiple myeloma [117, 118]. It was reported that bortezomib inhibits acute lethal GVHD in rodents after allogeneic BMT with no observed adverse effects on myeloid recovery and donor chimerism [119]. However, a later study by the same group reported that the time of administration of bortezomib after BMT is critical for the effects of the drug on the development of GVHD in mice. Early administration of bortezomib after BMT leads to
effective prevention of GVHD, but a 5-day delay in administration of the drug could lead to GVHD-related toxicity [120]. However, a subsequent clinical trial with bortezomib, TAC, and MTX for GVHD prophylaxis after reduced-intensity conditioning allogeneic stem cell transplantation (SCT) using human leukocyte antigen (HLA)-mismatched unrelated donors show that Bortezomib is a promising novel immunomodulatory agent in allogeneic transplantation [121].

Anti-CD3 mAbs have also been used in the treatment and prevention of GVHD. Even though anti-CD3 mAb was effective when used to treat acute GVHD in a dose-escalation trial, its use was associated with the risk of developing Epstein-Barr Virus (EBV)-associated lymphoproliferative disorders [122]. Some murine anti-CD3 antibodies can activate T cells in vivo and be counter-productive in the treatment of GVHD. Therefore, the use of genetically engineered anti-CD3 mAb became a more valid option to decrease side effects associated with murine anti-CD3 mAb. The nonmitogenic anti-CD3 antibody, BC3 improved the clinical manifestations of GVHD in a phase I-II clinical trial possibly by modulating T cell function [123]. Yu et al reported that non-FcR-binding anti-CD3 antibody induce apoptosis selectively in antigen-activated, cycling T cells [124]. Later, the Anasetti group conducted a phase I clinical trial with visilizumab (Nuvion) and reported that the humanized non-FcR-binding anti-CD3 mAb, which has the ability to selectively induce apoptosis in activated T cells was effective in the treatment of glucocorticoid-refractory acute GVHD [125]. Recent studies by Li et al indicate that preconditioning with anti-CD3 monoclonal antibody before conditioning with total body irradiation (TBI) prevents GVHD and retains the necessary GVL in a HCT model of MHC-mismatched rodents [126]. Anti-CD3 mAb still represents a viable
option for the prevention and treatment of GVHD. The level of disparity of MHC between donor and recipient is an indicator of the risk of GVHD development. It is hoped that with the advent of DNA-based tissue typing, increased accuracy and specificity of HLA typing will lead to closer HLA matching between donor and recipient, hence a reduction in GVHD [127].

**Regulatory T cells: cellular therapeutic for GVHD.**

Studies conducted with mixed lymphocyte reaction (MLR) experiments with both mouse and human cells demonstrate the ability of regulatory T cells to suppress the proliferative responses of alloreactive CD4$^+$ T cells [128, 129]. It was reported that Tregs are effective in suppressing autoimmune diseases [130] as well as solid organ transplantation [131]. These findings lead researchers to investigate the role of Tregs in GVHD. It was initially reported that depletion of CD4$^+$CD25$^+$ T cells from the donor graft accelerated GVHD and increased lethality [132]. Additionally, Tregs have been reported to be effective in preventing the development of GVHD across major and minor MHC barriers in various HCT models [34, 37, 133, 134]. These studies demonstrate an important role of Tregs in the development of GVHD. However, even though physiological levels of endogenous CD4$^+$CD25$^+$ T cells may contribute to the development and course of GVHD, their small number is likely insufficient to control the overwhelming alloreactive T cell responses involved in major MHC-mismatched BMT settings [16]. Furthermore, the use of Tregs in allogeneic HCT is very promising since it was reported that the Tregs can suppress GVHD while preserving the GVL activity.
However, three major issues still hinder the implementation of Tregs as immunotherapy in the clinic. These include the low circulating numbers of Tregs in the peripheral blood, the loss of suppressor activity following \textit{ex vivo} expansion and the lack of Treg-specific markers to purify \textit{ex vivo} expanded Tregs [12]. However, the \textit{in vivo} dynamics of Treg trafficking and survival predict effective strategies to control GVHD after allogeneic transplantation [135]. Despite these considerations, several clinical trials are ongoing that adoptively transfer Tregs as immunotherapy to prevent the development of GVHD. One group has transplanted freshly isolated donor Tregs while a second group has expanded Tregs from cord blood with anti-CD3 and anti-CD28 coated microbeads and utilized them in double umbilical cord blood transplantation [40, 41].

\textbf{Antigen-specific regulatory T cells.}

Tregs are effective in the prevention of GVHD and can spare GVL [35]. However, Tregs exert their suppression \textit{in vivo} in a non-antigen-specific manner [49]. Since the use of HCT is primarily for the maintenance of graft-versus-tumor (GVT) and/or GVL effect, non-antigen-specific suppression of Tregs included in the graft may not be entirely beneficial to the recipient since susceptibility to infection presents itself with the possibility of widespread suppression. The goal following HCT is for the recipient to maintain an immune response against infections, while enabling specific suppression of the deleterious GVHD-causing allo-reactive T cells. This may be achieved by the generation and use of antigen-specific Tregs. Several groups have used alloantigen-specific Tregs in the prevention of autoimmune diseases and have reported
their superiority to polyclonal Tregs. In experimental autoimmune encephalomyelitis (EAE) mouse model for multiple sclerosis. Hori et al demonstrate that the effect of myelin basic protein (MBP)-specific Treg cells was associated with specificity for MBP [136]. A recent study also reported that TGFβ-induced myelin peptide-specific regulatory T cells can mediate antigen-specific suppression in EAE [137]. Antigen-specific Tregs was also used in the NOD mouse model for type 1 diabetes and Tregs which were specific for a pancreatic autoantigen were more efficient at preventing diabetes than polyclonal Treg cells [138, 139]. In a more recent study, Tonkin et al reported that Tregs prevented diabetes in NOD mice only when their antigen was present in vivo in the pancreas [140]. Additionally, autoimmune gastritis was also prevented by transfer of stomach-specific Treg cells, but not with polyclonal Treg cells [141]. Our group had generated antigen-specific iTregs by Foxp3 transduction and has shown that these cells are more effective than polyclonal Tregs in preventing GVHD in a non-myeloblative murine BMT model [142]. However, this study used the non-myeloblative BMT model utilized gene transfection to generate the iTregs for the study. This is very difficult to translate to the clinic, and therefore a study using a myeloblative BMT model would be more representative of the human BMT. Also Foxp3 induction with the conversion of peripheral CD4+CD25− naïve T cells to CD4+CD25+ regulatory T cells by TGFβ and IL-2 [55] provides a more translatable method for Treg generation.

The H-Y antigen has previously been implicated in GVHD involving male recipients of female donors. miHAs have varied tissue distribution; some are restricted to hematopoietic tissues [143-147], while others have ubiquitous tissue expression [98]. Pidala and Anasetti have proposed that the varied tissue distribution of miHA may be
utilized for the production of antigen-specific iTregs in GVHD prevention [98]. The antigen-specific Tregs with specificity for minor antigens that have broad tissue distribution can be generated and these iTregs can selectively suppress donor alloreactive T cells with specificity for the broad distribution minor antigens. This would selectively abrogate the cells responsible for the development of aGVHD. However, these Tregs would have no effect on the alloreactive T cells which have specificity for minor antigens that are restricted to hematopoietic cells and thus preserve the GVL effect [98]. Specific GVHD prevention with corresponding sparing of the GVL effect has great potential for application to human transplantation.

**CD28 costimulation and GVHD development.**

Activation of naïve T cells by recipient alloantigen starts immediately after cell transplantation and results in the development of severe acute GVHD. The CD28 costimulatory molecule plays an important role in the development of GVHD, contributes to its pathogenesis and enhances its severity. Our group investigated the role of CD28 in the development of GVHD by comparing the ability of wild-type (WT) and CD28 deficient T cells to initiate GVHD. We previously reported that CD28 signals contribute to the pathogenesis of GVHD and enhance its severity [148]. CD28 costimulation is important for cytokine production in the pathogenesis of GVHD [148]. In contrast, the CTLA4-signal inhibits T cell response and reduces GVHD [149].
Central Hypothesis

Our preliminary data suggests that strong CD28 costimulation has a suppressive effect on the generation of iTregs in the periphery. We will therefore investigate which CD28-mediated signal(s) are responsible for suppressing iTreg generation. The central hypothesis is that strong CD28 costimulation has an important role in the suppression of iTregs in the periphery. It is not known whether suppression of iTreg generation mediated by CD28 costimulation contributes to development of GVHD. We will determine whether suppression of iTreg generation mediated by CD28 costimulation has a role in the T cell response \textit{in vivo}. Further, based on the superiority of antigen-specific iTregs to polyclonal Tregs in preventing autoimmune diseases, we also hypothesize that induced Tregs with specificity for an antigen expressed on the recipient would be more effective and specific than polyclonal nTregs in the prevention of GVHD. We will use the following specific aims to test our hypotheses:

Specific Aims

1) Determine the effects of CD28 costimulation on iTreg generation in the periphery

2) Determine the role of Antigen-specific TGFβ-induced Tregs in the prevention of GVHD after BMT.
CHAPTER 2.
MATERIALS AND METHODS

Experimental Mice

C57BL/6 (B6) and BALB/c mice were purchased from the National Cancer Institute (Bethesda, MD). Founders of CD28 WT and mutation Tg mice on B6 background, including CD28-WT, CD28-Lck, CD28-PI3K and CD28-Itk Tg strains, were provided by Drs. X. Tai and A. Singer at the National Cancer Institute [23]. Each of these 4 Tg strains was bred into CD28 knockout (KO) mice so that Tg CD28 molecules were the only CD28 receptors expressed by the Tg T cells. We screened CD28 Tg mice by FACS and selected the mice that expressed CD28 on CD4+ cells comparably among different strains of mice. C57BL/6 (B6, H-2b), B6 that express congenic Ly5.1 or Thy1.1, B6 bm12 and OT-II TCR transgenic (Tg) strains were purchased from the Jackson Laboratory (Bar Harbor, ME). Foxp3\textsuperscript{gfp} knock-in (KI) strain was obtained from Rudensky’s laboratory at University of Washington (Seattle, WA) [19, 150]. OT-II Foxp3\textsuperscript{gfp} KI and (B6.OVA x bm12)F1 strains were produced by cross-breeding. Luciferase-transgenic (Luc-Tg) strain on B6 background was kindly provided by Dr. R. Negrin (Stanford University, CA) [151]. B6 OVA Tg under β-actin strain was kindly provided by S. Schoenberger (La Jolla Institute for Allergy and Immunology, San Diego, CA). These strains of mice were bred and all mice used in this study were housed in a pathogen-free condition at H. Lee Moffitt Cancer Center & Research Institute (Tampa,
FL). All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

**Reagents and Antibodies**

Recombinant mouse IL-2 and human TGFβ-1 were purchased from R&D systems (Minneapolis, MN). Anti-mouse CD3 (clone 145.2C11) and CD28 (clone 37.51) mAbs were produced and purified in our laboratory. Purified PE-conjugated anti-mouse/rat Foxp3 (clone FJK-16s) was purchased from eBioscience (San Diego, CA). Other fluorochrome-conjugated mAbs were purchased from eBioscience or Becton Dickinson (San Jose CA).

