Scholar Commons
USF University of South Florida

University of South Florida
Scholar Commons

Graduate Theses and Dissertations

Graduate School

1-1-2015

Targeting T-bet for Prevention of Graft-Versus-Host Disease and Leukemia Relapse after Allogeneic Hematopoietic Stem Cell Transplantation

Jianing Fu
University of South Florida, jianing@mail.usf.edu

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the Cancer Biology Commons, Cell Biology Commons, and the Immunology and Infectious Disease Commons

Scholar Commons Citation

Fu, Jianing, "Targeting T-bet for Prevention of Graft-Versus-Host Disease and Leukemia Relapse after Allogeneic Hematopoietic Stem Cell Transplantation" (2015). *Graduate Theses and Dissertations.*
http://scholarcommons.usf.edu/etd/5828

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.

Targeting T-bet for Prevention of Graft-Versus-Host Disease and Leukemia Relapse after Allogeneic Hematopoietic Stem Cell Transplantation

by

Jianing Fu

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cell Biology, Microbiology and Molecular Biology
College of Arts and Sciences
University of South Florida

Major Professor: Xue-Zhong Yu, M.D., M.S.
Co-Major Professor: Claudio Anasetti, M.D.
Amer Beg, Ph.D.
Kenneth Wright, Ph.D.

Date of Approval:
March 12, 2015

Keywords: T cell differentiation, IFN-γ, hematopoietic antigen presenting cells, T cell apoptosis

Copyright © 2015, Jianing Fu

Dedication

This dissertation is dedicated to my mom Lixin Zhao and dad Qiang Fu, who are giving me their endless love and support sustained me through this journey. And to my grandparents who gave me full sweet memories in my childhood. Also to my aunts, uncles, and cousins who together define the feelings of home.

I want to specially thank Dr. Ting-Chao Chou and Dorothy Chou who inspire me and give me kindest support in both science and life.

I would like to thank my friends, Jie Chen, Ying Han, Valentina Schneeberger, Ye Zhang, Yanting Yang, Min Huang, Yongxia Wu, Jessica Heinrichs, Fang Gao, Marilena Tauro, Yu Yu, Kane Kaosaard, Dapeng Wang, Kelly Barrios, Kelley Haarberg, Jeremy Frieling, Xiaohong Zhao, Qian Wu, Lihua Ye, and Xiao Wan. You made my life colorful in these fleeting years.

Acknowledgments

I am very thankful to my mentor Dr. Xue-Zhong Yu for his careful guidance and strong support, which brought me into the transplant immunology world and inspired my interest. His knowledge, patience, and attitude towards science lead me to the current stage, and I feel my continuous growth and improvement. I really appreciate the help from my co-major professor Dr. Claudio Anasetti, who with unequalled knowledge and experience that made me have more stringent demands on myself. I would like to show my gratitude to all the current and previous members of my committee: Dr. Amer Beg, Dr. Kenneth Wright, Dr. Eduardo Sotomayor, and Dr. Alan List, for their kind and helpful assistance during my PhD study.

I would like to specially thank Dr. Pavan Reddy for taking the time to serve as my external committee chair.

A special ‘thank you’ is also extended to the past and present Yu lab members: Dapeng Wang, Yu Yu, Kane Kaosaard, Kelley Haarberg, Jessica Heinrichs, Yongxia Wu, Hung Nguyen, Steven Schutt, David Bastian, Anusara Daenthanasanmak, and Yuejun Liu. They have given me full support and their assistance to my study is greatly appreciated.

I also want to thank Dr. Tomomi Toubai, Dr. Chen Liu, Dr. Niklas Finnberg, and Dr. Youhai Chen, for providing the experimental design suggestions, pathology scoring, as well as the important reagents for my project.

Thanks for the technique support provided by the flow cytometry core, animal facility, molecular biology core, and bioinformatics core of H. Lee Moffitt Cancer Center, as well as by

the flow cytometry core, imaging core, and animal facility of Medical University of South Carolina.

Last but not least, I would like to thank Dr. Wright and Cathy, who have always given thoughtful replies towards my questions and helped me handle with all the policy changes and paper work due to my off campus international student status. I am honored to be one of the graduate students in our Cancer Biology PhD Program.

Table of Contents

List of Tables.. v

List of Figures. vi

Abstract viii

Chapter 1: Background 1

1.1 T-bet: a master regulator shaping immune responses 1

1.1.1 T-bet in innate immunity 1

1.1.2 T-bet in adaptive immunity 3

1.1.3 T-bet in disease 5

1.2 Allo-HSCT, GVHD, and GVL 7

1.3 Helper T-cell differentiation: general pathways and characteristics 10

1.3.1 The Th1/Th2 cell paradigm 12

1.3.2 Differentiation and characteristics of Th17 cells 13

1.3.3 Interaction of Th17 cells with Th1, Th2, and Tregs 14

1.4 Helper T-cell subsets in GVHD: experimental and translational evaluations 16

1.4.1 Transcription factors involved in T-cell differentiation in GVHD 16

1.4.1.1 Th1: T-bet, STAT1, STAT4, c-Rel 16

1.4.1.2 Th17: RORγt, STAT3, AhR, and IRF4 19

1.4.1.3 Tregs: Foxp3 20

1.4.2 Effector cytokines of distinct T-cell subsets: contribution to GVHD 21

1.4.2.1 Th1: IFN-γ, TNF-α, and IL-2 21

1.4.2.2 Th17: IL-17, TGF-β, IL-21, IL-23, and IL-6 23

1.4.3 Reciprocal differentiation of Th1, Th2, and Th17 cells in GVHD 26

1.5 Helper T-cell subsets in GVHD: clinical strategies 27

1.5.1 Current findings of the role of T-cell subsets in clinical GVHD 27

1.5.2 New approaches to target T-Cell differentiation for controlling GVHD in clinic 28

1.6 Roles of CD8 T cells in GVHD development and mediating GVL effect 33

1.7 Effect of APCs in GVHD and GVL 34
1.7.1 APCs populations..... 34
1.7.1.1 Hematopoietic APCs..... 34
1.7.1.2 Nonhematopoietic APCs..... 35
1.7.2 Pathways of antigen presentation after allo-HSCT..... 36
1.7.3 Effect of APCs in GVHD 36
1.7.3.1 DCs in GVHD..... 36
1.7.3.2 B cells in GVHD..... 37
1.7.3.3 Macrophages in GVHD 38
1.7.3.4 Nonhematopoietic APCs in GVHD..... 38
1.7.4 Roles of APCs in mediating GVL effect 39
1.7.5 Strategies to targeting APCs in allo-HSCT 39

Chapter 2: T-bet is Critical for the Development of Acute Graft-versus-Host Disease through Controlling T cell Differentiation and Function..... 41
2.1 Abstract..... 41
2.2 Introduction..... 42
2.3 Materials and methods..... 43
2.3.1 Mice 43
2.3.2 BMT models 44
2.3.3 Flow cytometry and serum cytokine detection..... 45
2.3.4 Microarray and real-time PCR..... 45
2.3.5 *In vitro* generation of Th17 cells and Th17-mediated GVHD model..... 47
2.3.6 Statistics..... 47
2.4 Results..... 48
2.4.1 T-bet is required for CD4 T-cell mediated acute GVHD 48
2.4.2 T-bet is important for GVHD induction in miHA-mismatched model..... 50
2.4.3 Differential gene profiles of T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells after allo-BMT 51
2.4.4 T-bet regulates T-cell pathogenicity through IFN-γ-independent manners. 53
2.4.5 T-bet controls the optimal function of Th17 cells in GVHD induction..... 56
2.4.6 T-bet$^{-/-}$ donor T cells largely preserve the GVL effect by neutralizing IL-17 in allo-BMT recipients..... 60
2.5 Discussion..... 63

Chapter 3: T-bet Promotes Acute Graft-versus-Host Disease by Regulating Hematopoietic Antigen Presenting Cells in Mice 70
3.1 Abstract 70
3.2 Introduction 71
3.3 Materials and methods 72
3.3.1 Mice 73
3.3.2 BMT models 73
3.3.3 Chimeras BMT model 74
3.3.4 GVL model 74
3.3.5 Flow cytometry 75
3.3.6 *In vivo* mixed lymphocyte reaction (MLR) 75
3.3.7 Statistics 75
3.4 Results 75
3.4.1 T-bet expressed on host contributes to aGVHD 76
3.4.2 T-bet on host determines alloresponse and impairs early apoptosis of allogeneic donor T cells 76
3.4.3 T-bet expressed on host promotes donor CD4 T-cell accumulation in spleen and infiltration to liver 80
3.4.4 T-bet expressed on host contributes to GVHD in CD4 but not CD8-dependent miHA-mismatched model 83
3.4.5 T-bet on host hematopoietic cells is primarily responsible for promoting GVHD 85
3.4.6 T-bet regulates antigen presenting ability of donor hematopoietic APCs to induce GVHD 86
3.4.7 T-bet$^{-/-}$ host DCs produce less IFN-γ and induce early apoptosis of donor T cells primarily through Trail/DR5 axis 86
3.4.8 T-bet$^{-/-}$ recipients partially maintain GVL effect 91
3.5 Discussion 93

Chapter 4: Conclusions and Future Directions 98

List of References 102

Appendices 131
Appendix A 132
Appendix B 133
Appendix C 134

Appendix D 135

About the author .... End Page

List of Tables

Table 1.1 Role of T-bet in regulating different diseases 6

Table 1.2 Interventional strategies on APCs in allo-HSCT 40

Table 2.1 Differential protein expression of T-bet$^{-/-}$ and IFN-$\gamma^{-/-}$ T cell after allo-BMT 55

List of Figures

Figure 1.1 Three phases of GVHD pathophysiology ... 9

Figure 1.2 Pathways of helper T-cell differentiation: cytokines, transcription factors and epigenetic modifications. ... 11

Figure 1.3 Therapeutic strategies to control GVHD by targeting of T-cell differentiation. ... 29

Figure 2.1 T-bet is required for CD4 T-cell mediated acute GVHD ... 50

Figure 2.2 T-bet is required for GVHD induction in miHA-mismatched BMT model. ... 51

Figure 2.3 Differential gene profiles of T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells after allo-BMT ... 52

Figure 2.4 T-bet regulates T-cell migration and cytokine production through IFN-γ-independent manners ... 54

Figure 2.5 T-cell differentiation from WT, T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells *in vitro* ... 56

Figure 2.6 T-bet controls the optimal function of Th17 cells in GVHD induction ... 58

Figure 2.7 T-bet regulates Th17 pathogenic or nonpathogenic signature genes after allo-BMT ... 59

Figure 2.8 T-bet and Eomes expression on WT, T-bet$^{-/-}$ and IFN-γ$^{-/-}$ T cells *in vitro* ... 62

Figure 2.9 T-bet$^{-/-}$ donor T cells largely preserve GVL effect upon neutralizing IL-17 in allo-BMT recipients ... 63

Figure 2.10 Gene profiles of IFNγR identified by microarray ... 66

Figure 3.1 Absence of T-bet expression on host ameliorates GVHD ... 78

Figure 3.2 Allogeneic donor T cells significantly reduce IFN-γ production and proliferation but increase early apoptosis in T-bet$^{-/-}$ recipients 79

Figure 3.3 T-bet expressed on host is dispensable for activation, proliferation, and apoptosis of donor T cells in syngeneic recipients 81

Figure 3.4 Allogeneic donor T cells significantly decrease accumulation in spleen and infiltration to liver in T-bet$^{-/-}$ recipients 82

Figure 3.5 Absence of T-bet expression on host ameliorates GVHD in CD4- not CD8-depedent miHA-mismatched BMT model 84

Figure 3.6 T-bet is critical for recipient hematopoietic APCs to induce GVHD.................... 86

Figure 3.7 Donor hematopoietic APCs deficient for T-bet induce attenuated GVHD 88

Figure 3.8 T-bet$^{-/-}$ host DCs induce early apoptosis of donor T cells primarily through Trail-DR5 pathway 88

Figure 3.9 DR5 expression on donor T cells in WT or T-bet$^{-/-}$ allogeneic recipients 90

Figure 3.10 *Ex vivo* MLR profile of WT or DR5$^{-/-}$ T cells 91

Figure 3.11 T-bet$^{-/-}$ recipients partially preserve GVL effect 92

Figure 3.12 T-bet on host is not required for CD107a and granzyme B production by donor CD8 T cells 93

Abstract

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective therapeutic option for many malignant diseases. However, the efficacy of allo-HSCT is limited by the occurrence of destructive graft-versus-host disease (GVHD). Since allogeneic T cells are the driving force in the development of GVHD, their activation, proliferation, and differentiation are key factors to understanding GVHD pathogenesis. On the other hand, antigen-presenting cells (APCs) are essential for allogeneic T-cell priming and the development of GVHD. The T-box transcription factor T-bet is a master regulator for IFN-γ production and Th1 differentiation. T-bet also regulates the functions of APCs including dendritic cells (DCs) and B cells. Therefore, we investigated the role of T-bet in T cell responses, as well as on APC functions, in acute GVHD (aGVHD) using murine models of allogenic bone marrow transplantation (allo-BMT).

In Chapter 2, we evaluated the roles of T-bet and IFN-γ in T-cell responses. T-bet$^{-/-}$ T cells induced significantly less GVHD compared with either wild-type (WT) or IFN-γ$^{-/-}$ counterparts in CD4-driven major histocompatibility complex (MHC)- or minor histocompatibility antigen (miHA)–mismatched models. We defined several T-bet-dependent but IFN-γ-independent molecules that may account for this distinct outcome. Further study indicates that T-bet also controls the optimal activity of Th17 cells to induce GVHD. Moreover, the compromised graft-versus-leukemia (GVL) effect of T-bet$^{-/-}$ T cells could be essentially reversed by IL-17 neutralization. Thus, targeting T-bet or regulating its downstream effectors independent of IFN-γ may be a promising strategy to control GVHD in the clinic.

In Chapter 3, we evaluated the role of T-bet on APCs and found that T-bet$^{-/-}$ recipients developed much milder GVHD than their WT counterparts in MHC-mismatched or CD4-depedent miHA-mismatched models. As the functional readout of APCs, allogeneic donor T cells, particular CD4 subpopulation, significantly reduced IFN-γ production, proliferation and migration, and caused less damage in liver and gut in T-bet$^{-/-}$ recipients. We further observed that T-bet on recipient hematopoietic APCs, particular DCs, was primarily responsible for donor T-cell response and pathogenicity in GVHD. In fact, Trail/DR5 interaction served as a major signaling pathway responsible for donor T-cell apoptosis and impaired GVHD development in T-bet$^{-/-}$ recipients. Furthermore, T-bet expression on the host is largely dispensable for the GVL effect.

Taken together, we propose that T-bet is a potential therapeutic target for the control of GVHD through regulating T cells as well as APCs. We believe further exploration and understanding of the immunobiology of T-bet in controlling the activities of T cells and APCs in GVHD will expand therapeutic options for the continuing success of allo-HSCT.

Chapter 1: Background[1]

1.1 T-bet: a master regulator shaping immune responses

T-bet is a T-box family transcription factor encoded by *Tbx21*, which denotes "T-box expressed in T cells" due to its original discovery in controlling the activity and function of T helper type 1 (Th1) cells (1). While accumulating studies have shown that T-bet is expressed in multiple immune cell lineages, including dendritic cells (DCs), natural killer (NK) cells, NKT cells, and certain subsets of innate lymphoid cells (ILCs) in the innate immune system, as well as CD4 and CD8 T cells, B cells, and γδ T cells in the adaptive immune system. Thus, T-bet serves as a master regulator to shape immune responses and has been considered as a bridge to link innate and adaptive immunity (2).

1.1.1 T-bet in innate immunity

The innate immune system provides the first line defense against infection. As one of the major professional antigen presenting cells (APCs) in the innate immune system, DCs always attract the attention of researchers. Shortly after T-bet was defined in Th1 cells (1), a different study demonstrated that the expression of T-bet in human myeloid DCs could be induced rapidly and directly by IFN-γ (3). The adjuvant activity of CpG DNA to control infection also requires T-bet expression in murine DCs (4). Functional studies both *in vitro* and *in vivo* further indicated

[1] Portions of this chapter have been previously published (Fu et al. Arch Immunol Ther Exp (Warsz). 2014 Aug; 62(4):277-301.) and are utilized with permission of the publisher (p130).

that T-bet is crucial for optimal IFN-γ production by DCs, and is required for antigen-specific CD4 T-cell priming (5). Mechanistically, several signaling pathways have been identified responsible for T-bet expression in DCs, including IFN-γ, IL-12, IL-18 and Toll-like receptor 9 (TLR9), among which the IFN-γ signaling seems to be the dominate pathway (4, 6, 7). Upon interacting with T-cell receptor (TCR) on T cells, the DCs will produce IFN-γ and IL-12, which then triggers T-bet-driven Th1 differentiation from naïve CD4 T cells (6). On the other hand, overexpression of T-bet in DCs can enhance the surface expression of CXCR3 and IL-12Rβ2 on CD4 T cells, and promote the granzyme B and IFN-γ production by CD8 T cells resulting in the augmented tumor antigen specific CD8 T-cell responses (7). Therefore, the potential positive feedback loop constituted by IFN-γ and T-bet in DCs play an important role to activate T cells, connecting the innate and adaptive immune responses.

NK cells are able to rapidly respond to viral infection and tumors, because they kill the target cells without recognizing MHC or producing antibodies. NKT cells are considered as a heterogeneous group of T cells sharing the characteristics of T cells and NK cells, thus involving both innate and adaptive immunity. The expression of T-bet is required for the development, maturation, and function of both NK and NKT cells (8-10). T-bet$^{-/-}$ NK cells are immature and sensitive towards apoptosis (8). Subsequent study revealed that the normal development and terminal maturation of NK cells relies on the combination regulation of both T-bet and another T-box transcription factor eomesodermin (Eomes) (9). The failure of IFN-γ production and impaired survival of T-bet$^{-/-}$ NKT cells are possibly due to the low response to IL-15 receptor signaling given the decreased expression of CD122 (IL-2 and IL-15 receptor β-subunit) (8). However, despite of reduction in number, T-bet$^{-/-}$ NKT cells, are sufficient to induce the airway hyperreactivity through the compensatory increase of IL-4 and IL-13 (11).

T-bet has also been shown to play a critical role to control the development and proper function of ILCs, a family of cells regulating homeostasis and remodeling at mucosal sites, particular group 1 and 3 with the dominate expression of transcription factor T-bet and RORγt, respectively (12). T-bet$^+$ ILC1s are responsive to IL-12 and IL-15, and can produce IFN-γ (13), which was found to correlate with mucosal tissue inflammation in human Crohn's disease (14). The plasticity of ILC3s seems to be controlled by T-bet. T-bet expression in ILC3s leads to the induction of IFN-γ and repression of IL-17 that is partially mediated through IL-7 receptor signaling (15). The expression of T-bet was then identified in CCR6$^-$, not CCR6$^+$ ILC3 subsets, which is positively related to the natural cytotoxicity receptor NKp46 expression and IFN-γ production that is essential for protecting epithelial barrier against infection (16).

1.1.2 T-bet in adaptive immunity

The adaptive immune system consists of multiple highly specialized cells to eliminate pathogens. The clonal expansion of T lymphocytes is initiated through TCR recognition of specific pathogen-derived antigens presented by APCs. Unlike in the innate immune system, effect of T-bet in the adaptive immune system has been extensively studied especially in CD4 and CD8 T cells (2).

Although naïve CD4 T cells do not express T-bet, it is actively induced through the stimulation of TCR and IFN-γR signaling as the first wave, and IL-12R signaling as the second wave (1, 17-19). T-bet self-induction did not occur when IFN-γ or IL-12 was present during Th1 differentiation, but this may happen through IFN-γ and IL-12-independent pathways (20). The regulation of T-bet expression in Th1 cells also occurs at post-transcriptional or post-

translational levels controlled by the microRNA-29 (21, 22) or the ubiquitin-dependent degradation pathway which involves the Lys-313 in the T-box domain (23), respectively.

T-bet has a unique role in the differentiation of all three subsets (Th1, Th2, Th17) of $CD4^+$ Th cells by promoting the Th1 differentiation, while simultaneously inhibiting the opposing Th2 and Th17 lineage commitment (24). This involves a complex epigenetic regulation network (25). The bivalent histone H3 lysine 4 (H3K4me3) and H3K27me3 modifications reveal the flexibility of master regulator expression in different lineages, particularly for the murine *Tbx21* locus (26). T-bet associates with RbBp5, a core component of H3K4 methyltransferase complexes, and with JMJD3, a H3K27 demethylase (27). Epigenetic studies showed that T-bet is able to mediate the removal of the repressive H3K27me3 mark at the *Ifng* and *Cxcr3* promoters and establish the permissive H3K4-methylation state in mice (27). The concomitant repression or interference of other lineage-specific transcription factors, on the other hand, helps the reinforcement of Th1 differentiation. For example, T-bet directly binds to *Gata3* locus (28), or indirectly binds to *Il4* silencer site by forming a T-bet-RUNX3 complex (29), effectively preventing Th2 differentiation. Similarly, T-bet binds to RORγt activator RUNX1 that prevents *Rorc* (which encodes RORγt) and *Il17* expression and impairs Th17 differentiation (30).

While T-bet predominately controls the production of IFN-γ by CD4 T cells (31), it has cooperative and partially redundant function with Eomes in regulating homeostasis and cytolytic activity of CD8 T cells (32-34). T-bet expression in CD8 T cells also relies on TCR and IL-12R signaling, which accounts for the early activation of antigen-specific CD8 T cells. Then Eomes is induced in a RUNX3-dependent manner, and substitutes T-bet to further produce IFN-γ and granzyme B (35, 36). Once the pathogen clearance is achieved, a reservoir of memory CD8 T cells is generated. T-bet expression is associated with effector memory T cells that are equipped

with a more differentiated phenotype (37), while Eomes is more important for the maintenance of central memory cells (38).

B cells can produce different antibodies in response to antigen stimulation, through a process that includes affinity maturation and class-switch recombination. Studies show that T-bet can regulate IgG class switching in B cells and thus control their autoantibody production (39). IFN- γ, TLR9 and CD40 signaling induces T-bet expression in B cells, and leads to subsequent induction of IgG2a, IgG2b and IgG3, but repression of IgG1and IgE (39-41). T-bet also regulates the persistence and survival of IgG2a^{+}CD38^{hi} memory B cells through transcriptional regulation of mature B cell receptor (42). Moreover, the migration of IgG2a^{+} memory B cells to inflammatory sites is driven by T-bet through positively controlling chemokine receptor CXCR3 (43) .

T-bet is inducibly expressed in γδ T cells, a subset of the non-conventional T cells, through TCR signaling (44). The cytokine production of γδ T cells, particularly IFN-γ, is regulated by both T-bet and Eomes (45).

1.1.3 T-bet in disease

Given T-bet regulates various cell types in both innate and adaptive immune systems, it is not surprising that T-bet plays a critical role in many diseases, which have been summarized in Table 1.1. The effect of T-bet could be either beneficial or detrimental, depending on the types of cells that are controlled by T-bet, and how they contribute to the pathogenesis of different diseases. Graft-versus-host disease (GVHD), which occurs after the allogeneic hematopoietic stem cell transplantation (allo-HSCT), is our absolute focus in this dissertation.

Table 1.1 Role of T-bet in regulating different diseases.

