

THE STIMULATORY ROLE OF ICOS IN THE DEVELOPMENT OF
CD146⁺CCR5⁺ T CELLS CO-EXPRESSING IFN- γ AND IL-17 DURING GRAFT-
VERSUS-HOST DISEASE

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Graft-versus-host disease (GVHD) remains the major complication after allogeneic hematopoietic stem cell transplantation (HSCT), resulting from immunological attack on target organs such as gastrointestinal (GI) tract, liver and skin from donor allogeneic T cells. The most common treatment for GVHD is immunosuppressive drugs such as corticosteroids, which may result in many side effects including the loss of the beneficial graft-versus-leukemia (GVL) effect and increased infection rates. However, GVHD-specific drugs have yet to be implemented. Here we show that by targeting on a novel pathogenic CD4⁺ T cell subpopulation that our lab previously found in patients with GI GVHD, we can develop new avenues to treat GVHD. This novel population is characterized as CD146⁺CCR5⁺ T cells, co-expressing IL-17A and IFN- γ . We found that the inducible T-cell costimulator (ICOS), which has been reported to be important for human Th17 differentiation *in vitro*, is critical for the development of this nonconventional T Helper 1 (Th1*)-polarized CD146⁺CCR5⁺ conventional T cells (Tconvs) population. Furthermore, we found that ICOS can induce the generation of Th1*-polarized CD146⁺CCR5⁺ regulatory T cells (Tregs) population, lowering the frequencies of phenotypic markers of functional Tregs. Our data also showed that inhibiting the major transcriptional factor of Th17, RAR-related orphan receptor gamma t (ROR γ t), could prevent the development of CD146⁺CCR5⁺

Tconvs *in vitro*. Our results demonstrate how pathogenic CD146⁺CCR5⁺ T cells are induced through ICOS or ROR γ t, suggesting new targets for GVHD treatment. We anticipate our assay to be a starting point for the development of novel GVHD-specific drugs. For example, the treatments that focus on inhibiting ROR γ would have fewer side effects than general immunosuppressive drugs that GVHD patients use today and inhibit GVHD while sparing the GVL effect. Furthermore, we expect the CD146⁺CCR5⁺ Tconvs and/or Tregs can be used as GVHD biomarkers. These biomarkers may guide preemptive treatments such as ROR γ t inhibitor.

Sophie Paczesny, M.D., Ph.D., Chair

TABLE OF CONTENTS

LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xi
INTRODUCTION	1
BACKGROUND	4
SIGNIFICANCE	13
INNOVATION	16
PRELIMINARY DATA	18
HYPOTHESIS 1	30
CHAPTER 1. The role of ICOS in the development of Th1*-polarized CD146 ⁺ CCR5 ⁺ Tconvs during GVHD	31
OVERVIEW AND RATIONALE	31
MATERIALS AND METHODS	32
RESULTS	36
DISCUSSION	51
HYPOTHESIS 2	53
CHAPTER 2. The role of ROR γ t in the development of Th1*-polarized CD146 ⁺ CCR5 ⁺ Tconvs during GVHD	54
OVERVIEW AND RATIONALE	54
MATERIALS AND METHODS	55
RESULTS	57

DISCUSSION	62
HYPOTHESIS 3	64
CHAPTER 3. The role of ICOS in the development of Th1*-polarized CD146+CCR5+ Tregs during GVHD	65
OVERVIEW AND RATIONALE	65
MATERIALS AND METHODS	66
RESULTS	68
DISCUSSION	71
FUTURE DIRECTION	72
REFERENCES	74
CURRICULUM VITAE	

LIST OF FIGURES

Figure 1. Overview of GVHD pathogenesis	9
Figure 2. Definition and gating strategy of human Tconvs and Tregs by flow cytometry	19
Figure 3. CD4 ⁺ CD146 ⁺ CCR5 ⁺ T cell subset is a biomarker of intestinal GVHD	21
Figure 4. CD146 ⁺ CCR5 ⁺ T cells are Th1* polarized	22
Figure 5. TMP778 inhibits IL-17A production in human CD4 T cells	25
Figure 6. CD146 ⁺ CCR5 ⁺ Treg frequency is increased during GVHD	29
Figure 7. Human CD146 expression in PBMCs from healthy donors	37
Figure 8. CD146 ⁺ CCR5 ⁺ Tconvs can be induced after allogeneic stimulation in MLRs	39
Figure 9. ICOS stimulation with Th17 polarization induces CD146 ⁺ CCR5 ⁺ Tconvs	41
Figure 10. ICOS stimulation with Th17 polarization induces Th1* cells	43
Figure 11. ICOS stimulation with Th17 polarization induces pathogenic Th17 markers	45
Figure 12. CD146 ⁺ Tconvs are Th1* prone	47
Figure 13. Adoptive transfer of ICOS stimulated T cells exacerbates xeno-GVHD	49
Figure 14. CD146 ⁺ CCR5 ⁺ Tconvs and Th1* are inhibited by a novel	

RORyt inhibitor, TMP778	58
Figure 15. RORyt inhibitor, TMP778, inhibits the generation of CD146 ⁺ CCR5 ⁺ Tconvs in <i>in vitro</i> MLRs	61
Figure 16. ICOS stimulation polarizes Tregs towards Th1*-polarized CD146 ⁺ CCR5 ⁺ cells	69

LIST OF ABBREVIATIONS

APCs	Antigen Presenting Cells
CCL14	CC Chemokine Motif Ligand 14
CCR5	CC Chemokine Receptor 5
CD25	IL-2 receptor α -chain
CFA	Complete Freund's Adjuvant
EAE	Experimental Autoimmune Encephalomyelitis
Foxp3	Forkhead box P3
GI	Gastrointestinal
GVHD	Graft-Versus-Host Disease
GVL	Graft-Versus-Leukemia
HD	Healthy Donors
HMGB-1	High Mobility Group Box 1
HSCT	Hematopoietic Stem Cell Transplantation
IBD	Inflammatory Bowel Disease
ICOS	Inducible T-cell COStimulator
IFN- γ	Interferon gamma
IL-17	Interleukin 17
iTregs	Induced Tregs
LPS	Lipopolysaccharide
MCAM	Melanoma Cell Adhesion Molecule

MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
NK cells	Natural Killer Cells
NRM	Non-Relapse Mortality
NSG	NOD/scid/ <i>IL-2Rγ</i> ^{-/-}
nTregs	Natural Tregs
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
ROR γ t	RAR-related Orphan Receptor gamma t
T-bet	T-box Expressed in T cells
Tconvs	T Conventional Cells
Tfh	T Follicular Helper
TGF- β	Transforming Growth Factor beta
Th	T Helper
Th1	T Helper 1
Th1*	Nonconventional Th1
Th2	T Helper 2
Th17	T Helper 17
TNF- α	Tumor Necrosis Factor α
Tregs	Regulatory T Cells
Xeno-GVHD	Xenogeneic GVHD

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative option for a variety of acquired hematological malignancies such as leukemia, myelodysplastic syndromes and multiple myeloma. Its use has also expanded beyond blood and bone marrow cancers, such as congenital immunodeficiency and autoimmune diseases [1]. However, its efficacy is limited by the life-threatening graft-versus-host disease (GVHD), which is caused by immunological attack from donor allogeneic T cells on target organs such as the gastrointestinal (GI) tract [2].

In spite of the advances in the diagnosis and treatment of acute and chronic GVHD, immunosuppressive drugs such as corticosteroids remain the gold standard for the therapy of GVHD. However, corticosteroid treatment has a response rate of less than 50% [3, 4] and its administration is associated with significant side effects such as the appearance of opportunistic infections [5] and loss of the beneficial graft-versus-leukemia (GVL) effect. Therefore, developing a specific treatment that targets GVHD pathogenic T cells and spares the GVL effect is needed.

Our laboratory has recently identified a novel T cell subpopulation in GVHD: CD146⁺CCR5⁺ CD4⁺ T cells. The frequency of these CD146⁺CCR5⁺

conventional T cells (Tconvs) is increased significantly in the cells from GI GVHD patients compared to HSCT patients without GVHD. Furthermore, CD146⁺CCR5⁺ Tconvs have a phenotype similar to T helper 1 (Th1) and T helper 17 (Th17) cells by co-expressing IL-17A and IFN- γ , which are also identified as nonconventional Th1* cells [6]. In our preliminary studies, we also found that the frequency of CD146⁺CCR5⁺ regulatory T cells (Tregs) is increased in GI GVHD patients while the frequency of total Tregs is decreased. Similar to CD146⁺CCR5⁺ Tconvs, CD146⁺CCR5⁺ Tregs co-express IFN- γ and IL-17A, indicating that they might be Th1*-polarized and have lost suppressive function after allo-HSCT. Th1 cells are reported to be an important driver in the pathogenesis of acute GVHD and there are also reports suggesting Th17 cells work synergistically with Th1 during GVHD [7]. Given these findings, I hypothesize that these Th1*-polarized CD146⁺CCR5⁺ Tconvs might be pathogenic in human GVHD and the inhibition of this population might be able to treat GVHD while sparing GVL. I also hypothesize that CD146⁺CCR5⁺ Tregs are less tolerogenic than classical Tregs and can also be targeted in GVHD treatment.

To inhibit the induction of CD146⁺CCR5⁺ CD4⁺ T cells during human GVHD, my research focuses on two molecules: inducible T-cell costimulator (ICOS) and RAR-related orphan receptor gamma t (ROR γ t). ICOS is a CD28-superfamily costimulatory molecule that is expressed on activated human T cells [8, 9]. ICOS is also reported to be critical for the development of IL-17 producing human Th17 cells [10]. ROR γ t is the master transcriptional factor for Th17 cells [11]. Chapter

1 elaborates how we induced the generation and development of CD146⁺CCR5⁺ Tconvs by ICOS co-stimulation during Th17 differentiation or mixed lymphocyte reaction (MLR) *in vitro*. In Chapter 2, I focus on inhibiting CD146⁺CCR5⁺ Tconvs by using a newly discovered ROR γ t inhibitor, TMP778. Chapter 3 shows that CD146⁺CCR5⁺ Tregs can also be induced by ICOS co-stimulation, co-expressing IL-17A and IFN- γ . The results suggest that both ICOS and ROR γ t are important for the development of Th1*-polarized CD146⁺CCR5⁺ CD4⁺ T cells and may represent new avenues to treat human GVHD more specifically and with fewer side effects.

BACKGROUND

T helper cell activation and differentiation

CD4 T helper (Th) cells are important cells in adaptive immunity. Naïve CD4 T cells are activated through interaction with antigen-major histocompatibility complex (MHC). They can differentiate into different subtypes of effector T cells depending on cytokine milieu, including Th1, T helper 2 (Th2), T helper 9 (Th9), Th17, T follicular helper (Tfh) and regulatory T cells (Tregs). My research focuses on Th1, Th17 and Tregs, which are all important cellular regulators during GVHD. My studies also focus on human samples and a recent study proposed to call the pathogenic Th17 co-expressing IFN- γ and IL-17A: nonconventional Th1 (Th1*) [6].

Th1 cells are differentiated through the upregulating of the transcriptional factor, T-box expressed in T cells (Tbet) in the presence of IL-2 and IL-12 and produce IFN- γ ; Th17 cells are differentiated through the upregulating of ROR γ t and produce IL-17A and IL-17F; induced Tregs (iTregs) are differentiated through the upregulating of the transcriptional factor, Forkhead box P3 (Foxp3) in the presence of IL-2 and produce IL-10 and TGF- β , while naturally occurring Tregs (nTregs) are developed in the thymus and express Foxp3 intrinsically, and they represent a mature T cell subpopulation critically involved in maintaining peripheral tolerance [12].

Th17

Human interleukin 17 (IL-17) was first described in 1995 [13, 14] and subsequent studies identified IL-17 secreting T helper cells as Th17. Since the first description of IL-17, different members of IL-17 cytokine family have been identified, including IL-17A (also known as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F. The functions of IL-17A and IL-17F are best understood as they mediate pro-inflammatory responses [15-17]. IL-25 plays an important role in Th2 immunity against parasites and allergy [18-20] while the functions of IL-17B, IL-17C and IL-17E are largely unknown [21-24]. Th17 cells, which are induced by transcriptional factor ROR γ t, have been reported to play an important role in human autoimmune diseases such as psoriasis [25], inflammatory bowel disease (IBD) [26] and ankylosing spondylitis [27]. The pathogenic role of IL-17 has also been evidenced by a series of mouse models of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) [28] and arthritis [29]. Recent success in clinical trials to treat psoriasis and rheumatoid arthritis by inhibiting Th17 pathways further emphasizes the pathogenic role of Th17 in human autoimmunity [30]. However, the clinical trials of IL-17A monoclonal antibody as treatment for human autoimmune diseases have shown variability in responses, suggesting that alternative pathways to block pathogenic Th17 need to be developed [31].

Pathogenic Th17 or also called Th1*

In many autoimmune diseases such as psoriasis and IBD, CD4 memory T cells can produce both IL-17A and IFN- γ [32]. A recent study done by Becattini and colleagues has shown that single human naïve CD4 T cells primed by a pathogen *in vitro* can give rise to multiple fates, including classical Th1, Th2, Th17 and nonconventional Th1* that co-express IFN- γ and IL-17A [6]. *C. albicans*-specific Th1* could be converted from plastic Th17 as they share extensive clonotype, while *M. tuberculosis*-specific Th1* could be generated from a different pathway besides Th17 as shared clonotype was not observed. My research focuses on how these preferential expansions shape the polarized (e.g. Th1, Th17 and Th1*) responses during GVHD.

Tregs

The regulatory T cells (Tregs) are a very important population in GVHD pathogenesis. They have been identified as a subpopulation of CD4 T cells that express high levels of the IL-2 receptor α -chain (CD25). Tregs also express the fork-head box transcription factor (Foxp3), which is crucial for their suppressive function [33].

GVHD pathogenesis and treatment

GVHD occurs in 25%-60% of HSCT patients depending on several factors such as donor type, donor age, recipient age, intensity of conditioning regimen and donor lymphocyte infusion [34]. It is caused by immunological attack from donor allogeneic T cells on target organs such as the GI tract, liver and skin [2]. Prior to HSCT, patients receive a conditioning regimen such as irradiation or chemotherapy, releasing pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), danger-associated molecular patterns (DAMPs) such as high mobility group box 1 (HMGB-1) [35], and cytokines such as IL-6, IL-1 and TNF- α from tissue damage. This phase is called the cytokine storm. After the introduction of allogeneic donor T cells, host antigen presenting cells (APCs) present antigens to donor T cells and prime them, resulting in the differentiation of pathogenic Th1 and Th17 cells. Donor APCs play a relatively minor role in GVHD initiation, they are important later for the GVL activity [36]. Along with the direct cytotoxicity of effector T cells on the target organs, natural killer cells (NK cells) and pro-inflammatory cytokines contribute all together to the end-organ damage, which is seen in the GI tract, liver and skin (Figure 1) [35].