**T cell Purification**

CD4⁺CD25⁻ T cells were purified through negative selection using biotinylated CD45R, CD11b, CD8a, TER, DX5 (eBioscience), CD25 and anti-biotin Micro Beads (Miltenyi Biotec). The purity of CD4+CD25- cells ranged from 85 to 95%, but CD4⁺CD25⁺ cells was always less than 1% among total CD4⁺ cells.

**Induced Regulatory T cell Generation**

With anti-CD3.

To generate polyclonal iTregs, CD4⁺CD25⁻ T cells were seeded at 2.5 x 10⁵/well in 48-well plates and stimulated with 0.5-1.0 µg/ml soluble anti-CD3 mAb in the presence of 1.25 x 10⁶ irradiated syngeneic T-cell depleted (TCD) splenocytes as APCs, with and without TGF-β1 and IL-2 both at 2 ng/ml. Alternatively, CD4⁺CD25⁻ T cells
were stimulated by plate-bound anti-CD3 mAb at 5 or 10 ng/ml without or with plate-bound anti-CD28 mAb at various concentrations in the absence of APCs. In some cases, additional pp1 was included in the culture at the concentrations indicated. After stimulation for 4 days, cells were harvested for the measurement of Foxp3 expression, or GFP expression if T cells from Foxp3<sup>gfp</sup> reporter mice were used.

**Antigenic stimulation with OVA peptide.**

To generate antigen-specific (OVA) iTregs, CD4<sup>+</sup>CD25<sup>−</sup> T cells from OT-II Foxp3<sup>gfp</sup> KI mice were seeded at 2.5 x 10<sup>5</sup>/well in 48-well plates and stimulated with 0.5 µg/ml OVA peptide in the presence of 1.25 x 10<sup>6</sup> irradiated syngeneic TCD-splenocytes as APCs, with 2 ng/ml TGF-β1 and IL-2 at 1 ng/ml. After incubation for 6 days, cells were harvested and sorted for measuring CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup> iTregs. Percentage of CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup> cells ranged from 20% to 60% among CD4<sup>+</sup> cells. CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>−</sup> cells were also sorted and used as controls.

**Allogeneic stimulation.**

To generate iTregs with allogeneic stimulation, B cells or bone marrow-derived DCs (BM-DCs) from BALB/c mice were used to stimulate C57BL/6 CD4<sup>+</sup>CD25<sup>−</sup> T cells with TGF-β and IL-2. CD4<sup>+</sup>CD25<sup>−</sup> T cells from the mutant strains were seeded at 200 x 10<sup>3</sup>/well in 96-well plates and stimulated with 40 x 10<sup>3</sup>/well LPS-activated B cells in the
presence of TGFβ and IL-2. After stimulation for 6 days, cells were harvested for the measurement of CD4, CD25 and Foxp3 expression by flow cytometry.

**Flow Cytometry Analysis**

**Surface staining.**

Two-, 3-, 4- or 5-color flow cytometry was performed to measure the expression of surface molecules according to standard techniques. Analysis was performed using a FACScan or FACS Calibur with CellQuest Pro version 5.2.2 (BD Biosciences) or the LSR II with DIVA software.

**Intracellular staining.**

Multi-color flow cytometry was performed to measure intracellular Foxp3 expression according to the manufacturer’s instruction (eBiosciences, San Diego, CA). Fluorescence conjugated-Abs were purchased from BD-Pharmingen (San Diego, CA) or eBioscience (San Diego, CA). Analysis was performed by using a FACS Calibur or LSR II instrument and CellQuest Pro version 5.2.1 (BD Biosciences) or FlowJo version 8.5.3 software (TreeStar, Ashland, OR). Other intracellular cytokines were measured after stimulation with PMA and ionomycin in vitro for 4-5 hours with the addition of Golgi Stop for the last 2 hours. The cells were then stained for surface expression of CD4, Ly5.1 and Thy1.1, and for intracellular expression of IFN-γ, IL-17 and IL-10.
**CFSE labeling.**

For measurement of proliferative response *in vitro* and *in vivo*, T cells were labeled with CFSE (Molecular Probes). The CFSE dilution in T cells was analyzed by flow cytometry. For measurement of proliferative response *in vivo*, the T cells were labeled with CFSE and then adoptively transferred to mice via tail vein injections. After 3 days, the spleens were harvested and stained for surface expression of CD4.

**Bone Marrow Transplantation**

As recipients, BALB/c mice were exposed to 800 - 900 cGy of total body irradiation (TBI), while (B6 x bm12)F1 were exposed to 1200 - 1300 cGy (2 doses of 600 - 650 cGy given 3 hours apart) of TBI. TCD-BM cells alone or in combination with purified CD25⁺ T cells from indicated donors were injected into recipients via tail vein within 24 hours of 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 sacrificed and counted as GVHD lethality. In separate experiments, cell expansion and iTreg generation of donor T cells were measured in recipient spleen at indicated times after BMT. In some experiments, T cells from *Luc-Tg* B6 donors were used as Teffs and transplanted into either BALB/c or (B6 x bm12)F1 recipients. The recipient mice underwent bioluminescence imaging (BLI) to tract the transplanted Teffs. Recipient mice were injected with luciferase substrate which allowed serial imaging of the *in vivo* bioluminescent signal using an IVIS200 charge-coupled device imaging system (Xenogen).
[3H] Thymidine Incorporation Assay

To access cell proliferative response, cultures were be pulsed with [3H] thymidine (1 µCi) 8 hours before collection. The cells were harvested (Cell Harvester) and counted in a liquid scintillation counter (Top Count, Perkin-Elmer) to measure proliferation. Testing was done in triplicate.

Statistical Analysis

The log-rank test was used to detect statistical differences in recipient survival in GVHD experiments. Student’s t test was used to compare percentages or numbers of donor T cells.
CHAPTER 3.

STRONG CD28 COSTIMULATION SUPPRESSES GENERATION OF
INDUCED TREGS THROUGH Lck SIGNALING

ABSTRACT

CD28 costimulation is required for the generation of naturally-derived regulatory T cells (nTregs) in the thymus through lymphocyte-specific protein tyrosine kinase (Lck) signaling. However, it is not clear how CD28 costimulation regulates the generation of induced Tregs (iTregs) from naïve CD4 T-cell precursors in the periphery. To address this question, we induced iTregs (CD25⁺Foxp3⁺) from naïve CD4 T cells (CD25⁻Foxp3⁻) by T-cell receptor stimulation with additional transforming growth factor β (TGFβ) \textit{in vitro}, and found that the generation of iTregs was inversely related to the level of CD28 costimulation independently of interleukin-2 (IL-2). By using a series of transgenic mice on CD28-deficient background that bears WT CD28 or mutated CD28 in its cytosolic tail incapable of binding to Lck, phosphoinositide 3-kinase (PI3K) or IL-2–inducible T-cell kinase (Itk), we found that CD28-mediated Lck-signaling plays an essential role in the suppression of iTreg generation under strong CD28 costimulation. Furthermore, we demonstrate that T cells with the CD28 receptor incapable of activating Lck were prone to iTreg induction \textit{in vivo}, which contributed to their reduced ability to cause graft-versus-host disease. These findings reveal a novel mechanistic insight into how CD28 costimulation negatively regulates the generation of iTregs, and provide a rationale for
promoting T-cell immunity or tolerance by regulating Tregs through targeting CD28-signaling.

**Background and Rationale**

Regulatory T cells (Tregs) play an essential role in the maintenance of immunological tolerance to prevent autoimmune disease. The development of Tregs in the thymus requires Foxp3, a member of transcription factors characterized by their winged helix-forkhead DNA-binding domain [152]. Although it is widely accepted that natural Tregs develop in the thymus (also termed nTregs), compelling evidence indicates that Tregs with an identical phenotype can be induced in the periphery from CD4\(^+\) non-Treg precursors under certain conditions. For example, all CD4\(^+\) cells from RAG\(^{-/-}\) TCR transgenic (Tg) mice are CD25\(^-\), but a small proportion of these cells convert to a CD25\(^+\) Treg phenotype after adoptive transfer into antigen-bearing mice or mice that have been administered a tolerizing dose of peptide antigen [153, 154]. Furthermore, *De novo* generation of CD4\(^+\)CD25\(^+\) Tregs from CD4\(^+\)CD25\(^-\) cells can also occur in thymectomized mice [155]. Such Tregs that are induced in the periphery are also termed induced Tregs (iTregs). Although our understanding of the microenvironment for iTreg development *in vivo* is still limited, it is clear that TCR stimulation, TGF\(\beta\) and IL-2 are required for their development [55, 60, 156, 157].