Disease	Role of T-bet	Cell Type Affected	Evidence	Refs
GVHD	detrimental	CD4 T, CD8 T; APCs	- Control T-cell differentiation and function through IFN-γ-independent manners; - Regulate hematopoietic APC function to promote allogeneic T-cell proliferation, migration, and survival.	(46) (47) (48)
Experimental autoimmune encephalomyelitis (EAE)	detrimental	Th1 and Th17	- T-bet-dependent pathway, such as IL-23R signaling, but not pathway-specific end products, such as IFN-γ and IL-17, contributes to CD4 T-cell encephalitogenicity.	(49) (50) (51)
	dispensable	Th17	- T-bet$^{-/-}$ Th17 cells maintain/enhance GM-CSF and IL-17 production, and are able to induce EAE.	(52) (53)
Crohn's disease	detrimental	Th1	- Increased T-bet expression and IFN-γ production in $CD4^{+}$ T cells correlates with disease development.	(54) (55)
		ILC1s	- T-bet^{+} ILC1s are responsive to IL-12 and IL-15, and can produce IFN-γ contributing to disease development.	(14)
Type 1 diabetes	detrimental	CD4 T	- IFN-γ production controlled by T-bet in T cells is a contributing factor.	(56)
		DCs	- T-bet expression in DCs and CD4 T cells is required for the initiation and later stage pathogenesis, respectively.	(57)
Inflammatory arthritis	detrimental	DCs	- T-bet$^{-/-}$ DCs have compromised ability to secrete proinflammatory mediators and to prime naive T cells.	(58)
Asthma	beneficial	Th2	- T-bet regulates the Th1/Th2 balance by inhibiting Th2 differentiation and IL-4 and IL-13 production to alleviate asthma.	(59) (60)
		B cells	- T-bet negatively regulates IgE production.	(41)
Allograft rejection	beneficial	Th17	- T-bet regulates the Th1/Th17 balance by inhibiting Th17 differentiation and IL-17 production to suppress allograft rejection.	(61)
		Th2, Th17	- T-bet$^{-/-}$ RORγt$^{-/-}$ CD4 T cells drive Th2-mediated allograft rejection.	(62)

1.2 Allo-HSCT, GVHD, and GVL

Allo-HSCT, previously known as allogeneic bone marrow transplantation (BMT), is a transplant between two genetically non-identical individuals (63), which serves as an important therapeutic option to treat various malignant and non-malignant diseases, including, acute and chronic leukemias, lymphomas, multiple myelomas, aplastic anemias, solid tumors, and severe immunodeficiency disorders (64). There is estimated to be more than 20,000 allo-HSCTs performed annually around the world. Some patients present with remission of primary diseases after allo-HSCT, however, in many cases patients develop a common secondary complication named GVHD, in which transplanted cells injury the host tissues. GVHD accounts for 15–30% of deaths following allo-HSCT and is a major cause of morbidity in up to 50% of transplant recipients (63). The effectiveness of allo-HSCT for many malignant diseases is due to T-cell mediated graft-versus-tumor (GVT) or graft-versus leukemia (GVL) effects. However, allo-HSCT benefits are frequently offset by the destructive GVHD which is also induced by donor T cells (65).

GVHD has a complex etiology but ultimately is the result of donor T-cells recognizing disparate histocompatibility antigens (HA) of the recipient and causing injuries to normal tissues (66). Murine models of GVHD are generally grouped into MHC-mismatched, or minor HA-mismatched situations. GVHD develops in response to a full (class I and II) MHC disparity is dependent on CD4 T cells and with additive pathology caused by CD8 T cells. Different from CD4-dependent GVHD, CD8-induced GVHD primarily depends on the cytolytic machinery. Either CD8 T cells, CD4 T cells, or both, may play a role in minor HA induced GVHD depending on the strain combination. A majority of clinical allo-BMT recipients are MHC matched but minor HA disparate with the donor, however, there is no one single mouse model to

fully represent clinical setting of allo-HSCT (67). There are two forms of clinical GVHD: acute GVHD (aGVHD) is characterized by damage to skin, liver, lung, and gastrointestinal tract. Patients with aGVHD have increased risk of morbidity and mortality following allo-HSCT. Chronic GVHD (cGVHD) has more diverse manifestations and many autoimmune-like characteristics. Moderate to severe cGVHD substantially diminishes long-term survival as well as quality of life for patients (68). GVHD has been postulated to occur in several stages (66, 69). The first stage involves the cytoreductive conditioning and/or immunosuppression, which allows for donor cell engraftment. Release of proinflammatory cytokines affects host APCs and helps fuel the alloreactive donor T-cell response through alloantigen recognition. This is followed by the "induction phase" in which TCR ligation and costimulation combine to achieve full T-cell activation and subsequent expansion. As the alloreactive T cells expand, they differentiate into different subsets; this is driven by the existence of certain transcriptional factors and cytokines. Activated T cells further home to various GVHD target organs, a process controlled by adhesion molecules, integrins, and chemokines. The production of chemokines in inflamed and injured tissue also results in the recruitment of other cell-types (neutrophils, NK cells, monocytes/macrophages) to the GVHD target organs further contributing to tissue injury. The "effector phase" is noted for the destruction of host tissue by immune effector molecules (e.g. FasL, perforin, granzymes, IFN-γ, and TNF-α) and results in the continued production of proinflammatory cytokines that continually fuel the entire GVHD process (Figure. 1.1).

The challenge of allo-HSCT for treatment of leukemia and other malignancies of the hematopoietic system is the prevention of GVHD without losing the GVL effect. GVL also depends on donor T cells that recognize alloantigens and tumor-associated antigens. Various strategies have been used in different murine models to successfully separate GVHD from GVL

such as: depletion of alloreactive T cells, inhibition of inflammatory cytokines, interfering with T-cell cytolytic pathways, co-stimulatory pathways and trafficking, infusing certain subsets of T cells, and using immunosuppressive cell populations, including Tregs and NKT cells (70-72). Despite its paradigmatic and clinical relevance, the mechanisms involved in the GVL effect are still not completely understood and many currently used strategies to prevent GVHD impair T-cell function with deleterious GVL effects. However, advances are being made gradually by documenting the potential of GVHD prevention and even with GVL augmentation, such as the strategy of adoptively transfer murine or human Trail^{+} T cells to induce the fratricide of alloactivated donor T cells (73).

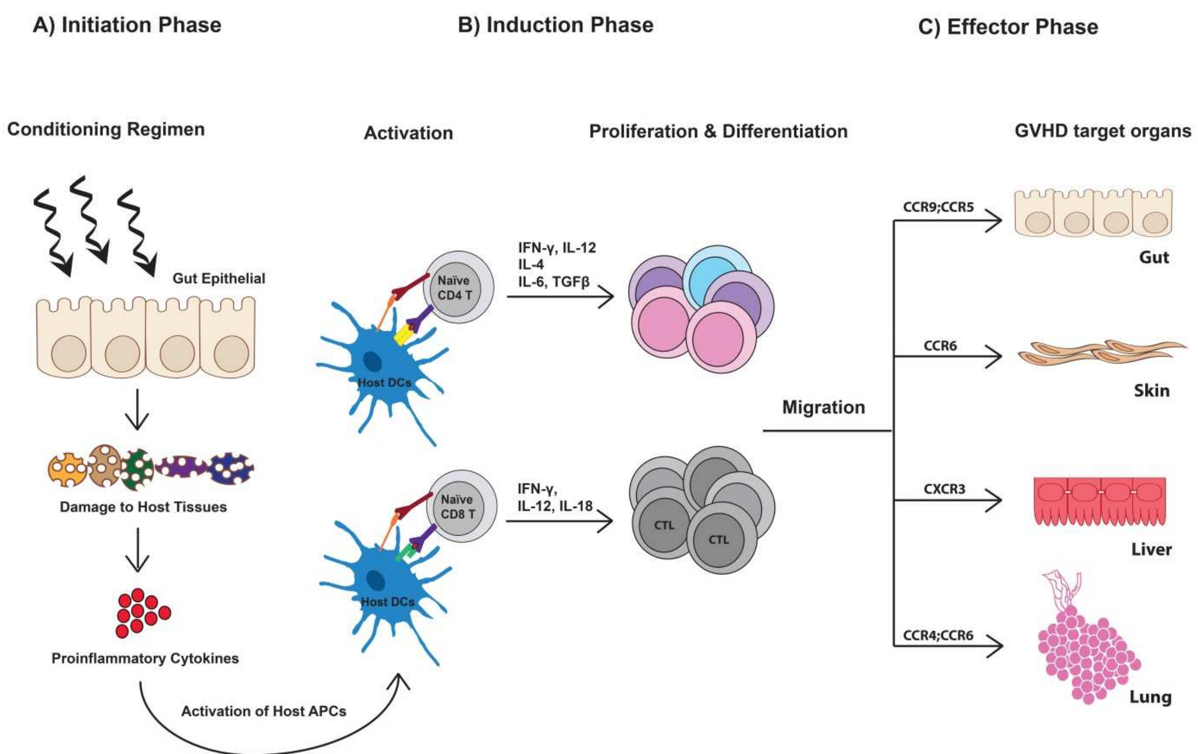
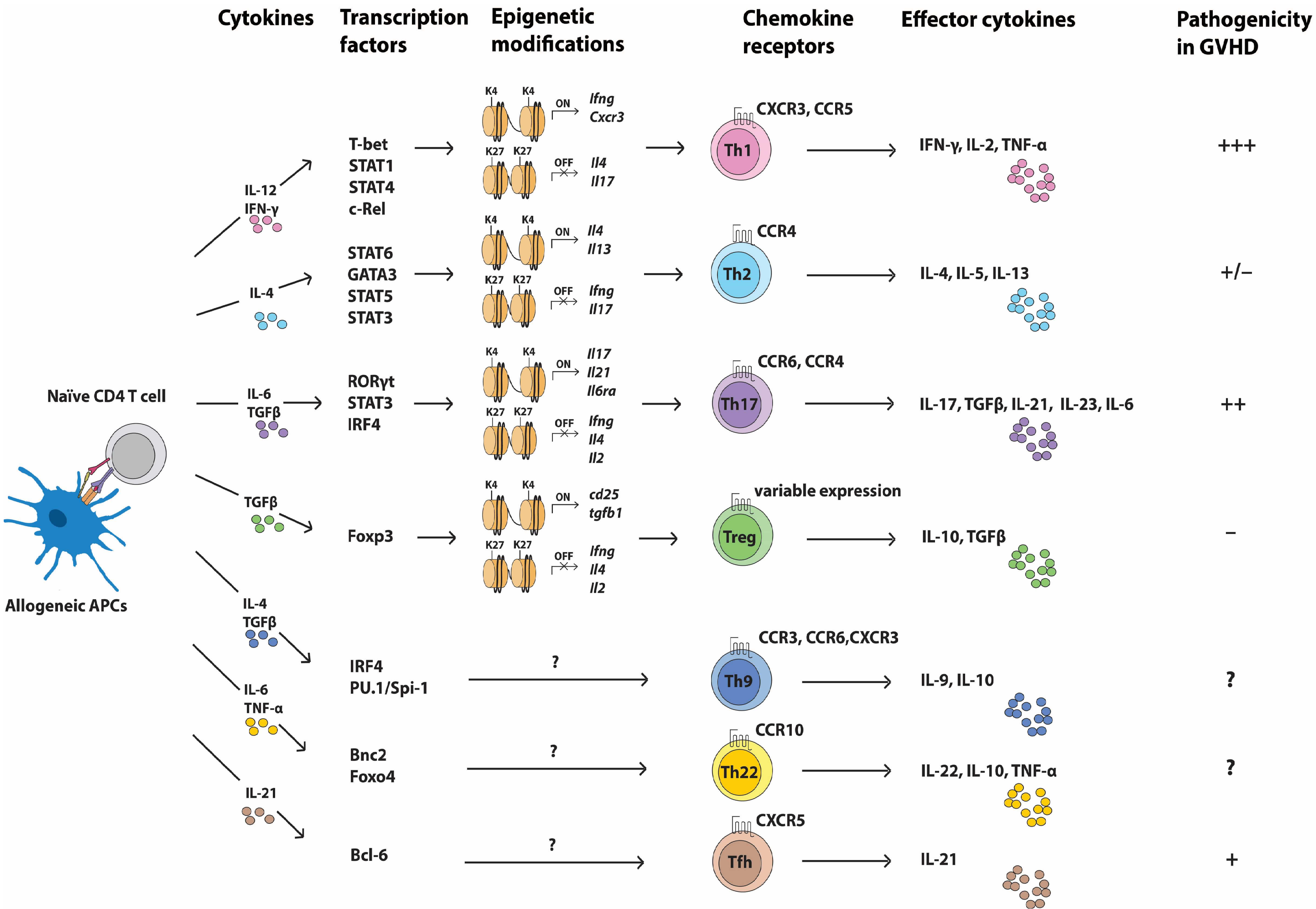


Figure 1.1 Three phases of GVHD pathophysiology. Acute GVHD has been postulated to occur in three sequential phases. (1) The initiation phase starts from conditioning regimen (e.g., irradiation) that makes “space” to allow for donor cell engraftment, but also causes host tissue damage. Release of proinflammatory cytokines leads to increased expression of MHC and adhesion molecules that enhance alloantigen presentation by host APCs to donor CD4 and CD8

T cells. (2) The induction phase involves TCR ligation and costimulation to achieve full T-cell activation and subsequent proliferation. As the alloreactive T cells expand, they differentiate into different subsets. This process is driven by the cytokine milieu through lineage-specific transcription factors. Activated T cells release additional inflammatory cytokines, express chemokine receptors, and migrate to various GVHD target organs. Chemokine production in inflamed tissues recruits other cell types (NK cells, neutrophils, monocytes) to the target organ sites, further contributing to GVHD pathology. (3) The effector phase is noted for the destruction of host tissue by immune effector molecules, such as perforin, granzymes, IFN-γ, and TNF-α, culminating in clinically evident GVHD.

1.3 Helper T-cell differentiation: general pathways and characteristics

T-cell activation, proliferation, and differentiation are considered determining factors for GVHD development. Naïve $CD4^+$ T cells can commit to particular lineages on the basis of different modes of stimulation, antigen concentration, costimulation, and cytokine milieu. It is well established that following activation, CD4 T cells will differentiate into either Th1 (secreting IL-2 and IFN-γ), Th2 (secreting IL-4, IL-5, and IL-13), Th17 (secreting IL-17, IL-21, and IL-22), or Tregs (secreting IL-10 and TGF-β) (74). Several newly reported subsets of Th cells, such as Th9, Th22, and T follicular helper cells (Tfh), also enriched our understanding about T-cell differentiation. (75-77). Acute GVHD has been considered a Th1-type disease based on the predominance of cytotoxic T lymphocyte (CTL)-mediated pathology and the increased production of Th1-type cytokines, including IFN-γ (78, 79). However, Th17 cells have also been implicated in the induction of experimental murine aGVHD (80, 81). In this section, we summarize the general paradigms and characteristics among Th cells, including Th1, Th2, Th17, and Tregs (Figure1.2).



Cytokines
Transcription factors
Epigenetic modifications
Chemokine receptors
Effector cytokines
Pathogenicity in GVHD
Naïve CD4 T cell
Allogeneic APCs
IL-12
IFN-γ
T-bet
STAT1
STAT4
c-Rel
K4
K27
ON
OFF
Ifng
Cxcr3
Il4
Il17
CXCR3, CCR5
Th1
IFN-γ, IL-2, TNF-α
+++
IL-4
STAT6
GATA3
STAT5
STAT3
Il4
Il13
Ifng
Il17
CCR4
Th2
IL-4, IL-5, IL-13
+/–
IL-6
TGFβ
RORγt
STAT3
IRF4
Il17
Il21
Il6ra
Ifng
Il4
Il2
CCR6, CCR4
Th17
IL-17, TGFβ, IL-21, IL-23, IL-6
++
TGFβ
Foxp3
cd25
tgfb1
Ifng
Il4
Il2
variable expression
Treg
IL-10, TGFβ
–
IL-4
TGFβ
IRF4
PU.1/Spi-1
?
CCR3, CCR6,CXCR3
Th9
IL-9, IL-10
?
IL-6
TNF-α
Bnc2
Foxo4
?
CCR10
Th22
IL-22, IL-10, TNF-α
?
IL-21
Bcl-6
?
CXCR5
Tfh
IL-21
+

Figure 1.2 Pathways of helper T-cell differentiation: cytokines, transcription factors and epigenetic modifications. Upon encountering cognate antigens presented by APCs, naïve CD4 T cells differentiate into different T helper subsets, including the classic Th1, Th2, Th17, Tregs, or more recently defined Th9, Th22, or Tfh effector cells. Cytokines secreted in the microenvironment during differentiation play a major role in dictating the phenotype of activated CD4 T cells. T-cell lineages are further defined depending on the expression of transcription factors, chemokine receptors, and effector cytokines, which are influenced by epigenetic modifications. In the standard model, CD4 T cells can differentiate into Th1, Th2, Th17, or Treg cells when activated in the presence of IL-12/IFN-γ, IL-4, IL-6/TGFβ, or TGFβ, respectively. T-bet, GATA3, RORγt, and Foxp3 have been well characterized and considered as the master lineage determinants of Th1, Th2, Th17 and Treg cells, accordingly. T-bet leads to gene silencing of Th2 and Th17 effector cytokines: IL-4 and IL-17 thus opening chromatin at Th1 cytokine or chemokine receptor gene loci, such as IFN-γ and CXCR3 activating transcription. Likewise, GATA3 causes gene silencing of Th1 and Th17 effector cytokines: IFN-γ and IL-17 thus activating Th2 effector cytokines IL-4 and IL-13. CD4 T cells may also differentiate into Th9, Th22, or Tfh subsets when activated in the presence of IL-4/TGFβ, IL-6/TNF-α, and IL-21, respectively. They express lineage specific transcription factors, chemokine receptors, and produce effector cytokines. Based on the respective cytokine profiles, responses to chemokines, and interactions with other cells; these fully functional Th cells exert specific functions and contribute to immune response. Different symbols represent the pathogenicity of different Th subsets in GVHD. "+": pathogenic effect; "-": protective effect; "?": non-conclusive effect.

1.3.1 The Th1/Th2 cell paradigm

Th1 and Th2 cells have two distinct cytokine secretion patterns that have been defined for both murine and human Th cells (82). Mosmann and Coffman initiated Th1/Th2 paradigm in 1986, when their seminal work was published, which showed that murine $CD4^+$ T-cell clones could be characterized not only on the basis of their antigen specificity but also by their functional skill sets, defined initially by differential cytokine secretion profiles (83). This paradigm was further strengthened by equivalent findings from other groups, proving Th1 cells distinctively produce IFN-γ, lymphotoxin, IL-2 and TNF-α. In contrast, Th2 cells produce their signature cytokines: IL-4, IL-5, and IL-13. However, Th2 cells do produce lower levels of TNF-α, GM-CSF, and IL-2 than Th1 cells (84, 85). Beyond the studies on lineage-specific cytokines, critically important work from the groups of Zheng and Flavell (86) and Szabo et al. (1) further identified lineage-specific transcription factors for Th1 and Th2 cells in mice. T-bet (*Tbx21*) was

found to be the critical transcription factor for the generation of Th1 cells, whereas GATA3 served that role for Th2 cells. Evidence provided by different studies strongly suggests that the cytokines play a major role in inducing the transcription factors that determine T-cell differentiation (18, 87). Th1 cells are triggered by IL-12 and IFN-γ, and Th2 cells are triggered by IL-4. With growing experience it has become clear that for Th1 and Th2 cells specialization in patterns of cytokine production, transcription factors and other phenotypic characteristics do occur in mice and in humans. The Th1/Th2 paradigm has been accepted for more than 20 years (85). The differential development of these subsets allows for protective immunity against specific pathogens. Th1 cells are important in mediating cellular immune responses and play a critical role in the clearance of intracellular pathogens, whereas Th2 cells are mainly involved in humoral immunity such as allergic immune responses and anti-parasitic immunity (88).

1.3.2 Differentiation and characteristics of Th17 cells

Th17 cells are highly proinflammatory cells that are part of the adaptive immune response mounted against extracellular pathogens and also have been implicated in the induction of several autoimmune diseases in mice (89). In the past nine years, an accumulation of information on this popular T-cell subset (Th17) has been witnessed in both humans and mice: The cytokines for its differentiation and expansion have been identified and the key transcription factors that are involved in its generation have been elucidated (90). Murine IL-23 has long been recognized to be an inducer of IL-17 (91). However, naïve murine T cells do not express the IL-23R and do not differentiate into Th17 cells in the presence of IL-23 in vitro (92). Therefore, IL-23 cannot be the sole inducer of Th17 differentiation although it appears to have a role in the maintenance of effector function once these cells are induced (93). Other cytokines such as IL-6

and IL-21 are implicated in the regulation and induction of IL-17 production by using gene deficient mice (94, 95). In vitro, IL-6 is a potent inducer of Th17 cells but only when combined with other cytokines including TGF-β (96). Thus, it appears the conjunction of TGF-β with IL-6 is the initial drivers of Th17 specification, and they can also activate the transcription factor STAT-3, which is required for murine Th17 differentiation (97). However, full expression of the Th17 phenotype depends on RORγt, which induces the expression of IL-17 and IL-17F, contributes to the generation of IL-23R, and mediates IL-22 production in mice (98). In the absence of IL-6, IL-21 in combination with TGF-β can function as an alternative signal for the induction of Th17 cells (95). In disease models such as EAE that depend on Th17 induction, IL-6 seems to be more relevant than IL-21 (99). Other proinflammatory cytokines such as TNF-α and IL-1β have also been shown to facilitate Th17 differentiation (100, 101).

Th17 cells are characterized by the production of several distinct cytokines that are not typically secreted by Th1 and Th2 cells. In addition to the signature cytokine IL-17, Th17 cells also preferentially secrete IL-17F, IL-21, IL-22, and, in humans, IL-26 (102). These cytokines play important and non-redundant roles in enhancing the host defense against extracellular pathogens. The receptors for IL-17 and IL-22 are broadly expressed on various epithelial tissues. Thus, Th17 cells play critical roles in tissue immunity by mediating the crosstalk between immune system and parenchymal tissues.

1.3.3 Interaction of Th17 cells with Th1, Th2, and Tregs

A balanced interaction among various T-cell subsets is very important in maintaining immune homeostasis. Th1 and Th17 cells have a proinflammatory role and have been implicated in many inflammatory conditions in humans and mice (103), while an anti-inflammatory role is

attributed to Th2 and Treg cells. Th1 and Th2 cells inhibit the development of each other through the actions of their lineage-specific cytokines IFN-γ and IL-4, respectively (104, 105). This principle fits for Th17 cells as well, since the differentiation of Th17 cells can be inhibited by IFN-γ or IL-4. However, once fully differentiated, Th17 cells are resistant to the suppressive effects of IFN-γ and IL-4 *in vitro* (106, 107). Coexistence of Th17 and Th1 cells in inflammatory lesions suggest their possible redundant function in pathology of murine experimental autoimmune uveitis (108).

Another important aspect of Th17 cell differentiation is their relationship with Tregs. Tregs can follow a developmental path similar to other T cells and emerge from the thymus (referred tTreg or frequently as nTreg, for natural) or differentiate extrathymically from naïve $CD4^+$ T cells; Tregs in the latter subset are known as peripherally induced Treg cells (referred pTreg, or frequently as iTreg, for induced) (109, 110). Tregs are critical for the maintenance of immunological homeostasis and self-tolerance in humans and mice (111). These cells have been classically defined as being $CD4^+$ $CD25^+$ and expressing the transcription factor Foxp3 (112). Development of Th17 or Treg cells from induced murine naïve T cells has been suggested under a mutually exclusive manner (113). IL-6 plays an important role in regulating the balance between Th17 cells and Tregs in humans and mice. IL-6 induces the development of Th17 cells from naïve T cells together with TGF-β; in contrast, IL-6 inhibits TGF-β-induced Treg differentiation (114). Thus, IL-6 acts as a potent proinflammatory cytokine through promotion of Th17 differentiation and inhibition of Treg differentiation indicating that this cytokine alters the balance between effector and regulatory T cells. Moreover, Foxp3 itself is able to associate with ROR-γt and inhibit ROR-γt transcriptional activation in mice (115).

1.4 Helper T-cell subsets in GVHD: experimental and translational evaluations

GVHD is a major complication of allo-HSCT. Most of the knowledge about the pathophysiology of GVHD has been derived from studies in mouse models. The differentiation of Th cells requires coordinated cytokine signaling that induces the activation of specific transcription factors and the production of linage specific cytokines to promote lineage-specific $CD4^+$ T-cell differentiation. Here we summarize the experimental and translational evaluations of Th-cell subsets in GVHD, including the roles of important transcription factors and effector cytokines, and their reciprocal differentiation features.

1.4.1 Transcription factors involved in T-cell differentiation in GVHD

The molecular mechanism by which a Th precursor adopts a Th1, Th2, Th17, or Treg phenotype involves a complex interplay between the induction of lineage specific transcription factors, such as T-bet, GATA3, RoRγt, and Foxp3, and the stimulatory effects of the cytokine milieu which are largely provided by host APCs (116).