While much progress has been made in our understanding of GVHD pathogenesis, there is still no specific therapy that targets the stimulatory or suppressive factors of the adaptive and innate immune system. Corticosteroids are nonspecific immunosuppressant, which remain the first line for GVHD treatment, and have side effects such as increased risk of infections and loss of

GVL effect. In addition, response to steroids is seen only in about 50% of patients and those patients with steroid-resistant GVHD have a mortality rate in excess of 90% [37]. Therefore, there is a gap of knowledge in this area that needs to be filled as the one proposed in my studies.

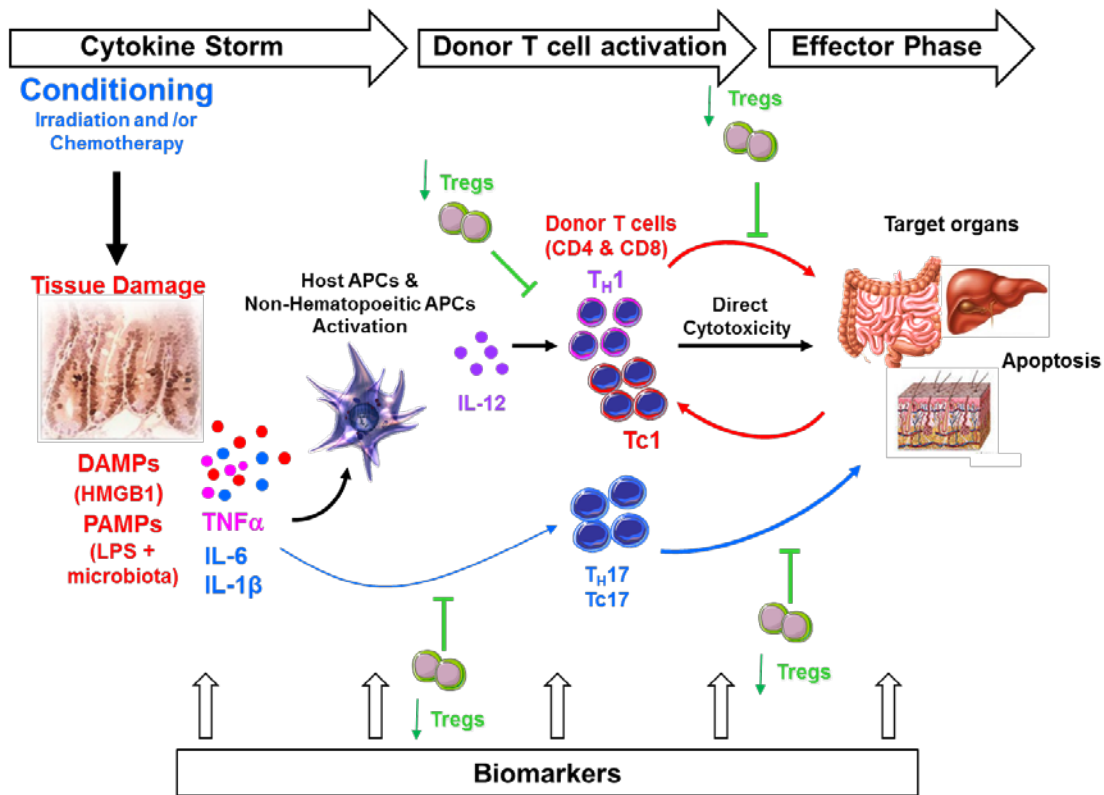


Figure 1. Overview of GVHD pathogenesis [35]. Conditioning regimen before HSCT causes damaged tissue to release DAMPs, PAMPs (LPS) and pro-inflammatory cytokines. Interactions between donor T cells and host APCs further increase the release of cytokines, creating a strong cytokine storm, which favors the differentiation of effector T cells, especially Th1 and Th17. Direct toxicity from effector T cells and indirect toxicity from pro-inflammatory cytokines, together result in end-organ damage in the GI tract, liver and skin. During GVHD, Treg frequency is decreased and Tregs play a suppressive role during Th1 and Th17 differentiation.

Th17 in GVHD

The role of Th17 in human and mouse GVHD has been controversial. Th17 cells in humans have not been extensively studied. Bossard and colleagues have shown a significantly higher numbers of Th17 cells in the intestinal mucosa of GVHD patients on a small cohort [38]. Other studies have also shown an increased level of circulating Th17 with an imbalance between Th17 and Tregs in GVHD patients [39-41]. The data from murine studies are debatable regarding their role in GVHD. The first murine GVHD study used IL-17A^{-/-} donor T cells and suggested that IL-17A attenuated severe acute GVHD due to the suppression of Th1 differentiation [42]. Another study by Carlson and colleagues showed that the differentiation of Th17 cells *in vitro* appears to cause lethal acute GVHD with severe cutaneous and pulmonary damage [43]. These differences may be due to variations among the murine models. The study by Yu and colleagues is more convincing as they used Tbet and ROR γ t single or double deficient donor T cells that have intrinsic defective differentiation toward Th1 and Th17, and showed that the double deficient donor T cells induced less severe GVHD than Tbet-deficient T cells [44, 45]. This result demonstrated that T cells expressing ROR γ t may work synergistically with T cells expressing Tbet to exacerbate murine GVHD. Another study confirmed the protective role of ROR γ t^{-/-} donor T cells in the development of murine acute GVHD [46]. A recent study also showed that adoptive transfer of Tbet-deficient Th17 cells could attenuate murine acute GVHD, further confirming that Tbet contributes to optimal function of Th17 cells [45].

Tregs in GVHD

Tregs are generally accepted as a beneficial population in GVHD [47], and many studies have shown that there is a persistent reduction of Tregs in GVHD patients, which causes an imbalance between the effector and regulatory arms of the immune system, resulting in an inflammatory cytokine storm in patients [48-50]. In addition to these studies that have examined Treg cells in peripheral blood of GVHD patients, Rieger and colleagues reported a decrease of mucosal Tregs in the intestinal biopsies of GVHD patients [51]. Furthermore, the adoptive transfer of Tregs to suppress GVHD in many murine models including xenogeneic GVHD (xeno-GVHD) models has already been shown [52-54], possibly sparing the GVL effect [55]. Clinical trials with Tregs as cellular therapy have also been shown to be effective in GVHD patients [56, 57]. As a novel GVHD therapy, low-dose IL-2 was reported to be correlated with the expansion of Tregs in patients [58]. However, the plasticity of human Tregs to Th17-polarized cells in a pro-inflammatory milieu has been reported [59-61]. It has been demonstrated that human Treg cells can convert into Th17 cells in patients with autoimmune diseases such as rheumatoid arthritis [62]. Global Mapping of H3K4me3 and H3K27me3 in iTregs and nTregs has also revealed the plasticity of Tregs to Th17 cells [63]. In many autoimmune murine models such as autoimmune arthritis and psoriasis, the conversion of Tregs to Th17 has been reported to explain the increased autoimmunity in mice [64, 65].

CD146 and CCR5

CD146 was first identified as an endothelial marker for melanoma (MCAM) [66]. It has been known as endothelial receptor overexpressed during inflammation [67]. Human CD146 is expressed on activated T cells [68] and its expression increases in patients with IBD [67] and mice with EAE [69]. Xing and colleagues have also shown that endothelial CD146 allows entry of CD146+ T cells in the gut [70]. CCL14 is a human CC chemokine that binds to the CC chemokine receptor 5 (CCR5) on T cells [71]. CCR5 has been shown to play an important role in GVHD pathogenesis in mice [72] and its expression on T cells can facilitate its infiltration in the gut [72, 73]. Reshef et al. also show that the blockade of CCR5 by maraviroc can inhibit lymphocyte trafficking and alleviate GVHD in patients [74].

SIGNIFICANCE

One contribution of our research is to determine the cellular phenotype of CD146⁺CCR5⁺ CD4⁺ T cells, including CD146⁺CCR5⁺ Tconvs and Tregs. Both of their frequencies are found elevated in GI GVHD patients, leading us to the hypothesis that CD146⁺CCR5⁺ CD4⁺ T cells are pathogenic during human GVHD. For CD146⁺CCR5⁺ Tconvs, our preliminary data suggested a Th1*-polarized phenotype and we continued to test it by *in vitro* differentiation of human Tconvs from healthy donors. Th1* cells can produce both Th1 and Th17 phenotypic cytokines, IL-17A and IFN- γ . Their appearance in different types of infections and autoimmune diseases are reported [32] while their frequencies in the blood of healthy donors are close to zero. Although IL-17A can be protective against intracellular pathogens [75], its pathogenic role in murine EAE models and human autoimmune diseases has also been recognized [28]. If CD146⁺CCR5⁺ Tconvs can be proven to have a Th1*-polarized phenotype and produce pathogenic cytokines, they might be a novel therapeutic target to treat GVHD. For CD146⁺CCR5⁺ Tregs, we found that their frequency was increased in GVHD patients while the frequency of total Tregs was decreased. Our clinical data also suggests a Th1* phenotype of these cells. Tregs are generally accepted as a beneficial population in GVHD, and many clinical studies have already begun to employ Tregs as a form of adoptive cellular therapy to prevent GVHD after HSCT [56, 57]. However, the plasticity of Tregs to Th17 has been

reported both *in vitro* and in many autoimmune murine models. My hypothesis is that CD146⁺CCR5⁺ Tregs are more plastic and less suppressive than non-CD146⁺CCR5⁺ Tregs during GVHD. If we demonstrate that CD146⁺CCR5⁺ Tregs in patients following HSCT are less functional and/or readily converted to Th17, they will become a novel target when treating GVHD. We designed both *in vitro* and *in vivo* experiments to investigate the function of these Tregs. If their role in the development of GVHD can be proven, CD146⁺CCR5⁺ Tregs can be the next population to be targeted as a supplement for Treg cellular therapy for GVHD.

Another contribution of our research is to propose potential therapeutic targets: ICOS and ROR γ t for GVHD treatment. This contribution is also significant, because it can lead to the development of GVHD treatments, which are specific to Th17 type immunity. Because GVL effect is mediated by Th1 cells and Th17 cells mediate mostly GVHD, treatments that specifically target Th17 immunity would ameliorate GVHD, without loss of the GVL effect. Anti-ICOS and ROR γ inhibitors have potential for more targeted drug therapy for GVHD in the future. Both aim to inhibit the development of Th1^{*}-polarized CD146⁺CCR5⁺ T cells. ICOS is a co-stimulatory molecule that has been shown to be critical in the differentiation of human Th17 cells [10]. Anti-murine-ICOS has been successfully used to alleviate murine GVHD [76] but only the Th1 effect was studied at the time. ROR γ is an important transcriptional factor of Th17. A recent study discovered a novel ROR γ inhibitor, which can inhibit Th17 differentiation [77, 78]. If these two treatments can successfully inhibit Th17, they may increase GVHD

while preserving GVL, ultimately increasing the survival and success rate of patients receiving allogeneic HSCT.

INNOVATION

GVHD treatments targeting Th1 immune response have been studied for decades, but the known functions are limited by the loss of the GVL effect. IFN- γ produced by Th1 cells is very important for promoting the GVL effect and Th1 inhibition will result in decreased IFN- γ production thereby decreasing the GVL effect following allogeneic HSCT [79]. Unlike Th1 specific treatment, targeting Th17 that does not mediate GVL is likely to separate GVHD and GVL effects. Our research is innovative because Th17 targeting has never been performed in human GVHD. Different from previous studies using regular murine models, xeno-GVHD models are more clinically relevant for human disease and may ultimately guide development of novel GVHD therapeutics. Anti-ICOS treatment has been reported to alleviate murine GVHD, but its cellular mechanism of ICOS co-stimulation is still unknown and has not been studied in xeno-GVHD models. TMP778 is a novel ROR γ inhibitor, which can inhibit Th17 differentiation and thus attenuate EAE [77]. We explored for the first time the function of TMP778 in GVHD *in vitro* and hopefully will explore it *in vivo* in the near future. Our research is also innovative because CD146⁺CCR5⁺ Tregs were only recently discovered in my laboratory as playing a role in GVHD. Exploring this novel population's plasticity and using it as a therapeutic target could offer promising treatment for GVHD. If CD146⁺CCR5⁺ Tregs are also Th1* polarized, I hypothesized that they can be targeted at the same time that the CD146⁺CCR5⁺ Tconvs by either anti-

ICOS and/or ROR γ t inhibitor. These novel treatments are expected to overcome the current problems with non-specific GVHD treatment and could potentially benefit more allo-HSCT patients.

PRELIMINARY DATA

To identify early GVHD biomarkers, our laboratory performed proteomic analysis using plasma taken 14 days prior to clinical manifestations of GI GVHD. We selected candidate markers that had at least a 1.5 fold increase in plasma levels in GI GVHD patients compared to HSCT patients without GVHD. The CC chemokine motif ligand 14 (CCL14) and CD146 were the two lead candidates. We hypothesized that the increase of CD146, CCR5, or both can serve as GVHD biomarkers with diagnostic or prognostic value.

In previous experiments, our laboratory has found a novel T cell subpopulation in GVHD: CD146⁺CCR5⁺ CD4⁺T cells. Our preliminary clinical data shows that: (1) CD146⁺CCR5⁺ Tconv frequency is a cellular biomarker of GI GVHD; (2) CD146⁺CCR5⁺ Tconv population is Th1* polarized; and (3) CD146⁺CCR5⁺ Treg frequency is increased in GVHD patients while the overall Treg frequency is decreased.

Definition and Gating Strategy of Tconvs and Tregs

In our studies, Tconvs are defined as $CD4^+CD25^{lo}CD127^+$ human T cells; Tregs are defined as $CD4^+CD25^+CD127^{lo}$ human T cells (Figure 2) (Unpublished data from Gomez A).

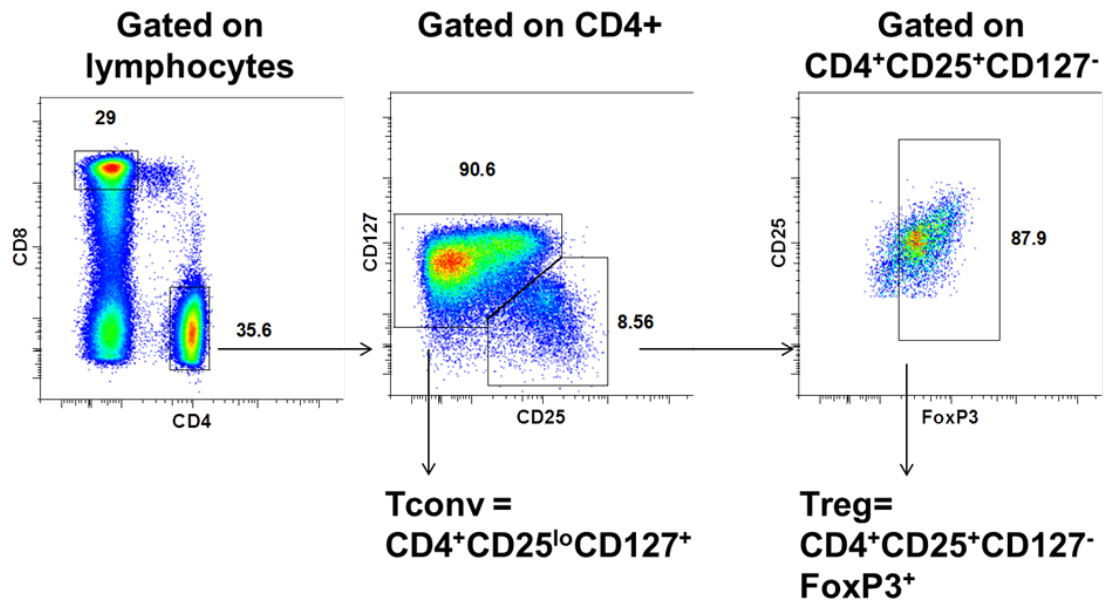


Figure 2. Definition and gating strategy of human Tconvs and Tregs by flow cytometry. Human CD4 T cells were gated on lymphocytes; Tconvs were gated on CD4 T cells as $CD4^+CD25^{lo}CD127^+$ population; Tregs were gated on CD4 T cells as $CD4^+CD25^+CD127^{lo}$ population (Unpublished data from Gomez A).