A crucial regulator of Tregs is the CD28 receptor, a dominant costimulatory molecule for T cell activation. The first clue to the critical role of the CD28 family in nTreg function was the observation that prevention of CD28 ligation with CTLA4-Ig
exacerbated autoimmune disease in NOD mice [74]. Mice deficient for CD28 or its ligands (B7, CD80 and CD86) have substantially reduced number of nTregs [23, 74]. As a consequence, NOD mice lacking CD28 develop more rapid and severe autoimmune diabetes compared to wild-type (WT) mice. Recent studies indicate that CD28 is essential for nTreg development in the thymus and for nTreg survival and homeostasis in the periphery [23, 158]. Thus, while these mice are lacking potent costimulation (CD28) for T effector cells (Teffs), they are also lacking nTregs, the most effective mediators of self-tolerance, yielding a balanced deficit that results in the preservation of antigen-mediated activation [75].

A potential role of CD28 in the generation of iTreg has not been rigorously investigated. Conversion of conventional CD4⁺CD25⁻ T cells into Tregs occurs in thymectomized mice and requires B7 costimulation [155]. Furthermore, CD28 costimulation is required for the generation of iTregs from naïve CD4⁺CD25⁻ T cells through production of IL-2 [77]. However, there is also scattered evidence suggesting that high levels of CD28 costimulation reduce Foxp3 expression and limit iTreg generation through undefined mechanism(s) [13, 159]. In this study, we clearly demonstrate that the high levels of CD28 costimulation suppress generation of iTregs from naïve CD4 T cells while promoting expansion of Teffs. By using a series of Tg mice on CD28-deficient background that bears either WT CD28 or mutated CD28 in its cytosolic tail that is incapable of binding to Lck, PI3K or Itk, we found that strong CD28 costimulation suppresses iTreg induction through Lck-signaling but independent of IL-2 production.
CD28 Costimulation through Soluble anti-CD28 mAb

CD28 is required for the full development of nTregs in the thymus and for homeostasis of nTregs in the periphery. Besides developing in the thymus, Tregs can also be generated or differentiated from naïve CD4 T cells in the periphery, and these Tregs are termed iTregs. How CD28 costimulation modulates the generation of iTregs is unclear. We investigated the effect of CD28 costimulation on iTreg generated from naïve CD4+CD25-Foxp3- cells in vitro during TCR-stimulation in the presence of TGFβ. CD4+CD25-GFP cells were purified from Foxp3\textsuperscript{gfp} reporter mice and were stimulated with soluble anti-CD3 mAb plus various concentrations of agonistic anti-CD28 mAb. After culturing for 4 days, iTregs (CD4+GFP\textsuperscript{+}) were generated in the presence of TGFβ (Fig. 1A). Anti-CD28 mAb inhibited iTreg generation in a dose dependent manner, in percentage (Fig. 1B) as well as in absolute cell number (Fig. 1C). In contrast, anti-CD28 stimulation increased the numbers of Teffs (CD4+GFP-) (Fig. 1C) and IL-2 production (Fig. 1D) in a dose-dependent manner, which is consistent with the established concept that agonistic anti-CD28 mAb provides T-cell costimulation.
Figure 1. Soluble anti-CD28 mAb reduces iTreg generation in vitro. CD4+GFP+ cells were purified by FACS sorting from Foxp3<sup>gfp</sup> reporter mice and stimulated with anti-CD3 mAb plus irradiated TCD splenocytes as APCs in the absence or presence of TGFβ. Soluble anti-CD28 mAb was added at different concentrations into the culture. Four days after stimulation, cultured cells were harvested and measured for CD4, GFP and intracellular Foxp3 expression. A. The data show the percentage of GFP<sup>+</sup> (upper panel) or Foxp3<sup>+</sup> (lower panel) cells on gated CD4<sup>+</sup> cells. B. The data are presented as the mean of percentage of Teffs (GFP<sup>+</sup>) and Tregs (GFP<sup>+</sup>) cells in total CD4<sup>+</sup> cells in triplicate wells. C. The data are presented as the mean ± 1 SD of absolute numbers of Teffs (CD4<sup>+</sup>GFP<sup>+</sup>) and Tregs (CD4<sup>+</sup>GFP<sup>+</sup>) cells in triplicate wells. D. In separate experiments, cultured cells were harvested and measured for CD4 and GFP expression whereas culture supernatant was measured for IL-2 production. The data show the percentage of GFP<sup>+</sup> on gated CD4<sup>+</sup> cells (left Y-axis), and IL-2 production (right Y-axis). The data are presented as the mean ± 1 SD in triplicate wells, and represent 1 of 3 replicate experiments.
CD28 Costimulation through Plate-bound anti-CD28 mAb

Because T cells receive other co-stimulatory signals provided by APCs in the culture, it is possible that signals other than CD28 were also required for the inhibition of iTreg generation. To address this possibility, purified CD4^+CD25^- T cells were stimulated with cross-linking anti-CD3/anti-CD28 plus TGFβ in the absence of APCs (Fig. 2A). Under this culture condition, we also observed that anti-CD28 inhibited iTreg generation in a dose-dependent manner and increased expansion of Teffs (Fig. 2C), indicating that other signals from APCs may not have a significant effect in this phenomenon. Because CD28 costimulation promotes IL-2 production, it is possible that strong CD28 costimulation may suppress iTreg generation through high levels of IL-2. However, when a high concentration of exogenous IL-2 at 2 ng/ml was added to the culture, it did not suppress iTreg generation and did not affect the suppression of iTreg generation by strong CD28 costimulation (Fig. 2). We therefore conclude that strong CD28 costimulation suppresses the generation of iTregs independently of IL-2 production. However, since there was a difference both in percentage and absolute number of the Tregs generated without and with IL-2 in the presence of low but not high levels of anti-CD28 (Fig. 2), it is possible that IL-2 can circumvent inhibition mediated by lower levels of CD28 costimulation.
Figure 2. Plate-bound anti-CD28 mAb reduces iTreg generation in vitro. CD4^+CD25^- cells were purified from spleen and lymph node cells from normal B6 mice and stimulated with plate-bound anti-CD3 mAb plus anti-CD28 mAb in the absence of APCs. Exogenous TGFβ was also included in the culture with or without additional IL-2. Four days after stimulation, cultured cells were harvested and measured for CD4, CD25 and intracellular Foxp3. A. The expression of CD25 and Foxp3 is shown on gated CD4^+ cells. B. The data are presented as the mean of percentage of Teffs (CD25^-Foxp3^-) and Tregs (CD25^-Foxp3^+) cells in total CD4^+ cells in triplicate wells. C. The data are presented as the mean ± 1 SD of absolute numbers of Teffs (CD4^-CD25^-Foxp3^-) and Tregs (CD4^-CD25^-Foxp3^+) cells in triplicate wells. The data represent one of 4 replicate experiments.
Lck-signal is Critical for CD28-mediated Suppression of iTreg Generation

Through its cytoplasmic motifs, CD28 can recruit and activate several kinases including Lck, PI3K and Itk [82]. To explore the question of which CD28-mediated signal(s) are responsible for suppressing iTreg generation, we utilized a set of CD28 Tg mice that express CD28 receptors with different mutations in the CD28 cytosolic tail: CD28 with an unmutated cytosolic tail (hereafter CD28-WT); CD28 with mutations in P187A and P190A that abrogates Lck binding (CD28-Lck); CD28 with a mutation in Y170F that abrogates PI3K binding (CD28-PI3K); and CD28 with mutations in P175A and P178A that abrogates Itk binding (CD28-Itk) (Fig. 3). To identify the CD28 kinase-activating domains (cytoplasmic motifs) required for suppressing iTreg generation, purified CD4<sup>+</sup>CD25<sup>+</sup> T cells from CD28-WT, CD28-Lck, CD28-PI3K or CD28-Itk Tg mice were stimulated with cross-linking anti-CD3/anti-CD28 plus TGFβ without APCs. In the absence of exogenous IL-2, additional anti-CD28 inhibited iTreg generation from CD28-WT naïve CD4 T cells in a dose-dependent manner (Fig. 4A). iTreg generation from CD28-Itk cells was also inhibited by anti-CD28. These data showed that strong CD28 costimulation suppressed iTreg generation, and Itk activation mediated by CD28 did not play a significant role in this process. In contrast to CD28-WT or CD28-Itk cells, anti-CD28 did not inhibit iTreg generation from CD28-PI3K cells and even increased iTreg generation from CD28-Lck cells (Fig. 4A), indicating that CD28-mediated PI3K and Lck signals are required for the suppression of iTreg generation.
Figure 3. CD28 transgenic mice. All the CD28 transgenes were bred into CD28--/ mice to eliminate the endogenous CD28 expression. These CD28 transgenes were introduced in a human CD2C-based transgenic vector, which drives expression in immature thymocytes and mature T cells. The point mutations (bold and underlined) in the cytosolic tails were generated by changing Y (tyrosine) to F (phenylalanine) and P (proline) to A (alanine).
Because CD28-mediated Lck activation is critical for IL-2 production [23, 160, 161] and CD28-mediated PI3K activation also contributes to IL-2 production, [162-164] it was possible that IL-2 produced by CD28-WT or CD28 Itk T cells stimulated by high concentrations of anti-CD28 was responsible for the suppression of iTreg generation, whereas CD28-PI3K or CD28 Lck T cells were unable to produce high levels of IL-2. In the presence of exogenous IL-2, anti-CD28 suppressed iTreg generation similarly from CD28-WT or CD28-Itk T cells in a dose-dependent manner, but suppression was less on CD28-PI3K T cells and was minimal on CD28-Lck T cells (Fig. 4B). These results demonstrate that strong CD28 costimulation suppresses the generation of iTregs through Lck- and PI3K-signaling independently of IL-2 production. Because CD28-mediated Lck-signaling plays the most important role in this process, we focused on the Lck-pathway for further studies.
Figure 4. CD28-mediated Lck and PI3K signals contribute to the suppression of iTreg generation. CD4^{+}CD25^{−} cells were purified from a series of Tg mice on CD28-deficient background that bear WT-CD28 or unmutated CD28 in its cytosolic tail incapable of binding to Lck (CD28-Lck), PI3K (CD28-PI3K) or Itk (CD28-Itk). Purified CD4^{+}CD25^{−} cells were then stimulated with plate-bound anti-CD3 and indicated concentrations of anti-CD28 in the presence of TGFβ. The culture was without (A) or with additional IL-2 (B). Four days after stimulation, cultured cells were harvested and measured for expression of surface CD4, CD25 and intercellular Foxp3, and CD4^{+}CD25^{−}Foxp3^{+} cells were considered as iTregs. The data show percent relevance of iTreg generation with anti-CD28 to that without anti-CD28. The numbers were presented as the mean ± 1 SD of percent relevance as pooled data from triplicate experiments.
CD28- and TCR-mediated Lck Signaling Contribute to iTreg Generation Differently