1.4.1.1 Th1: T-bet, STAT1, STAT4, c-Rel. Several transcription factors in coordination induce full Th1 differentiation, including T-bet, STAT1, STAT4, and c-Rel. As one of the master regulators that shape immune responses, T-bet has a unique role in the differentiation of all three subsets (Th1, Th2, Th17) of $CD4^+$ Th cells by promoting the Th1 differentiation, while simultaneously inhibiting the opposing Th2 and Th17 lineage commitment (24). T-bet also controls the expression of a subset of genes encoding molecules that influence the migration and homeostasis of Tregs during Th1 cell-mediated immune responses (24). T-bet is a transcriptional activator of IFN-γ (1), and the key regulator for the Th1 differentiation program. T-bet orchestrates Th1 migration by directly controlling expression of chemokine receptor CXCR3 and

chemokines CCL3 and CCL4 (117, 118). T-bet can also function as a repressor of certain genes such as *Socs1*, *Socs3,* and *Tcf7*, that promotes the development of Th1 fate. T-bet physically associates and functionally recruits the transcriptional repressor Bcl-6, to the *Socs1*, *Socs3*, and *Tcf7* promoter regions and represses their transcription (119). The repression of *Socs* family members *Socs1* and *Socs3* is necessary to create functional Th1 cells. The repression of *Tcf7* will inhibit Th2 fate by sequestering the Th2-specific transcription factor GATA3 away from the *Il5* and *Il13* promoters. Runt-related transcription factors also participate in the differentiation process. Runx1 and Runx3 were found to promote Th1 cell differentiation in coordination with T-bet (29, 120). T-bet and Runx3 bind to *Il4* silencer and prevent *Il4* expression (24). In Th17 cells, T-bet interacts with Runx1 and blocks Runx1-mediated transactivation of *Rorc*, then suppresses the Th17 lineage development (24). Our group previously showed targeting T-bet and RORγt can prevent GVHD without offsetting GVL activity in allo-BMT murine models (47). Our recent study also found that T-bet is critical for CD4 T-cell-mediated GVHD by controlling T-cell differentiation and function, also the involvement of T-bet-dependent but IFN-γ-independent molecules can partially explain the compromised ability of T-bet deficient but IFN-γ sufficient CD4 T cells in murine GVHD induction (46).

STAT1 is another main signal transducer for IFN-γ (121). In response to IFN-γ stimulation, STAT1 is activated and then initiates Th1 development. T-bet is induced by IFN-γ and STAT1 signal pathway upon T-cell activation. Once triggered, T-bet in turn activates IFN-γ expression, leading to autocrine and paracrine positive feedback effects on IFN-γ/STAT1 signaling and further controls Th1 differentiation (17). STAT1 activation in GVHD target tissue and secondary lymphoid organs is one of the earliest events in GVHD induction (122). Ma et al.

(123) showed that absence of STAT1 in donor $CD4^+$ T cells promotes the expansion of Tregs and reduces GVHD in mice.

STAT4 is another important transcription factor involved in the Th1 cell differentiation (124). By inducing IFN-γ production, STAT4 creates a positive feedback loop for further T-bet and IL-12Rβ2 expression. At later stages of differentiation, IL-12/STAT4 pathway upregulates expression of IL-18Rα. IL-12 along with IL-18 induces IFN-γ production, independent of TCR activation, thus creating a pathway for enhancing Th1 response (125). Nikolic et al. (126) evaluated the role of STAT4 or STAT6 in the induction of aGVHD using gene deficient mice as donors, and found that $STAT4^{-/-}$ donor T cells mediated aGVHD with delayed kinetics compared with wild-type (WT) or $STAT6^{-/-}$ T cells. On the other hand, STAT6 was indispensable for liver and skin GHVD.

c-Rel controls CD4 T-cell differentiation during diverse immune responses by using both T-cell autonomous and nonautonomous mechanisms (127). Hlx, another transcription factor and Th1 cell-specific homeobox protein, has been shown to synergize with T-bet in promoting the transcription of IFN-γ (128). One explanation is that c-Rel might indirectly regulate the expression of IFN-γ by controlling the levels of Hlx. The defect in IFN-γ production by c-Rel-deficient Th1 cells could also be due to a remodeling failure at IFN-γ locus (129). Alternatively, c-Rel could be involved in a pathway parallel to T-bet or serve as a common effector molecule downstream of T-bet and STAT4 pathways. Thus, the precise position of c-Rel in the hierarchy of the Th1 transcriptional cascade leading to the production of IFN-γ has not been defined yet. Our group recently found that (130) T cells deficient for c-Rel have a dramatically reduced ability to cause aGVHD after allo-BMT by using major and minor histocompatibility mismatched murine models. Our data showed that c-$Rel^{-/-}$ T cells had a reduced ability to

expand in lymphoid organs and to infiltrate in GVHD target organs in allogeneic recipients. c-Rel$^{-/-}$ T cells were defective in the differentiation into Th1 cells after encountering alloantigens, but were enhanced in the differentiation toward Foxp3^{+} Treg cells. Furthermore, c-Rel$^{-/-}$ T cells had largely preserved activity to mediate GVL response. Thus, c-Rel plays an essential role in T cells in the induction of aGVHD.

1.4.1.2 Th17: RORγt, STAT3, AhR, and IRF4. The development of Th17 cells is mainly dependent on the transcription factors RORγt, STAT3, AhR, and IRF4 (98, 131). ROR*γ*t together with ROR*α* synergistically directs the differentiation program of Th17 lineage and their absence completely aborted Th17 development (131). Fulton and colleges (132) have evaluated the role of Th17 cells in murine aGVHD by infusing donor T cells lacking *Rorc*. Their data suggests that *Rorc* deficient T cells have reduced ability to induce aGVHD. However, our study demonstrated that (47) RORγt alone plays little role on donor T cells in the induction of aGVHD, but RORγt significantly control the T-cell pathogenesis in aGVHD in the absence of T-bet. Thus, targeting both T-bet and RORγt can effectively prevent GVHD without offsetting GVL activity in murine models of allo-BMT (47).

As one of GVHD-specific signaling pathways, STAT3 plays an important role in the differentiation process by activating downstream IL-6, IL-21, and IL-23 signaling. It can also induce ROR*γ*t expression by binding to IL-17A and IL-17F promoters (133). Deficiency of STAT3 causes enhanced expression of T-bet and Foxp3, which are involved in the development of opposing Th1 and Treg lineages, respectively (134). Ma et al. (135) reported that, STAT3 activation in the spleen correlated with high levels of IL-6 and IL-10. The marked change in IL-6/IL-10 ratio during the development of GVHD could suggest that STAT3 may act as a promoter of inflammation during the early priming and induction phase of GVHD (high IL-6/IL-10 ratio)

and may mediate anti-inflammatory signals at later time points (low IL-6/IL-10 ratio). A study from Lu et al. (136) also provides evidence that STAT3 and ERK1/2 phosphorylations are critical for T-cell alloactivation and GVHD. Furthermore, by using conditional STAT3 knockout mice as donors, Radojcic et al. (137) demonstrated that STAT3 is critical for CD4-driven cGVHD.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with important physiological roles in biological responses, such as controlling cell proliferation and inflammation. Studies suggest that AhR promotes Th17 differentiation through the inhibition of STAT1 and STAT5. However, absence of AhR did not completely abort Th17 differentiation, but was associated with inability to produce IL-22 (138, 139). Activation of AhR by its prototypic ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), induces potent suppression of aGVHD in mice. Suppression is associated with development of a regulatory population of donor $CD4^+$ $CD25^+$ T cells, but is independent of CTLA4-IFN-γ-IDO pathway (140).

BATF/IRF4 complexes are also involved in the transcriptional network to control Th17 differentiation. Cooperatively bound BATF and IRF4 contribute to the initial chromatin accessibility and play a central role in RORγt-mediated activation of Th17 molecular signatures (141). $IRF4^{-/-}$ mice failed to enhance expression of RORγt and subsequently did not develop EAE as a result of impaired Th17 response (142). However, the role of IRF4 in the pathogenesis of GVHD needs to be further investigated.

1.4.1.3 Tregs: Foxp3. As an important subset of Th cells, roles of Tregs in GVHD have been widely investigated by us and others (143-146). Accumulating studies clearly indicate that regulatory $CD4^+$ T cells play a vital role in attenuating GVHD and are critically important for the maintenance of tolerance (147-149). Tregs appear to uniquely express the Foxp3 transcriptional

repressor. Foxp3 expression negatively correlated with the severity of GVHD in patients (150). Clinical studies on recipients receiving BMT suggest that antigen-specific $CD4^{+}CD25^{+}Foxp3^{+}$ T cells play a critical role in the regulatory control of GVH reactions mediated by both alloreactive and autoreactive lymphocytes (151).

1.4.2 Effector cytokines of distinct T-cell subsets: contribution to GVHD

1.4.2.1 Th1: IFN-γ, TNF-α, and IL-2. Acute GVHD has been proposed to be induced by Th1 cells based on the predominant increased production of Th1-type cytokines, including IFN-γ, TNF-α, and IL-2, which are detected in serum or donor T cells during aGVHD in both humans and mice (152, 153). Paradoxically, the principal Th1 cytokine IFN-γ appears to be unnecessary for GVHD induction and it can even inhibit the development of GVHD in lethally irradiated allo-HSCT recipients (154-157). One study (155) showed that $CD4^{+}$ T cells from IFN-$\gamma^{-/-}$ mice resulted in accelerated GVHD after lethal total body irradiation (TBI) using a single MHC- II mismatch model. In sharp contrast, the use of these same IFN-$\gamma^{-/-}$ $CD4^{+}$ donor cells in combination with sublethal TBI significantly ameliorated GVHD-associated mortality. Administration of anti-IFN-γ antibodies to sublethally irradiated recipients of WT donor cells confirmed the role of IFN-γ neutralization in $CD4^{+}$ T-cell-mediated GVHD. Thus, opposite roles of IFN-γ on CD4-mediated GVHD were shown to depend on different irradiation conditions. The absence of IFN-γ is protective against GVHD in models using sublethal TBI, whereas deleterious GVHD occurs in lethal TBI conditioning. Further studies indicate that IFN-γ plays a protective role in lung, but a detrimental role for gut tissues (158, 159). IFN-γ specifically suppresses lung GVHD through IFN-γR signaling in lung parenchymal cells (160, 161).

TNF-α has been implicated to be involved in the pathophysiology of GVHD at several steps. It participates in the initiating stage and amplifies the disease progression once established. A series of clinical experiments demonstrated the strong correlation between TNFR1 levels and GVHD supporting the importance of TNF-α in this process (162). Cooke et al. (163) found that donor resistance to lipopolysaccharide significantly reduces the severity of aGVHD after allogeneic BMT and that attenuation of early intestinal toxicity mediated by TNF-α is responsible for this effect. TNF-α has both direct and indirect effect on GVHD: TNF directly induce apoptosis on target tissues, and TNF indirectly activates T-cell proliferation pathways. TNF-α has been used as a therapeutic target in preclinical GVHD models with promising results. Soluble TNF receptors or monoclonal antibodies can be used to inhibit TNF-α pharmacologically. Korngold et al. (164) have found that a chimeric rat/mouse anti-TNF-α mAb could reduce the development of GVHD but preserve the GVL responses in murine models, with a more significant effect in CD4- rather than CD8-mediated disease. Infliximab is a genetically constructed IgG1 murine-human chimeric monoclonal antibody against TNF-α. Binding of infliximab to the soluble subunit of TNF-α blocks the interaction of TNF-α with its receptors and causes cell lysis. Infliximab was proven to be well tolerated and active for the treatment of steroid-resistant aGVHD in patients (165). Further investigations are ongoing to define the optimal dose and duration of TNF-α inhibition for GVHD prevention and treatment.

IL-2 was initially described as a T-cell growth factor. Given this knowledge, IL-2 treatment was initially used as a strategy to augment immune responses in cancer. Despite high doses of IL-2 this treatment only induced modest antitumor responses; it also caused substantial toxicities in patients with metastatic melanoma (166). Because IL-2 is a stimulant in the immune environment, it initially has been expected to exacerbate GVHD rather than relieve it. But earlier

studies found that IL-2 inhibits GVHD-promoting activity of $CD4^+$ cells while preserving CD4- and CD8-mediated GVL effects in mice (167). Two recent clinical studies (168, 169) using much lower doses of IL-2 in different immune-mediated diseases suggesting that IL-2 may have a dominant role in immunosuppression rather than in immune stimulation. It may also explain the equivocal effects of IL-2 treatment seen in earlier clinical trials. Low dose of IL-2 administration in patients after allo-HSCT suppressed the occurrence of cGVHD (168) and improved vasculitis arising from chronic HCV infection (169). Increased numbers of Tregs were observed after IL-2 treatment in both studies. Given that Tregs have a high affinity for IL-2, meaning they bind especially well to IL-2 at low concentrations, researchers theorized that low doses of IL-2 could activate Tregs to a far greater extent than conventional effector T cells, thereby reducing the immune response causing cGVHD (168). Another preclinical study showed the synergistic effects of rapamycin and IL-2, displayed by increased expansion of donor-derived Tregs and reduced lethal aGVHD in mice (170). IL-2 sets an example to represent the complex dynamic state of the immune system involving both suppression and activation.

1.4.2.2 Th17: IL-17, TGF-β, IL-21, IL-23, and IL-6. Among investigators, a conclusive role of IL-17 in GVHD development has not yet reached a consensus. Adoptive transfer of highly purified *ex vivo* polarized murine Th17 cells results in a more aggressive disease that is dependent on IL-17 (80, 81). However, IL-$17^{-/-}$ donor $CD4^+$ T cells could attenuate, exacerbate, or had no effect on GVHD depending on the model and knockout mouse strains used (159, 171, 172). Kappel et al. (171) found that IL-$17^{-/-}$ total T cells induced similar GVHD responses as WT cells, although, when purified $CD4^+$ IL-$17^{-/-}$ T cells were given, there was improved outcomes regarding the prolonged median survival. They conclude that IL-17 contributes to the early development of $CD4^+$ T-cell-mediated GVHD by promoting production of

proinflammatory cytokines; however, it is dispensable for GVHD and GVT activity by unfractionated T cells. Yi et al. (172) obtained substantially different results using a similar murine BMT model. IL-17$^{-/-}$ T cells were found to exacerbate GVHD and to accelerate recipient mortality compared with WT T cells. Administration of exogenous IL-17 or neutralizing IFN-γ could prevent the deleterious outcomes seen with IL-17$^{-/-}$ cells. This leads the investigators to conclude that GVHD is exacerbated due to the augmentation of Th1 differentiation.

TGF-β plays critical roles in two antagonistically related Th cell subsets, Th17 and iTreg cells. The conjunction of TGF-β with IL-6 is the initial driver of Th17 specification. Hill's group demonstrated that (173) neutralization of TGF-β after allo-BMT significantly increased aGVHD severity in mice and the concurrent prevention of IL-10 production further exaggerated this effect. Early after allo-BMT, donor T cells were the predominant source of TGF-β and were able to alleviate aGVHD in a TGF-β-dependent manner. Although the neutralization of TGF-β augmented the proliferation and expansion of donor T cells after allo-BMT, it paradoxically impaired cellular cytotoxicity to host antigens and associated GVL effects. Different from the protective effects in aGVHD, TGF-β contributed to the severity of cGVHD after allo-BMT.

IL-21 is produced by CD4^{+} T cells, especially Th17 cells (174) and NKT cells (175) which signals through the IL-2Rγc and IL-21R complex. Recent reports show that inhibiting IL-21 decreases disease severity in murine models of GVHD (176, 177). Genetically disrupting IL-21 signaling or by using neutralizing mAbs could reduce transplant-related weight loss, tissue pathology, and mortality. Disease amelioration correlated with decreased numbers of CD4^{+} T cells secreting IFN-γ and a concomitant increase in the proportion of CD4^{+} T cells expressing Foxp3, which is due to the conversion of CD4^{+} CD25^{-} T cells into iTregs rather than preferential expansion of nTregs themselves (176). Critical differences exist in how IL-21 functions in mouse

and humans. IL-21 can act as the second signal required for Th17 polarization in humans (178), whereas only IL-6 but not IL-21, has this function in mice (113). Blazar's group established a human xenogeneic GVHD model (179), and found that treatment of neutralizing mAb of human IL-21 lead to significantly reduced GVHD-associated weight loss, mortality, increased Tregs, and a decrease in T cells secreting IFN-γ or granzyme B in recipient mice. Based on these findings, anti-IL-21 mAb could be considered for GVHD prevention in clinic.

By using genetic and antibody-based strategies, Das et al. (180) examined the effect of blocking of IL-23 signaling on GVH and GVL activity after allo-BMT in mice. They demonstrated that IL-23 blockade selectively protects the colon, specifically by attenuating gut injury while still preserving the GVL effect. Interestingly, the colon did not serve as a sanctuary site for subsequent systemic relapse in GVHD-protected mice since a potent GVL response could be mounted in this organ under conditions where tumor cells migrated to this site. Thus, blockade of IL-23 signaling is an effective strategy for separating GVH and GVL responses. IL-23 could be a potential therapeutic target for the regulation of alloresponses in humans.

As a pivotal inflammatory cytokine in immune system, IL-6 plays important roles in altering the balance between the effector and regulatory arms. It also drives a proinflammatory phenotype that defines GVHD characteristics. Studies show that inhibition of the IL-6 signaling pathway, by antibody-mediated blockade of the IL-6 receptor (IL-6R), markedly reduces GVHD-caused pathologic damage in mice (181). This is accompanied by a significant increase in the absolute number of Tregs and a significant reduction of Th1 and Th17 cells in GVHD target organs. This demonstrates that blockade of IL-6 signaling decreases the ratio of proinflammatory T cells to Tregs. However, another group (182) showed that a brief duration of IL-6 inhibition in recipient mice did not increase the absolute numbers of mature donor Tregs.

This could be a consequence of the several key differences between the models, including the dose of radiation, the infusion of unsorted splenocytes, and the duration of IL-6 blockade. Thus, blockade of IL-6 with anti–IL-6R mAb therapy may be testable in clinical trials to prevent GVHD in allo-BMT.

1.4.3 Reciprocal differentiation of Th1, Th2, and Th17 cells in GVHD

In addition to the well-established balance between regulatory and effector T cells, the balance among Th1, Th2, and Th17 effector subsets also plays an important role in regulating T-cell immune response. In aGVHD, naïve donor $CD4^+$ T cells recognize alloantigens on host APCs and differentiate into Th subsets, but the role of Th subsets in GVHD pathogenesis was incompletely characterized until six years ago when Yi et al. (159) published their study in which they discussed the reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in GVHD. They found that in an MHC-mismatched murine model (C57BL/6 → BALB/c), donor $CD4^+$ WT T cells predominantly differentiated into Th1 cells and preferentially mediated tissue damage in the gut and liver. However, deficiency of IFN-γ in $CD4^+$ T cells enhanced Th2 and Th17 differentiation and exacerbated tissue damage in the lung and skin. Absence of both IL-4 and IFN-γ augmented Th17 differentiation and caused preferential skin damage. Deficiency of both IFN-γ and IL-17 led to further augmentation of Th2 differentiation and idiopathic pneumonia. The tissue specific GVHD pathology elicited by Th1, Th2, and Th17 cells was partially associated with their tissue-specific migration, which is dependent upon differential expression of chemokine receptors. Several studies have described the increased expression of chemokines and chemokine receptors in GVHD (183, 184). The profile of chemokine and chemokine receptor expression is diverse in different target organs of GVHD.

The results obtained by Yi et al. (47) indicate that donor $CD4^+$ T cells can reciprocally differentiate into Th1, Th2, and Th17 cells, and each Th subset contributes to specific GVHD target organ tissue damage. This study further enriches the content of the standard model for Th differentiation, especially in aGVHD settings. Although Th2 cells may still be pathogenic to certain tissues (e.g. lung), our recent work demonstrated that Th1 and Th17 subsets are the primary driving force to induce aGVHD in mice, thus targeting these two subsets while preserving or promoting Tregs may represent an effective strategy to control aGVHD (47).

1.5 Helper T-cell subsets in GVHD: clinical strategies

1.5.1 Current findings of the role of T-cell subsets in clinical GVHD

The pathogenesis of GVHD involves various Th-cell subsets. Each individual disease manifestation appears to have a distinct T-cell milieu, which is determined by target structure and inflammatory environment. Several aspects contribute to the understanding of T-cell subset function in humans including cytokine quantifications in peripheral blood, lesion tissue biopsies, or from single nucleotide polymorphisms (SNPs) of cytokine genes and their association with GVHD (185-187). Much of the information about the role of T-cell subsets in clinical GVHD is confusing and contradictory, which indicates that, the picture of GVHD etiology is likely more complex in humans.

Despite the perceived skewing of GVHD toward a Th1 process, no study has found increased serum levels of IL-12 in the patients after allo-HSCT compared with healthy donors (188). Consistently, investigators have found increased expression for IL-6, TNF-α, and IL-1β, which correlated with either disease severity (189, 190) or disease onset (186). Zhao et al. (191)

found that, donor-derived Th17 cells in the allografts emerged as the independent risk factor during the course of aGVHD in human, which suggests that IL-17-producing cells might be a type of initiating cell in aGVHD. Recently, The Japan Donor Marrow Program analyzed rs2275913, a SNP within the promoter region of the IL-17 gene and found that patients with this SNP had increased IL-17 secretion increasing their risk of aGVHD (192). G-CSF treatment *in vivo* reduced the occurrence of aGVHD through decreasing IL-17 production by T cells. In accordance with the study reported by Zhao et al. (191), Dander et al. (193) found that the peripheral blood of patients with ongoing GVHD showed an increased number of IL-17-secreting T cells after transplantation, not surprisingly this increase was associated with the clinical course of aGVHD. In contrast, Ratajczak et al. (194) demonstrated that Th17 cells in human GVHD was not associated with any evidence of severe tissue damage at the onset of disease, but the Th17/Treg ratio detected in lesion biopsies from gastrointestinal tract and skin is positively correlated with the severity of aGVHD and serves as a sensitive and specific marker of human GVHD. In addition, they detected significantly more IFN-γ producing T cells in biopsies from affected areas. This data suggests that detection of individual T-cell subsets might not be sufficient to reflect the complicated immune processes in human GVHD.

1.5.2 New approaches to target T-Cell differentiation for controlling GVHD in clinic

Tocilizumab, a humanized anti-IL-6R mAb, has been approved in several countries for the treatment of rheumatoid arthritis and other inflammatory diseases. Administration of tocilizumab has been reported to effectively reduce GVHD severity in the gastrointestinal tract as determined by a marked reduction in the volume of diarrhea (195). A small-scale clinical study in GVHD patients indicates that tocilizumab has activity in the treatment of steroid

refractory GVHD (196). Tocilizumab is currently under phase I clinical trial and the expected effects are to decrease Th17 differentiation while increasing Treg induction in GVHD patients (149). However, Betts et al. (197) did not observe an effect of tocilizumab on human monocyte-derived dendritic cells maturation and activation, alloreactive T-cell proliferation, Treg expansion, or allogeneic Th1/Th17 responses *in vitro*, despite its on-target suppression of IL-6R signaling (Figure 1.3). Therefore, the efficacy of tocilizumab on GVHD regulation warrants further evaluation in clinic.