CD146⁺CCR5⁺ Tconvs in GI GVHD patients

Gomez from our laboratory first analyzed peripheral blood cells from 214 HSCT patients including 71 GI GVHD, 48 no GVHD and 33 non-GVHD enteritis patients at onset of symptoms. The frequency of CD146⁺CCR5⁺T cells was significantly increased in GI GVHD patients compared to patients without GVHD ($p < 0.001$) or non-GVHD enteritis ($p < 0.001$) (Figure 3). Our laboratory then further characterized this CD146⁺CCR5⁺ T cell population using nanostring technology to define differential transcriptomes between CD146⁺CCR5⁺ Tconvs and non-CD146⁺CCR5⁺ Tconvs. Interestingly, ROR γ t and Tbet, two transcriptional factors essential for Th1* development were among the most upregulated transcripts. We then confirm these findings at the protein level in patient samples with intracellular staining of ROR γ t, Tbet and IL-17. These data suggest that in GI GVHD patients, CD146⁺CCR5⁺ T cells had a Th1* phenotype (ROR γ ⁺Tbet⁺) compared to non-CD146⁺CCR5⁺ T cells (Figure 4).

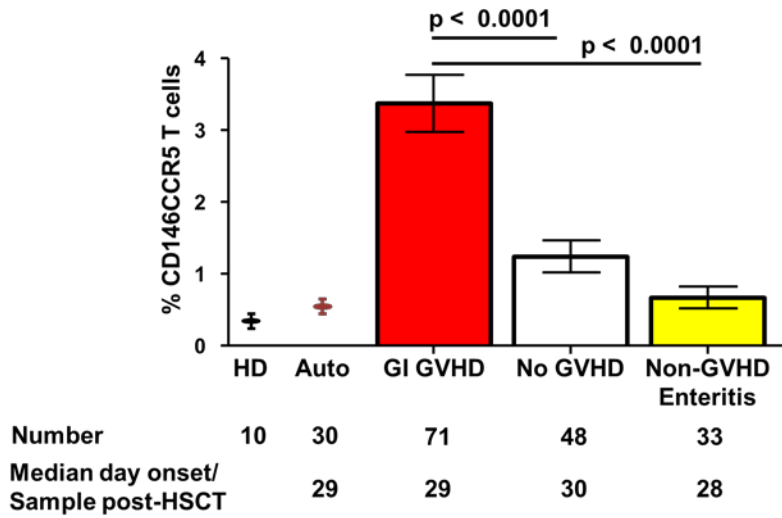
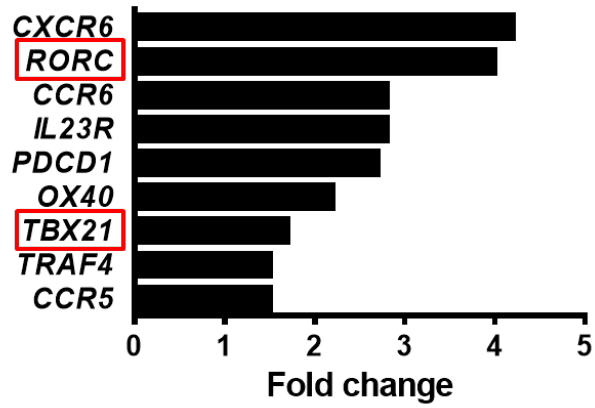
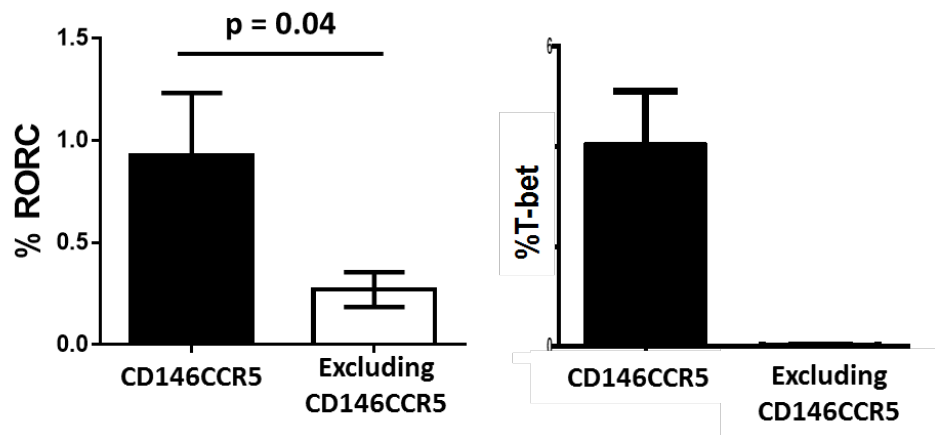


Figure 3. CD4⁺CD146⁺CCR5⁺ T cell subset is a biomarker of intestinal GVHD. CD146⁺CCR5⁺ Tconv frequencies on CD4⁺ T cell in healthy donors (HD), auto-transplant patients (Auto) and allogeneic patients (all others) were measured by flow cytometry. (Unpublished data from Gomez A, student t test, mean \pm SEM, significance for $p < 0.05$)

A Transcriptome (nanosttring)



B Intracellular Proteins (Flow Cytometry)



C

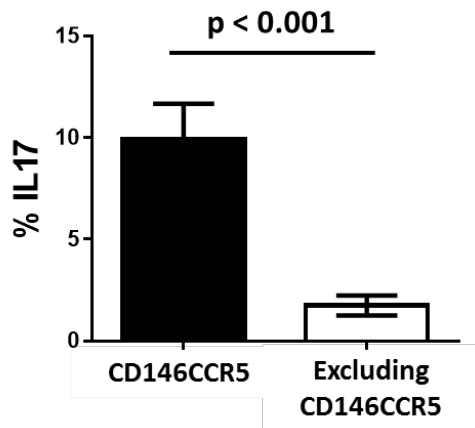


Figure 4. CD146⁺CCR5⁺ T cells are Th1* polarized. (A) Transcriptional differences were compared between CD146⁺CCR5⁺ Tconvs and non-CD146⁺CCR5⁺ Tconvs by using nanostring technology. The expressions of (B) transcriptional factor ROR γ t, Tbet and (C) cytokine IL-17 on CD146⁺CCR5⁺ Tconvs and non-CD146⁺CCR5⁺ Tconvs in GVHD patients were measured by flow cytometry. (Unpublished data from Gomez and Braun, student t test, mean \pm SEM, significance for $p < 0.05$)

TMP778 as a novel ROR γ t inhibitor

Skepner and colleagues have recently identified a novel small-molecule ROR γ t inhibitor: TMP778 [78]. They reported that IL-17 and IL-17 producing cells induced by ROR γ t in human T cells can be inhibited by TMP778 (Figure 5A, 5B). In addition, TMP778 can inhibit Th17 signature gene expression by cells isolated from psoriatic patient [78]. A subsequent study also showed that ROR γ t small-molecule inhibitor TMP778 could suppress Th17 cell responses *in vivo* and thus ameliorated EAE (Figure 5C) [77]. We hypothesize that Th1* cells can be targeted in GVHD treatment with TMP778. Therefore, TMP778 could also be a promising GVHD-specific drug.

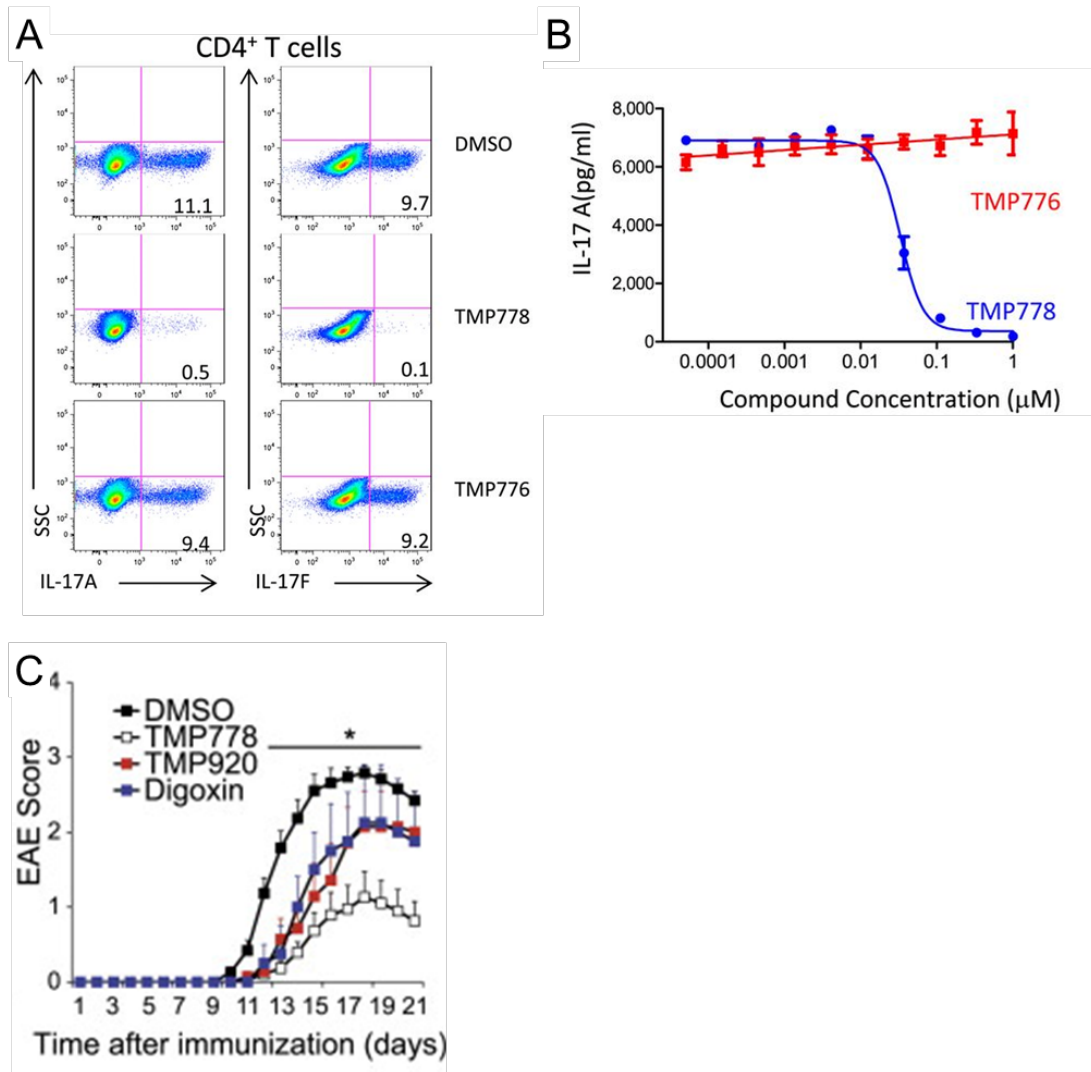


Figure 5. TMP778 inhibits IL-17A production in human CD4 T cells.

(A, B) Naive CD4⁺ T cells were transduced with RORγt lentivirus and then stimulated with CD3/CD28 beads in the presence of DMSO, 0.1 μM TMP778 or its diastereomer TMP776 for ten days. (A) After the infection, IL-17 expression was measured by flow cytometry. (B) Cells were then harvested and restimulated with CD3/CD28 beads in the presence of TMP778 or TMP776 for 48 h. IL-17

titers in the supernatants were determined by using Meso Scale Discovery (MSD) assays. Data are representative of two to three separate experiments.

(C) C57BL/6 mice were immunized with MOG₃₅₋₅₅ plus complete Freund's adjuvant (CFA), and injected with TMP778, TMP920, digoxin or DMSO subcutaneously twice daily. Mice were evaluated daily for signs of EAE. (Figures from Skepner J et al., JI, 2014 and Xiao S et al., Immunity, 2014, mean \pm SD, *p < 0.05).

CD146⁺CCR5⁺ Tregs in GVHD patients

Magenau and colleagues published previously that Treg frequency is correlated with GVHD severity at onset [50]. In cohort 1, Treg frequency decreased with each increasing grade of GVHD (Figure 6A). Patients with GVHD whose Treg frequency was less than the median had a significantly greater non-relapse mortality (NRM) at one year than patients with Treg frequency equal to or greater than the median (Figure 6B).

In unpublished work by Gomez, our laboratory analyzed peripheral blood cells from a second cohort of 214 HSCT patients including 71 GI GVHD, 48 No GVHD and 33 non-GVHD enteritis patients at onset of symptoms. As many studies reported previously [80-82], the Treg frequency was significantly decreased in GI GVHD patients as compared to patients without GVHD ($p=0.02$) or non-GVHD enteritis ($p=0.04$) (Figure 6C). Surprisingly, the frequency of CD146⁺CCR5⁺ Tregs was significantly increased in GI GVHD patients as compared to patients without GVHD ($p=0.04$) or non-GVHD enteritis ($p=0.03$) (Figure 6D, 6E).

Transcriptome analysis with Nanostring technology and flow data of these CD146⁺CCR5⁺ Tregs showed that they expressed some levels of IFN- γ and IL-17A as compared to non-CD146⁺CCR5⁺ Tregs that expressed none (data not shown), indicating that the CD146⁺CCR5⁺ Tregs might be Th1*-polarized and may have altered suppressive function after allo-HSCT.