Because Lck activation can be induced by TCR- or CD28-engagement among others, we investigated the contribution of CD28- and TCR-mediated signaling in the generation of iTregs. This was addressed by using Src-specific inhibitor (pp1) in cultures of CD28-WT or CD28-Lck T cells stimulated by anti-TCR with or without anti-CD28 mAb in the absence of APCs. Lck signaling comes from the TCR in CD28-Lck T cells, whereas Lck signaling comes from both CD28 and TCR in CD28-WT T cells. In the absence of anti-CD28, pp1 reduced the generation of iTregs from CD28-WT or CD28-Lck T cells to a similar extent (Fig. 5A), indicating that TCR-mediated Lck signaling promotes the generation of iTregs in the absence of CD28 costimulation. On the other hand, in the presence of anti-CD28, pp1 restored the generation of iTregs from CD28-WT cells thereby neutralizing the anti-CD28 suppressive effect, whereas the inhibitor had no effect on the generation of iTregs with CD28-Lck cells (Fig. 5B). It is of interest that pp1 diminished iTreg generation from CD28-Lck T cells in the absence (Fig. 5B) but not in the presence of anti-CD28 mAb (Fig. 5B). The reason is not clear, but other signals (i.e. PI3K) derived from CD28 ligation through anti-CD28 might overcome the inhibition through Lck blockade as data in figure 4 suggest.
Figure 5. Lck-activation through TCR and CD28 regulates iTreg generation. CD4⁺CD25⁺ cells isolated from CD28-WT and CD28-Lck Tg mice were stimulated with plate-bound anti-CD3 in the presence of TGFβ, IL-2 and indicated concentrations of pp1. Cells were also stimulated without (A) or with (B) 40 µg/ml plate-bound anti-CD28 mAb. Four days after stimulation, cultured cells were harvested and measured for expression of surface CD4, CD25 and intracellular Foxp3. The data show mean ± 1 SD of percent Tregs (CD25⁺Foxp3⁺ among gated CD4⁺ cells) in triplicate wells, and the data represent one of 3 replicate experiments.
Effect of Inhibition of Lck Signaling on T-cell Division

Inhibition of Lck signaling might affect T-cell division and therefore interfere with iTreg generation. To address this issue, we labeled T cells with CFSE and tested how pp1 affected TCR-driven cell division. Under the culture condition with exogenous IL-2, we did not observe a significant effect on T-cell division with pp1 at the concentrations tested (Fig. 6). These data indicate that CD28-mediated Lck signaling primarily contributes to the suppression of iTreg generation, in which TCR-mediated Lck signaling played a very little role.
Figure 6. Lck activation regulates T-cell division and iTreg generation in vitro.
CFSE labeled CD4⁺CD25⁺ T cells from CD28-WT mice were stimulated with plate-bound anti-CD3 mAb in the presence of TGFβ, IL-2 and indicated concentrations of pp1. Cells were also stimulated without (upper panel) or with (lower panel) plate-bound anti-CD28 mAb. Four days after stimulation, cultured cells were harvested and measured for CD4 and Foxp3 expression and CFSE profile. The data show Foxp3 expression and CFSE profile on gated CD4⁺ cells, and represent 1 of 2 replicate experiments.
Strong CD28-signal Derived from B7 Suppresses iTreg Generation

As strong CD28 costimulation with an agonistic anti-CD28 mAb could suppress iTreg generation, we further investigated whether strong CD28 costimulation elicited by its natural ligands B7 (CD80 and CD86) would also suppress the generation of iTregs in vitro. CD4^+CD25^- T cells were purified from Foxp3^gfp^ reporter mice and stimulated with naïve or LPS-activated allogeneic B cells from BALB/c mice. As expected, LPS activated B cells expressed much higher levels of CD86 than naïve B cells (Fig. 7A). Under the culture condition with TGFβ and IL-2, ~70% of iTregs (GFP^+CD25^+) were generated from naïve CD4 T cells stimulated by allogeneic naïve B cells, but a much lower percentage of iTregs was generated when stimulated with allogeneic, LPS-activated B cells (Fig. 7B). Moreover, the addition of CTLA4-Ig to block B7:CD28 interactions increased iTreg generation in a dose-dependent manner (Fig. 7C). These results confirm that CD28-costimulation elicited by high expression of B7 on activated B cells was responsible for the suppression of iTreg generation in vitro.

We asked further questions whether iTregs induced after stimulation with B cells were suppressive and whether their activities were different when generated with resting versus activated B cells. To this end, polyclonal nTregs and iTregs generated with allogeneic B cells were isolated by FACS sorting and used as Tregs to suppress the allogeneic response of naïve B6 T cells to BALB/c APCs. As expected, nTregs suppressed alloresponse in high ratios of Treg:Teff up to 1:16. The iTregs generated with allogeneic B cells suppressed alloresponse even in low ratios up to 1:64 regardless of resting or activated B cells (Fig. 7D), confirming the function and specificity of iTregs generated in vitro.
In separate experiments, we further tested whether iTreg generation would be reduced through high levels of CD28 costimulation \textit{in vivo}. To do so, we used adoptive cell transfer model that was established previously in our laboratory [77]. Purified CD4\textsuperscript{+}CD25\textsuperscript{−} T cells from Ly5.1\textsuperscript{+} B6 Foxp3\textsuperscript{gfp} reporter mice were transferred into sublethally irradiated syngeneic WT B6 or B7.1/7.2 double deficient recipients. The percentage of iTregs generated in WT recipients was significantly higher than in B7-deficient recipients (p < 0.001), confirming our previous observation that CD28-signal at base levels is required for optimal generation of iTregs \textit{in vivo}. Under this situation, iTreg generation was significantly reduced with LPS stimulation in WT but not B7-deficient recipients (Fig. 7E). These results suggest that inflammation caused by LPS limited iTreg generation from naive CD4 T cells \textit{in vivo}, and B7/CD28 interaction was largely attributed to reduced iTreg generation. These data extended our \textit{in vitro} observations that LPS-stimulation increased B7 expression by B cells (Fig. 7 B and C) or dendritic cells (Fig. 8), and those activated APCs induced iTreg generation at much lower levels compared with non-activated APCs. Taken together, we concluded that strong CD28 costimulation signals derived from the B7:CD28 ligation suppress iTreg generation both \textit{in vitro} and \textit{in vivo}. 
Figure 7. Strong CD28-signal derived from B7 suppresses iTreg generation. A. CD86 expression is shown on resting and LPS-activated B cells (B220⁺). B. CD4⁺CD25⁺ cells isolated from Foxp3gfp reporter mice on B6 background were stimulated with resting or activated allogeneic B cells from BALB/c mice in the presence of TGFβ and IL-2. Six days after stimulation, cultured cells were harvested and measured for expression of CD4, CD25 and GFP. The data show mean ± 1 SD of percent Tregs (% CD25⁺GFP⁺ on gated CD4⁺ cells) in triplicate wells. C. The culture condition was the same as (B), except that LPS-activated B cells were used and CTLA4-Ig at various concentrations was added into the culture. The data present fold of increase in the generation of iTregs (CD25⁺GFP⁺ cells among gated CD4⁺ cells) in the culture with CTLA4-Ig relevant to that without CTLA4-Ig. The numbers were presented as the mean ± 1 SD of fold increase in triplicate wells. The data presented in each panel were reproduced in at least 3 replicate experiments.
Figure 8. Effect of CD28-mediated signaling on Treg generation. Naïve CD4⁺CD25⁻ T cells from CD28-WT and CD28-Lck mice were stimulated with BMDC in the presence of TGFβ and IL-2. CD4 and Foxp3 expression were measured 6 days after stimulation. Percentage of Foxp3 cells were shown on gated CD4 cells.
Suppression of iTreg Generation Through CD28-mediated Lck-Signal Contributes to T-cell Pathogenicity In Vivo