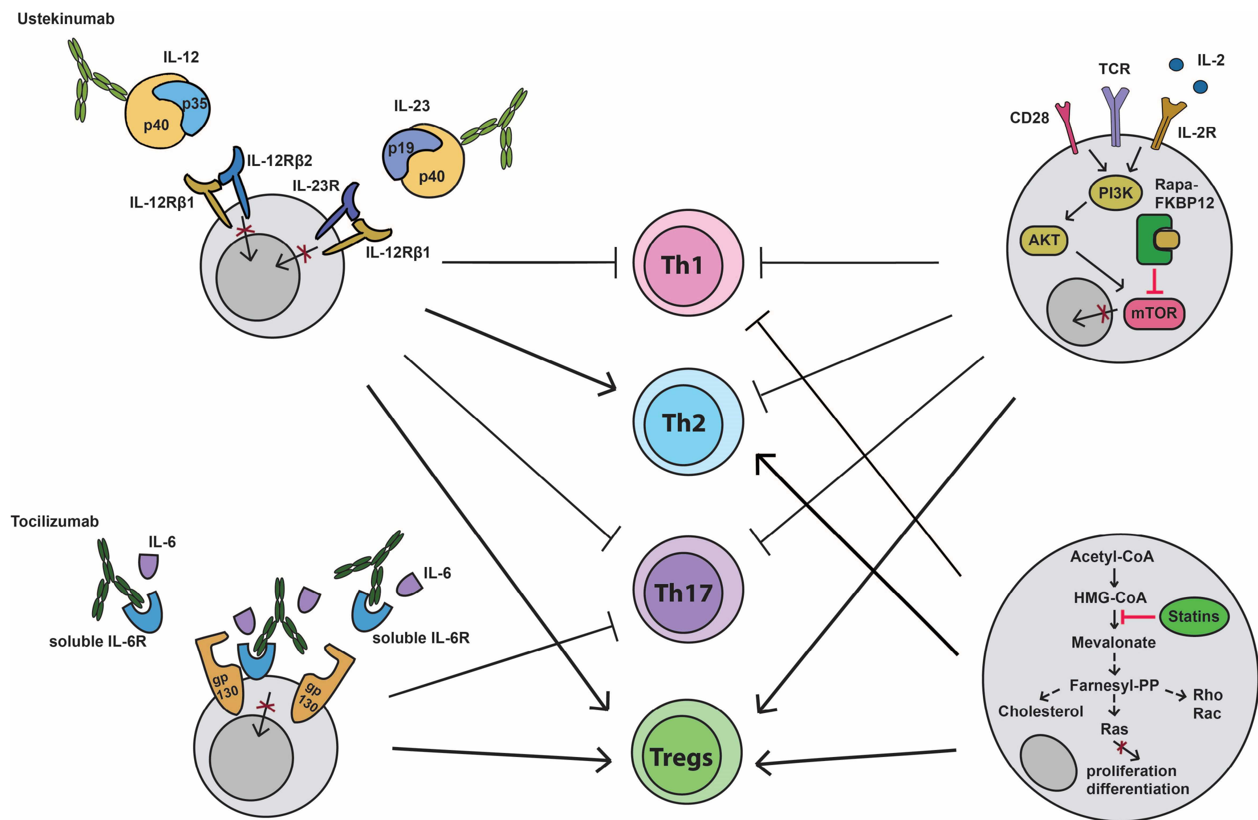


Figure 1.3 Therapeutic strategies to control GVHD by targeting of T-cell differentiation. (1) Ustekinumab, anti-p40 mAb, binds to p40 subunit of IL-12 and IL-23 preventing their interaction with IL-12Rβ1 on the T cell surface, thus, inhibiting IL-12 and IL-23 signaling. As the consequence, Ustekinumab is expected to reduce the differentiation to Th1 and Th17 while promoting the differentiation towards Th2 and Tregs. (2) Tocilizumab, anti-IL-6R mAb, interferes IL-6 binding to IL-6R either membrane bound or soluble form, thus preventing the dimerization of signal transducer gp130 and its interaction with IL-6/IL-6R complex. Treatment

is expected to result in blocking Th17 differentiation and enhancing Treg generation. (3) Rapamycin inhibits mTOR activation. In conventional T cells, diverse signals determine mTOR activity, including ligated costimulatory molecule CD28, TCR, the cytokine IL-2, and its receptor. Among the complex networks, signals are transduced through PI3K–AKT-mediated pathway to activate mTOR. Upon activation, mTOR promotes Th1, Th2, and Th17 differentiation but reduces Treg differentiation by regulating their lineage specific transcription factors. mTOR functions as part of the complexes mTORC1 and mTORC2. Rapamycin in complex with the immunophilin FK506-binding protein 1A, 12 kDa (FKBP12) inhibits mTORC1 and decreases the T-cell differentiation towards Th1, Th2, and Th17 while enhancing Tregs. (4) Statins, HMG-CoA reductase inhibitor, blocks the conversion of HMG-CoA to mevalonate and thereby prevents the formation of cholesterol and isoprenoids, which leads to the inhibition of isoprenylation of small GTPases such as Ras, Rho, and Rac. This influences multiple intracellular signaling pathways and cellular functions including proliferation and differentiation. Statins causes preferential development of Th2 cells, as well as Tregs, and inhibition of Th1-driven responses.

Ustekunimab (Stelara) is a humanized monoclonal antibody that directly binds to p40, a subunit shared by IL-12 and IL-23. As a treatment utilized to regulate the immune-mediated inflammatory disorders such as psoriasis, ustekunimab had not been previously used for GVHD until Pidala et al. (198) observed its activity in a patient with advanced refractory aGVHD. Ustekinumab was successful in inducing clinical remission of aGVHD in that patient and the correlative data on peripheral blood serum cytokine production indicated the skewing of $CD4^+$ T-cell repertoire from Th1 to Th2 and Tregs. Given that IL-12 and IL-23 are key drivers of the differentiation of Th1 and Th17 cells responsible for GVHD (199, 200), use of ustekunimab to target against both Th1 and Th17 may provide a powerful novel approach for GVHD prevention (Figure 1.3). A comparative trial, lead by Dr. Pidala, is currently ongoing to assess the biologic and clinical activity of ustekinumab in GVHD patients when given in concert with the established regimen of sirolimus/tacrolimus.

Rapamycin (Sirolimus), a macrolide antibiotic produced by *Streptomyces hygroscopicus*, is the prototypical inhibitor of the molecular target of rapamycin (mTOR). It has been used as a prophylaxis and treatment in a number of immune disorders including GVHD (201, 202). Pidala

et al. (203) provided preliminary evidence for the efficacy of sirolimus as a sole primary therapy in the treatment of aGVHD. In a series of ten patients at high risk for corticosteroid toxicity or leukemia relapse who developed grade II–III aGVHD after allo-HSCT, it was reported that primary therapy with sirolimus resulted in durable complete remission of aGVHD in 5 (50%) without requirement for glucocorticoids. Projected overall survival at 18 months is 79% and projected relapse-free survival at 15 months is 70%. Several studies recently have reported the use of gene targeting to disrupt mTOR function in lymphocytes confirming an essential role for mTOR signaling in T-cell differentiation (204). Complete deficiency of mTOR signaling (both mTORC1 and mTORC2) abrogated the differentiation of Th1, Th2, and Th17 lineages, and instead T cells were diverted into a Foxp3^{+} regulatory phenotype. This was associated with reduced levels of activation of STAT transcription factors and subsequent failure to induce expression of the Th1, Th2, and Th17 master regulators T-bet, GATA3, and RORγt (205). Different groups report distinct roles for mTORC1 and mTORC2 in T cells although with discrepancies (205, 206). Rapamycin induces *in vitro* expansion of nTreg (207), which has also been used recently to establish good manufacturing practice for expansion protocols for human CD4^{+}CD25^{+} T cells (208). Rapamycin and IL-2 enable the expansion of donor-derived nTreg and conversion of CD25^{-} T cells to iTreg, which potently inhibits GVHD development *in vivo* (170). Thus, the application of rapamycin in combination with IL-2 to get higher yields of nTreg and iTreg represents a promising strategy of pharmacologic Treg modification to treat or prevent GVHD (Figure 1.3). The main oral alternative to sirolimus is everolimus with slightly improved bioavailability and the use of everolimus in refractory cGVHD has been reported by Lutz et al. during the 38th Annual Meeting of the European Group for Blood and Marrow Transplantation in Switzerland (209). Sirolimus and everolimus are currently under phase II/III or phase I clinical

trials, respectively, and the expected effects are to decrease Th1 cell differentiation and retain or increase Treg cells in GVHD patients (149).

HMG-CoA reductase inhibitors (statins) are prescribed worldwide for the prevention and treatment of dyslipidemia and atherosclerosis (210). Statins can bind to HMG-CoA reductase and displace its natural substrate HMG-CoA, leading to inhibition of mevalonate and cholesterol biosynthesis. Cholesterol metabolism has been indicated to play an important role in modulating T-cell proliferation and differentiation through liver X receptor signaling (211, 212). Stains can also indirectly inhibit several posttranslational modification proteins such as Ras, Rho, and Rac, through reduced mevalonate production (210). Thus, statins can further effect multiple intracellular signaling pathways and cellular functions including differentiation, motility, secretion, and proliferation (213). Atorvastatin is the prototypic statin drug that has a proven safety track record (214). Inhibiting Ras leads to preferential development of Th2 cells and inhibition of proinflammatory Th1-driven responses (213). This change in intracellular signaling may also effect the development of Treg cells, as exposure of human $CD4^+$ T cells to Atorvastatin *in vitro* increased expression of Foxp3. Furthermore, patients with hyperlipidemic who took statins up to eight weeks had more circulating $Foxp3^+$ Treg cells (215). In addition to direct effects on T cells, statins inhibit IFN-γ-induced expression of MHC-II and block upregulation of a variety of costimulatory molecules and cytokines in APCs (213). Hamadani et al. (216) performed a retrospective review of 67 individuals who had received allo-HSCT containing T cells. They found that the rate of grade II–IV aGVHD in patients receiving statins was 10%, compared with 40% in non-treated group. Also the progression-free survival at three years in acute myeloid leukemia patients with or without statin use were 54 and 28%, respectively, which indicates the preserved GVL effect in patients using statins at the time of

allografting. Extensive preclinical and retrospective clinical data suggest that statins can prevent aGVHD without decreasing GVL effects. In order to prospectively validate these observations, phase-II clinical trials evaluating the role of Atorvastatin in preventing aGVHD is currently ongoing. The expected effects are to inhibit development of Th1 cells and enhance the induction of Th2 and Treg cells in GVHD patients (149, 217).

1.6 Roles of CD8 T cells in GVHD development and mediating GVL effect

T-cell alloreactivity is the key factor for the pathology of GVHD, and involves both helper CD4 and cytotoxic CD8 T-cell subsets. Unlike CD4 T cells that are activated by exogenous antigens presented by MHC-II molecules on recipient APCs, CD8 T cells are activated by endogenous antigens presented by MHC-I molecules. GVHD which developes across a full MHC disparity commonly requires both CD4 and CD8 T cells, with the dominant contribution of CD4 T cells and the additive pathology provided by CD8 T cells (218). GVHD can also develop even with matched MHCs but mismatched miHAs, where either CD8, or CD4 T cells, or both are able to induce the disease, depending on the donor/recipient strain combinations (218). In MHC-mismatched systems, the GVHD target organ damages can occur without the direct interaction between cognate T cells and the MHC expressed on tissues, particularly in CD4-, to a lesser extent in CD8-, mediated GVHD which occurs at least partially as a result of cytokine production (219). However, in miHA-mismatched systems, CD8 but not CD4 T cells require TCR binding to MHC on target tissues in order to cause GVHD (220). Only host APCs are able to stimulate CD8 T cells to induce GVHD in CD8-dependent systems (221). Activated CD8 T cells will release perforin, granzymes and upregulate FasL to cause GVHD (222, 223).

In the aspect of mediating GVL effect, both CD4 and CD8 T cells require direct leukemic contact (220). Among various types of cells (NK cells, CD4 T cells and CD8 T cells) that are involved in GVL responses, CD8 T cells are considered as the major contributor due to their potent alloantigen specificity (224, 225) and cytolytic activity (226, 227). The helper CD4 T cells release cytokines and interact with APCs through costimulatory molecules and then stimulate those APCs. The activated APCs provide a "license to kill" to cytotoxic CD8 T cells, which is also important for the GVL effect (71). After donor CD8 T cells recognize the antigen expressed by the host malignant cells, either alloantigens or tumor-associated antigens, they will further lyse the target cells through similar mechanisms involved in GVHD, including the perforin-granzyme pathway alone, or in combination with FasL-Fas pathway (228). Exposure and mobilization of lysosomal-associated membrane protrin-1 (LAMP-1/CD107a) during perfoin/granzyme-mediated lysis is well correlated with cytotoxic activity of CD8 T cells (229, 230).

1.7 Effect of APCs in GVHD and GVL

APCs are essential to induce GVHD and mediate GVL reactions due to their ability to integrate and prime T cells. Multiple cell types from either the hematopoietic or nonhematopoietic compartments may participate in antigen presentation process with distinct efficiencies (231, 232).

1.7.1 APCs populations

1.7.1.1 Hematopoietic APCs. DCs, B cells, and macrophages are the major hematopoietic APCs that are also considered as the professional APCs with a specialized ability

to process and present antigens onto MHC molecules. These professional APCs have distinct circulatory patterns and occupy different locations. Conventional DCs can be divided into migratory DCs and resident DCs. Migratory DCs include the skin epidermal Langerhans cells and dermal DCs that uptake antigen from peripheral tissue and present it in the lymph nodes. Resident DCs, such as splenic or thymic DCs, load and process antigen within lymphoid organs (233, 234). Overall, DCs are the most effective APCs to prime T cells although depending on their origin and activation status. The activated conventional myeloid DCs can produce IL-12 and promote Th1 differentiation, as well as cytotoxic CD8 T-cell responses (233). Naïve B cells recirculate between peripheral blood and secondary lymphoid organs without presenting in the skin or majority of the mucosal sites. They uptake antigens through B-cell receptor (BCR) and present on MHC-II molecules that interact with CD4 T cells, and generate efficient antibody responses (235). Macrophages are strategically stationed at various locations throughout the body and perform their immune surveillance function. When danger signals such as tissue damage occur, they are actively recruited to the inflammatory sites and prepare to stimulate lymphocytes to combat pathogens (236).

1.7.1.2 Nonhematopoietic APCs. Nonhematopoietic APCs include diverse types of cells, such as epithelial cells, endothelial cells and myofibroblasts, which can be induced to express MHC-II and develop antigen-presenting abilities under specific conditions. IFN-γ is the major effector cytokine to induce MHC-II expression both *in vitro* and *in vivo*, and likely enable those nonhematopoietic APCs to modulate either effector or memory CD4 T cell responses under inflammatory conditions (237). Nonhematopoietic APCs are distributed in different organs including the liver, intestine, and lung that are frequently targeted by allogeneic T cells during GVHD development (174, 237).

1.7.2 Pathways of antigen presentation after allo-HSCT

There are two pathways of antigen presentation after allo-HSCT, direct presentation mediated by host APCs and indirect presentation mediated by donor APCs (238). Host alloantigens, either endogenous or exogenous, can be directly presented by host APCs to donor CD8 or CD4 T cells. Alternatively, host allogeneic MHC antigens or miHAs may be crosspresented by donor APCs to donor T cells. Direct presentation is likely to be predominant during early stages of aGVHD, given that no GVHD was induced in mice lacking MHC-I on host APCs despite the capacity of donor APCs to perform cross-presentation (221). However, indirect presentation driven by donor APCs may contribute to further tissue injury thus intensifying the aGVHD (239, 240). Moreover, CD4-dependent GVHD could be induced through either host or donor APCs (241), whereas only host APCs will initiate CD8-dependent GVHD (221).

1.7.3 Effect of APCs in GVHD

1.7.3.1 DCs in GVHD. After lethal conditioning, radiosensitive DCs and precursor cells derived from the host are lost within the first few days after allo-BMT. However, that period of time is long enough for host DCs to prime donor T-cell activation, and initiate their proliferation and differentiation. Studies show that host CD11c^{+} DCs were activated and accumulated in the T cell areas of the spleen within 6 hours of lethal irradiation. After 5 days, even though less than 1% of host DCs were detectable, the primed donor CD8 T cells had undergone seven divisions which is sufficient to trigger GVHD in miHA-mismatched recipients (242). Adding back MHC-II$^{+/+}$ host-derived DCs, but not B cells, into MHC-II$^{-/-}$ mice could effectively break the resistance

of GVHD development in CD4-dependent model. Similar results were seen in CD8-dependent model although to a lesser extent (243). This demonstrates that host DCs rather than host B cells are critical in priming donor CD4 and CD8 T cells to induce GVHD. Moreover, persistence of host DCs is positively correlated with aGVHD (244, 245).

After the first few days post-transplant, the vacancy in host APC numbers is filled by differentiated APCs contained within the donor graft or by other donor BM progenitor-differentiated cells (238). Although the kinetics of full functional reconstitution are not clear, numerical recovery of DCs and macrophages has been shown to be more rapid than that of B cells (246). In preclinical murine BMT models, conventional DCs have been proved to be the critical donor APCs to induce GVHD, compared with macrophages or B cells (247). Clinical studies have documented that low numbers of circulating $CD11c^{+}$ DCs in peripheral blood at the time of engraftment were associated with the increased risk for relapse, aGVHD and death aftter allo-HSCT, which may be explained by the increased DC homing to lymphoid tissues or GVHD target organs, and the lack of regulatory DCs (248).

However, recent studies suggested that absence of recipient DCs using induced or constitutive depletion of $CD11c^{+}$ conventional DCs exacerbates aGVHD when all other APC subsets are intact (249, 250). These findings have refined our understanding of APCs in GVHD, and warn against the strategies of simply targeting APCs especially DCs as a means to alleviate GVHD (233, 251).

1.7.3.2 B cells in GVHD. The effect of B cells in aGVHD is still controversial. The effectiveness of B-cell depletion to reduce aGVHD in both mice (252) and humans (253-255) has demonstrated the pathogenic role of B cells, either donor-or host-derived. The timing of early

B-cell depletion by administrating rituximab seems crucial for its beneficical effects in aGVHD (256). In contrast, other studies have shown the protective effect of B cells. Host B cells, but not DCs within the spleen, are able to secret IL-10 after total body irradiation (TBI), thereby inhibiting the expansion of allogeneic T cells and the induction of aGVHD (257). Donor B cells have also been shown to alleviate aGVHD through a Treg-dependent mechanism (247, 258).

1.7.3.3 Macrophages in GVHD. Similar to B cells, a consensus on the roles of macrophages in GVHD has not been reached. A study evaluating human sequential biopsy indicated that host macrophages contributed to GVHD through antigen presentation and cytokine production, leading to the activation and proliferation of CD8 T cells, and cytokine expression of memory CD4 T cells. The persistence of host macrophages is likely contributing to GVHD by maintaining the activated T-cell responses (259). However, a later study suggested that host macrophages which persist after conditioning can engulf donor alloreactive T cells and prevent their proliferation. As a result, preemptive CSF-1 therapy can ameliorate GVHD by expanding host suppressive or regulatory macrophages (260).

1.7.3.4 Nonhematopoietic APCs in GVHD. Recent studies provide compelling evidence that hematopoietic APCs may not be essential for aGVHD induction, due to host nonhematopoietic APCs being sufficient to induce lethal aGVHD in mice (249, 250, 261). It has been known that nonhematopoietic cells could express MHC-II occasionally, such as within the gut and thymus (262, 263). The potential nonhematopoietic APCs that initiate GVHD include epithelial cells, endothelial cells and myofibroblasts, as suggested by different studies (249, 261). However, many aspects are still unknown regarding the mechanism of nonhematopoietic APCs to induce GVHD. For instance, where does the nonhematopoietic antigen presentation and alloactivation occur? Within the target tissues or in the context of stromal cells within secondary

lymphoid organs? Another possibility has been suggested by a recent study, where the donor DCs could incorporate the recipient MHC into their own membranes and indirectly mediate the nonhematopoietic antigen presentation (264).

1.7.4 Roles of APCs in mediating GVL effect

Both alloantigens and tumor-specific antigens (TSAs) can initiate GVL responses (70, 265). APCs are well known for inducing alloantigen-specific responses, demonstrating their importance for mediating GVL (266). Host APCs are predominated for full GVL effects, while donor APCs can also initiate GVL activity with low tumor bulk (239, 266). Given the host tumors express both alloantigens and TSAs, even if they perform antigen-presenting features, it is likely undergoing immune editing that results in ineffective T-cell response through multiple suppressive mechanisms (267). Recently, a subset of host–derived $CD8^+$ DCs is suggested to play a critical role in enhancing TSA responses, and is required for optimal GVL effects (268). In the settings of delayed lymphocyte infusion (DLI), GVL can occur without GVHD (269). Here the GVL activity is dependent on MHC alloreactivity and little or no GVL activity against miHAs or TSAs is detected (269).

1.7.5 Strategies to targeting APCs in allo-HSCT

The strategies to manipulate the functions and/or numbers of APCs to achieve therapeutic benefit of allo-HSCT have been reported and are still under active investigation (233, 238, 270). Below is a summary of major interventional strategies on APCs, particular DCs, during experimental or clinical allo-HSCT (Table 1.2).

Table 1.2 Interventional strategies on APCs in allo-HSCT.

Therapy	Target	Outcome	Evidence	Refs
Alemtuzumab (Campath-1H)	$CD52^+$ DCs and T cells	- Alloreactive stimulatory activity *in vitro* ↓ - Human aGVHD↓	- Depletion of $CD52^+$ myeloid DCs from peripheral blood but not epidermis or gut. - Depletion of $CD52^+$ T cells.	(271) (272) (273) (274) (275)
CMRF-44 IgM Ab	Langerhans cells (LC) and peripheral blood DC express CMRF-44 antigen	- Number of migratory LC ↓ -Allostimulation by LC *in vitro* ↓	- Induces LC-depletion by complement-mediated cytotoxicity *in vitro*	(276)
Anti-CD83 polyclonal Ab	$CD83^+$ activated DCs	- Allogeneic T cell proliferation ↓ - aGVHD ↓ - GVL preserved	- Induce lysis of activated $CD83^+$ DCs. -Spare pre-existing donor T cells, including those target viruses and malignant cells.	(251)
IMMU-114 mAb	$HLA\text{-}DR^+$ APCs (B cells, monocytes, DCs)	- APCs apoptosis↑ - Alloreactive T-cell proliferation ↓	- A humanized anti- HLA-DR mAb, efficiently deplete all APCs with little direct effect on T cells.	(277)
SAHA or ITF2357	Histone deacetylase (HDAC) in DCs	- Allostimulatory responses of DC ↓ - Murine aGVHD ↓ - GVL preserved	- Reduce proinflammatory cytokine production, costimulatory molecule expression by DCs. - Increase expression of IDO.	(278)
Bortezomib	Proteasome in immature DCs	- Allogeneic T-cell proliferation ↓; Th1 ↓ - Immature DCs apoptosis ↑ - Murine or human aGVHD ↓ - GVL preserved	- Inhibit phenotypic and functional maturation and induce more potent apoptosis in immature DCs through suppression of NF-κB activity. - Decrease allogeneic T-cell proliferation and increase their apoptosis.	(279) (280) (281)
Regulatory DCs (rDCs)	DC/T-cell interaction micro-environment	- Murine aGVHD and cGVHD↓ - GVL preserved	- rDCs displayed high levels of MHC molecules and extremely low levels of costimulatory molecules. - Generation of donor inducible Tregs and anergic, Ag-specific $CD4^+$ T cells.	(282) (283) (284)
Genetically modified Trail-DCs	DC/T-cell interaction micro-environment	- Murine aGVHD ↓ - GVL preserved	- Induce alloreactive T-cell and tumor-cell apoptosis through Trail-DR5 pathway.	(285)

Chapter 2: T-bet is Critical for the Development of Acute Graft-versus-Host Disease through Controlling T cell Differentiation and Function[2]

2.1 Abstract

T-bet is a master regulator for IFN-γ production and Th1 differentiation. We evaluated the roles of T-bet and IFN-γ in T cell responses in acute GVHD and found that T-bet$^{-/-}$ T cells induced significantly less GVHD compared with wild-type or IFN-γ$^{-/-}$ counterparts in both MHC-mismatched and MHC-matched but miHA–mismatched models driven by CD4 T cells. T-bet$^{-/-}$, but not IFN-γ$^{-/-}$, CD4 T cells had a markedly reduced ability to cause tissue damage in liver and gut. This distinct outcome is reflected by the differential gene expression on donor CD4 T cells deficient for T-bet or IFN-γ. At mRNA and protein levels, we defined several T-bet-dependent molecules that may account for the impaired ability of T-bet$^{-/-}$ T cells to migrate into target organs and to produce Th1-related cytokines. Moreover, these molecules were independent of either endogenous IFN-γ, such as CXCR3 and programmed death-1 (PD-1), or systematic IFN-γ, such as NKG2D, I-Ab, and granzyme B (GZMB). Although both T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells are prone to differentiate into Th17 cells, polarized Th17 cells deficient for T-bet but not for IFN-γ had a significantly reduced ability to cause GVHD. Finally, T-bet$^{-/-}$ T cells had a compromised GVL effect, which could be essentially reversed by neutralization of IL-17 in the recipients. We conclude that T-bet is required for Th1 differentiation and migration, as well as

---

[2] This chapter have been previously published (Fu et al. J Immunol. 2015 Jan 1;194(1):388-97.) and are utilized with permission of the publisher (p131).

for optimal function of Th17 cells. Thus, targeting T-bet or regulating its downstream effectors independent of IFN-γ may be a promising strategy to control GVHD in the clinic.

2.2 Introduction

GVHD is a major limitation for the efficacy of allo-HSCT in the treatment of hematologic malignancies because it leads to significant morbidity and mortality (63). The cytokine storm caused by conditioning and Th1-cell cytokines produced by allogeneic T cells are the driving forces for the initiation and development of GVHD (78, 152, 153, 286). Paradoxically, the principal Th1 cytokine, IFN- γ, plays a dispensable role for GVHD development in some experimental murine BMT models (154-157, 287, 288), where exacerbated GVHD was observed in hosts receiving IFN-$\gamma^{-/-}$ grafts (155, 157, 287, 288) or after IFN-γ neutralization (287) following lethal irradiation. On the other hand, administration of recombinant IFN-γ showed a protective effect for CD4 T-cell mediated GVHD (154) .