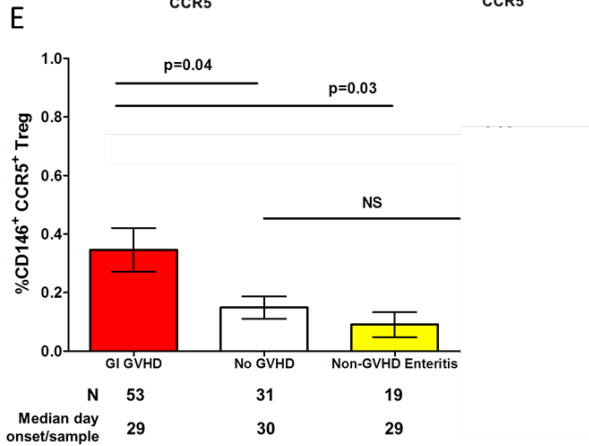
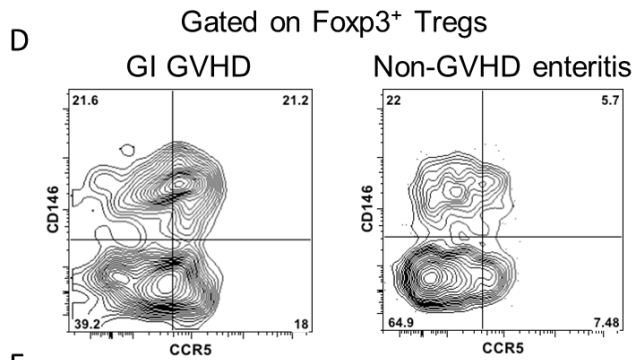
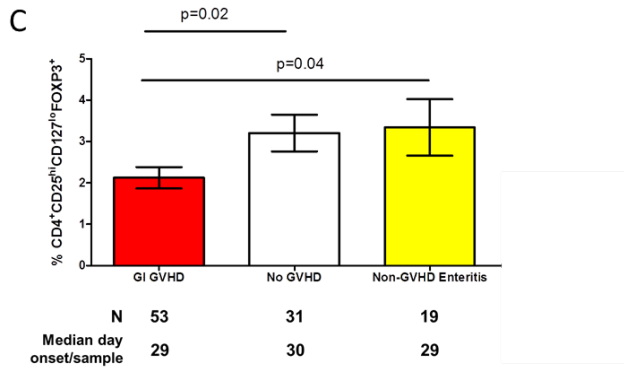
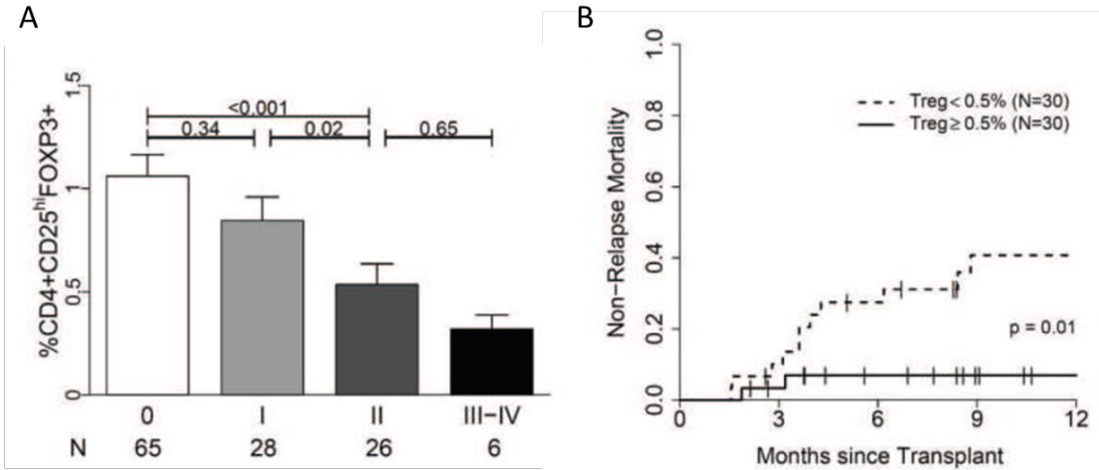


Figure 6. CD146⁺CCR5⁺ Treg frequency is increased during GVHD.

In cohort 1 (N = 60), fresh blood samples from allogeneic transplant patients with GVHD were acquired within 24hrs of acute GVHD onset and analyzed according to GVHD severity: (A) Mean Treg frequencies by grade of GVHD at onset. (B) NRM in patients with GVHD divided according to the median Treg frequency (high Treg $\geq 0.5\%$ (N=30) or low Treg < 0.5 (N=30)) (Figures from Magenau, et al., Biol Blood Marrow Transplant, 2010).

In cohort 2 (N=214), (C, D, E) the frequencies of CD25⁺CD127⁻Foxp3⁺ Tregs and CD146⁺CCR5⁺ Tregs on CD4 T cell in GI GVHD patients, HSCT patients without GVHD (No GVHD) and patients with non-GVHD enteritis were measured by flow cytometry. (Unpublished data by Gomez A, student t test, mean \pm SEM, significance for $p < 0.05$)

HYPOTHESIS 1

**ICOS co-stimulation is important for the development of Th1*-polarized
CD146⁺CCR5⁺ Tconvs during GVHD**

CHAPTER 1. The role of ICOS in the development of Th1*-polarized CD146⁺CCR5⁺ Tconvs during GVHD

OVERVIEW AND RATIONALE

Since ICOS is critical for human Th17 development [10] and our clinical data shows that CD146⁺CCR5⁺ Tconvs are a Th1* polarized population, I investigated the role of ICOS in CD146⁺CCR5⁺ Tconvs generation *in vitro* and also tested the hypothesis that ICOS stimulation is important in the early development of CD146⁺CCR5⁺ Th1* in a xenogeneic GVHD (xeno-GVHD) model. The rationale for using a xeno-GVHD model rather than a classical murine model is due to the fact that murine donor T cells do not express CD146 [83], while human donor T cells express CD146. Xeno-GVHD can be induced by transferring human PBMCs or purified CD4 T cells to immunocompromised mice. I chose NOD/scid/*IL-2R γ* ^{-/-} (NSG) mice as recipients. NSG mouse is a non-obese diabetic (NOD)-scid mouse bearing mutations in the IL-2 receptor common γ -chain (IL-2R γ chain). IL-2 receptor is responsible for various signaling through the IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors, so the lack of this gene results in significantly impaired development and function of innate and adaptive immunity [84]. Previous studies have shown that this model will have a consistent development of xeno-GVHD within 20 days and a mortality rate of 90% by 2 months [85].

MATERIALS AND METHODS

Human blood and peripheral blood mononuclear cells (PBMCs)

Peripheral blood buffy coats from healthy adult volunteer donors were commercially obtained from the Indiana Blood Center. PBMCs were prepared from buffy coats by Ficoll-Paque density grade centrifugation (GE Amersham) and extensive washing with PBS.

Human T cell and PBMCs culture media

Human Th1, Th17 and PBMCs were cultivated in both T cell expansion medium (Invitrogen) and complete RPMI that was composed of RPMI 1640 complete tissue culture medium supplemented with L-glutamine, 10 U/ml penicillin/streptomycin, 20 mM HEPES, 0.1 mM NEAA, 1 mM sodium pyruvate (all from Invitrogen) plus 10% FBS (Hyclone), and 0.05 mM 2-ME (Sigma).

Human T cell polarization

T cells were isolated from fresh buffy coat. Naïve CD4⁺ T cells were negatively isolated using naïve CD4⁺ T cell isolation kit (Miltenyi). Total CD4⁺ T cells were negatively isolated using CD4⁺ T isolation kit (Miltenyi). CD146⁺CD4⁺ T cells and

CD146⁻CD4⁺ T cells were isolated from total CD4⁺ T cells using CD146 microbeads (Miltenyi). Purification of isolation is >95%.

For T cell activation, 0.5×10^6 T cells were plated in 48-well flat bottom plate with CD3/CD28 or CD3/ICOS coated M-450 Tosylactivated Dynabeads (Invitrogen). The bead-to-cell ratio was 1: 5. Cells were cultivated in T cell expansion medium (Invitrogen) in a 37°C and 5% CO₂ incubator.

For Th1 polarization, IL-2 (2 ng/ml), IL-12 (10 ng/ml) (R&D system), and neutralizing antibodies against IL-4 (10 µg/ml) (eBioscience) were added on day 0. For Th17 polarization, IL-1β (20 ng/ml), IL-6 (30 ng/ml), IL-23 (30 ng/ml), TGF-β (2 ng/ml) (R&D system) and neutralizing antibodies against IL-4 (5 µg/ml) and IFN-γ (2 µg/ml) (eBioscience) were added on day 0. The polarizing cytokines and antibodies were maintained throughout the cell culture.

Mixed lymphocyte reaction

The MLR was performed with responder cells and stimulator cells from two MHC-mismatched healthy donors in a 96-well round bottom plate. The responder was 1.5×10^6 /ml whole PBMCs isolated from fresh buffy coat. The stimulator was 1.5×10^6 /ml PBMCs that were irradiated by 3000 cGy irradiation, leaving mostly APCs in the stimulator. The culture of the responder and stimulator from the

same donor were used as an autologous control. After 10 days of culture, the cells were harvested and processed to flow cytometry analysis.

Flow cytometry

For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml) (Sigma), Ionomycin (1 µg/ml) (Sigma) and Brefeldin A (3 µg/ml) (eBioscience) for 5 hours in a 37°C and 5% CO₂ incubator. Intracellular cytokines and transcriptional factors were stained with FoxP3 staining Kit (eBioscience). Stained cells were analyzed with Attune (Invitrogen) Flow Cytometer and FlowJo software.

Statistical analysis

Data were analyzed by unpaired student t test. Value of P= 0.05 or less was considered to be statistically significant.

Mice

Immunodeficient NSG mice were used as recipient mice. Mice were obtained from the In Vivo Therapeutics Core at the Indiana University Simon Cancer Center, housed under specific pathogen-free conditions in sterile ventilated racks. All mice were maintained on the food supplemented with Uniprime and

acidic water throughout the experiments. All procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Xeno-GVHD model

Human CD4 T cells were isolated from fresh buffy coat from healthy donors and cultured in the presence of beads coated with either CD3/CD28 or CD3/ICOS *in vitro* for 6 days. NSG mice were conditioned with 350 cGy total body irradiation 24 hours before transplantation (Day -1). Equal numbers of mice were intravenously injected with approximately 1.5×10^6 CD28 activated or 1.5×10^6 ICOS activated human CD4 T cells on Day 0. Survival, weight loss and GVHD clinical score were monitored every other day after transplantation. GVHD signs (body weight loss, hunched posture, reduced motility, hair loss and ruffed fur) were scored twice a week by using the grading system developed by Cooke et al. [86].

RESULTS

***In vitro* Th17 polarization increases CD146 expression on human T cells**

Unstimulated human CD4 T cells from PBMCs of healthy donors expressed approximately 4% CD146 (Figure 7A showing percentage on total T cells). Since CD146⁺CCR5⁺ T cells from GVHD patients are Th1* polarized co-expressing IFN- γ and IL-17A, I wanted to measure the expression of CD146 on human CD4 T cells differentiated under Th1 and Th17 *in vitro* polarization. I isolated CD4 T cells from PBMCs and stimulated the cells with CD3/CD28 Dynabeads in either Th1 polarizing condition or Th17 polarizing condition for 7 days. Consistent with patients' data, *in vitro* Th17-differentiated cells expressed more CD146 than Th1-differentiated cells (Figure 7B). These data suggests that the frequency of CD146 on human CD4 T cells can be induced by TCR stimulation and Th17 polarizing condition *in vitro*.

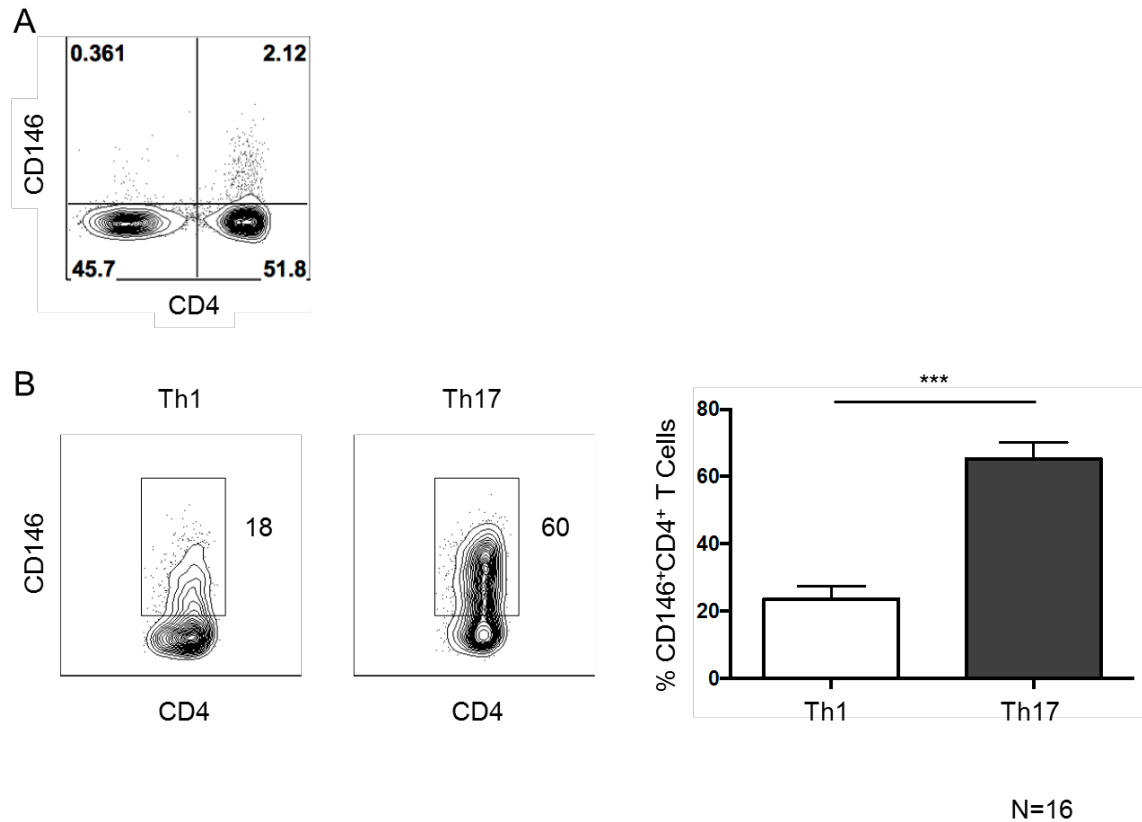


Figure 7. Human CD146 expression in PBMCs from healthy donors.