Lastly, we addressed whether suppression of iTreg generation mediated by CD28-costimulation would be relevant to T-cell responses *in vivo*. To this end, we compared the ability of CD4 and CD8 T cells isolated from CD28-WT and CD28-Lck mice in the induction of acute graft-versus-host disease (GVHD). Because development of nTregs is severely impaired in CD28-Lck mice, nTregs (CD4<sup>+</sup>CD25<sup>+</sup>) were removed from donor T-cell graft for a fair comparison. We observed that all the recipients of CD28-WT T cells died within 5 weeks after BMT, but the recipients of CD28-Lck T cells died significantly later and 40% survived long-term (*p* = 0.005) (Fig. 9 A and B). We hypothesized that increased iTreg generation from CD28-Lck CD4 T cells might be accounted for by the reduced GVHD mediated by CD28-Lck T cells. To test this hypothesis, we tested the ability of CD28-WT or CD28-Lck CD4 T cells in the induction of GVHD in the combination of WT CD8 T cells, where the only difference was CD4 cells. Under this situation, CD28-WT but not CD28-Lck CD4 T cells were capable of causing severe GVHD (Fig. 9C and D). To directly test the role of CD28-mediated Lck-signal in iTreg generation *in vivo*, purified CD4<sup>+</sup>CD25<sup>-</sup> T cells from CD28-WT and CD28-Lck mice were transferred to irradiated BALB/c mice. After 2 weeks, significantly higher percentages of iTregs were generated from CD28-Lck T cells than CD28-WT T cells *in vivo* (Fig. 9E). The absolute number of iTregs generated from CD28-Lck T cells was also substantially higher than that from CD28 WT T cells (2.5 ± 0.1 x 10<sup>5</sup> vs. 0.6 ± 0.5 x 10<sup>5</sup> per spleen). Thus, elevated iTreg generation was associated with diminished GVHD development. Additional or alternative mechanism
which CD28-Lck T cells induced significantly less GVHD could be resulted from impaired activation and expansion of Teffs without CD28-mediated Lck signaling. Using CFSE-labeling, we observed that both CD28-WT and CD28-Lck T cells divided rapidly in irradiated allogeneic BALB/c recipients (Fig. 9G). As expected, a subset of donor T cells produced IFNγ in the allogeneic recipients, but CD28-WT T cells had slightly higher percentage of IFNγ-producing cells than CD28-Lck T cells on CD4⁺ but not CD8⁺ cells (Fig. 9H). Taken together, these data support the hypothesis that enhanced iTreg generation with CD28-Lck T cells contributes to the decreased ability of these T cells to induce acute GVHD.
Figure 9. CD28-mediated Lck signaling regulates GVHD development and iTreg generation in vivo. BALB/c mice were lethally irradiated (800 cGy) and transplanted with $5 \times 10^6$ TCD-BM cells alone or plus $1 \times 10^6$ T cells (CD4$^+$ or CD8$^+$ CD25$^+$) from CD28-WT or CD28-Lck mice. Recipient mice were monitored throughout experimental period for survival (A) and weight change (B). Using the same BMT setting as A and B, $0.5 \times 10^6$ CD8$^+$ cells from normal B6 donors alone or plus $0.5 \times 10^6$ CD4$^+$ cells from CD28-WT or CD28-Lck mice were transplanted. Data show recipient survival (C) and weight changes (D) days after BMT. E. BALB/c mice were lethally irradiated (800 cGy) and transplanted with $5 \times 10^6$ TCD-BM cells alone or plus $1 \times 10^6$ T cells (CD4$^+$CD25$^+$) from CD28-WT and CD28-Lck mice. Two weeks after transplantation, recipient spleens were harvested and measured for expression of surface CD4, CD25 and intracellular Foxp3. Percentage of Tregs (CD25$^+$Foxp3$^+$) on gated donor T cells (H2K$^b$CD4$^+$) is shown in each mouse of total 3 mice per group, and the data represent 1 of 3 replicate experiments. In separate experiments, CFSE-labeled T cells (CD4$^+$ or CD8$^+$ CD25$^+$) isolated from CD28-WT and CD28-Lck mice were transplanted into lethally irradiated (800 cGy) BALB/c mice at $2 \times 10^6$ per mouse. Four days after transplantation, recipient spleens were harvested and measured for CFSE profile, expression of surface CD4 and H2K$^b$, and intracellular IFN$\gamma$. Expression of CD4 and H2K$^b$ was shown on live spleen cells (F). CFSE profile (G) and intracellular IFN$\gamma$ expression (H) were shown on gated donor CD4 (H2K$^b$CD4$^+$) or CD8 (H2K$^b$CD4$^+$) T cells. The data represent 1 of 2 replicate experiments.
Discussion and Conclusion

The data presented in this report indicate that strong CD28 costimulation suppresses the generation of iTregs from naïve CD4 T cells, primarily through Lck-signaling. It is well established that CD28 costimulation positively regulates T-cell response by promoting IL-2 production, cell cycle entrance, activation and survival of T cells [82, 165]. On the other hand, CD28 costimulation plays a critical role in development of nTregs in thymus and in maintenance of nTregs in the periphery, by which CD28 contributes to maintaining self-tolerance [166]. In addition, our previous work indicates that low or base level of CD28 costimulation is required for iTreg generation through IL-2 production primarily mediated by CD28-mediated Lck [77]. The current finding adds a new facet to the regulatory function of CD28 costimulation, which at high levels can promote T-cell response by shutting down iTreg generation from naïve CD4 T cells. This new concept fits well with the overall mission of T-cell responses. Under acute infection, high levels of CD28 costimulation not only promote activation and expansion of effector T cells, but also suppress generation of iTregs that permits effective immune responses against infectious agents. In contrast, under quiescent situations or in cancer, low levels of CD28 costimulation not only limit activation and expansion of effector T cells, but also promote homeostasis of nTregs and generation of iTregs permitting immune tolerance to self or tumor antigens.

Efficient development of nTregs in the thymus relies on CD28 costimulation through its cytosolic Lck-binding motif and c-Rel leading to activation of NF-κB [78-81]. Although the same motif is required with CD28 costimulation for IL-2 production, CD28-mediated nTreg development is independent of IL-2 production [23]. Ironically,
our current work demonstrates that the same Lck-binding motif is again required for the suppression of iTreg generation mediated by strong CD28-costimulation independently of IL-2 production. Thus, the same CD28-mediated Lck-signal exclusively of IL-2 production is responsible for efficient development of nTregs in the thymus, but also for the control of iTreg generation in the periphery. Taken together, we propose that, through Lck- and Rel-dependent signaling pathway, CD28 costimulation at base level is required for nTreg development in the thymus and iTreg generation under quiescent situation, whereas CD28 costimulation at high levels limits iTreg generation during active immune responses. How the CD28-mediated Lck-signal regulates these two distinct processes is currently unclear, although apparently T cells are in different stages (immature vs. mature) and CD28-costimulation is at different levels (base vs. elevated).

Interestingly, although TCR-ligation also activates Lck, the TCR-mediated Lck-signal promoted rather than suppressed the generation of iTregs (Fig. 5). Under the culture condition specified in this report, high levels of TCR-stimulation (i.e. high concentrations of anti-CD3 mAb) without CD28-costimulation were not suppressive to iTreg generation (data not shown). The different outcomes may have resulted from the distinguished patterns of Lck activation mediated by TCR and CD28, respectively. Upon TCR engagement, Lck is recruited and remains only transiently in the immunologic synapse. Subsequently, Lck's SH3 domain interacts with C-terminal proline motif of CD28, which disrupts formation of potentially inhibitory intramolecular Lck interactions between the Lck SH3 domain and its kinase domain that interfere with Lck activity [167]. In this way, CD28 costimulation greatly increases both the intensity and duration of Lck
activity at the immunological synapse of TCR-engaged T cells, which may be critically important for suppressing the generation of iTregs.

A recent study by Gottschalk et al elegantly demonstrate that TCR ligand density and potency determine induction of iTregs in the periphery, and conclude that a low density of a strong TCR agonist is optimal to induce a persistent generation of iTregs in vivo [168]. Their study was focused on T cell responses under non-inflammatory condition, where CD28 costimulation was at low or base level. Because CD28 costimulation reduces the extent of TCR ligation required for effective T-cell responses likely by lowering the threshold of TCR signal transduction for T-cell activation and promoting the formation of an immunological synapse [169, 170]. CD28 regulates iTreg induction could be through modifying TCR signaling. However, we propose that the combined signals through TCR and CD28 determine the proper levels of iTreg induction and Teff generation from naïve CD4 T cells.

Using a GVHD model, we showed that CD28-Lck donor T cells caused significantly less GVHD than WT donor T cells (Fig. 7A and 7B). Furthermore, in combination with WT CD8 T cells, WT CD4 T cells induced severe GVHD whereas CD28-Lck CD4 T cells failed to do so (Fig. 7C and 7D). Because significantly higher rate of iTregs were generated from CD28-Lck than WT CD4 T cells (Fig. 7E), we conclude that enhanced generation of iTregs contributed to decreased GVHD induced by CD28-Lck donor T cells although lower effector function might still be a part of the mechanism. Our data differ from the observation that CD28 costimulation was paradoxically inhibitory in MHC class II-mismatched murine cardiac graft rejection and in the development of autoimmune diabetes in NOD mice [74, 171]. The outcomes in
these 2 studies reflect the important role of CD28 costimulation at base levels in the
generation and maintenance of Tregs under non-inflammation situation. In contrast,
inflammation is created under myeloablative allogeneic BMT, in which CD28
costimulation presumably at high levels would in turn limit the generation of iTregs and
facilitate GVHD development.

In summary, in understanding how CD28 costimulation promotes productive T-
cell response, our study reveals a novel mechanism that CD28 costimulation promotes
immunity by suppressing the generation of iTregs in the periphery. CD28 costimulation
executes this negative regulation on iTregs through its Lck-binding motif independently
of IL-2 production. Given CD28 provides a predominant costimulation and Tregs is one
of the most critical regulatory components in many if not all aspects of immunology, the
current finding may provide the rationale for promoting T-cell immunity or tolerance by
regulating iTreg induction through targeting CD28-signaling in many immunologic
responses under autoimmunity, transplantation and cancer development.
CHAPTER 4.