T-bet, the T-box transcription factor, has a unique role in the differentiation of all three subsets (Th1, Th2, Th17) of $CD4^+$ helper T cells by promoting Th1 differentiation, while simultaneously inhibiting Th2 and Th17 lineage commitment (24). T-bet target genes have been identified in primary human T cells, which show that T-bet is associated with genes of various functions in Th1 cells, including those with roles in transcriptional regulation, chemotaxis, and adhesion (117). T-bet is a transcriptional activator of IFN-γ (1) and orchestrates the cell-migratory program by directly controlling expression of the chemokine receptors CXCR3 and CCR5, as well as the chemokines CCL3 and CCL4 (117, 118). T-bet also has cooperative and

partially redundant functions with Eomes, another T-box transcription factor, to control CD8 T cell cytotoxicity and IFN-γ production (32, 33).

Previously, we observed that T cells deficient for T-bet are impaired in the induction of acute GVHD (47). However, the effect and mechanism of T-bet on T cells to induce GVHD and mediate the GVL effect has not been thoroughly studied, particularly the reason for the paradoxical outcomes of GVHD caused by T-bet$^{-/-}$ or IFN-γ$^{-/-}$ T cells. We therefore utilized T cells from T-bet$^{-/-}$ or IFN-γ$^{-/-}$ mice as donors and tested whether T-bet could be a potential target for preventing GVHD after allo-BMT. We then elucidated the underlying mechanisms by which T-bet or IFN-γ differentially regulates allogeneic T-cell response after allo-BMT. We identified several molecules that depend on T-bet, but not on endogenous IFN-γ produced by donor T cells or systematic IFN-γ produced by any type of cell, which may be responsible for T-cell pathogenicity in GVHD induction. Furthermore, we define the role of T-bet in Th17 function related to GVHD and its impact on the GVL effect. Our study provides new biological insight on T-bet, as well as the rationale to target T-bet or its downstream effectors, to control GVHD after allo-BMT.

2.3 Materials and methods

2.3.1 Mice

C57BL/6 (B6; H-2^b, CD45.2), B6.Ly5.1 (CD45.1) and BALB/c (H-2^d) were purchased from National Cancer Institute (NCI, Frederick, MD). T-bet$^{-/-}$, IFN-γ$^{-/-}$ and IFN-γR$^{-/-}$ mice on B6 background and founders of C.B10-H2^b/LilMcdJ (BALB.B; H-2^b) mice were purchased from

The Jackson Laboratory (Bar Harbor, ME). BALB.B mice were bred at H. Lee Moffitt Cancer Center (Moffitt, Tampa, FL). All animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at Moffitt or Medical University of South Carolina (MUSC, Charleston, SC). Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of University of South Florida (Tampa, FL) or MUSC.

2.3.2 BMT models

T-cell purification from whole spleen and lymph nodes was done by negative depletion using magnetic beads as previously described (47). MHC- and miHA-mismatched (B6→BALB/c) or MHC-matched but miHA-mismatched (B6→BALB.B) BMT models were used as previously established (289). Briefly, 8-10 week old recipient mice were conditioned with total body irradiation (TBI) based on weight at 750 to 850 cGy (single dose) for BALB/c and 900 to 950 cGy (split 3 hours apart) for BALB.B using a Shepherd Mark I Cesium Irradiator (J. L. Shepherd and Associates, San Fernando, CA) at Moffitt, or at 650 to 750 cGy (single dose) for BALB/c using an X-RAD 320 X-ray Irradiator (Precision X-ray Inc., North Branford, CT) at MUSC. Within 24 hours post-conditioning, recipients were intravenously injected with T-cell depleted (TCD) BM alone with or without T cells from different strains of donors. Recipient mice were monitored for weight loss and other clinical signs of GVHD twice/week. Clinical scores were tabulated as 5 parameters: weight loss, posture, activity, fur texture, and skin integrity. Individual mice were scored 0 to 2 for each criteria and 0 to 10 overall (290). A GVL model was established by intravenously injecting luc/neo plasmid-transduced A20 B-cell lymphoma cells (A20-luc) on BALB/c background the day of BMT (2×10^3 A20 cells/mouse). In

addition, recipients were treated with anti-IL-17 mAb (clone: 17F3; Bio X Cell, West Lebanon, NH) or PBS control at doses and schedules specified. Tumor growth was measured with bioluminescent imaging (BLI) using Xenogen-IVIS ® 200 Pre-clinical In vivo Imaging System (Perkin-Elmer, Waltham, MA) and analyzed by Living Image Software (Perkin-Elmer) as previously described (291). Tumor and GVHD mortality were distinguished by BLI signal intensity and clinical manifestation of GVHD. Recipients at pre-moribund stage were euthanized and counted for lethality. Representative samples of GVHD target organs were excised from recipients 14 days post-BMT and subjected to pathology scoring as previously described (47, 291).

2.3.3 Flow cytometry and serum cytokine detection

Mononuclear cells were isolated from recipient spleen or liver as previously described (47, 291, 292) and stained for surface receptors and intracellular cytokines using standard flow cytometric protocols. Stained cells were analyzed using Diva software, LSR II (BD Biosciences, San Jose, CA), and FlowJo (TreeStar, Ashland, OR). Cytokine levels in recipient serum were quantified using a cytometric bead assay (BD Biosciences, San Jose, CA) (293).

2.3.4 Microarray and real-time PCR

Lethally irradiated BALB/c mice were transplanted with WT Ly5.1^{+} B6 TCD-BM (5 × 10^{6}/mouse) plus WT, T-bet$^{-/-}$ or IFN-$\gamma^{-/-}$ naïve CD4^{+} Ly5.1^{-} T cells (1 × 10^{6}/mouse). Recipients were euthanized on day 7. Donor-derived T cells (CD4^{+} H-2K^{b+} Ly5.1^{-} DAPI^{-}) in recipient splenocytes were isolated by cell sorting (BD FACSAria II Cell Sorter), and lysed in TRIzol (Life technologies, Grand Island, NY) to extract total RNAs. Microarray analysis was performed

using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Array images were analyzed and processed by robust multi-array average (RMA) procedure (294, 295). Heat map was generated by using Cluster 3.0 and Java TreeView. In order to identify potential genes significantly changed (either increased or decreased) in the T-bet$^{-/-}$ versus WT group, but not shared by the IFN-γ$^{-/-}$ group, three sets of genes were selected: 1) signal fold change of T-bet$^{-/-}$ vs. WT >2; 2) signal fold change of T-bet$^{-/-}$ vs. IFN-γ$^{-/-}$ >2; and 3) signal fold change of IFN-γ$^{-/-}$ vs. WT >2. Fold change calculation was based on the mean value of individual values from three independent experiments. The genes overlapped in set 1 and set 2 were further selected, and genes from set 3 were excluded from those selected genes. The signal value below 500 is considered as background noise. Thus, the primary pool of T-bet-dependent but endogenous IFN-γ-independent genes was established. Additional criteria were added when heat map analysis was performed. When selecting T-bet positively regulated genes, the signal fold change of T-bet$^{-/-}$ vs. WT, T-bet$^{-/-}$ vs. IFN-γ$^{-/-}$, and IFN-γ$^{-/-}$ vs. WT is >4, >3 and <2, respectively, and the lowest signal value limit of WT group is set at 1675 ($2^{10.71}$). When selecting T-bet negatively regulated genes, the signal fold change of T-bet$^{-/-}$ vs. WT, T-bet$^{-/-}$ vs. IFN-γ$^{-/-}$, and IFN-γ$^{-/-}$ vs. WT is >2, >2 and <2, respectively, and the lowest signal value limit of T-bet$^{-/-}$ group is set at 1024 (2^{10}). In heat map scale, signal value of 1024 (2^{10}) was termed "0.00" and represented by black color; signal value of 8192 (2^{13}) was termed "3.00" and represented by red color; signal value of 128 (2^{7}) was termed "-3.00" and represented by green color. Mouse array data can be accessed at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-2198.

To quantify mRNA using real-time PCR, extracted total RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit. All PCR experiments were performed using TaqMan gene expression assays (Hopx, Mm00558630_m1; Slamf1, Mm00443316_m1; Serpinb9, Mm00777163_m1; Igf2r, Mm00439576_m1; Klrd1, Mm00495182_m1; Klrc1, Mm00516111_m1; H-2Aa, Mm00439211_m1; H-2Ab1, Mm00439216_m1; Retnla, Mm00445109_m1; GAPDH, Mm99999915_g1), universal PCR Master Mix and the ABI-PRISM 7900 Sequence Detection System (Applied Biosystems, Life technologies, Grand Island, NY). Target gene expression was calculated using the comparative method for relative quantitation upon normalization to the internal GAPDH expression control.

2.3.5 *In vitro* generation of Th17 cells and Th17-mediated GVHD model

$CD4^{+}CD25^{-}$ cells isolated from WT, T-bet$^{-/-}$ or IFN-$\gamma^{-/-}$ mice on B6 background were stimulated in the presence of syngeneic APCs with 2 μg/mL anti-CD3 mAb, 5 ng/mL TGFβ, 10 ng/mL IL-6 and 5 μg/mL anti-IFN-γ mAb. On day 3, 50U/mL mIL-2 was added. Cell phenotype ($CD4^{+}$ IL-17^{+} %) was confirmed on day 4 by intracellular cytokine staining of IFN-γ and IL-17. On day 5, polarized T cells were collected and dead cells were removed using Ficoll separation. Equal numbers of live $CD4^{+}$ IL-17^{+} cells derived from WT, T-bet$^{-/-}$ or IFN-$\gamma^{-/-}$ T cells were used to induce GVHD to compare pathogenicity.

2.3.6 Statistics

For comparison of recipient survival among groups in GVHD experiments, the log-rank test was used to determine statistical significance. To compare GVHD clinical scores, pathology scores, weight loss, cytokine levels, and gene expression levels, a Student *t* test was used.

2.4 Results

2.4.1 T-bet is required for CD4 T-cell mediated acute GVHD

Our previous study indicated that T-bet$^{-/-}$ total T cells are less pathogenic than their WT counterparts in inducing GVHD in fully MHC-mismatched BMT models (47). In contrast, IFN-γ, a major cytokine regulated by T-bet, is dispensable or even protective against GVHD development (154-157, 287, 288). Considering Eomes is preferentially expressed on CD8 T cells (32, 37) and CD4 T-cell differentiation is better defined (286, 296), we first focused our studies on CD4-mediated GVHD and aimed to further understand how T-bet and IFN-γ differentially affect the development of acute GVHD. As shown in figure 2.1, IFN-γ$^{-/-}$ CD4 T cells mediated comparable GVHD to WT CD4 T cells, whereas T-bet$^{-/-}$ CD4 T cells caused significantly milder GVHD. Recipients of T-bet$^{-/-}$ donor T cells showed significantly higher long-term survival rates (Figure 2.1A), less weight loss (Figure 2.1B) and lower GVHD clinical scores after 4 and 8 weeks following allo-BMT (Figure 2.1, C and D) compared with the recipients of either WT or IFN-γ$^{-/-}$ CD4 T cells. Alleviated GVHD caused by T-bet$^{-/-}$ CD4 T cells was also supported by significantly lower pathological scores of those recipients 14 days after allo-BMT. Recipients of T-bet$^{-/-}$ CD4 T cells had markedly reduced T-cell infiltration and tissue damage in GVHD target organs including the liver, gut, and skin (Figure 2.1, E and F). However, T-bet$^{-/-}$ CD4 T cells induced similar severity of pulmonary GVHD as WT CD4 T cells (Figure 2.1, E and F), consistent with the previous report that pulmonary GVHD is not associated with Th1-mediated response (297). Serum cytokines detected 14 days post-transplant revealed that T-bet$^{-/-}$ CD4 T cells produced significantly lower levels of IFN-γ compared to WT CD4 T cells

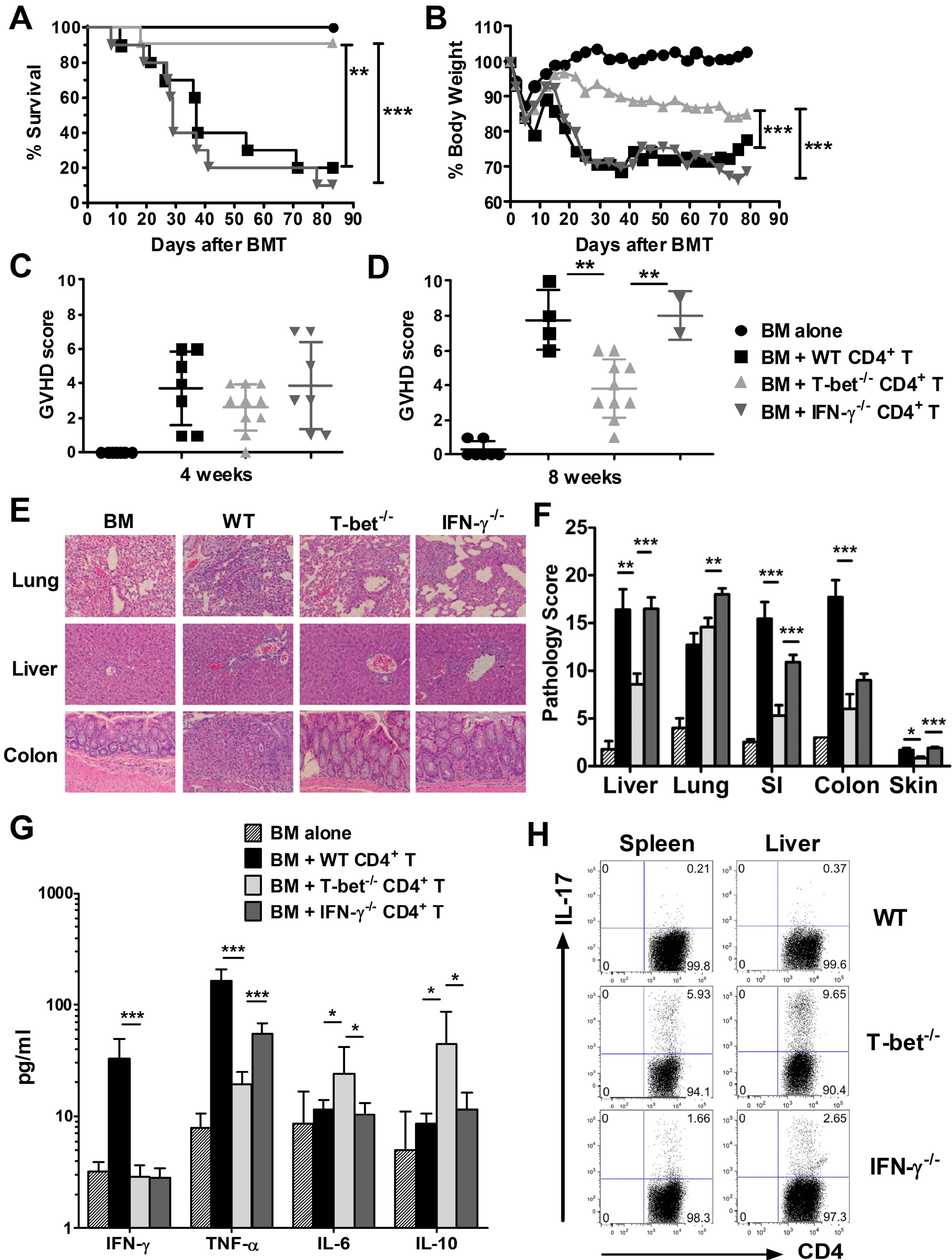

A
% Survival
Days after BMT
B
% Body Weight
Days after BMT
C
GVHD score
4 weeks
D
GVHD score
8 weeks
BM alone
BM + WT CD4+ T
BM + T-bet-/- CD4+ T
BM + IFN-γ-/- CD4+ T
E
BM
WT
T-bet-/-
IFN-γ-/-
Lung
Liver
Colon
F
Pathology Score
Liver
Lung
SI
Colon
Skin
G
BM alone
BM + WT CD4+ T
BM + T-bet-/- CD4+ T
BM + IFN-γ-/- CD4+ T
pg/ml
IFN-γ
TNF-α
IL-6
IL-10
H
Spleen
Liver
IL-17
CD4
WT
T-bet-/-
IFN-γ-/-

Figure 2.1 T-bet is required for CD4 T-cell mediated acute GVHD. Lethally irradiated BALB/c mice were transplanted with 5×10^6/mouse TCD-BM from WT B6 donor alone (n=7), or plus WT (n=10), T-bet$^{-/-}$ (n=11), or IFN-γ$^{-/-}$ (n=10) CD4$^+$ T cells at 1×10^6/mouse. Recipient mice were monitored throughout the experimental period for survival (A), weight change (B), GVHD clinical scores at 4 weeks (C) and 8 weeks (D) post-transplant. Lethally irradiated BALB/c mice were transplanted with 5×10^6/mouse TCD-BM from WT B6 donor alone (n=4), or plus WT (n=7), T-bet$^{-/-}$ (n=7), or IFN-γ$^{-/-}$ (n=8) naïve CD4$^+$ T cells at 1×10^6/mouse. Recipients were euthanized 14 days post-transplant and samples of liver, lung, small intestine, colon and skin were collected for H&E staining (E) and scored for microscopic GVHD severity by a pathologist blinded to the treatment groups. Photomicrographs depicting the average disease score morphology from one representative experiment out of two separate experiments (Original magnification ×200). Pathological score mean ± SE (F) of GVHD target organs across two separate experiments are depicted. Sera collected at necropsy 14 days post-transplant were subjected to cytokine bead analysis (G) to quantify serum cytokine concentrations of IFN-γ, TNF-α, IL-6, and IL-10. Cytokines that were undetectable are not graphically represented. (H) Recipients were euthanized 14 days post-transplant and samples of spleen and liver were collected for intracellular staining of IL-17. FACS plots show CD4$^+$IL-17$^+$ cells from representative recipients in each group. Data are shown from two replicate experiments combined. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

(Figure 2.1G). Consistent with the pathological scores, T-bet$^{-/-}$ CD4 T cells also produced lower levels of TNF-α, but higher levels of IL-10 and IL-6 compared to either WT or IFN-γ$^{-/-}$ CD4 T cells (Figure 2.1G). Furthermore, intracellular IL-17 expression was significantly higher in T-bet$^{-/-}$ or IFN-γ$^{-/-}$ donor T cells than in WT donor T cells in recipient spleens and livers (Figure 2.1H). Altogether, T-bet deficient CD4 T cells produced lower levels of inflammatory cytokines and induced less damage in the recipient liver, gut and skin, indicating that T-bet, not IFN-γ, is required for CD4 T-cell mediated acute GVHD.

2.4.2 T-bet is important for GVHD induction in miHA-mismatched model

miHA mismatches play a critical role in clinical GVHD development in human leukocyte antigen (HLA)-identical BMT conditions (298). Given the effect of T-bet$^{-/-}$ or IFN-γ$^{-/-}$ total T cells in fully MHC-mismatched models has been reported previously by us and others (47, 288, 299), we utilized a clinically relevant MHC-matched, multiple miHA-mismatched CD4-

dependent BMT model, B6→BALB.B, and found that the recipients which received splenocytes from T-bet$^{-/-}$ mice demonstrated prolonged survival with decreased GVHD severity compared with those that received WT or IFN-γ$^{-/-}$ splenocytes (Figure 2.2). These results suggested that T-bet plays a critical role in the development of acute GVHD regardless of BMT model.

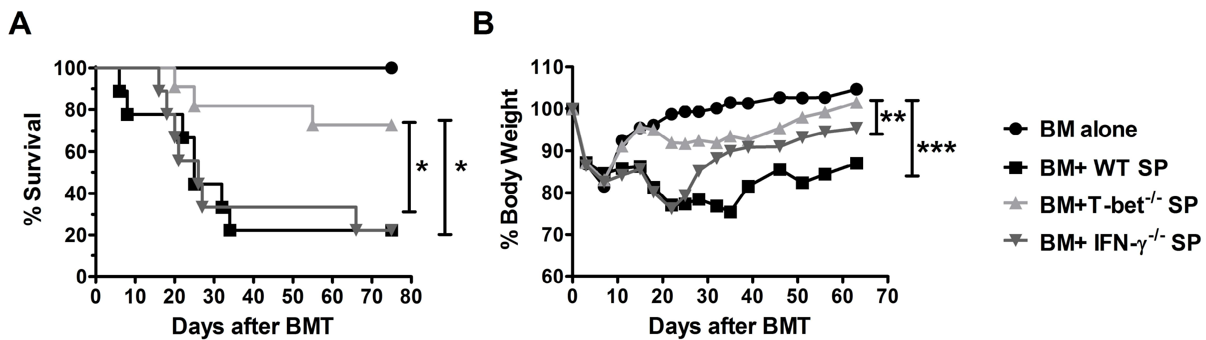


Figure 2.2 T-bet is required for GVHD induction in miHA-mismatched BMT model. Lethally irradiated BALB.B mice were transplanted with 5×10^6/mouse TCD-BM from WT B6 donor alone (n=6), or plus WT (n=9), T-bet$^{-/-}$ (n=11), or IFN-γ$^{-/-}$ (n=9) splenocytes (SP) at 20×10^6/mouse. Recipient mice were monitored throughout the experimental period for survival (A) and weight change (B). Data are shown from two replicate experiments combined. Asterisk indicates statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

2.4.3 Differential gene profiles of T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells after allo-BMT

To determine the mechanism of how T-bet affects T-cell pathogenicity in the induction of GVHD, we hypothesized that the molecules which are regulated by T-bet but not required for IFN-γ production are responsible for GVHD development. To identify these molecules, we isolated donor CD4 T cells from spleens of recipients at 7 days following allo-BMT (B6→BALB/c) and analyzed the gene profiles by microarray. Internal controls, *Tbx21* (gene encoding T-bet) and *Ifng* (gene encoding IFN-γ), were essentially absent in T-bet$^{-/-}$ CD4 T cells and IFN-γ$^{-/-}$ CD4 T cells, respectively (Figure 2.3, A and B). The microarray heat map showed

that, a total of 28 genes were down-regulated and 8 genes were up-regulated only in T-bet$^{-/-}$ but not in IFN-γ$^{-/-}$ CD4 T cells compared to WT CD4 T cells (Figure 2.3A). Among them, we chose to present those genes potentially relevant to GVHD in a bar graph, including *Cxcr3, Ccr5, Ccl3, Ccl4, Klrc1, Klrd1, Nkg7, and Pdcd1* that were positively regulated, whereas *H2-Aa* and *H2-Ab1* were negatively regulated by T-bet independent of endogenous IFN-γ (Figure 2.3B). Changes in representative genes (*Hopx*, *Slamf1*, *Serpinb9*, *Igf2r*, *Klrd1*, *Klrc1, H2-Aa, H2-Ab1 and Retnla*) were confirmed by real time PCR (Figure 2.3C). We reason that at least some of those genes we identified are responsible for the compromised ability of T-bet$^{-/-}$, not IFN-γ$^{-/-}$, CD4 T cells in the induction of GVHD.