(A) CD146 expression on unstimulated PBMCs from healthy donors was measured by flow cytometry, gated on total T cells. (B) Naïve CD4⁺ T cells were isolated from PBMCs of healthy donors and cultured under Th1 or Th17 polarizing conditions for 7 days. CD3/CD28 Dynabeads were added on day 0 to activate the cells. CD146 expression on CD4⁺ T cells of different culture conditions was measured by flow cytometry on day 7. (Statistical data were pooled from 16 independent experiments, student t test, mean \pm SEM, * $p < 0.05$)

Th1*-polarized CD146⁺CCR5⁺ Tconvs can be induced upon allogeneic stimulation

To further investigate the role of CD146⁺CCR5⁺ Tconvs in the development of human GVHD, I performed *in vitro* MLRs to mimic the allogeneic reaction in GVHD patients. I cultured lymphocytes-free PBMCs (the responder) from one healthy donor with whole PBMCs (the stimulator) from another MHC-mismatched healthy donor. After culturing for 10 days, the frequency of CD146⁺CCR5⁺ Tconvs in the allogeneic culture was significantly higher than the frequency of CD146⁺CCR5⁺ Tconvs in the autologous control (Figure 8A, 8B). To determine if these CD146⁺CCR5⁺ Tconvs are Th1* polarized as we observed in PBMCs from GVHD patient, we measured the cytokine expression and found that the percentage of IL-17A⁺IFN- γ ⁺ coproducing T cells was much higher on the CD146⁺CCR5⁺ Tconvs compared to that on the non-CD146⁺CCR5⁺ Tconvs (Figure 8C). These results indicate that CD146⁺CCR5⁺ Tconvs can be generated from allogeneic stimulation and possess a pathogenic Th1* phenotype.

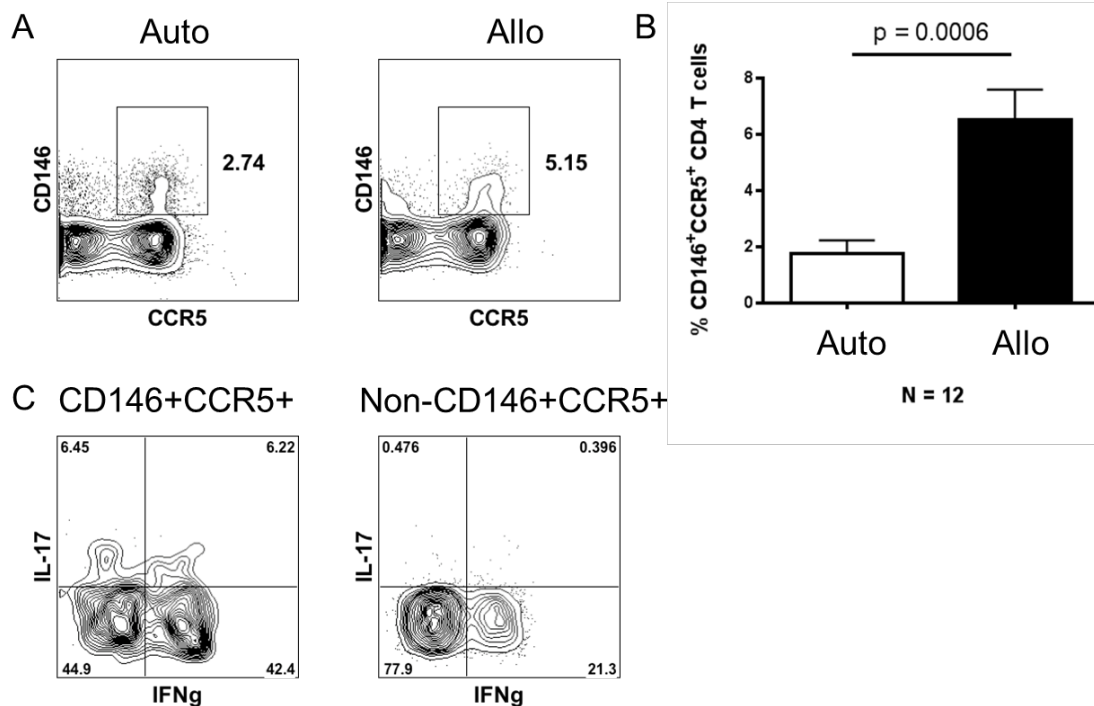


Figure 8. CD146⁺CCR5⁺ Tconvs can be induced after allogeneic stimulation in MLRs. PBMCs from one healthy donor were irradiated by 3000 cGy before culture. The irradiated PBMCs were cultured with whole PBMCs either from the same donor (autologous) or another MHC-mismatched healthy donor (allogeneic) for 10 days. (A, B) The frequency of CD146⁺CCR5⁺ Tconvs on total CD4 T cells was measured by flow cytometry on day 10. (C) The cytokine production of CD146⁺CCR5⁺ Tconvs and non-CD146⁺CCR5⁺ Tconvs was measured by flow cytometry on day 10. (Statistical data were pooled from 12 independent experiments, student t test, mean ± SEM, *p < 0.05)

ICOS is important for the development of the CD146⁺CCR5⁺ Tconvs

ICOS stimulation has been shown to be critical for the development of human Th17 in the study by Paulos et al. [10]. I hypothesized that ICOS stimulation can also induce the generation of Th1^{*}-polarized CD146⁺CCR5⁺ Tconvs. I investigated the role of ICOS in CD146⁺CCR5⁺ Tconvs generation. I found that naïve T cells from healthy donors, differentiated with both Th17 inducing cytokines and ICOS stimulation, showed increased percentage of CD146⁺CCR5⁺ T cells compared to naïve T cells differentiated with Th1 and CD28, Th17 and CD28 or Th1 and ICOS (Figure 9).

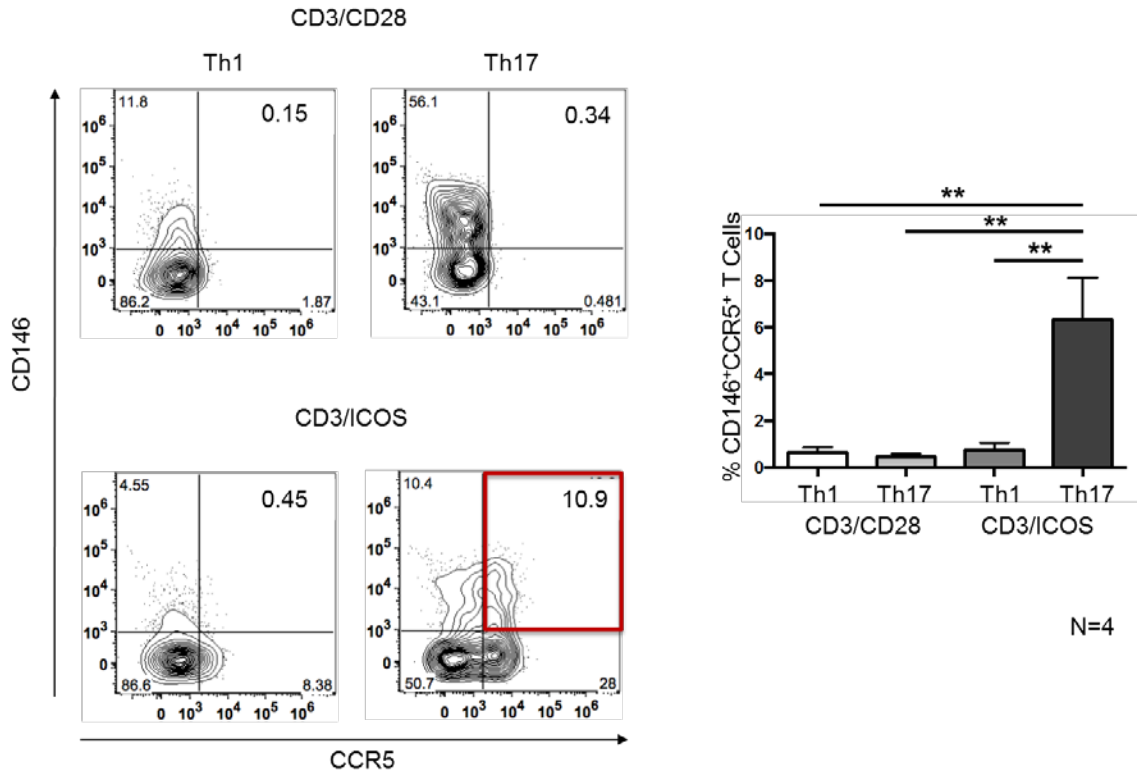


Figure 9. ICOS stimulation with Th17 polarization induces CD146⁺CCR5⁺ Tconvs. Naïve CD4 T cells were isolated from PBMCs from healthy donors and cultured in the presence of CD3/CD28 or CD3/ICOS Dynabeads under Th1 or Th17 polarizing conditions. After 7 days, the frequency of CD146⁺CCR5⁺ Tconvs was measured by flow cytometry. (Statistical data were pooled from 4 independent experiments, student t test, mean \pm SEM, *p < 0.05)

Th17 polarization with ICOS stimulation induces the generation of Th1* cells

Naïve T cells differentiated with both Th17 and ICOS also co-expressed more Th1 and Th17 cytokines (IL-17A⁺IFN- γ ⁺) than those differentiated with Th1 and CD28, Th17 and CD28 or Th1 and ICOS (Figure 10). These results indicate that Th1* cells can be induced by ICOS stimulation in the presence of Th17 polarizing condition.

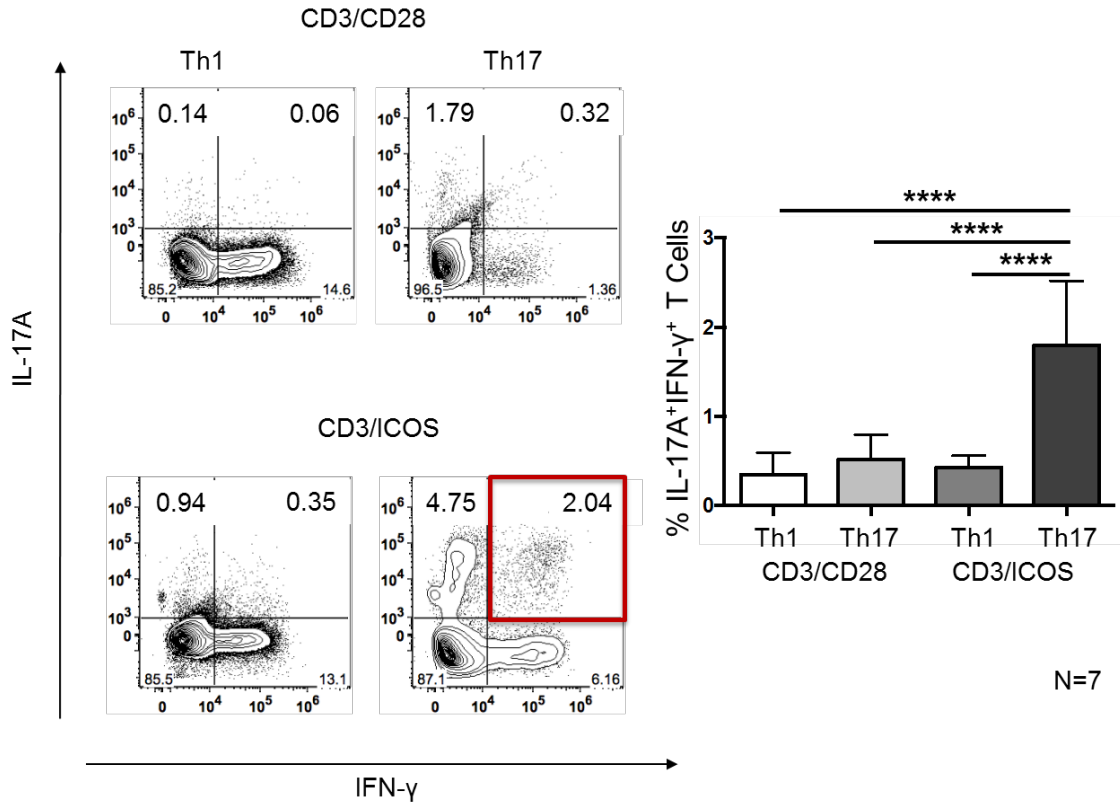


Figure 10. ICOS stimulation with Th17 polarization induces Th1* cells.

Naïve CD4 T cells were isolated from PBMCs from healthy donors and cultured in the presence of CD3/CD28 or CD3/ICOS Dynabeads under Th1 or Th17 polarizing conditions. After 7 days, the frequency of IL-17A⁺IFN-γ⁺ Tconvs was measured by flow cytometry. (Statistical data were pooled from 7 independent experiments, student t test, mean ± SEM, *p < 0.05)

Th17 polarization with ICOS stimulation induces the pathogenic Th17 surface markers on Tconvs

Th17 polarization and ICOS stimulation also increased other pathogenic Th17 markers such as CD161, IL-23R and CXCR6 (Figure 11).

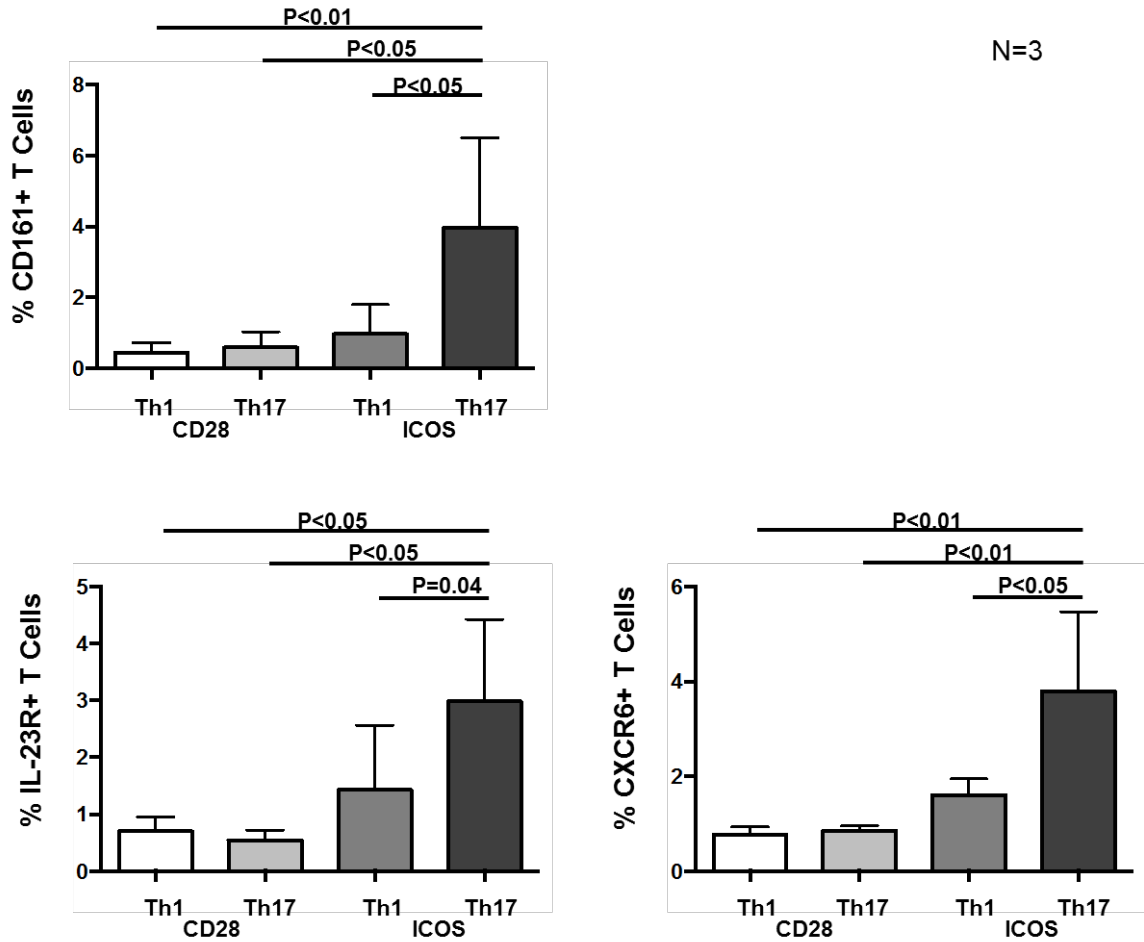


Figure 11. ICOS stimulation with Th17 polarization induces pathogenic

Th17 markers. Naïve CD4 T cells were isolated from PBMCs from healthy donors and cultured in the presence of CD3/CD28 or CD3/ICOS Dynabeads under Th1 or Th17 polarizing conditions. After 7 days, the frequencies of CD161⁺, IL-23R⁺ and CXCR6⁺ Tconvs were measured by flow cytometry.