EFFICIENT AND SELECTIVE PREVENTION OF GRAFT–VERSUS-HOST DISEASE BY ANTIGEN-SPECIFIC INDUCED TREGS VIA LINKED SUPPRESSION

Abstract

Naturally occurring regulatory T cells (nTregs) suppress the development of GVHD and may spare graft-versus-leukemia (GVL) effect [35]. Because nTreg is a rare population in a healthy individual, the limited source and the non-selective suppression are major hurdles towards the application of nTregs in the control of clinical GVHD after allogeneic HCT. An alternative approach is to generate induced Tregs (iTregs) from naïve CD4 precursors, but the effectiveness of iTregs in the control of GVHD is highly controversial and requires further investigation. The other critical but unsolved issue on Treg therapy is how to achieve antigen (Ag)-specific tolerance that distinguishes GVHD and GVL effect. To address the important issues on the effectiveness of iTregs and Ag-specificity of Tregs, we generated Ag-specific iTregs and tested their potential in the prevention of GVHD in pre-clinical BMT model. CD4⁺CD25⁺Foxp3⁺ iTregs generated from OT-II TCR transgenic T cells specific for OVA target Ag efficiently prevented GVHD induced by polyclonal T effector cells (Teffs) only in the allogeneic recipients that express OVA protein but not in OVA⁻ recipients. The efficacy of these Ag-specific
iTregs was significantly higher than polyclonal iTregs. As controls, OT-II CD4\(^+\)Foxp3\(^-\) cells had no effect on GVHD development in OVA\(^-\) recipients and exacerbated GVHD in OVA\(^+\) recipients when transplanted together with polyclonal Teffs. Because the iTregs recognize OVA whereas Teffs recognize alloAg bm12, our data reveal for the first time that Tregs prevent GVHD through a linked suppression. Mechanistically, OT-II iTregs expanded extensively, and significantly suppressed expansion and infiltration of Teffs in OVA\(^+\) but not in OVA\(^-\) recipients. These results demonstrate that Ag-specific iTregs can prevent GVHD efficiently and selectively, providing a proof of principle that Ag-specific iTregs may represent a promising cell therapy for their specificity and higher efficacy in allogeneic HCT.

**Background and Rationale**

Allogeneic BMT or HCT offers great promise for the treatment of a variety of diseases including cancer, autoimmunity, aplastic anemia, and other hematopoietic diseases. However, GVHD remains the major complication following this therapeutic procedure because it leads to high morbidity and mortality in patients [84, 108]. Despite the magnitude of this complication and the extensive efforts to overcome this problem, no clinical strategy has been established to efficiently prevent GVHD without producing a broad immune suppression. Recent evidence indicates that the use of Tregs (CD4\(^+\)Foxp3\(^+\)) is one of the promising approaches to control GVHD in numerous mouse models [34-39] in addition to early clinical trials [40, 41].

Although it is widely accepted that natural CD4\(^+\)Foxp3\(^+\) Tregs are developed in
the thymus (termed nTregs), accumulating evidence suggests that T cells with regulatory function may also arise in the periphery under certain conditions and are termed induced Tregs (iTregs). The full extent of differences and similarities between iTregs and nTregs has not yet been defined [172]. Due to the infrequency of nTregs in the peripheral blood and the difficulty in isolating sufficient nTregs with adequate purity, much attention has been placed on the use of in vitro-expanded nTregs with emphasis on retaining their regulatory capabilities. Other studies have focused on iTregs generated from naive CD4+CD25- cells to obtain a regulatory cell population to suppress immune responses in vitro and in vivo. However, the use of iTregs as an immunotherapy is still controversial concerning their stability in Foxp3 expression [142, 173-176].

Because Tregs need to be activated by their specific antigen (Ag) to exert their suppressive function, it is understood that polyclonal populations of Tregs will only have limited efficacy on a per cell basis to regulate allogeneic responses due to the low frequency of alloantigen-reactive Tregs within the whole population. Although large numbers of polyclonal Tregs are capable of preventing GVHD in rodents, broad polyclonal suppression is expected. Therefore, Ag specificity of Tregs is critical to selective suppression mediated by these cells. In experimental autoimmune disease models, Ag-specific Tregs are highly effective in controlling autoimmune diabetes, gastritis and encephalomyelitis [137, 138, 177]. However, the advantage of using Ag-specific Tregs in the prevention of GVHD has not yet been investigated.

We previously generated Ag-specific iTregs by foxp3 transduction and demonstrated that they persist long-term in vivo and suppress GVHD in a non-
myeloablative BMT model when activated by the cognate Ag; either constitutively expressed or introduced via immunization [142]. In our previous study, however, a non-myeloablative BMT model was used that is not representative of clinical HCT, and iTregs were generated through gene transfection. In the current study, we addressed these two important issues and demonstrate that TGFβ-induced, Ag-specific iTregs efficiently and selectively prevent GVHD in a murine model of myeloablative BMT.

**TGFβ-induced, Ag-specific iTregs Prevent GVHD in an Ag-dependent Manner**

Recent progress made by many groups including ours indicates that iTregs can be generated from naïve CD4 T cells upon TCR stimulation in the presence of TGFβ [55, 77, 157]. iTregs are effective in suppressing autoimmune diseases, but their effect in controlling GVHD is controversial and remains to be further investigated. For this reason, we generated OT-II TCR Tg and foxp3/gfp KI mice by cross-breeding. OVA-specific iTregs were then generated from OT-II Tg and foxp3/gfp KI CD4⁺CD25⁺ T cells by stimulating them with OVA peptide in the presence of TGFβ (Fig. 10).

We then tested whether OVA-specific iTregs (CD4⁺CD25⁺GFP⁺) were able to prevent GVHD induced by polyclonal T cells in a B6 → (B6 x bm12)F1 BMT model, in which donor CD4⁺ T cells (Teffs) recognize mismatched recipient MHC II alloAg (H₂bm12). To specifically activate iTregs, (B6.OVA x bm12)F1 mice were used as recipients that ubiquitously express OVA. The bm12 mutation can present OVA peptide, but OT-II T cells cannot recognize this MHC/peptide complex. In this setting, Teffs at indicated dose induced 50% GVHD lethality. Similar numbers of OVA⁺ and OVA⁻
recipients were used for the Teff alone group, but the same results was observed in survival or weight loss regardless of OVA expression (data not shown). Additional iTregs completely prevented GVHD lethality in OVA\(^+\) (p = 0.01) but not in OVA\(^-\) recipients (p = 0.8) (Fig. 11), indicating that activation of iTregs was required for their suppressive function. CD4\(^+\)GFP\(^-\) control cells had no effect on GVHD in OVA\(^-\) recipients, or even accelerated GVHD in OVA\(^+\) recipients as Teffs (Fig. 11). These results demonstrate that Ag-specific iTregs are potent in suppressing GVHD in an activation-dependent manner. Because the iTregs recognize OVA whereas Teffs recognize alloAg bm12, these data reveal that Tregs prevent GVHD through a linked suppression.
**Figure 10. Isolation of iTregs and nTregs.**

A, generation and purification of iTregs. CD4⁺CD25⁻ cells were purified from spleen and lymph node of OT-II TCR Tg and foxp3/gfp KI mice. These purified T cells were stimulated with OVA peptide at 0.5 µM in the presence of irradiated TCD-splenocytes. TGFβ was added in the culture at 2 ng/ml for Treg generation. Four to six days after culture, cells were harvested and stained for CD4, CD25 and GFP expression. The phenotype of cultured cells is shown on gated live cells (2 left panels). CD4⁺ CD25⁺GFP⁺ and CD25⁺GFP⁻ cells were separated by FACS sorting (2 right panels).

B, purification of nTregs. CD4⁺ cells were isolated through negative selection from spleen and lymph node of B6 foxp3/gfp KI mice. These CD4⁺ enriched cells were stained for CD4 and CD25 expression. The phenotype of these cells is shown on gated live cells (2 left panels). CD4⁺ CD25⁺GFP⁺ and CD25⁺GFP⁻ cells were separated by FACS sorting (2 right panels).
Figure 11. The effect of TGFβ-induced Tregs in GVHD. OVA⁺ or OVA⁻ (B6 x bm12)F1 mice were lethally irradiated and transferred with 5 x 10⁶ TCD-BM alone or plus 1 x 10⁶ CD4⁺ T cells (Teffs) from B6 donors. OVA-specific iTregs (CD4⁺CD25⁺GFP⁺) were generated and purified by FACS sorting as shown in figure 1. OVA-specific iTregs or controls at 0.5 x 10⁶/mouse each were added into donor graft. Recipient survival (A) and body weight changes (B) are shown. Ten recipients were included in each group except that 5 mice were used in GFP⁺ or GFP⁻ cells to OVA⁻ groups. The data are pooled from 2 replicate experiments using a similar setting.
TGFβ-induced, Ag-specific iTregs are Significantly More Effective in the Prevention of GVHD than Polyclonal iTregs

To further evaluate the potency of OVA-specific iTregs in the prevention of GVHD, these iTregs were used at 1:4 or 1:8 ratio of Treg: Teff. We found that GVHD lethality was completely prevented at either cell dose (Fig. 12 A and B). To compare the potency of Ag-specific versus non Ag-specific iTregs, polyclonal iTregs were generated from CD4<sup>+</sup>CD25<sup>-</sup> cells of B6 foxp3/gfp KI mice by stimulating with anti-CD3 mAb in the presence of TGFβ as shown in our previous work [77]. In contrast to Ag-specific iTregs, the polyclonal iTregs had a partial effect only at 1:2 ratio of Treg:Teff in suppressing GVHD (Fig. 12C and D). These data indicate that Ag-specific iTregs are ~ 8-fold more effective than polyclonal iTregs in GVHD prevention.
Figure 12. The potency of Ag-specific and polyclonal iTregs in suppressing GVHD. A and B, OVA\(^+\) (B6 x bm12)F1 mice were lethally irradiated and transferred with TCD-BM alone or plus 1.6 x 10\(^6\) CD4\(^+\)CD25\(^-\) T cells (Teffs alone) from B6 donors. OVA-specific iTregs (CD4\(^+\)CD25\(^+\)GFP\(^+\)) were generated from OT-II T cells and were added at 0.2 or 0.4 x 10\(^6\) each into donor graft. C and D, OVA\(^-\) (B6 x bm12)F1 mice were lethally irradiated and transferred with TCD-BM alone or plus 1 x 10\(^6\) CD4\(^+\)CD25\(^-\) T cells (Teffs alone) from B6 donors. Polyclonal iTregs (CD4\(^+\)CD25\(^+\)GFP\(^+\)) generated from WT B6 T cells with anti-CD3 stimulation plus TGFβ were added at 0.25 or 0.5 x 10\(^6\) each into donor graft. Recipient survival (A and C) and body weight changes (B and D) are shown. Five or six recipients were included in each group for both experiments.
Ag-specific iTregs Suppress the Expansion, Activation and Infiltration of Teffs In Vivo