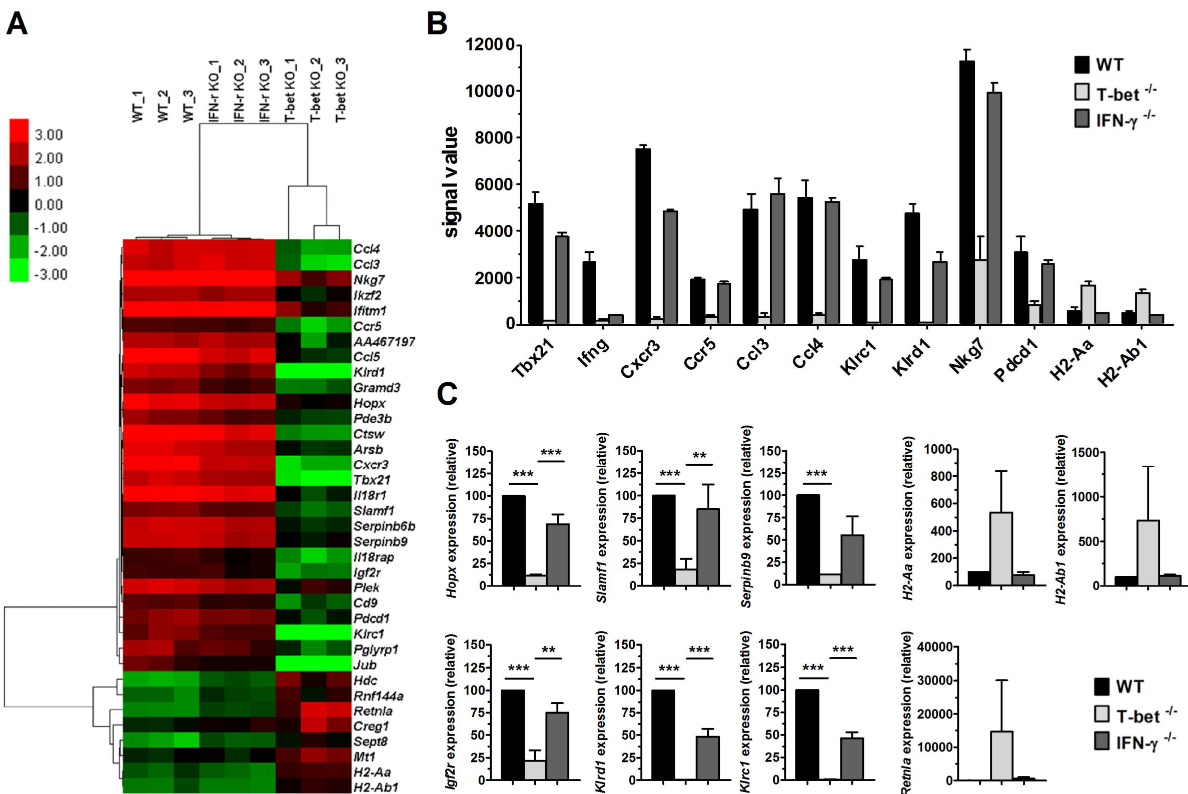


Figure 2.3 Differential gene profiles of T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells after allo-BMT. Lethally irradiated BALB/c mice were transplanted with 5×10^{6}/mouse TCD-BM from WT Ly5.1^{+} B6 donor plus WT, T-bet$^{-/-}$ or IFN-γ$^{-/-}$ naïve CD4^{+} Ly5.1^{-} T cells at 1×10^{6}/mouse (n=4 per

group per experiment). Recipients were euthanized 7 days post-transplant and their spleens were collected. By sorting different strains of donor-derived T cells ($CD4^+$ H-$2K^{b+}$ Ly5.1^- $DAPI^-$) from recipient splenocytes, $1\sim2\times10^6$ cells were obtained per group, and total RNAs were extracted for microarray and real-time PCR analysis. The genes shown are significantly changed in the T-$bet^{-/-}$ group but not shared by the IFN-$\gamma^{-/-}$ group. Heat map (A), and mean signal value ± SE (B) of representative genes identified by microarray using either WT, T-$bet^{-/-}$, or IFN-$\gamma^{-/-}$ donor CD4 T cells were shown. Selected gene expressions were confirmed by real-time PCR (C). Pooled data from three independent experiments are presented. Asterisk indicates statistical significance: **p<0.01, ***p<0.001.

2.4.4 T-bet regulates T-cell pathogenicity through IFN-γ-independent manners

Our microarray data provides potential explanations for why T-$bet^{-/-}$ and IFN-$\gamma^{-/-}$ T cells induce distinct outcomes of GVHD at the gene level. However, protein expression is typically better correlated with biological function. To confirm our microarray study and further investigate the underlying mechanism, we utilized a MHC-mismatched BMT model (B6→BALB/c), and examined the expression of several molecules, which represent multiple aspects of allogeneic T-cell function: activation (I-A^b and NKG2D), migration (CXCR3), cytotoxicity (CD94 and GZMB), and exhaustion (PD-1), of donor T cells by flow cytometry 7 days after allo-BMT. We observed that T-$bet^{-/-}$ donor T cells significantly impaired proliferation in secondary lymphoid organs (spleen) and migration into target organs (liver), when compared to their WT counterparts (Figure 2.4A). This was consistent with their significantly decreased IFN-γ production, but increased IL-17 and IL-10 secretion (Figure 2.4B). T-$bet^{-/-}$ donor T cells also showed significantly less infiltration into the liver compared with IFN-$\gamma^{-/-}$ T cells (Figure 2.4A). The expansion defect of T-$bet^{-/-}$ T cells in the spleen is mostly displayed in the CD8 but not CD4 subpopulation (Figure 2.4A), which indicated that absence of T-bet affects multiple aspects of CD4 T cell function independent of cell expansion. Moreover, in contrast to WT or IFN-$\gamma^{-/-}$ T cells, T-$bet^{-/-}$ T cells significantly decreased the expression of CXCR3, NKG2D, PD-1,

and CD94, but increased the expression of I-A^b and GZMB in CD4 or CD8 constituent subpopulations (Table 2.1), or both (Figure 2.4C).

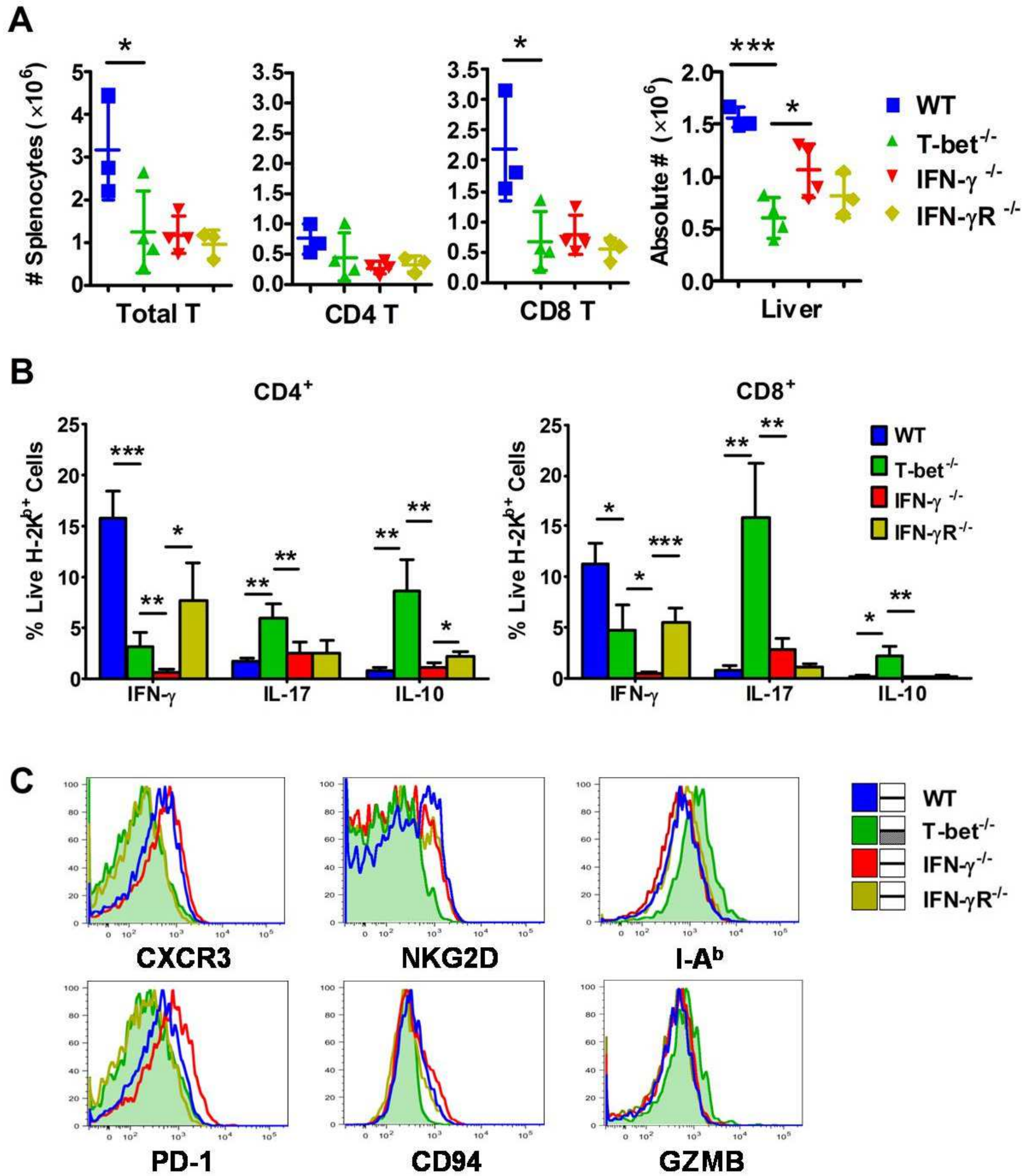


Figure 2.4 T-bet regulates T-cell migration and cytokine production through IFN-γ-independent manners. Lethally irradiated BALB/c mice were transplanted with 5×10^6/mouse TCD-BM from

WT Ly5.1$^+$ B6 donor plus WT, T-bet$^{-/-}$, IFN-γ$^{-/-}$ or IFN-γR$^{-/-}$ naïve CD4$^+$ Ly5.1$^-$ T cells at 1×10^6/mouse (n=3~4 per group). Recipients were euthanized 7 days post-transplant and their spleens and livers were collected and absolute numbers of donor total T cell, CD4$^+$ or CD8$^+$ T cell were shown (A). Production of IFN-γ, IL-17 and IL-10, and expression of CXCR3, NKG2D, I-A^b, PD-1, CD94 and granzyme B (GZMB) by WT, T-bet$^{-/-}$, IFN-γ$^{-/-}$ or IFN-γR$^{-/-}$ donor T cell in recipient spleen were shown in bar graph (B), and histograms (C), respectively. Data from one representative experiment were presented. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

Table 2.1 Differential protein expression of T-bet$^{-/-}$ and IFN-γ$^{-/-}$ T cell after allo-BMT.

Mean ± SD %	CD4$^+$					CD8$^+$			
	WT	**T-bet$^{-/-}$**	**IFN-γ$^{-/-}$**	**IFN-γR$^{-/-}$**		**WT**	**T-bet$^{-/-}$**	**IFN-γ$^{-/-}$**	**IFN-γR$^{-/-}$**
CXCR3	44.9±8.6	30.2±3.0	57.5±4.3	24.2±2.0		73.6±6.1	48.0±3.4	80.3±3.6	56.8±1.0
NKG2D	22.1±0.7	20.9±2.1	18.8±1.5	14.9±0.5		60.9±1.5	29.3±2.0	47.7±2.3	52.4±4.9
I-A^b	17.1±3.7	54.6±9.1	15.2±3.5	26.9±3.4		29.7±4.6	55.8±4.1	25.7±5.5	37.0±4.9
PD-1	15.5±2.5	12.0±1.8	17.0±4.5	5.9±1.0		43.4±5.7	30.9±3.0	62.3±7.3	24.3±5.4
CD94	10.3±0.8	2.4±1.3	3.3±0.8	2.6±1.3		26.4±0.8	4.9±1.1	35.6±3.6	34.0±5.7
GZMB	1.6±0.6	6.3±2.9	1.8±1.0	2.0±0.4		0.6±0.4	3.0±2.1	1.3±0.3	0.5±0.3

Given IFN-γ can be produced by other cell types besides donor T cells, we aimed to distinguish the dependence of endogenous or systematic IFN-γ by including IFN-γR$^{-/-}$ T cells as additional controls, which are able to produce IFN-γ (Figure 2.4B) but unable to respond to IFN-γ. We found that CXCR3 and PD-1 were expressed in similarly low levels on the T cells deficient for T-bet or IFN-γR, but not IFN-γ. This suggests that their protein expression depends on T-bet, not endogenous IFN-γ, although it can be regulated by IFN-γ produced by other types of cells in the recipient. On the other hand, the expression of NKG2D, CD94, I-A^b and GZMB depends on T-bet, but not systematic IFN-γ, because their protein profiles are distinct on the T cells deficient for T-bet versus either IFN-γ or IFN-γR. Therefore, we have identified several T-

bet-dependent, but endogenous or systematic IFN-γ-independent molecules, that likely contributed to the impaired target organ migration and Th1-cytokine production by T-bet$^{-/-}$ T cells.

2.4.5 T-bet controls the optimal function of Th17 cells in GVHD induction

It is known that Th1-differentiation antagonizes Th17-differentiation (30, 300). Our data shows that either T-bet$^{-/-}$ or IFN-γ$^{-/-}$ T cells are prone to differentiate into Th17 cells both *in vitro* (Figure 2.5) and *in vivo* (Figure 2.1H), which is consistent with previous reports (47, 159).

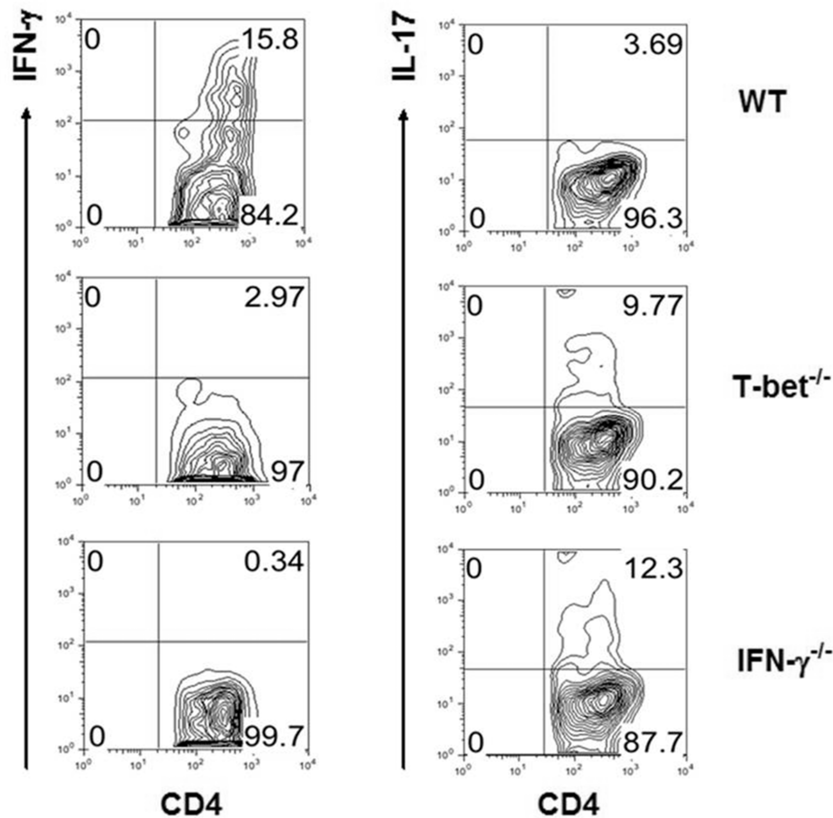


Figure 2.5 T-cell differentiation from WT, T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells *in vitro*. Isolated CD4^{+} CD25^{-} T cells (5×10^{5}) from WT, T-bet$^{-/-}$, and IFN-γ$^{-/-}$ mice on B6 background were co-

cultured with enriched CD11c^{high} (5×10^4) allogeneic bone marrow-derived dendritic cells (BM-DCs) for 5 days and the difference in IFN-γ and IL-17 production were shown by FACS plots.

Given the Th17 subset, per se, is capable of causing GVHD (80, 81), we hypothesized that optimal activity of Th17 cells may require T-bet but not IFN-γ, which may attribute to a distinct pathogenicity of T-bet versus IFN-γ in GVHD induction. Therefore, we examined the role of T-bet or IFN-γ in the pathogenicity of Th17 cells to cause GVHD using optimized Th17-polarizing culture conditions. We generated > 70% CD4$^+$ IL-17$^+$ cells from naïve CD4 T cells of WT, T-bet$^{-/-}$ or IFN-γ$^{-/-}$ B6 mice (Figure 2.6A) and then transferred equal numbers of these T cells into lethally irradiated BALB/c recipients. We found that T-bet$^{-/-}$, not IFN-γ$^{-/-}$, Th17 cells had a significantly reduced ability to induce GVHD. This is reflected by ameliorated morbidity and mortality of the recipients (Figure 2.6, B and C). Because bone marrow stromal niche is considered a sensitive target of allogeneic T cells, and the defective donor bone marrow-derived B lymphopoiesis is correlated with GVHD severity (301), we examined the percentage of donor-derived B cells at the end of observation periods (around day 85 after allo-BMT), which was found to be significantly higher in the recipients of T-bet$^{-/-}$ Th17 cells than those of WT or IFN-γ$^{-/-}$ Th17 cells (Figure 2.6 E), despite a similar number of total splenocytes among these groups (Figure 2.6 D). These data strongly suggest that T-bet also affects the pathogenicity of Th17 cells in GVHD induction. Moreover, Th17 pathogenic and nonpathogenic signature molecules are previously defined in autoimmune models, such as experimental autoimmune encephalomyelitis (EAE) (302-304). Based on the similar requirement of Th1 and/or Th17 cells and on T-bet expression in the induction of EAE and GVHD (47, 49), we used the genes identified in EAE model as a reference, to indirectly reflect the effects of T-bet on regulating Th17 pathogenicity in allo-BMT settings. Our microarray results indicate that T-bet positively regulated Th17

pathogenic signature genes on CD4 T cells after allo-BMT, such as *Lrmp*, *Ccl5*, *ICOS*, *Stat4*, *Lgals3*, *Malt1* and *GM-CSF*, while negatively regulating Th17 nonpathogenic signature genes such as *IL-10* (Figure 2.7). The observation is in-line with our hypothesis that T-bet contributes to the optimal function of Th17 cells after allo-BMT.

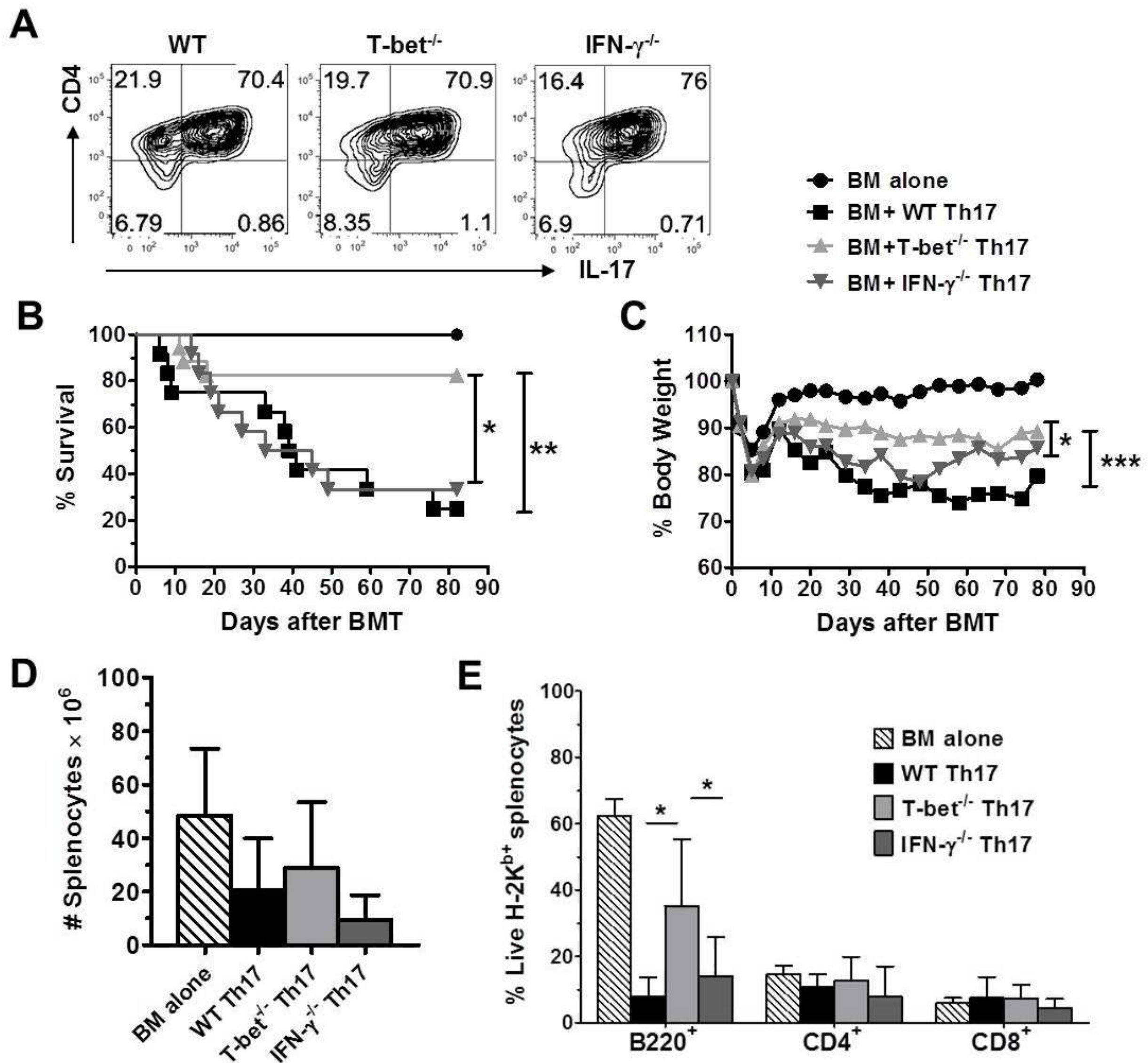


Figure 2.6 T-bet controls the optimal function of Th17 cells in GVHD induction. Lethally irradiated BALB/c mice were transplanted with 5×10^6/mouse TCD-BM from WT B6 donor alone (n=8), or plus *in vitro* polarized WT (n=12), T-bet$^{-/-}$ (n=17) or IFN-γ$^{-/-}$ (n=12) Th17 cells (A) at $0.5\sim1\times10^6$ /mouse after normalization of IL-17 producing CD4$^+$ cells. Recipient mice

were monitored throughout the experimental period for survival (B) and weight change (C). Upon completion of the experiment on day 80-90, absolute number (D) and percentage of donor-derived B-cell ($B220^{+}$) and T-cell ($CD4^{+}$ or $CD8^{+}$) reconstitution (E) of the spleen cells from remaining recipients were analyzed. Pooled data from three independent experiments are presented. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

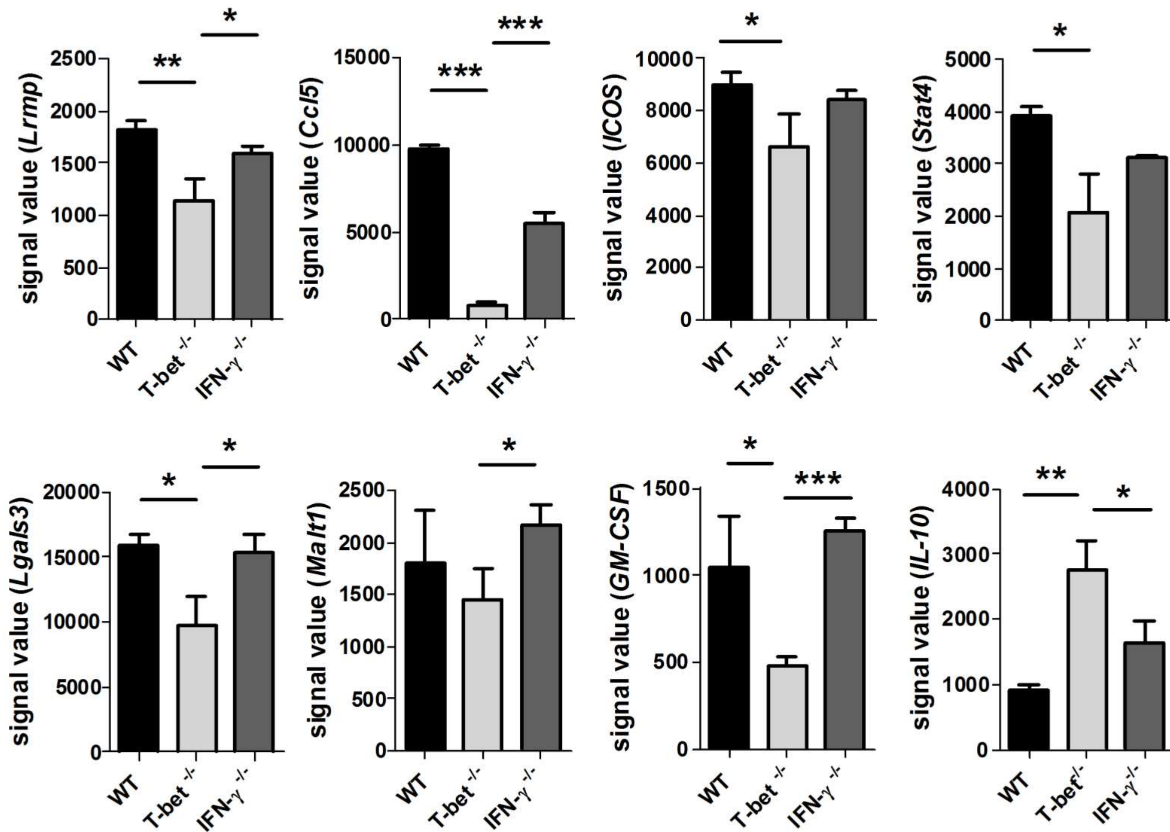


Figure 2.7 T-bet regulates Th17 pathogenic or nonpathogenic signature genes after allo-BMT. Mean signal value ± SE of selected Th17 pathogenic or nonpathogenic genes including Lrmp, Ccl5, ICOS, Stat4, Lgals3, Malt1, GM-CSF and IL-10, identified by microarray using either WT, T-bet$^{-/-}$, or IFN-$\gamma^{-/-}$ donor CD4 T cells 7 days post allo-BMT were shown. Pooled data from three independent experiments are presented. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

2.4.6 T-bet$^{-/-}$ donor T cells largely preserve the GVL effect by neutralizing IL-17 in allo-BMT recipients

The ultimate goal of allo-HSCT is to prevent GVHD while preserving the GVL effect. Cytotoxic CD8 T cells are known to play a predominate role in mediating GVL effects (265, 305). GVL effects of IFN-$\gamma^{-/-}$ T cells have been shown to correlate inversely with their GVHD-inducing activity in a CD8-dependent model (157). We hypothesized that T-bet$^{-/-}$ T cells may maintain their GVL activity due to the presence of Eomes, which preserves CD8 T-cell cytotoxic activity (32, 33). We tested the expression of Eomes and IFN-γ on CD4 and CD8 T cells in the presence or absence of T-bet and found that Eomes was mainly expressed on CD8 T cells compared to CD4 T cells. T-bet$^{-/-}$ CD8 T cells highly expressed Eomes and maintained IFN-γ production (Figure 2.8). To determine whether T-bet is required for T-cell mediated GVL effect, we used a B6 → BALB/c BMT model and infused a low dose of A20-luc B-cell lymphoma on the day of allo-BMT, which mimics clinical setting where a small number of malignant cells survive in patients after pre-conditioning regimen. We found that WT T cells at 0.25~0.5 × 10^6/mouse could effectively reject tumor, but the majority of recipients (79%) quickly died of GVHD (Figure 2.9 A, B and C). However, the recipients of 0.25~0.5 × 10^6 T-bet$^{-/-}$ T cells showed delayed tumor growth compared to those of BM alone, but eventually the majority of these recipients (86%) died of tumor relapse (Figure 2.9 A, B and C). These data suggest that although T-bet deficient T cells still express Eomes and had partially preserved ability to produce IFN-γ, their GVL activity was largely compromised.