(Statistical data were pooled from 3 independent experiments, one way ANOVA, mean ± SEM, *p < 0.05)

CD146⁺ Tconv are Th1* prone

When we gated on CD146 after culturing naïve T cells under Th17 polarization and ICOS stimulation for 7 days, I noticed that compared to CD146⁻ T cells, CD146⁺ T cells co-expressed more Th1* cytokines (IL-17A⁺IFN- γ ⁺) (Figure 12A) and pathogenic Th17 (Th1*) markers (GM-CSF, BATF, IL-23R) (data not shown), suggesting that they might be Th1* prone. I was able to further confirm this hypothesis by culturing under Th1 or Th17 polarization and CD28 or ICOS stimulation, sorted CD146⁺ and CD146⁻ T cells from mature total T cells, separately. CD146⁺ T cells co-expressed more Th1 and Th17 cytokines in all the differentiation culture conditions (Th1 or Th17 with CD28 or ICOS stimulation) (Figure 12B), suggesting that CD146⁺ T cells are already prone to a Th1* phenotype and additional polarization or TCR co-stimulation does not change their fate. Overall, these data show that CD146⁺ T cells have the capacity to exhibit a Th17 pathogenic cytokine profile that is independent of the effect of exogenous cytokines and TCR co-stimulation when CD146⁺ T cells are mature.

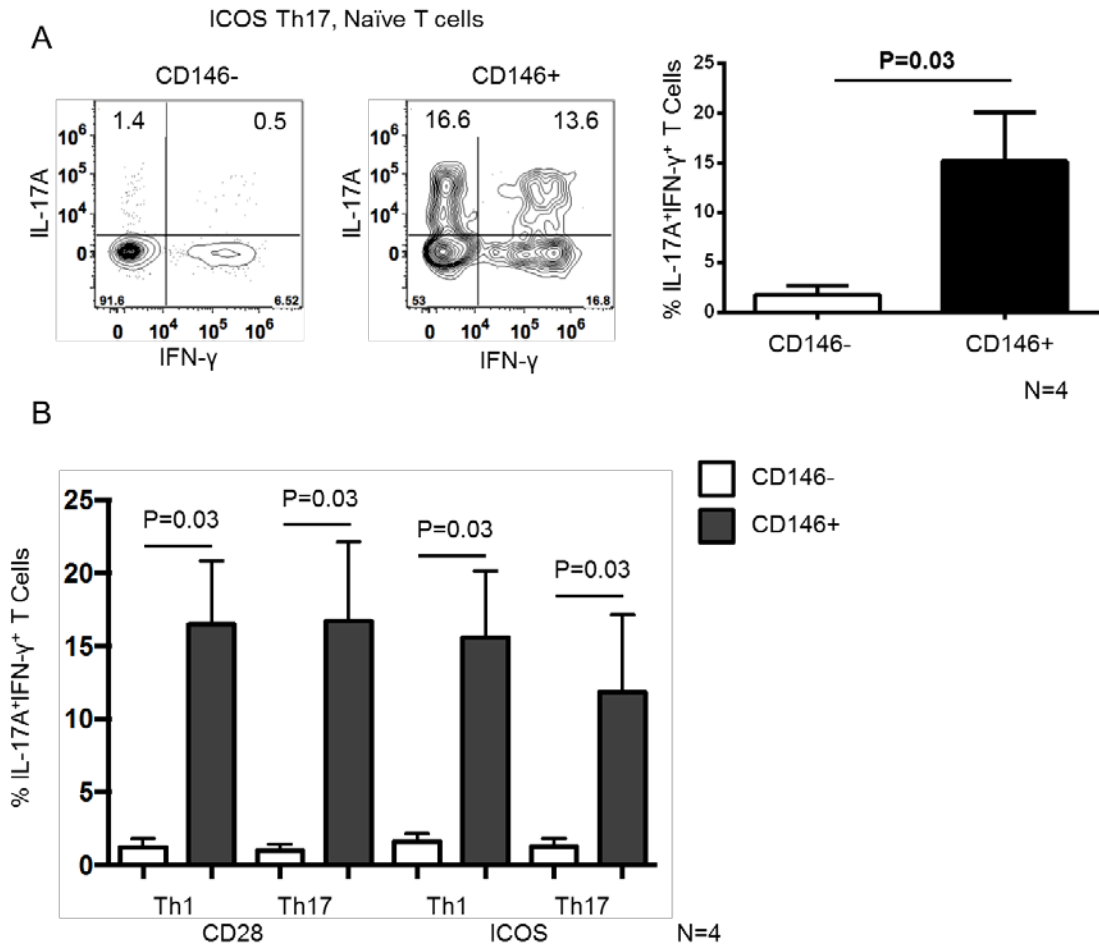


Figure 12. CD146⁺ Tconvs are Th1* prone. (A) Naïve CD4⁺ T cells or (B) mature CD146⁻CD4⁺ and CD146⁺CD4⁺ T cells were isolated from total T cells and cultured under Th1 or Th17 polarizing conditions for 7 days. CD3/CD28 or CD3/ICOS coated Dynabeads were added on day 0 to activate the cells. The frequency of IL-17A⁺IFN- γ ⁺ Tconvs on CD4⁺ T cells was measured by flow cytometry. (Statistical data were pooled from 4 independent experiments, student t test, mean \pm SEM, *p < 0.05)

Adoptive transfer of T cells simulated by ICOS induces more severe xeno-GVHD in an *in vivo* xenogeneic model

To further investigate the role of ICOS in the development of CD146⁺CCR5⁺ Tconvs during GVHD, I used a xenogeneic model and transferred human CD4 T cells stimulated *ex vivo* with CD28 or with ICOS to NSG mice. First, I demonstrated that in the presence of ICOS stimulation, human CD4⁺ T cells can be polarized towards CD146⁺CCR5⁺ Th1* even without Th17 skewing condition (Figure 13A). After human T cell xenogeneic transplantation (Figure 13B), I monitored the survival, body weight loss and GVHD clinical scores of the mice and found that mice injected with ICOS stimulated cells had more severe xeno-GVHD than mice injected with CD28 stimulated cells, which trended toward significance (Figure 13C). This result suggests that ICOS stimulation might exacerbate GVHD *in vivo*.

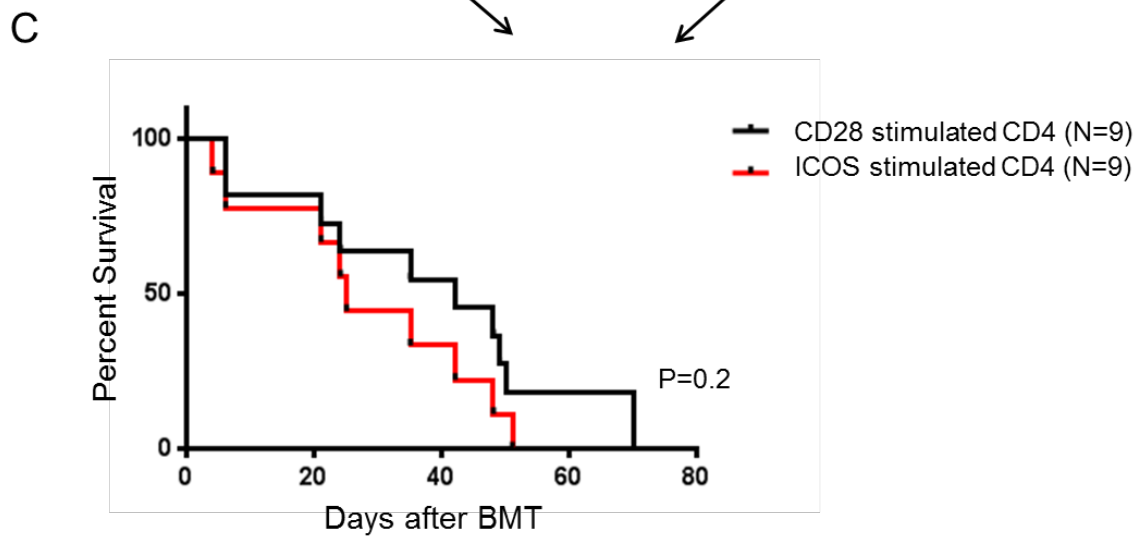
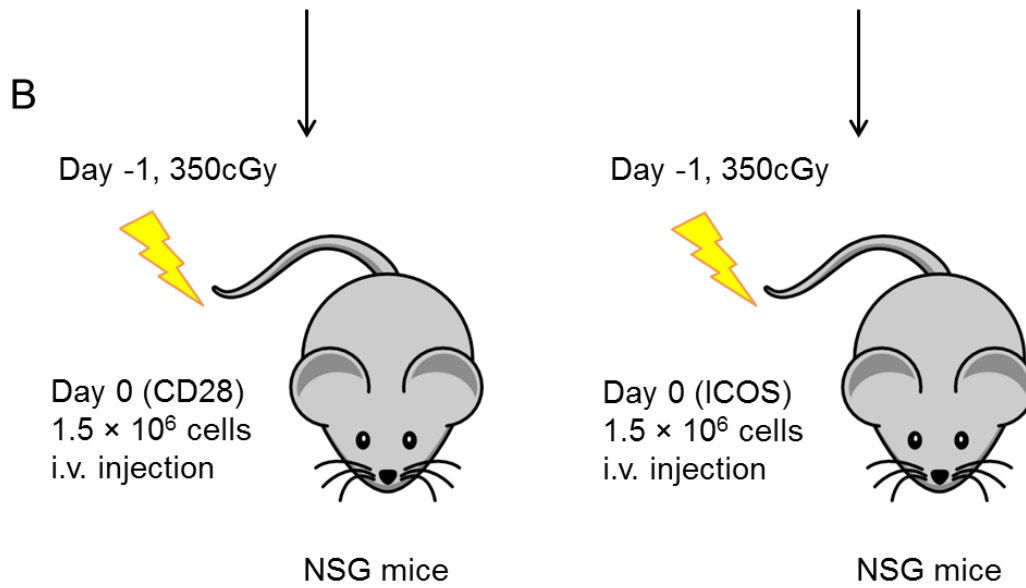
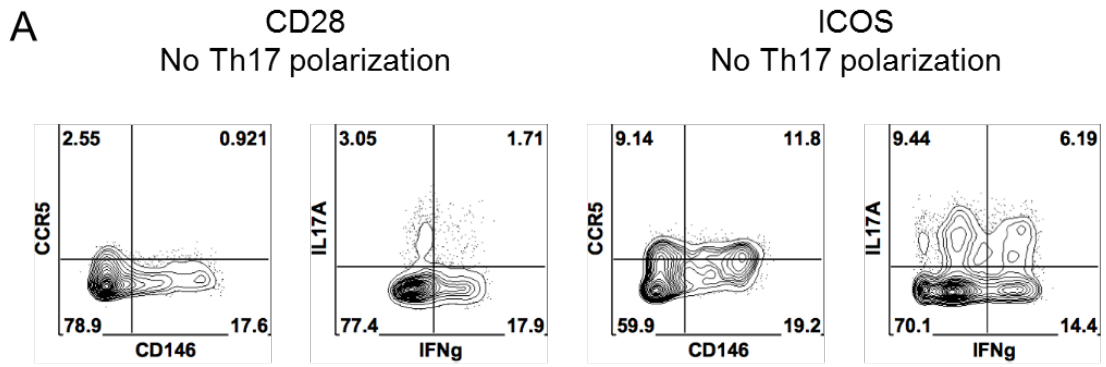


Figure 13. Adoptive transfer of ICOS stimulated T cells exacerbates xeno-GVHD. (A) Total CD4⁺ T cells were isolated from PBMCs and cultured in the presence of either CD3/CD28 or CD3/ICOS Dynabeads without polarizing conditions for 6 days. The expressions of CD146, CCR5, IFN- γ and IL-17A were measured by flow cytometry. (B) NSG mice were irradiated by 350 cGy on day -1 and injected with 1.5×10^6 /mouse stimulated T cells. (C) Percent survival was measured from day 0 up to day 70. Data were pooled from two independent experiments.

DISCUSSION

Here I show that CD146⁺CCR5⁺ Tconvs are Th1* polarized in GVHD and ICOS is important in the development of CD146⁺CCR5⁺ Tconvs. Indeed, I showed that CD146⁺ Tconvs could be induced after *in vitro* stimulation with ICOS and Th17 polarizing condition. I also provided *in vivo* evidence suggesting the pathogenic role of ICOS stimulated Tconvs.

The xeno-GVHD model also has the advantage to allow fast bench to bedside translation as targeted human neutralizing antibodies can be used. As I showed evidence of ICOS to induce pathogenic Th1* and CD146⁺CCR5⁺ Tconvs, I next hope to investigate the effect of ICOS inhibition in xeno-GVHD models. I tested a human ICOS blocking antibody from Ancell (ANC6C6) *in vitro* [87], but this antibody does not seem to neutralize the ICOS co-stimulation and thus Th1* differentiation. Unfortunately, we also failed to obtain the authorization to use another promising human ICOS neutralizing antibody (314.8), which has been reported to block the ICOS/ICOS-L interaction for GVHD studies [88]. An alternative to this pitfall is to target ICOS-L that has been reported to be up-regulated on APCs and induced by TNF- α or LPS [89, 90]. Therefore, I hypothesize that ICOS-L on host APCs could be targeted by ICOS-L neutralizing antibody during xeno-GVHD in future studies. However, targeting host APCs *in vivo* has been proven more challenging than targeting donor T cells in GVHD

studies. Therefore, my laboratory will focus, as priority, in future studies on the second therapeutic target ROR γ t as explained in chapter 2.