We next assessed the suppressive effects of Ag-specific iTregs on 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. Because low dose of Teffs (5 x 10⁵/mouse) was transferred into B6 mice (black) that are less sensitive for signal detection, no significant BLI signal was detected on day 7. The BLI detected on day 17 and 28 demonstrate that additional OT-II iTregs significantly reduced Teff expansion in OVA-expressing recipients (Fig. 13A and B). The distribution of the BLI signal suggests that the Teffs infiltrated more broadly to liver and gut without iTregs whereas Teffs were more constrained in spleen with iTregs (Fig. 13A).
Figure 13. The effect of Ag-specific iTregs on expansion and infiltration of Teffs. Lethally irradiated OVA⁺ (B6 x bm12)F1 mice were transplanted with B6 TCD-BM plus 0.5 x 10⁶/mouse Teffs (CD4⁺CD25⁺) isolated from Luc-Tg mice on B6 background. One group of recipients was also transferred with additional 0.25 x 10⁶/mouse OT-II iTregs (CD4⁺CD25⁺GFP⁺). Donor Teffs were monitored in recipient mice 17 and 28 days after BMT. A, animals were imaged from the ventral position for quantification of donor T cells. B, the average of relative signal intensity of 4 mice per group, and the data represent one of 2 replicate experiments.
To further evaluate the effect of iTregs on expansion and activation of Teffs, we transferred Teffs isolated from B6 Ly5.1+ mice and iTregs generated from Thy1.1+ OT-II CD4 precursor (1:2 ratio of Treg: Teff) along with TCD-BM isolated from normal B6 donors into OVA+ or OVA− (B6 x bm12)F1 recipients. Seven days after BMT, we measured Teffs (CD4+Ly5.1+) in recipient spleen and liver (Fig. 14A and D). There was an average of 1.9 ± 0.4 x10^6/mouse Teffs in the spleen of the recipients transferred with Teffs alone, 0.9 ± 0.1 x10^6 in the OVA+ recipients transferred with Teffs plus iTregs, and 1.8 ± 0.8 x10^6 in the OVA− recipients transferred with Teffs plus iTregs, respectively (Fig. 14B). The data indicate that iTregs significantly reduced Teff expansion in the OVA+ (p = 0.005) but not the OVA− recipients (p = 0.8). In the liver, the number of Teffs was also significantly lower in the OVA+ recipients transferred with Teffs plus iTregs than those with Teffs alone (Fig. 14E, p = 0.004), suggesting that iTregs reduced Teff expansion and/or infiltration in recipient liver, a major GVHD target organ. Because peripheral lymphoid organs are important for T cell activation, we examined the migration of iTregs to recipient lymph nodes and spleen relevant to antigen stimulation in vivo. In a separate experiment, we observed that the percentages of iTregs among CD4+ T cells were 36.3 ± 5.3% vs. 17.1 ± 3.1% in lymph nodes and spleen of OVA+ recipients, respectively (n = 4, p = 0.0007). However, the percentages of iTregs among CD4+ T cells were less than 1% in lymph nodes and spleen of OVA− recipients (n = 4, p = 0.08). These results suggest that Tregs preferentially reside in lymph nodes upon antigen stimulation.

To evaluate the activation of Teffs, we measured intracellular expression of IFNγ and IL-17, and calculated the numbers of IFNγ- and IL-17-producing Teffs in the
recipient spleen. The number of IFNγ-producing Teffs in the OVA⁺ recipients transferred with Teffs plus iTregs was significantly lower than that in the recipients of Teffs alone (p = 0.005), whereas there was no difference between the recipients with Teffs alone and those OVA⁻ recipients with Teffs plus iTregs (p = 0.9) (Fig. 14C). There were very few Teffs that produced IL-17 (< 2%) and no significant difference among those groups (data not shown). These results indicate that iTregs also reduced Teff activation when iTregs were activated by specific Ags.
Figure 14. Effects of Ag-specific iTregs on expansion and activation of Teffs. Teffs cells (CD4⁺CD25⁺) were isolated from WT Ly5.1⁺ donors and transferred at 1 x 10⁶/mouse together with TCD-BM into lethally irradiated OVA⁻ or OVA⁺ (B6 x bm12) F1 mice. The other 2 groups were transferred with OT-II Thy1.1⁺ iTregs at 0.5 x 10⁶/mouse into OVA⁻ or OVA⁺ (B6 x bm12) F1 recipients. Seven days after BMT, recipient spleen (A-C) and liver (D and E) were harvested for measuring expansion and activation of donor Teffs. A, top panels show percentages of CD4⁺ cells in live cells, and bottom panels show expression of Ly5.1 (Teffs’ marker) and Thy1.1 (iTregs’ marker) on gated CD4⁺ live cells in recipient spleen. B, absolute numbers of Teffs (CD4⁺Ly5.1⁺) are shown in average ± 1 SD. C, spleen cells were also measured for intracellular expression of IFNγ, and absolute numbers of IFNγ⁺ Teffs (CD4⁺Ly5.1⁺) are shown in average ± 1 SD. D, top panels show percentages of CD4⁺ cells in live cells, and bottom panels show expression of Ly5.1 (Teffs’ marker) and Thy1.1 (iTregs’ marker) on gated CD4⁺ live cells in recipient liver. E, absolute numbers of Teffs (CD4⁺Ly5.1⁺) in the liver are shown in average ± 1 SD. Each group includes 3 or 4 mice, and the data represent 1 of 3 replicate experiments.
After Adoptive Transfer In Vivo, iTregs Expanded to Higher Numbers While nTregs had More Stable Expression of Foxp3

We assessed the expansion and stability of iTregs in vivo. In experiments with the same setting as in figure 14, OT-II iTregs (CD4+Thy1.1+) expanded extensively in OVA+ but not OVA− recipients (Fig. 15A and B, p < 0.001). To further compare the expanding potential between iTregs and nTregs, we isolated polyclonal nTregs (CD4+CD25+GFP+) from naïve B6 foxp3/gfp KI mice (Ly5.2+) as standard controls (Fig. 9B). The expansion levels for OT-II iTregs in OVA+ recipients were significantly higher than that of nTregs (Fig. 15A and B, p = 0.001), indicating that Treg expansion depended on Ag-stimulation in vivo.

Recent publications suggest that iTregs are less stable than nTregs in maintaining Foxp3 expression. To address this concern, we gated on CD4+Thy1.1+ Tregs and analyzed their Foxp3/GFP expression. Because Tregs were highly purified through FACS sorting for GFP expression (Fig. 9A), the percentage of GFP− cells in gated CD4+Thy1.1+ cells would reflect the loss of Foxp3 expression. Polyclonal nTregs (CD4+CD25+GFP+) from naïve B6 foxp3/gfp KI mice were also used as standard controls. Under myeloablative allogeneic BMT, average of 43.6 ± 5.4% nTregs kept their GFP expression 7 days after cell transfer, whereas 29.4 ± 2.8% and 24.8 ± 2.8% iTregs kept their GFP in OVA+ and OVA− recipients, respectively (Fig. 15D and E). Foxp3 expression was less stable in iTregs than nTregs (p = 0.003), whereas the stability of iTregs was similar in the recipients regardless of OVA expression (Fig. 15D and E). To measure activation of Tregs, intracellular IFNγ and IL-10 were measured. We found that 7 days after BMT
there was an average of 20.0 ± 3.4% and 4.1 ± 0.5% IFNγ+ cells among Ag-specific iTregs and polyclonal nTregs, respectively. Furthermore, the number of IFNγ+ Ag-specific iTregs was significantly more in the OVA+ than OVA- recipients and significantly more than that of nTregs in recipient spleen (Fig. 15C, p < 0.001). In conclusion, Treg expansion depended on Ag-stimulation and iTregs were activated and expanded more extensively than nTregs, but iTregs were less stable than nTregs in Foxp3 expression upon Ag-stimulation under myeloablative allogeneic BMT.
Figure 15. Expansion and stability of Tregs in the recipients after allogeneic BMT. Experimental setting is the same as described in figure 5. One additional group of recipients was transferred with 0.5 x 10^6/mouse nTregs isolated from naïve B6 foxp3/gfp KI mice (Ly5.2^+Ly5.1^−). A, percentages of Thy1.1^+Ly5.1^− (iTregs) or Ly5.2^+Ly5.1^− cells (nTregs) on gated CD4^+ live cells in recipient spleen. B, absolute numbers of iTregs or nTregs are shown. C, absolute numbers of IFNγ^+ iTregs or nTregs are presented per spleen. D, GFP expression on gated iTregs or nTregs in recipient spleen. E, percentages of GFP^+ cells among gated iTregs or nTregs are shown in average ± 1 SD. Each group includes 3 or 4 mice, and the data represent 1 of 3 replicate experiments.
Discussion and Conclusion

Besides regulating autoimmunity, CD4⁺CD25⁺ Tregs also control allogeneic responses. Therefore, research on understanding and applying Tregs in the setting of HCT has been an active field in recent years. Due to low frequency of nTregs, current approaches in attempt to apply Tregs in clinical HCT are focused on adoptive transfer of polyclonal, *ex vivo* expanded, nTregs into transplant recipients before or after stem cell transplantation. Isolating and expanding polyclonal nTregs is feasible [178, 179]; however, questions remain about their efficacy and the consequences of broad immune suppression *in vivo*. E.g. these polyclonal nTregs may have a low potency in controlling GVHD and produce non-selective immune suppression without discriminating for GVH and GVL reactions.