T cells deficient for T-bet produce elevated levels of IL-17 (Figure 2.1H and 2.4B). Substantial evidence supports that IL-17 enhances tumor resistance to anti-angiogenesis therapy (306), promoting tumor progression through an IL-6/Stat3 signaling pathway (307, 308). Our

previous study indicates that additional RORγt (transcription factor of Th17 cells) deficiency reverses the enhanced ability of T-bet-deficient T cells to produce IL-17. Furthermore, T-bet/RORγt-deficient T cells have largely preserved GVL activity (47). Therefore, we further hypothesized that elevated IL-17 contributed to the compromised GVL effect of T-bet$^{-/-}$ T cells. To test this, we used the same MHC-mismatched B6→BALB/c model with IL-17 neutralizing mAb (clone: 17F3), and found that treatment with anti-IL-17 mAb significantly reduced tumor relapse in the recipients of T-bet$^{-/-}$ T cells. This is reflected by improved survival (Figure 2.9A) and reduction of tumor signal intensity by BLI (Figure 2.9 C and D). In contrast, the same treatment did not affect the survival of the recipients transplanted with WT T cells. Our data suggests that the compromised GVL activity of T-bet$^{-/-}$ T cells was, at least in part, due to elevated IL-17 produced by these T cells.

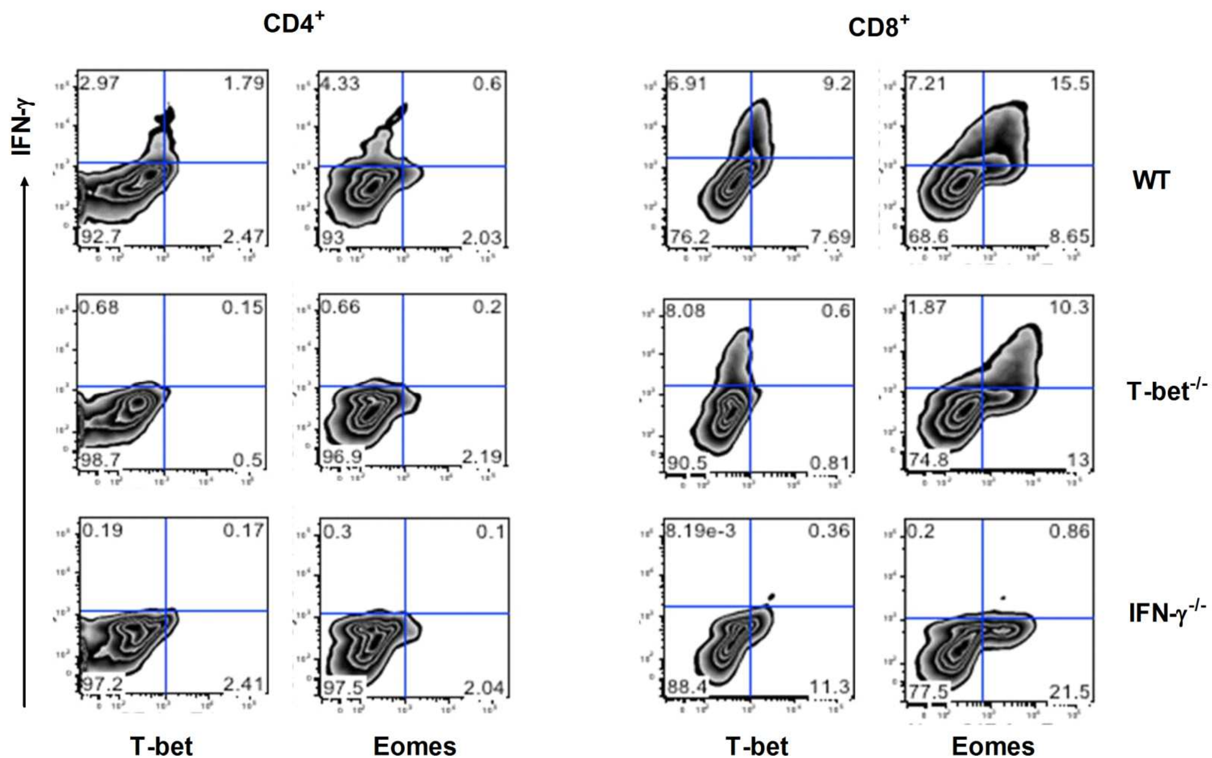


Figure 2.8 T-bet and Eomes expression on WT, T-bet$^{-/-}$ and IFN-γ$^{-/-}$ T cells *in vitro*. WT, T-bet$^{-/-}$ or IFN-γ$^{-/-}$ splenocytes were harvested and stimulated with anti-CD3 Ab for 3 days. PMA and ionomycin were added on day 3 to further stimulate the cells for intracellular staining of IFN-γ. Expression of T-bet, Eomes and production of IFN-γ by CD4 or CD8 T cells were shown.

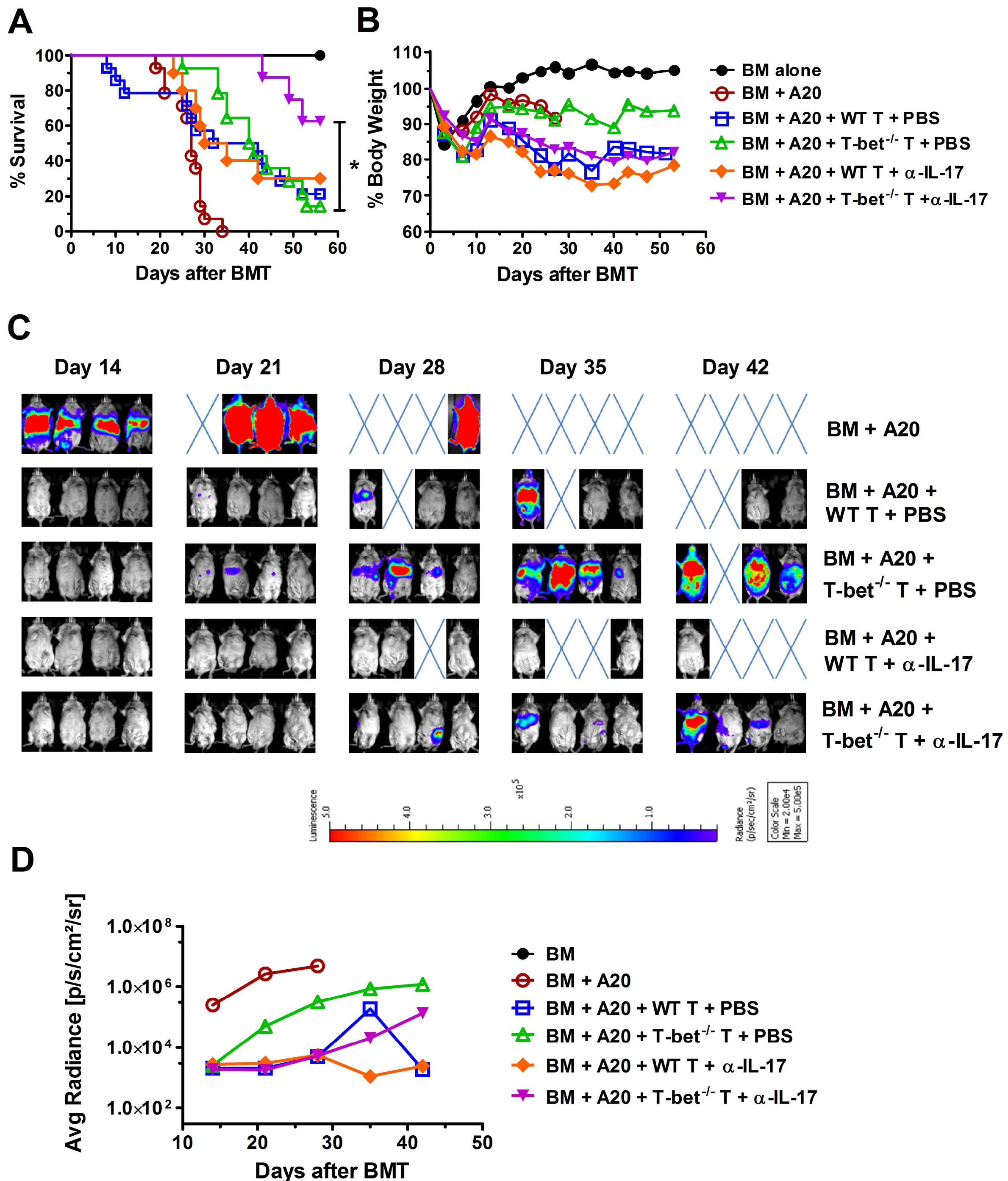


Figure 2.9 T-bet$^{-/-}$ donor T cells largely preserve GVL effect upon neutralizing IL-17 in allo-BMT recipients. BALB/c mice were lethally irradiated and transplanted with 5×10^6/mouse TCD-BM from WT B6 donor alone, or plus WT or T-bet$^{-/-}$ purified T cells at 0.25~0.5×10^6/mouse. Additionally, recipients were intravenously injected with 2×10^3/mouse A20 luciferase-transduced lymphoma cells at the time of BMT. Recipients were i.p. injected with α-IL-17 mAb (500µg/mouse for first dose, and 200µg/mouse for the following doses) or PBS control for 4 weeks (at day 0, 3, 7, 10, 14, 18, 21, 25 and 28), and were monitored throughout the experimental period for survival (A), weight change (B), and tumor growth by luciferin i.p. injection and whole body BLI (n=4~7 per group per experiment). Recipient BLI image (C) represents one of the 2 replicated experiments and average radiance intensity of BLI (D) pooled from all experiments was shown. Asterisk indicates statistical significance: *p<0.05.

2.5 Discussion

Here we show that T-bet deficient donor T cells, in contrast to IFN-γ deficient donor T cells, are impaired in their ability to induce acute GVHD in allogeneic recipients in fully MHC-mismatched (Figure 2.1 and 2.6) or MHC-matched but miHA-mismatched (Figure 2.2) murine BMT models. We identified distinct genetic profiles of T cells deficient for T-bet or IFN-γ to account for this difference (Figure 2.3) and further elucidated several T-bet downstream molecules, independent of either endogenous or systematic IFN-γ, contributed to GVHD pathogenicity (Figure 2.4). Furthermore, we found that T-bet deficient donor T cells have preserved GVL activity when the resulting increased IL-17 is neutralized (Figure 2.9).

Acute GVHD has been considered a Th1-type disease dominated by cytotoxic T cell-mediated pathology and increased production of Th1-type cytokines, including IFN-γ (78, 152, 153). Our data indicates that T-bet is required for CD4 T-cell mediated GVHD by controlling the differentiation and migration of Th1 cells (Figure 2.1) and the optimal function of Th17 cells (Figure 2.6). T-bet$^{-/-}$ CD4 T cells produced significantly lower levels of the pathogenic cytokine TNF-α, but higher levels of the anti-inflammatory cytokine IL-10 in recipient sera after adoptive transfer, which lead to reduced tissue damage in the liver and gut, compared to either WT or IFN-γ$^{-/-}$ CD4 T cells (Figure 2.1). Conversely, the Th17-related cytokine IL-6 was elevated in

recipient sera if the donor CD4 T cells were T-bet$^{-/-}$ (Figure 2.1G). In addition, increased IL-17 production by T-bet$^{-/-}$ donor CD4 T cells was present in recipient spleens and livers (Figure 2.1H). Our previous study also indicates that T-bet$^{-/-}$ CD4 T cells produced higher levels of Th2-related cytokines, IL-4 and IL-5, in recipient spleens, livers, and lungs (47). Consistent with previous reports, donor CD4 T cells can reciprocally differentiate into Th1, Th2, and Th17 cells, and each Th subset contributes to specific GVHD target organ tissue damage. Liver and gut are the primary target organs for Th1 cells, and skin is the primary target organ for Th17 cells, whereas pulmonary GVHD is mainly mediated by Th17 and Th2 cells (159). We observed that T-bet$^{-/-}$ CD4 T cells induced severe pulmonary GVHD similar to WT counterparts (Figure 2.1, E and F). We attribute the severe lung pathology mediated by T-bet$^{-/-}$ CD4 T cells to the augmented Th2 and Th17 cells and to the minimal IFN-γ production which leads to decrased PD-L1 expression on lung parenchyma (159). Moreover, due to the direct cytotoxicity of IFN-γ to the gastrointestinal tract (156), absence of T-bet or IFN-γ in donor CD4 T cells induced similar severity of colon GVHD but was less than that induced by WT CD4 T cells (Figure 2.1, E and F).

Multiple preclinical studies have investigated a paradoxical protective role of IFN-γ in GVHD development under lethal conditioning (154-157, 287, 288). In our current settings, IFN-γ$^{-/-}$ T cell induced comparable, not more severe GVHD, as their WT counterparts, which is likely due to the type and dose of donor T cells given was different from other studies (154-157, 287, 288). Taken together, the consensus is that IFN-γ produced by donor T cells is not required for the development of acute GVHD after lethal TBI and allo-BMT. However, the current work showed that T-bet, a transcriptional activator of IFN-γ, was required for GVHD induction. To further define the underlying mechanisms, we identified the differential gene profiles of donor T

cells deficient for T-bet or IFN-γ after allo-BMT. The potential key mediators include but are not limited to the following genes: *Cxcr3*, *Ccr5*, *Ccl3*, *Ccl4*, *Klrc1*, *Klrd1*, *Nkg7*, *Pdcd1*, *H2-Aa* and *H2-Ab1* (Figure 2.3). These targets are either positively or negatively regulated by T-bet and represent different aspects of allogeneic T-cell activity: activation (*H2-Aa* and *H2-Ab1*), migration (*Cxcr3*, *Ccr5*, *Ccl3*, and *Ccl4*), cytotoxic function (*Klrc1*, *Klrd1*, and *Nkg7*) or exhaustion (*Pdcd1*).

It is interesting that expression of MHC-II genes *H2-Aa* and *H2-Ab1* were increased on T-bet$^{-/-}$ CD4 T cells. The protein level of I-Ab (gene: *H2-Ab1*) was also significantly increased in T-bet$^{-/-}$ T cells, compared to either WT, IFN-γ$^{-/-}$, or IFN-γR$^{-/-}$ T cells (Figure 2.4C and Table 2.1), which we classified as a T-bet-dependent but systematic IFN-γ-independent molecule. Given that transfer of MHC molecules from APCs to T cells does exist, and the MHC-II-expressing T cells can engage in T: T interactions leading to increased apoptosis and hyporesponsiveness (309, 310), it is possible that overexpression of MHC-II molecules (such as I-Ab) on T-bet$^{-/-}$ donor T cells may result in increased cell death due to fratricide of allogeneic T cells. Alternatively, those MHC-II-expressing T-bet$^{-/-}$ T cells may compete with professional dendritic cells to present antigen, but not effectively induce T-cell proliferation due to lack of costimulatory signals; the regulatory mechanism utilized by T-bet^{-}NKp46^{-}RORγt^{+} innate lymphoid cells as demonstrated in a recent report (311).

Chemokine receptors and chemokines play important roles in T-cell migration to GVHD target organs (312, 313). CXCR3 is a direct target of T-bet (117), and targeting CXCR3 using its mAb can inhibit CD8-mediated GVHD in murine allo-BMT models (314). Targeting CCR5 using a small molecule inhibitor (Maraviroc) has recently been reported to be beneficial in patients with visceral GVHD in early clinical trials (315). Additionally, gene expression of

CCL3 and CCL4, ligands of CCR5 (316), were also found to be positively regulated by T-bet. Our data further indicates that CXCR3 is likely one of the key effectors contributing to the severe GVHD caused by IFN-$\gamma^{-/-}$ but not T-bet$^{-/-}$ T cells (Figure 2.1); in that the presence of systematic IFN-γ preserves the high expression of CXCR3 in IFN-$\gamma^{-/-}$ T cells via IFN-γR signaling, which was significantly reduced in T-bet$^{-/-}$ T cells at the gene level (Figure 2.10). This was also consistent with previous reports that the addition of exogenous IFN-γ to cultures of IFN-$\gamma^{-/-}$ T effector cells rescued CXCR3 expression (299). Therefore, in contrast to WT or IFN-$\gamma^{-/-}$ T cells, T-bet$^{-/-}$ T cells expressed significantly lower levels of Th1-related chemokine receptor genes (*Cxcr3* and *Ccr5*) and chemokine genes (*Ccl3* and *Ccl4*), as well as the CXCR3 protein. This impedes the migration and infiltration of allogeneic T cells to GVHD target organs (Figure 2.1 and 2.4).

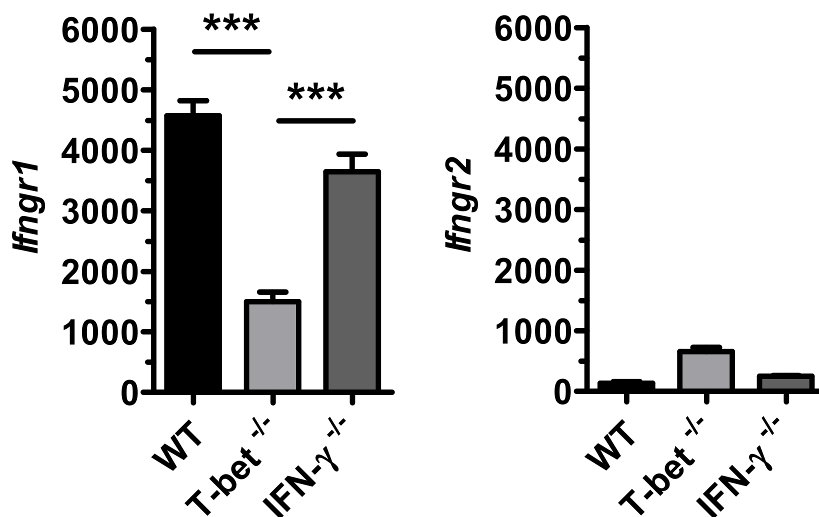


Figure 2.10 Gene profiles of IFN-γR identified by microarray. Mean signal value ± SE of genes *Ifngr1* and *Ifngr2* identified by microarray using either WT, T-bet$^{-/-}$, or IFN-$\gamma^{-/-}$ donor CD4 T cells 7 days post allo-BMT were shown. Pooled data from three independent experiments are represented. Asterisk indicates statistical significance: ***$p<0.001$.

Administration of an agonistic mAb of NKG2A inhibited donor T cell expansion and ameliorated acute GVHD in mice (317). However, we show that T-bet$^{-/-}$ donor T cells express extremely low levels of CD94/NKG2A genes (*Klrd1*and *Klrc1*, respectively) (Figure 2.3), and CD94 protein (Figure 2.4), in concert with ameliorated GVHD, suggesting that CD94/NKG2A could be biomarkers to positively predict GVHD severity. Lower expression levels of inhibitory molecules CD94, NKG2A, and T cell exhaustion marker PD-1 (Figure 2.3 and 2.4), on the other hand, may suggest that T-bet$^{-/-}$ T cells can still preserve their cytotoxic function to overcome tumor growth, which was additionally supported by enhanced production of GZMB by T cells deficient for T-bet but not IFN-γ (Figure 2.4C). Nkg7, a promoter of the T and NK cell surface cytotoxic molecule (318), has been considered as a Th1 cell-specific gene of which expression was previously shown to be regulated by T-bet (20, 117), and is positively correlated to cytotoxic T-cell destruction of epidermal cells in human GVHD (319).

In murine EAE models, deficiency in IFN-γ leads to exacerbated disease (320, 321). However, T-bet-deficient mice are protected from developing EAE (49, 50). We have observed similar results in GVHD models, which suggest that silencing *Tbx21* has therapeutic potential. Because transcription factors are difficult to be pharmacologically targeted, an alternative approach is targeting T-bet downstream molecules that are independent of endogenous or systematic IFN-γ (Figure 2.4), such as NKG2D, which is expressed on a variety of immune cells and plays a costimulatory role in activating CD8 T cells (322). Indeed, we classified NKG2D as a T-bet-dependent but systematic IFN-γ-independent molecule (Figure 2.4), consistent with the unpublished data from us and others (323), which indicates that blockade of NKG2D alleviated GVHD induced by CD8 or total T cells.

Th17 cells are capable of inducing GVHD in mice (80, 81). Both T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells are prone to Th17-differentiation with high levels of IL-17 production (Figure 2.5). In contrast to *in vitro* polarized WT or IFN-γ$^{-/-}$ Th17 T cells, T-bet$^{-/-}$ Th17 cells caused very mild GVHD (Figure 2.6, B-E). Consistently, our microarray data reveals that T-bet controls the optimal function of Th17 cells in GVHD possibly through regulating the expression of multiple genes representing Th17 pathogenic or nonpathogenic signatures (Figure 2.7). Our study showed T-bet$^{-/-}$ and IFN-γ$^{-/-}$ CD4 T cells have similar expression patterns of IFN-γ and IL-17 but opposing GVHD severities, which indicated that the culprit for GVHD development may not be IFN-γ or IL-17, but likely downstream effectors of T-bet we identified.

Although IL-17 seems to have less effect on GVHD development, it influences the tumor microenvironment, especially when shielding the GVL response of T-bet deficient donor T cells (Figure 2.9). T-bet$^{-/-}$ T cells produce more IL-17 than WT T cells (Figure 2.1H and 2.5), and IL-17 promotes tumor progression in a variety of tumors (306-308). Consistently, neutralizing IL-17 benefits overall survival of recipients transplanted with T-bet$^{-/-}$, not WT T cells. However, the GVL preservation of T-bet$^{-/-}$ T cells is reduced after stopping anti-IL-17 treatment. The tumor gradually relapsed, which further supports the detrimental role of IL-17 against preserved GVL effects of T-bet$^{-/-}$ T cells. We conclude that T-bet deficient T cells induce less GVHD and may still preserve GVL effects if the elevated levels of IL-17 are reversed. This is also supported by our previous findings that double deficiency of T-bet and RORγt prevents GVHD while sparing the GVL effect (47). Targeting T-bet itself is still far from translational application due to a lack of a specific inhibitor. Recently, the small molecule inhibitors for other transcription factors, such as c-Rel and RORγt, have been developed and showed promising results in alleviating GVHD (324) or cutaneous inflammatory disorders (325), respectively. The c-Rel inhibitor also

permits the maintenance of the GVL effect. Once T-bet inhibitors are developed, the mechanism we defined in the current study together with our previous report (47) will become applicable, e.g. a combinational treatment of T-bet inhibitor and anti-IL-17 mAb may be beneficial in controlling GVHD while maintaining the GVL effect.