HYPOTHESIS 2

**ROR γ t inhibition can prevent the development of Th1*-polarized
CD146⁺CCR5⁺ Tconvs during GVHD**

CHAPTER 2. The role of ROR γ t in the development of Th1*-polarized CD146⁺CCR5⁺ Tconvs during GVHD

OVERVIEW AND RATIONALE

ROR γ t is known as the master transcriptional factor of Th17 [11] and its expression on CD4⁺ T cell has been shown to be important in the development of acute GVHD [44, 46]. A recent study discovered a novel, potent, and selective ROR γ t inhibitor, TMP778, which can inhibit human and mouse Th17 differentiation *in vitro* and thus attenuate EAE *in vivo* [77, 78]. Therefore, the goal of this chapter was to investigate the role of TMP778 in inhibiting the development of Th1*-polarized CD146⁺CCR5⁺ Tconvs *in vitro*. I found that TMP778 can block the differentiation of pathogenic Th1* and CD146⁺CCR5⁺ Tconvs under either Th17 polarizing conditions or allogeneic reactions, suggesting that ROR γ t inhibition may represent a novel therapy for human GVHD.

MATERIALS AND METHODS

Human blood and PBMCs

Same as described in Chapter 1.

Th17 and PBMCs culture media

Same as described in Chapter 1.

TMP778 reconstitution

TMP778 powder was obtained from Dr. Jianfei Yang and synthesized by Tempero Pharmaceuticals (a GlaxoSmithKline company). TMP778 (stock solution) was diluted in pure DMSO at 808.76 mM and stored at -80°C. TMP778 (working solution1) were diluted in pure DMSO at 10 mM and stored at 4°C. TMP778 (working solution2) was then diluted in cRMPI to reach final working concentration of 0.01 μ M, 0.1 μ M, 1 μ M or 10 μ M. The percentage of DMSO contained in each concentration of TMP778 was 0.0001%, 0.001%, 0.01%, 0.1%, respectively.

Human Th17 differentiation and treatment with TMP778

Naive or total CD4⁺ T cells were isolated as previously described in Chapter 1. To study the effect of TMP778 on Th17 differentiation, naive or total CD4 T cells (2×10^6 cells/ml) were stimulated with pre-coated anti-CD3/anti-ICOS Dynabeads in complete RPMI under Th17 polarizing conditions (20 ng/ml IL-1 β , 30 ng/ml IL-6, 30 ng/ml IL-23, 2 ng/ml TGF- β , 5 μ g/ml anti-IL-4 and 2 μ g/ml anti-IFN- γ) in the presence of different doses of TMP778 (0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M) or DMSO control (0.0001%, 0.001%, 0.01%, 0.1%). After 7 days of culture, cells were harvested and processed to flow cytometry analysis.

MLR and treatment with TMP778

The MLR was performed as previously described in Chapter 1. Different doses of TMP778 (0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M) or DMSO (0.0001%, 0.001%, 0.01%, 0.1%) were added at the beginning, and the concentration of both was maintained throughout the culture.

Flow cytometry

Same as described in Chapter 1.

Statistical analysis

Same as described in Chapter 1.

RESULTS

TMP778 suppresses the development of pathogenic Th1* and CD146⁺CCR5⁺ Tconvs *in vitro*

To determine if TMP778 can block the development of Th1*-polarized CD146⁺CCR5⁺ Tconvs, I cultured either naïve CD4 T cells or total CD4 T cells under Th17 polarizing condition in the presence of different doses of TMP778 or DMSO and measured the expressions of IL-17A and IFN- γ and the frequency of CD146⁺CCR5⁺ Tconvs. TMP778, but not DMSO, started to inhibit the IL-17A production and also IL-17A⁺IFN γ ⁺ Th1* differentiation at 0.01 μ M and blocked up to 80% of pathogenic Th1* at 10 μ M (Figure 14A, 14B). The frequencies of CD146⁺CCR5⁺ Tconvs in the culture with TMP778 were also lower than those in the culture without TMP778 in a dose-dependent manner (Figure 14B). These results indicate that TMP778 can suppress not only the differentiation of Th17 but also the differentiation of Th1* polarized-CD146⁺CCR5⁺ Tconvs.

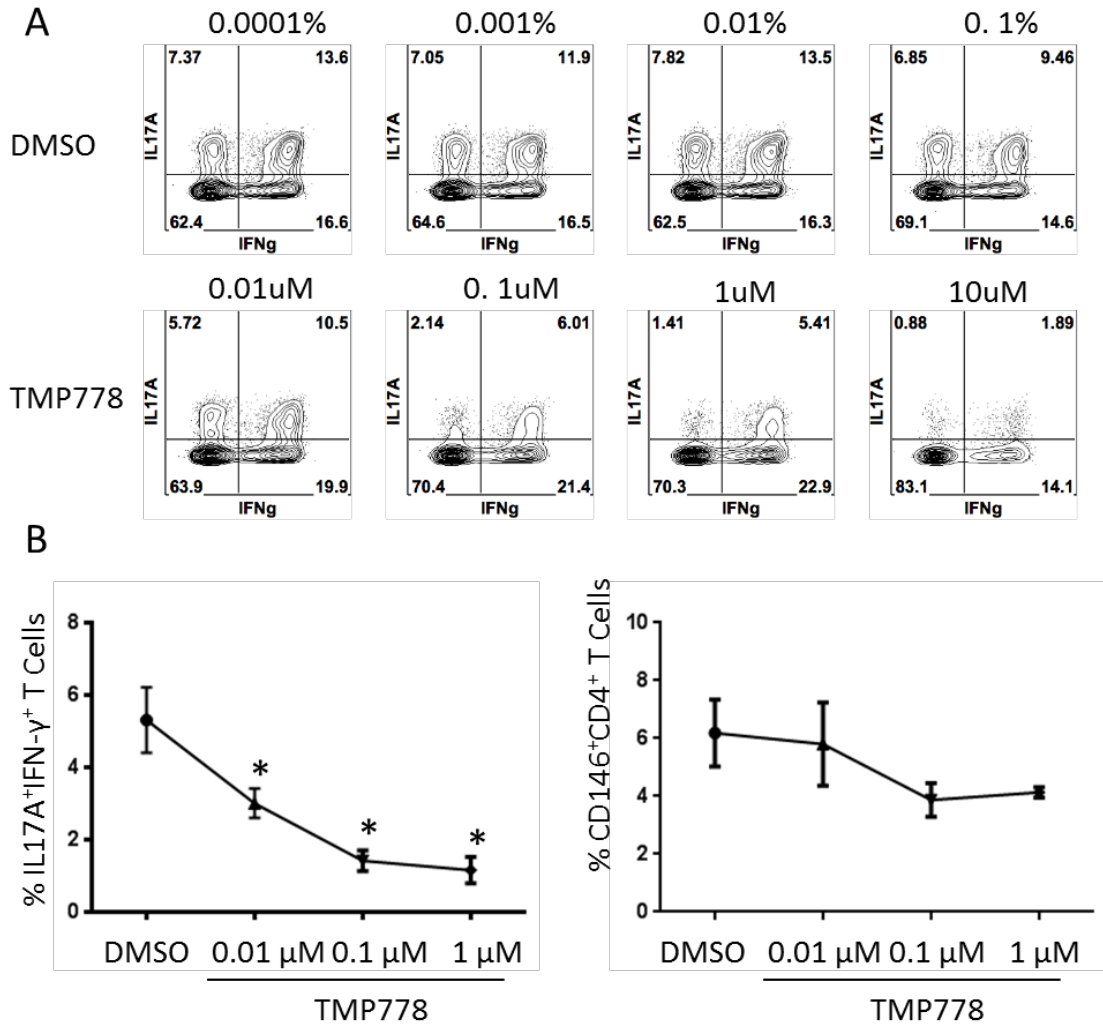


Figure 14. CD146⁺CCR5⁺ Tconvs and Th1* are inhibited by a novel ROR γ t inhibitor, TMP778. (A) Total CD4⁺ T cells or (B) naïve CD4⁺ T cells were isolated from PBMCs and cultured under Th17 polarizing conditions for 7 days in the presence of DMSO or different doses of TMP778. CD3/ICOS coated Dynabeads were added on day 0 to activate the cells. The frequencies of IL-17A⁺IFN- γ ⁺ Th1* and CD146⁺CCR5⁺ Tconvs were measured by flow cytometry.

(Statistical data were pooled from 3 independent experiments, student t test,
mean \pm SEM, * $p < 0.05$)

TMP778 suppresses the development of CD146⁺CCR5⁺ Tconvs in allogeneic reactions

To further determine the role of ROR γ t in human allogeneic responses, I performed *in vitro* MLRs to mimic the allogeneic reaction in GVHD patients with or without TMP778. The frequencies of CD146⁺CCR5⁺ Tconvs in the allogeneic groups with TMP778 were decreased in a dose-dependent manner (Figure 15). These data suggest that TMP778 can suppress the development of Th1*-polarized CD146⁺CCR5⁺ Tconvs in allogeneic reactions in *in vitro* and possibly *in vivo* settings planned for future studies.

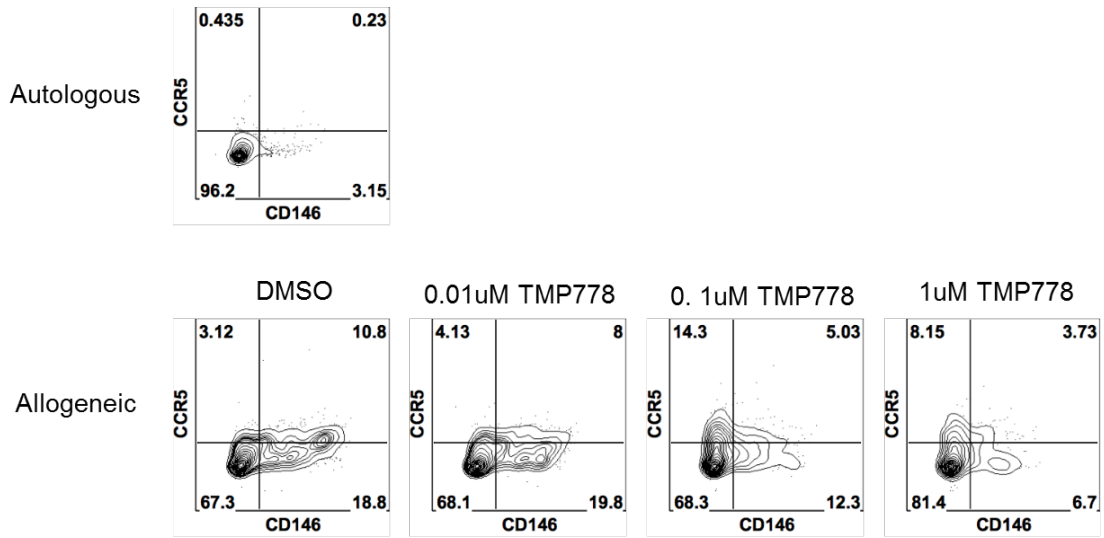


Figure 15. RORγt inhibitor, TMP778, inhibits the generation of CD146⁺CCR5⁺ Tconvs in *in vitro* MLRs. PBMCs from one healthy donor were irradiated by 3000 cGy before culture. The irradiated PBMCs were cultured with whole PBMCs either from the same donor (Autologous) or another MHC-mismatched healthy donor (Allogeneic) for 10 days. 0.01% DMSO and different doses of TMP778 (0.01 μM, 0.1 μM, 1 μM) were added to different allogeneic cultures on day 0. The frequency of CD146⁺CCR5⁺ Tconvs on total CD4 T cells was measured by flow cytometry on day 10.

DISCUSSION

I reported here that TMP778, a novel small-molecule ROR γ t inhibitor could inhibit the development of Th1*-polarized CD146⁺CCR5⁺ Tconvs *in vitro*. Our data in the previous chapter suggested that the frequency of the novel CD146⁺CCR5⁺ Tconvs population, which was identified as a GVHD biomarker, was increased significantly after ICOS stimulation *in vitro*. Here we show that the effect of ICOS co-stimulation and Th17 polarizing condition to drive the development of CD146⁺CCR5⁺ Tconvs can be counteracted by TMP778. Thus, the ROR γ t inhibitor, TMP778, can not only inhibit the *in vitro* differentiation and expansion of Th17 as previously reported [77, 78], but can also inhibit the differentiation of pathogenic Th1* as well as the Th1*-polarized CD146⁺CCR5⁺ Tconvs. These data also suggest that similar to classic IL-17 producing Th17 cells, IL-17A and IFN- γ co-producing Th1* cells could be transcriptionally regulated by ROR γ t.

I also tested the effect of TMP778 in allogeneic reaction. During the MLR from two MHC-mismatched human PBMCs, TMP778 was able to decrease the allo-reactivity by inhibiting the generation of Th1*-polarized CD146⁺CCR5⁺ Tconvs. These findings underline the potential of TMP778 as a treatment for GVHD.

For future studies, our laboratory wants to test the effect of TMP778 in the xeno-GVHD model described in the previous chapter. By blocking ROR γ t by TMP778

in vivo, we expect to see ameliorated xeno-GVHD. We also expect to see that ROR γ t blockade by TMP778 limits the development of pathogenic Th1* as we observed in *in vitro* studies.

HYPOTHESIS 3

**ICOS co-stimulation is important for the development of Th1*-polarized
CD146⁺CCR5⁺ Tregs during GVHD**

CHAPTER 3. Role of ICOS in the development of Th1*-polarized CD146⁺CCR5⁺ Tregs during GVHD

OVERVIEW AND RATIONALE

Our laboratory's clinical data suggested altered function of CD146⁺CCR5⁺ Tregs in GVHD patients by showing that CD146⁺CCR5⁺ Treg frequency was found to be elevated in GVHD patients while the frequency of total Tregs was decreased. Furthermore, CD146⁺CCR5⁺ Tregs in GVHD patients co-expressed IFN- γ and IL-17A, indicating that they might be Th1* polarized and lose suppressive function after allo-HSCT. The plasticity of Tregs to Th17 has been reported *in vitro* [91] and in many autoimmune murine models [64] as well as recently in murine GVHD studies [46, 92]. Therefore, I hypothesized that CD146⁺CCR5⁺ Tregs were more plastic and ready to convert to pathogenic Th17 during GVHD [92] and did research to investigate the role of CD146⁺CCR5⁺ Tregs *in vitro*.

MATERIALS AND METHODS

Human blood and PBMCs

Same as described in Chapter 1.

Tregs culture media

Human Tregs were cultivated in complete RPMI that was composed of RPMI 1640 complete tissue culture medium supplemented with L-glutamine, 10 U/ml penicillin/streptomycin, 20 mM HEPES, 0.1 mM NEAA, 1 mM sodium pyruvate (all from Invitrogen) plus 10% FBS (Hyclone), and 0.05 mM 2-ME (Sigma).

Treg Expansion

Natural regulatory T cells were isolated from PBMCs using human CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II (Miltenyi) and cultured in 96-well round bottom plate. 1×10^5 Tregs were expanded with either CD3/CD28 or CD3/ICOS coated M-450 Tosylactivated Dynabeads (Invitrogen) in the presence of 500 U/ml recombinant human IL-2 (R&D). The bead-to-cell ratio was 1: 5. Cells were cultivated in complete RPMI in a 37°C and 5% CO₂ incubator for 7 days.