The current study is aimed at increasing the potency and selectivity of Treg therapy. By using TGFβ-induced Ag-specific iTregs, we showed that Ag-specific iTregs were highly effective in preventing GVHD in a clinically relevant murine model of allogeneic BMT in an Ag-dependent manner (Fig. 11). The current study substantially extended the previous work by us and others showing that *in vitro* generated iTregs were effective in suppressing allogeneic responses in bone marrow or solid organ transplantation [142, 173-175]. However, our result is in contrast to a recent report by Konencke et al. that TGFβ-induced polyclonal iTregs were not effective in preventing GVHD presumably due to the instability of Foxp3 expression [176]. We interpret that the differences in the protocol of generating iTregs, the specificity of iTregs and GVHD model may contribute to the distinct outcome in these two studies. Higher levels in
expression of Ag-specific iTregs were likely resulted from higher levels of Ag-driven proliferation and less dependent on Ag and cytokine signals in recipients of pre-activated and dividing iTregs versus resting nTregs.

A potential concern is that iTregs may not have stable Foxp3 expression due to their status of epigenetic modification and lose their suppressive activity in vivo [180]. In fact, some studies have showed that in vitro generated iTregs were less suppressive than nTregs [181, 182]. However, there is also substantial evidence in the literature supporting that iTregs were as or more effective than nTregs in suppressing immune responses in vivo [55, 66, 137, 177, 183-186]. To address this concern on iTreg stability, we directly compared Foxp3 stability of iTregs and nTregs and observed that iTregs were less stable than nTregs in Foxp3 expression under allogeneic BMT (Fig. 6D and E). However, iTregs underwent substantially higher levels of Ag-driven expansion than nTregs (Fig. 15B), which may compensate for their inferior stability relative to that of nTregs. Remarkably, we showed that Ag-specific iTregs were able to prevent GVHD in 100% recipients at 1:8 ratio of Tregs to Teffs (Fig. 11A). In contrast, using the same murine BMT model where BM plus CD4+ T cells were transplanted into lethally irradiated bm12 recipients, Taylor et al. indicated that in vitro activated and expanded, polyclonal CD62L^{high} nTregs could prevent GVHD in nearly 100% at 3:1 ratio of Tregs to Teffs [38]. Taken together, these data indicate that Ag-specific iTregs can be ~24-fold more effective than the most potent polyclonal nTregs tested so far. Considering the frequency of alloreactive T cells, we observed that significantly more Ag-specific iTregs produced IFNγ after activation by cognate Ag than polyclonal nTregs after activation by alloantigens (20% vs. 4%), confirming that Ag-specific iTregs were more activated than
polyclonal nTregs. Because IFNγ production by Tregs is critical for their suppressive function \textit{in vivo} [187], high level of IFNγ production by Ag-specific iTregs also correlated with their superior suppressive activity to polyclonal nTregs.

A fundamental issue regarding Treg-mediated suppression not yet being addressed is whether Tregs execute their regulatory function through Ag-specific, Ag-linked or bystander suppression \textit{in vivo}. The current study made it clear that iTregs must be activated by their cognate Ag \textit{in vivo} in order for them to exert their suppressive function and to control GVHD (Fig. 11 and 12). Because iTregs recognize nominal Ag (OVA) whereas Teffs recognize allo-Ags (bm12), the results indicate that iTregs do not have to recognize the same Ag as Teffs for Tregs to suppress the responses elicited by the Teffs \textit{in vivo} and strongly support the notion that linked suppression is operational under allogeneic BMT settings. Our data are consistent with the results reported by Tang et al. that monoclonal Tregs (BDC2.5 TCR Tg) specific for an islet Ag are highly effective in controlling experimental diabetes induced by polyclonal diabetogenic Teffs [138]. These studies indicate that Treg-mediated immunosuppression does not have to be exclusively Ag-specific, which seems contradictory with the results observed by Joffre et al [96] or those by Zhang et al [137]. Using BM rejection model, Joffre et al. showed that Tregs specific for donor alloAgs selectively prevent rejection of donor BM but not third-party BM, both of which were transplanted into the same recipient [96], suggesting that Treg-mediated suppression is Ag-specific. Likewise, using an EAE model, Zhang et al. showed that myelin proteolipid protein (PLP)\textsubscript{139-151}-specific iTregs were effective at suppressing EAE induced by the cognate (PLP)\textsubscript{139-151} peptide, but not by (PLP)\textsubscript{178-191} peptide or even a mixture of the 2 peptides [137]. It is not clear why Tregs mediated suppression with
exquisite Ag-specificity in some studies but not the others. What is clear is that Tregs can induce Ag-specific or Ag-linked suppression but not bystander suppression in vivo. No bystander suppression in vivo is also evident in which the generation of donor-reactive iTregs prevents graft rejection without compromising immunity to a viral pathogen [188].

Isolating and expanding polyclonal nTregs has been shown to be feasible [178, 179]; however, questions remain about their efficacy and Ag specificity in vivo. E.g. although they can be expanded multi-fold in vitro, generating the absolute number of Tregs needed to treat a patient successfully may still be a challenge [189]. We want to emphasize that, unlike polyclonal alloreactive Tregs expanded with allogeneic APCs in vitro [133, 190, 191] or induced in vivo [12, 192, 193], the Ag-specific Tregs investigated in the current study are monoclonal and each of them specifically recognizes the cognate Ag, which likely contributes to the high efficacy of these cells in suppressing GVHD. In this proof-of-concept study the iTregs are monoclonal and uniformly recognize the cognized antigen with high affinity, thus caution should be noted from a translational perspective, as the results could be different with a population of polyclonal antigen-specific iTregs. Our current effort focuses on evaluating the effects of polyclonal iTregs specific for MHC or miHA antigens for better translational potential. The current study also provides evidence that iTregs prevent GVHD through linked suppression in an Ag-activation dependent manner, which likely has a broad impact in understanding how Tregs execute their suppressive function under biological or pathological situations. In clinical application, this finding indicates that iTregs specific for a miHA restricted on parenchymal tissues can distinguish GVHD versus GVL. Although creating Ag-specific Tregs is facilitated by the use of TCR Tg cells in mice, this approach will be more
challenging in humans. However, the approach can be applied in the clinic to treat hematological tumors by generating and using iTregs specific for restricted miHAs on GVHD target tissues, because human T cells can be primed by miHAs in vitro. In conclusion, this study provides a proof of principle that Ag-specific iTregs may represent a promising Treg therapy for their specificity and higher efficacy in allogeneic HCT.
CHAPTER 5:
CONCLUDING REMARKS

CD28 costimulation through Lck signaling is required for nTreg generation in the thymus [23] and for iTreg generation in the periphery [77]. In the current study we found that strong CD28 costimulation suppresses the generation of iTregs from naïve precursors through Lck signaling. The Lck signaling pathway is present both in TCR signaling and in CD28 costimulation. We have shown that CD28-mediated Lck signaling is required for the suppression of iTreg generation in the periphery, while the TCR-mediated Lck signaling is primarily associated with iTreg generation (Fig. 5). We have also shown that Teffs from mice with abrogated CD28-mediated Lck signaling induce less GVHD than mice with intact CD28-mediated Lck signaling, which indicates an important role of CD28-mediated Lck signaling in GVHD induction. Recipient mice that received Teffs from CD28-Lck mice exhibited increased iTreg generation compared with Teffs from CD28-WT mice (Fig. 9), which indicate that diminished GVHD development is associated with elevated iTreg generation. These findings have implications for therapy since the Lck signaling can be targeted to regulate immunity or tolerance. Drugs targeting kinases for the treatment of inflammation and autoimmune disorders have become a focus for some pharmaceutical and biotech companies [194]. It was reported that inhibition of Lck with small molecules has significant potential for therapeutic immunosuppression [195]. Several studies have been done using small molecule Lck
inhibitors. One such study used AA-770041, a novel and selective small molecule inhibitor of Lck which prevents heart allograft rejection across major histocompatibility barriers for at least 60 days [196]. Dasatinib is an oral small molecule inhibitor of Abl and Src family tyrosine kinases (SFK), including p56<sup>Lck</sup> (Lck) and was recently approved for the treatment of adults with chronic myeloid leukemia (CML) and resistance or intolerance to prior therapy, including imatinib [197-199]. It was demonstrated that Dasatinib inhibits TCR-mediated signal transduction, cellular proliferation, cytokine production, and in vivo T cell responses. However, in this study, the signal transduction and proliferative responses via IL-2 remained essentially intact, suggesting that Dasatinib displays specificity for TCR signaling [200]. The Lck signaling pathway plays an important role in CD28 co-stimulation as well as in TCR signaling. The Lck kinase activity is critical for TCR-mediated signaling, leading to normal T-cell development and activation [201]. Inhibition of Lck is expected not only to suppress activation of Teffs, but also to increase the generation of iTregs. Thus, given the potent ability and specificity of Dasatinib to inhibit Lck activity, the effect of Dasatinib in the development of GVHD should be investigated.

Our data indicates that CD28-mediated PI3K signaling may play a role in the suppression of iTreg generation in the periphery (Fig.4). The PI3K-regulated pathway is one of the major signaling pathways and is activated by TCR, IL-2R, and CD28 stimulation, leading to T-cell activation, proliferation, and cell survival [83]. Akt, a serine-threonine kinase, is a key downstream effector of the PI3K pathway which in response to PI3K activation, phosphorylates and regulates the activity of various targets including kinases, transcription factors and other regulatory molecules. Akt functions as a
key regulator of various critical cell functions including glucose metabolism, cell proliferation and survival. Therefore, the role of PI3K in iTreg suppression should be investigated.

The application of alloantigen-specific iTregs for the prevention of GVHD following allogeneic HCT seems very promising. Our results show a proof-of-principle that Tregs that are specific for peptides in the recipient can efficiently protect against polyclonal Teffs. The focus must now be on generating Tregs with specificity for miHA present on the epithelial tissues of recipients and testing their effects in similar transplantation models. The goal is to generate Tregs that are specific for ubiquitous alloantigens present on epithelial tissues but will spare hematopoietic antigens. This system can be easily translated to the clinic and holds promise to prevent GVHD and spare the GVL effect. The challenge will be to identify miHA that are present on epithelial tissues but absent on hematopoietic tissues.
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