Flow Cytometry

Same as described in Chapter 1.

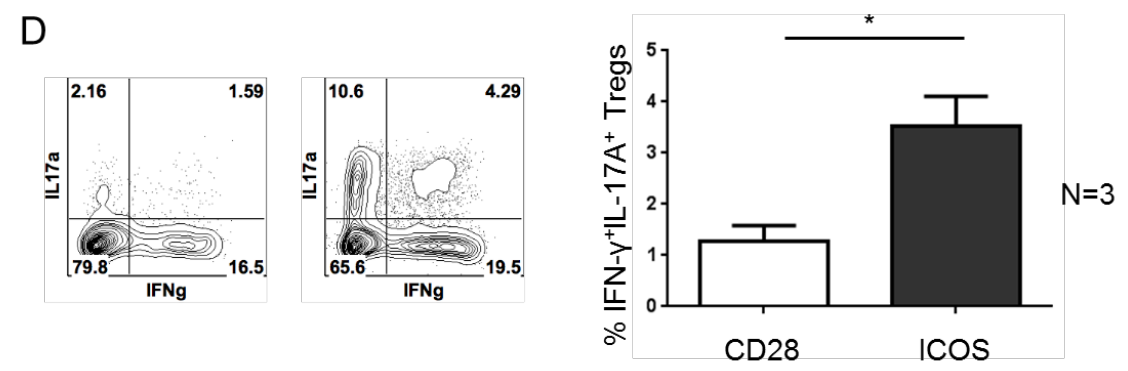
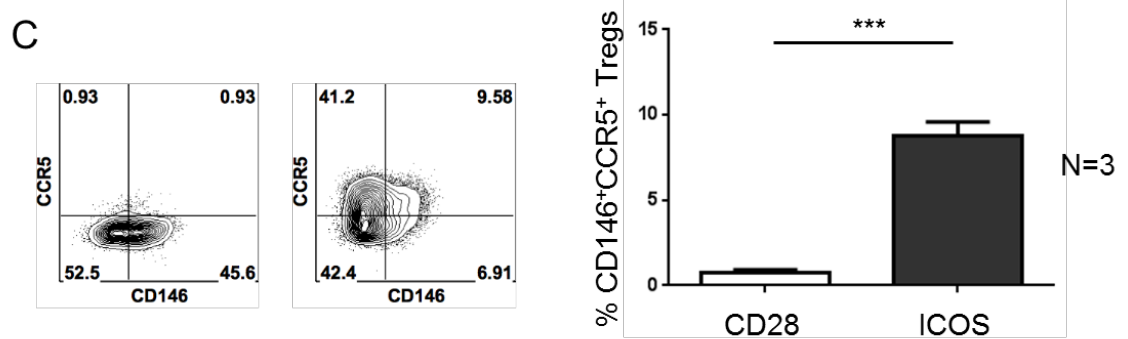
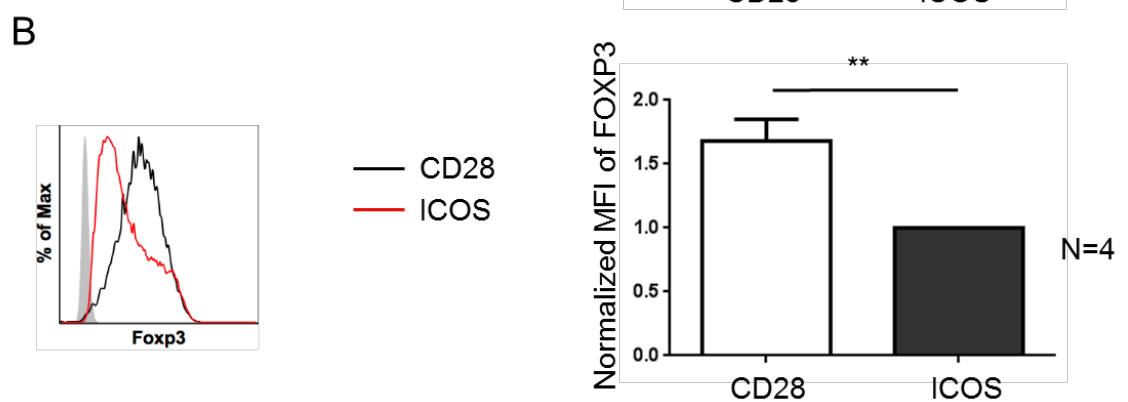
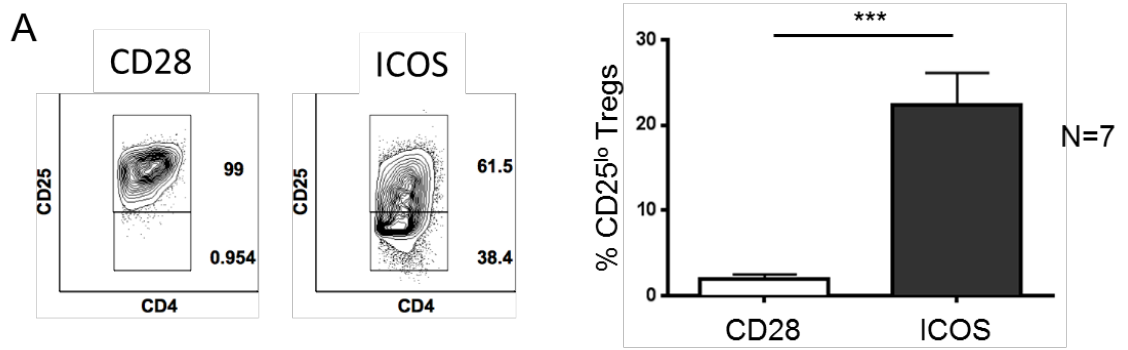
Statistical Analysis

Same as described in Chapter 1.

RESULTS

ICOS is important for the development of the Th1*-polarized CD146⁺CCR5⁺ Tregs

I cultured nTregs that were isolated from human PBMCs with either CD3/CD28 or CD3/ICOS stimulation beads in the presence of high-dose (500U/ml) of recombinant IL-2. After 7 days of culture, the flow analysis showed that ICOS activated Tregs expressed significantly lower levels of markers of functional Tregs such as CD25 and Foxp3 as compared to CD28 activated Tregs (Figure 16A, 16B), suggesting an altered suppressive function in ICOS activated Tregs. With ICOS stimulation, Tregs also showed a Th1*-polarized phenotype by expressing more CD146⁺CCR5⁺ and IL-17A⁺IFN- γ ⁺ Th1* cells (Figure 16C, 16D). These data suggest that ICOS stimulation can increase the frequency of CD146⁺CCR5⁺ Tregs that are Th1*-polarized *in vitro*. Tregs were reported to have the ability to convert to IL-17 secreting cells, which plays an important role in the pathogenesis of autoimmune diseases [64]. It is possible that these ICOS induced CD146⁺CCR5⁺ Tregs are Th1*-polarized and thus less suppressive, while *in vitro* Treg suppression assay showed that both of CD28 and ICOS stimulated Tregs retained suppressive function (Figure 16E). This observation warrants further analysis in the future.



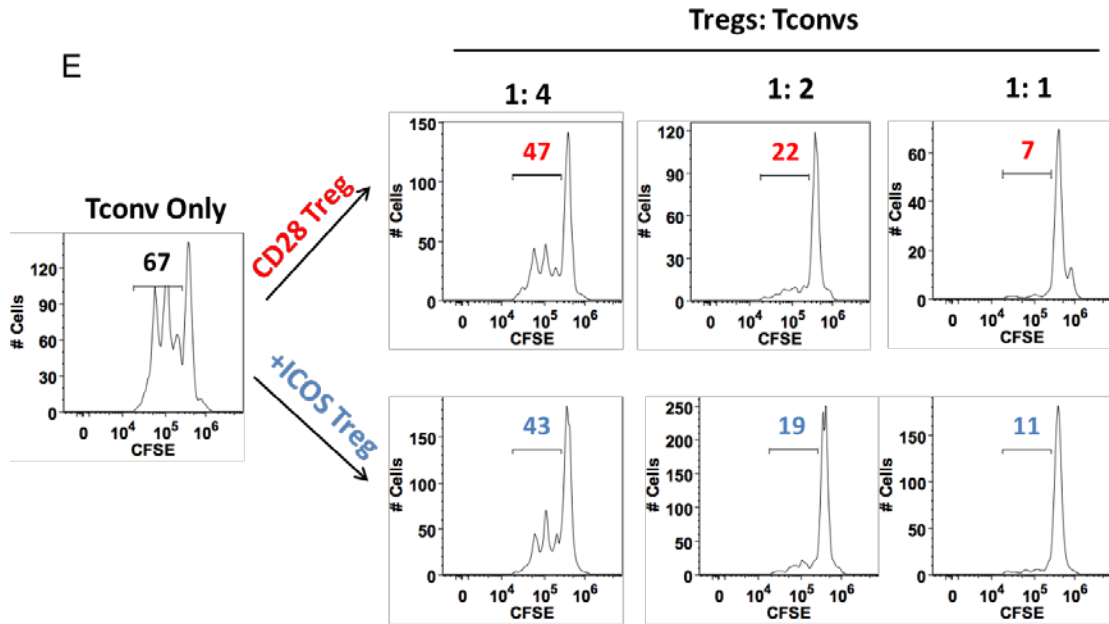


Figure 16. ICOS stimulation polarizes Tregs towards Th1*-polarized CD146⁺CCR5⁺ cells. nTregs were isolated from human PBMCs and cultured with CD3/CD28 or CD3/ICOS in the presence of 500 U/ml rhIL-2. The expressions of (A) CD25, (B) Foxp3, (C) CD146, (D) CCR5, IFN- γ and IL-17A were measured by flow cytometry (Statistical data were pooled from 3 independent experiments, student t test, mean \pm SEM, *p < 0.05). (E) After CFSE staining, CD28 or ICOS stimulated Tregs were restimulated with CD3/CD28 beads in the presence of different ratios of Tconvs as indicated above. After 3 days of culture, the frequencies of CFSE⁺ Tregs were measured by flow cytometry.

DISCUSSION

Our laboratory's clinical data suggested altered function of CD146⁺CCR5⁺ Tregs in GVHD patients. In addition, the results here indicate that ICOS stimulation increases the frequency of CD146⁺CCR5⁺ Tregs that are Th1*-polarized *in vitro*. The ICOS induced CD146⁺CCR5⁺ Tregs population decreased CD25 and Foxp3 expression, which is important for maintaining the suppressive function and stability of Tregs [93]. Tregs were reported to have the ability to convert to IL-17 secreting cells, which play an important role in the pathogenesis of autoimmune diseases [64]. To determine if these ICOS induced CD146⁺CCR5⁺ Tregs are less tolerogenic than other Tregs, I performed a suppressive functional assay to compare the function of CD28 and ICOS stimulated Tregs. Unfortunately, at this point, I didn't observe any significant differences of the suppressive ability between CD28 and ICOS stimulated Tregs *in vitro*. However, this could be explained by the discrepancies between *in vitro* studies and what really happens *in vivo* and future studies involving murine models are worth exploring.

FUTURE DIRECTION

GVHD remains the most common and fatal complication post-HSCT. In the future, our laboratory expects to develop novel treatments by inhibiting CD146⁺CCR5⁺ Tconvs and/or CD146⁺CCR5⁺ Tregs. If successful, it may have an impact on our understanding of the role of Th1* in GVHD. The implementation of xeno-GVHD model would be more clinically relevant and could ultimately explain the discrepancy between human and murine GVHD. Our laboratory has planned to perform xeno-GVHD experiments with TMP778 treatment as compared to DMSO treatment. We hypothesize that TMP778 can prevent the induction of pathogenic Th1*-polarized CD146⁺CCR5⁺ cells and we expect to see attenuated acute GVHD in the TMP778 treated group.

In future studies, our laboratory has also planned to target the plasticity of CD146⁺CCR5⁺ Tregs with the ROR γ t inhibitor. We will first do *in vitro* expansion of Tregs with TMP778 treatment or DMSO by using the protocol described in Chapter 2 and 3. We expect to see less CD146⁺CCR5⁺ Tregs generated with TMP778 in a dose-dependent manner. We will also measure the frequencies of total Tregs and CD146⁺CCR5⁺ Tregs to see the influence of ROR γ t blockade in xeno-GVHD models.

Overall, we expect that the treatment that focus on inhibiting ROR γ would have fewer side effects than general immunosuppressive drugs that GVHD patients use today and inhibit GVHD while sparing the GVL effect. We expect that the frequencies of CD146⁺CCR5⁺ Tconvs and/or Tregs can be used as GVHD biomarkers. These biomarkers may guide preemptive treatments such as ROR γ t inhibitor. Physicians will be able to treat post-HSCT patients with ROR γ t inhibitor when a high level of CD146⁺CCR5⁺ T cells is found. The combination of CD146⁺CCR5⁺ Tconvs and/or Tregs as a monitoring tool and ROR γ inhibitor as a treatment will better alleviate GVHD in the future.

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CURRICULUM VITAE

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Education

- 2006-2011: The Second Medical Institute, Southern Medical University (the former First Military Medical University), majoring in Clinical Medicine.
Degree Awarded: Bachelor of Medicine (June, 2011).
- 2012-2015: Indiana University-Purdue University Indianapolis (IUPUI), majoring in Microbiology and Immunology.
Degree Awarded by Indiana University: Master of Science (June, 2015).

Research Experience

- 2008-2010: Summer Intern, Department of Pathophysiology, Key Lab for Shock and Microcirculation Research, Southern Medical University.
- 2011-2012: Research Assistant, Laboratory of Cancer Epigenetics, Key Laboratory of Biotherapy in Zhejiang Province, Biomedical Research Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University.
- 2012-2015: Research Student, IBMG Program, Indiana University School of Medicine Graduate Division.

Academic Interests

- Human Immunology, Translational sciences, Graft-versus-host Disease.

Teaching Experience

- 2015.1-2015.5: Teaching assistant in Microbiology and Immunology, Department of Microbiology and Immunology, Indiana University-Purdue University Indianapolis.

Peer-Reviewed Publications

- Feng L, Pan M, Sun J, Lu H, Shen Q, Zhang S, Jiang T, **Liu L**, Jin W, Chen Y, Wang X, and Jin H. Histone deacetylase 3 inhibits expression of PUMA in gastric cancer cells. *J Mol Med (Berl)*, 91(1):49-58, January 2013.
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Hematology Meeting, San Francisco, CA, 2014. * **Plenary session (one of the top 6 abstracts among 6000).**

- Li W, **Liu L**, Gomez A, Zhang J, Ramadan A, Zhang Q, Mumaw C, Greenson J, Hammer S, Braun T, Jiang D, Virts E, Kelich S, Chu H, Hanash S, Hanenberg H and Paczesny S. Proteomics analysis of pre-symptomatic graft-versus-host disease plasma leads to discovery of a gut-tropic Th17-prone CD146CCR5 T cell population. (Manuscript in preparation)