Collectively, using genetic knockout mice, we prove that T-bet is critical for the development of aGVHD through controlling the differentiation and migration of Th1 cells as well as the pathogenicity of Th17 cells. Given that T-bet is a transcription factor which is unavailable to be pharmacologically targeted currently, we identify potential molecular targets downstream of T-bet, and define the underlying mechanisms accounting for the distinct GVHD outcomes caused by T-bet-versus IFN-γ-deficient donor T cells. This provides the rationale to target those T-bet-dependent, but endogenous or systematic IFN-γ-independent molecules, for the control of aGVHD in clinical settings.

In the study presented in Chapter 2, we investigated the effect and mechanism of T-bet in regulating T cell activity and function in the development of aGVHD after allo-BMT. Pharmacological blockade of T-bet, once available, is expected to affect other cell compartments in addition to donor T cells as far as they express T-bet. Because APCs also express T-bet and play critical roles in the induction of aGVHD, we therefore studied how T-bet regulates APC functions in mediating aGVHD and GVL effect, and we will report our findings in the next chapter.

Chapter 3: T-bet Promotes Acute Graft-versus-Host Disease by Regulating Hematopoietic Antigen Presenting Cells in Mice

3.1 Abstract[3]

Beyond its critical role in T cells, T-bet regulates the functions of APCs including DCs and B cells. Given that APCs are essential for priming allogeneic T cells and the development of GVHD, we evaluated the role of T-bet on APCs in acute GVHD using murine models of allo-BMT. The recipients deficient for T-bet developed much milder GVHD than their WT counterparts in MHC-mismatched or CD4-depedent miHA-mismatched models. As the functional readout of APCs, allogeneic donor T cells, in particular CD4 subset, significantly reduced IFN-γ production, proliferation and migration, and caused less damage in liver and gut in T-bet$^{-/-}$ recipients. We further observed that T-bet on recipient hematopoietic APCs was primarily responsible for the donor T-cell response and pathogenicity in GVHD, while T-bet also contributes to the function of donor hematopoietic APCs. T-bet$^{-/-}$ DCs, the most potent hematopoietic APCs, produced less IFN-γ and expressed higher levels of Trail, but not FasL or TNF, to induce significantly higher levels of apoptosis on donor T cells prior to their massive proliferation. In fact, Trail/DR5 interactions served as a major signaling pathway responsible for donor T-cell apoptosis and impaired GVHD development in the recipients deficient for T-bet. Furthermore, while T-bet on hematopoietic APCs was required for GVHD development, it was

[3] This abstract have been previously published (Fu et al. 56th ASH Annual Meeting, Blood, 2014, 124 (21), 846-846.) and are utilized with permission of the publisher (p132).

largely dispensable for the GVL effect. Taken together with our previous findings, we propose that T-bet is a potential therapeutic target for the control of GVHD through regulating T cells as well as APCs.

3.2 Introduction

GVHD remains to be a major obstacle for the efficacy and continuing success of allo-HSCT in the treatment of various malignant and non-malignant diseases (63). Activation of antigen presenting cells (APCs) plays a crucial role in priming alloreactive donor T cells to induce and intensify GVHD (149, 221, 239, 240). After conditioning, temporarily survived recipient APCs are essential for aGVHD initiation, especially in MHC-mismatched transplants and in CD8-mediated aGVHD across only miHAs (68). Donor APCs also contribute to the increased intensity of aGVHD by priming donor T cells (239, 240), and may perpetuate cGVHD (238). APCs include diverse types of cells that have the common ability to prime T cells, such as DCs, B cells and macrophages derived from the hematopoietic system. Hematopoietic APCs clearly contribute to the development of GVHD (221), although recently have been proved dispensable since recipient nonhematopoietic APCs, such as myofibroblasts, endothelial cells, and epithelial cells, are sufficient to induce lethal GVHD in mice (249, 250). Among all APCs, DCs are considered as the most efficacious APCs and potent inducers of GVHD due to their superior ability to take up antigen, express co-stimulatory molecules, and produce proinflammatory cytokines to polarize T cells (233, 326).

Our group and others previously reported the fundamental role of the T-box transcription factor T-bet on T cells in GVHD, inflammatory diseases or autoimmune diseases (46, 47, 49, 54, 327). T-bet also regulates the activation and function of many APCs, such as DCs (4, 5, 58) and

B cells (41, 328). Although the development, differentiation and activation of bone marrow derived DCs and splenic DCs were unimpaired in mice lacking T-bet, T-bet is required for optimal production of IFN-γ and antigen-specific T-cell activation by DCs (5), which is highly correlated with GVHD induction. The study showed that T-bet$^{-/-}$ DCs failed to induce inflammatory arthritis due to the compromised ability to secrete proinflammatory mediators and to prime naive T cells (58). However, microbiome-dependent spontaneous colitis can occur in the absence of T-bet as a result of the derepression of TNF-α in mucosal DCs (329). Therefore, the effect of T-bet on DCs in the development of different diseases may depend on the differential microenvironment.

In the current study, by using several well-defined, clinically relevant murine models of allo-BMT, we found that T-bet deficiency on hematopoietic APCs, both recipient and donor origin, attenuates aGVHD. The proliferation and IFN-γ production of allogeneic donor T cells were significantly impaired in T-bet$^{-/-}$ recipients, but more Foxp3^{+} Tregs were present in their spleens. Additionally, T-bet$^{-/-}$ APCs enhanced early apoptosis of allogeneic donor T cells within lymphoid organs primarily through the Trail-DR5 axis, thus contributing to their decreased infiltration and tissue damage to GVHD target organs. Furthermore, allogeneic donor T cells in T-bet$^{-/-}$ recipients largely preserved GVL effect. Our data demonstrate T-bet is a promising therapeutic target for the control of GVHD through regulating APCs functions.

3.3 Materials and methods

3.3.1 Mice

C57BL/6 (B6; H-2^b), congenic B6.Ly5.1^+ (CD45.1^+; H-2^b), and FVB/N (FVB; H-2^q) were purchased from National Cancer Institute. 129S2/Sv (H-2^b) were purchased from Charles River Laboratories. T-bet$^{-/-}$ mice on B6 or BALB/c (H-2^d) background, and C3H.SW (H-2^b) were purchased from The Jackson Laboratory. All animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at H. Lee Moffitt Cancer Center or Medical University of South Carolina (MUSC). Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of University of South Florida or MUSC.

3.3.2 BMT models

T-cell purification from whole spleen and lymph nodes was done by negative depletion using magnetic beads as previously described (47). MHC- and miHA-mismatched (FVB→ B6; B6→BALB/c) or MHC-matched but miHA-mismatched (129S2/Sv→ B6; C3H.SW→ B6) BMT models were used as previously established (46, 289). Briefly, different strains of recipient mice were conditioned with a lethal dose of total body irradiation (TBI). Within 24 hours post-conditioning, recipients were i.v. injected with T-cell depleted (TCD)-BM alone or plus various numbers of T cells purified from donor mice. Recipients were monitored for weight loss and other clinical signs of GVHD twice/week (163). Spleens of DR5$^{-/-}$ mice on B6 background and their wild type (WT) counterparts were kindly provided by Dr. Niklas Finnberg from Penn State Hershey Cancer Institute. H&E staining and a semi-quantitative scoring system were used to account for histologic changes consistent with GVHD in the target organs as previously described (46, 47, 291).

3.3.3 Chimeras BMT model

WT-B6 Ly5.1^{+} or T-bet$^{-/-}$ B6 mice were lethally irradiated with 1000-1200 cGy and infused with TCD-BM cells from syngeneic T-bet$^{-/-}$ B6 or WT-B6 Ly5.1^{+} donors such that T-bet deficiency is confined to only the hematopoietic cells or the nonhematopoietic cell compartments, respectively. Control chimeras were also established by using lethally irradiated WT-B6 Ly5.1^{+} or the T-bet$^{-/-}$ B6 mice as recipients, and transferred with TCD-BM cells from syngeneic WT or T-bet$^{-/-}$ B6 donors respectively. Chimerism analyses of the donor hematopoietic cells were complete donor types (>95%) 2-3 months after first syngeneic BMT (data not shown). The [B6→ B6 Ly5.1^{+}], [B6 T-bet$^{-/-}$ → B6 T-bet$^{-/-}$], [B6 T-bet$^{-/-}$ → B6 Ly5.1^{+}] and [B6 Ly5.1^{+} → B6 T-bet$^{-/-}$] mice were then used as recipients in an allo-BMT. The chimeric mice received 1000-1200 cGy irradiations and were i.v. injected with purified T cells along with TCD-BM cells from allogeneic FVB donors.

3.3.4 GVL model

In the B6→BALB/c model, recipients received 2×10^{3} luc/neo plasmid-transduced A20 B-cell lymphoma cells (A20-luc) on BALB/c background, while in the FVB→B6 model, recipients received 0.1×10^{6} C1498 luciferase transduced atypical myeloid leukemia cells (C1498-luc) generated in B6 mice at the time of BMT. Tumor mortality and GVHD mortality were distinguished by BLI signal intensity and clinical manifestation of GVHD (46, 291).

3.3.5 Flow cytometry

Mononuclear cells were isolated from recipient spleen and liver and stained and analyzed for surface receptors and intracellular cytokines using standard flow cytometric protocols as previously described (47, 291, 292).

3.3.6 *In vivo* mixed lymphocyte reaction (MLR)

T cells isolated from donor mice were gently mixed and incubated with 2 μM CFSE in PBS at 37°C for 7 minutes. The CFSE-labeled T cells were i.v. injected into lethally irradiated recipients. After 4 days, recipient splenocytes were stained and analyzed using flow cytometry. Soluble DR5 (sDR5) protein is a human truncated DR5, which has been shown to effectively block Trail induced apoptosis in both human and mouse systems (330). sDR5 used in certain experiments was kindly provided by Dr. Youhai Chen from University of Pennsylvania.

3.3.7 Statistics

For comparison of recipient survival or tumor relapse rate, the log-rank test was used to determine statistical significance ($p<0.05$). To compare GVHD clinical scores, pathology scores, weight loss, cytokine levels, as well as *in vivo* and *in vitro* T-cell proliferation, a Student *t* test was used.

3.4 Results

3.4.1 T-bet expressed on host contributes to aGVHD

Development of GVHD is initiated by host APCs in presenting alloantigens to activate donor T cells (286). To determine the effect of T-bet on host APCs *in vivo*, we utilized a fully MHC-mismatched model (FVB→B6) of allo-BMT, where host APCs are essential for GVHD development (68). Compared to WT, T-bet$^{-/-}$ recipients demonstrated significantly lower mortality from GVHD after allo-BMT (Figure 3.1A) with less weight loss (Figure 3.1B), and displayed significantly better donor-derived B220^{+} B-cell and CD4 and CD8 T-cell reconstitution in spleen 80 days post-transplant (Figure 3.1, C-F). Because GVHD impairs donor B- and T-cell reconstitution, these results reflect that T-bet$^{-/-}$ recipients suffered only mild GVHD. The reduced mortality of T-bet$^{-/-}$ recipients was associated with less severe pathologic injuries in liver and gastrointestinal (GI) tract on day 14 post-transplant as compared with their WT counterparts. T-bet$^{-/-}$ recipients showed better maintenance of normal cell morphology in GVHD target organs without severe lymphocyte infiltration (Figure 3.1, G and H). Thus, T-bet expression in the host contributes to GVHD, suggesting a potential role of T-bet in regulating recipient APCs.

3.4.2 T-bet on host determines alloresponse and impairs early apoptosis of allogeneic donor T cells

APCs may affect the pathogenicity of donor T cells after allo-BMT in various ways, including alloreactivity, homeostasis, and/or migration. We next evaluated these aspects as the indirect functional readout of host APCs to understand the cellular mechanisms by which T-bet regulates GVHD through host APCs. Donor CD4 and CD8 T cells showed significantly reduced proliferation (CFSE staining) and activation (IFN-γ production) in T-bet$^{-/-}$ as compared to WT allogeneic recipients, in which more defects were observed in CD4 than CD8 T cells (Figure 3.2,

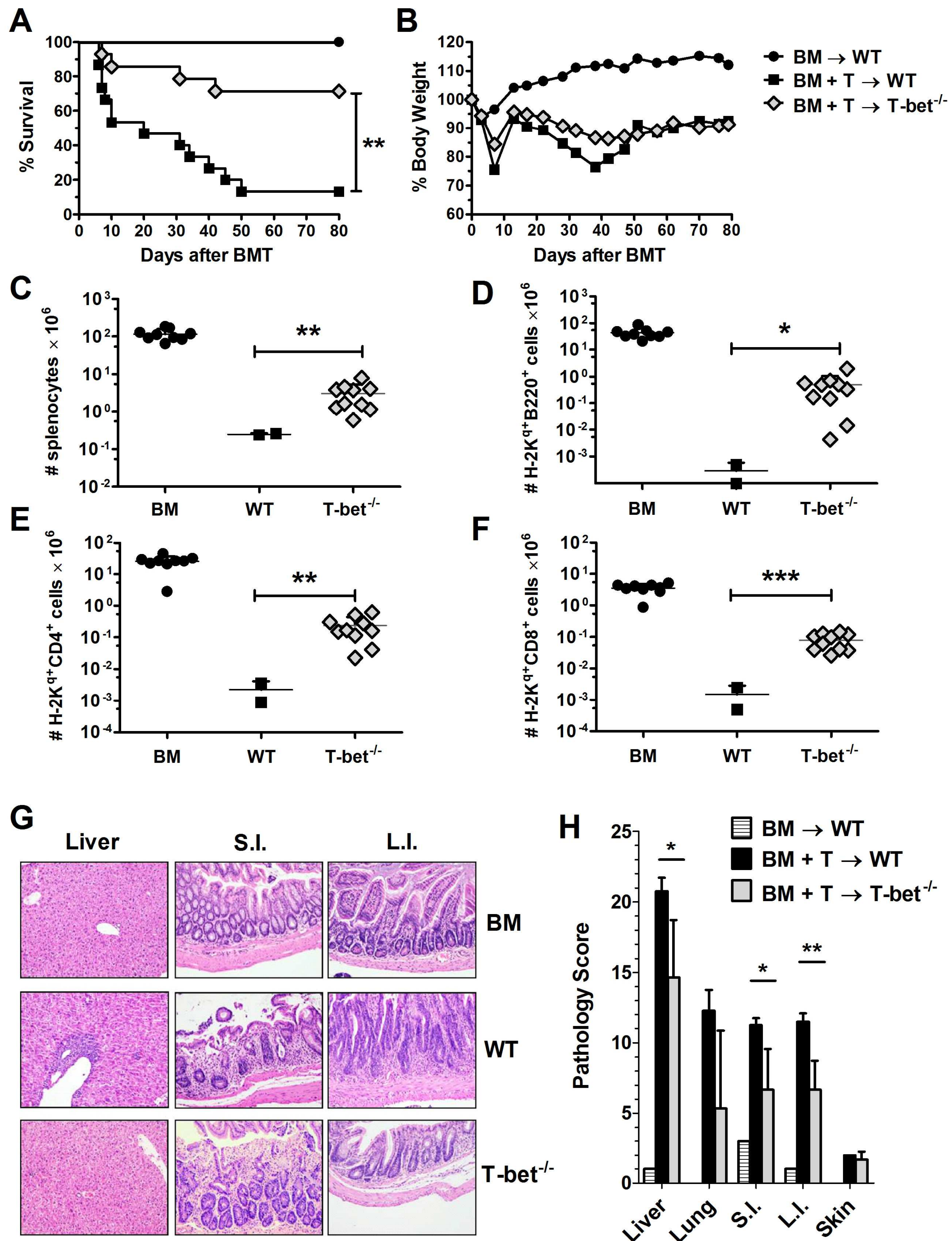

A
B
C
D
E
F
G
H
% Survival
Days after BMT
% Body Weight
BM → WT
BM + T → WT
BM + T → T-bet-/-
splenocytes × 10^6
H-2K^q+B220+ cells ×10^6
H-2K^q+CD4+ cells × 10^6
H-2K^q+CD8+ cells ×10^6
BM
WT
T-bet-/-
Liver
S.I.
L.I.
Pathology Score
Lung
Skin

Figure 3.1 Absence of T-bet expression on host ameliorates GVHD. Lethally irradiated WT or T-bet$^{-/-}$ mice on B6 background (n=9~15 per group) were transplanted with 5×10^6/mouse TCD-BM from FVB donors alone, or plus purified FVB T cells at 2×10^6/mouse. Recipient mice were monitored throughout the experimental period for survival (A) and weight change (B) post-transplant. Upon completion of the experiment on day 80, phenotypes of the spleen cells from survived recipients were analyzed. Expression of H-2K^q, B220, CD4 and CD8 was detected by flow cytometry and absolute number of total splenocytes (C), donor-derived B cells (D), CD4 (E) and CD8 (F) T cells were calculated. Pooled data from three separate experiments are presented. In separate experiments with the same setting as A-E, recipients were euthanized 14 days post-transplant and samples of liver, lung, small intestine, large intestine, and skin were collected for H&E staining and scored for microscopic GVHD severity by a pathologist blinded to the treatment groups (G). Photomicrographs depict the average disease score morphology from one representative experiment (n=3~4 per group). Pathological score mean ± SE of GVHD target organs are depicted (H). Asterisk indicates statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

A-C). In those allogeneic recipients, absolute numbers of donor T cells (H-2K^{q+}) and CD4 T cells were significantly reduced in the T-bet$^{-/-}$ recipients compared to the WT counterparts, whereas the number of donor CD8 T cells were comparable (Figure 3.2 D). The impact of T-bet on APCs specific to T-cell proliferation and activation was dependent on alloantigen recognition because such an impact on T cells was not seen in syngeneic recipients (Figure 3.3A). We also noticed that apoptosis (Annexin V$^+$) was significantly increased in CD4 and CD8 donor T cells in allogeneic T-bet$^{-/-}$ vs. WT recipients among CFSE^{high} but not CFSE^{low} population (Figure 3.2 E and F). No difference in apoptosis of donor T cells was seen in syngeneic recipients regardless of T-bet expression (Figure 3.3B). Collectively, these data suggest that the deficiency of T-bet on host APCs mitigates allogeneic T-cell proliferation and IFN-γ production while enhancing apoptosis before they undergo massive expansion.

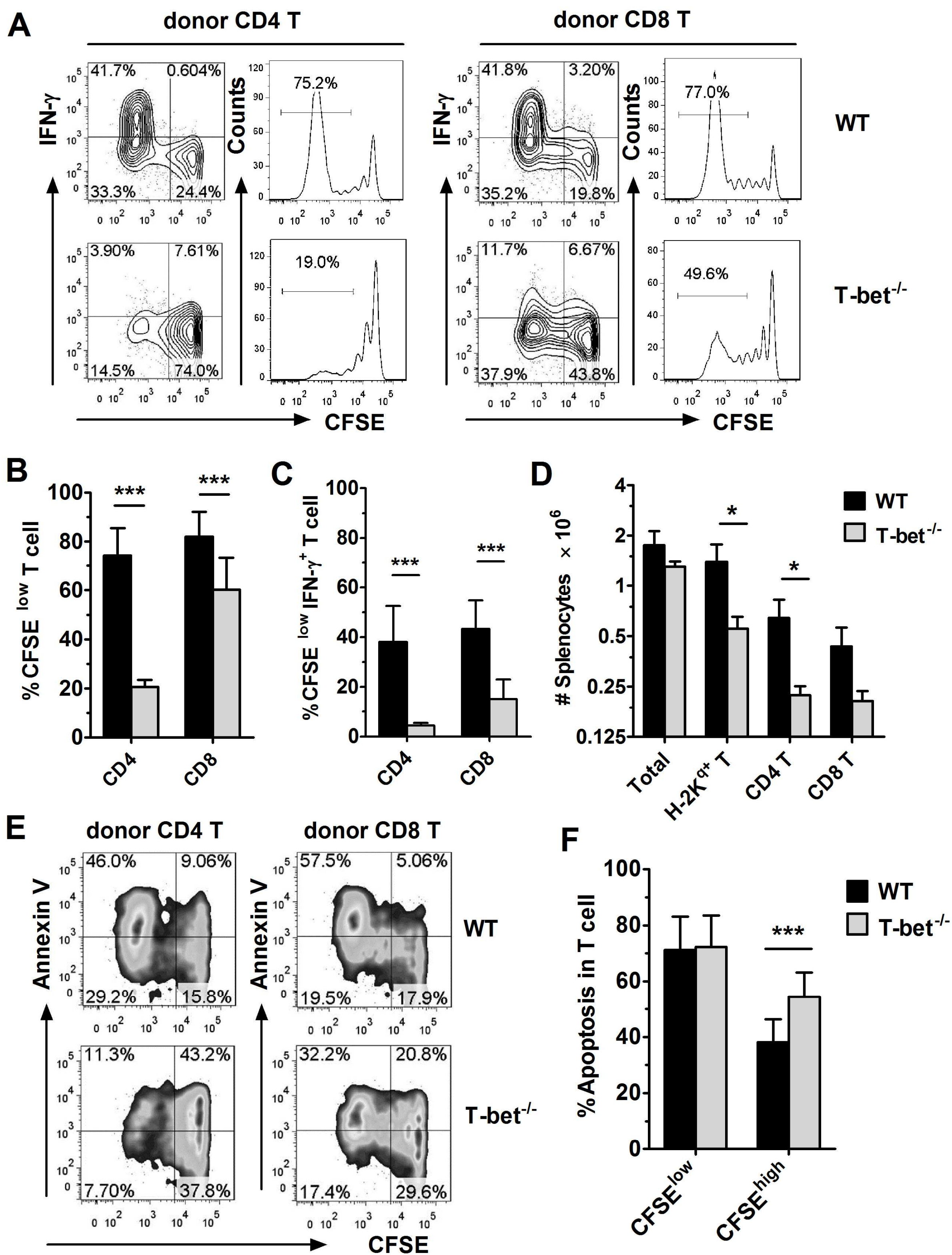


Figure 3.2 Allogeneic donor T cells significantly reduce IFN-γ production and proliferation but increase early apoptosis in T-bet$^{-/-}$ recipients. WT or T-bet$^{-/-}$ B6 mice were lethally irradiated and

transplanted with 3×10^6/mouse purified CFSE-labeled T cells from FVB donors (n=10 per group, pooled from three experiments). Profiles of CFSE and IFN-γ in donor $CD4^+$ or $CD8^+$ T cells were shown from representative recipients in each group 4 days post-transplant (A). Percentages of $CFSE^{low}$ (B) and $CFSE^{low}$ IFN-γ^+ donor T cells (C) are shown, and the absolute numbers of total splenocytes, H-$2K^{q+}$, $CD4^+$ or $CD8^+$ T cells are presented (D). Profiles of CFSE and Annexin V in donor CD4 or CD8 T cells are shown from representative recipients in each group (E). Percentages of total T-cell apoptosis in $CFSE^{low}$ or $CFSE^{high}$ population are shown (F). Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

3.4.3 T-bet expressed on host promotes donor CD4 T-cell accumulation in spleen and infiltration to liver

To delineate allogeneic donor T-cell expansion and migration capacity, we utilized congenic marker Ly5.1 to distinguish the donor T cells ($Ly5.2^+$) from donor BM cells ($Ly5.1^+$). We found that, 7 days post-transplant, T-$bet^{-/-}$ recipients had higher numbers of total splenocytes, but significantly decreased percentage of H-$2K^{b+}$ $Ly5.2^+$ donor T cells, resulting in the comparable absolute number of donor T cells in the spleens of WT and T-$bet^{-/-}$ recipients (Figure 3.4 A and C).

However, in T-$bet^{-/-}$ recipients, there were significantly fewer total CD4 donor T cells (Figure 3.4C) which also produced less IFN-γ (Figure 3.4D) or expressed lower CXCR3 (Figure 3.4E), while recruiting significantly more $CD25^+Foxp3^+$ Tregs in the spleen compared to WT recipients (Figure 3.4 B and C). Moreover, fewer total and IFN-γ-producing donor CD4 T cells were detected in the liver of T-$bet^{-/-}$ recipients (Figure 3.4 G and H). On the other hand, no significant differences in absolute number were observed on donor CD8 T cells in either the spleens or livers of T-$bet^{-/-}$ vs. WT recipients (Figure 3.4 C-E, G and H). Therefore, we conclude that increased expansion and/or generation of Tregs but decreased expansion and infiltration of CD4 effector T cells were likely accounted for attenuated GVHD in T-$bet^{-/-}$ recipients (Figure 3.1).

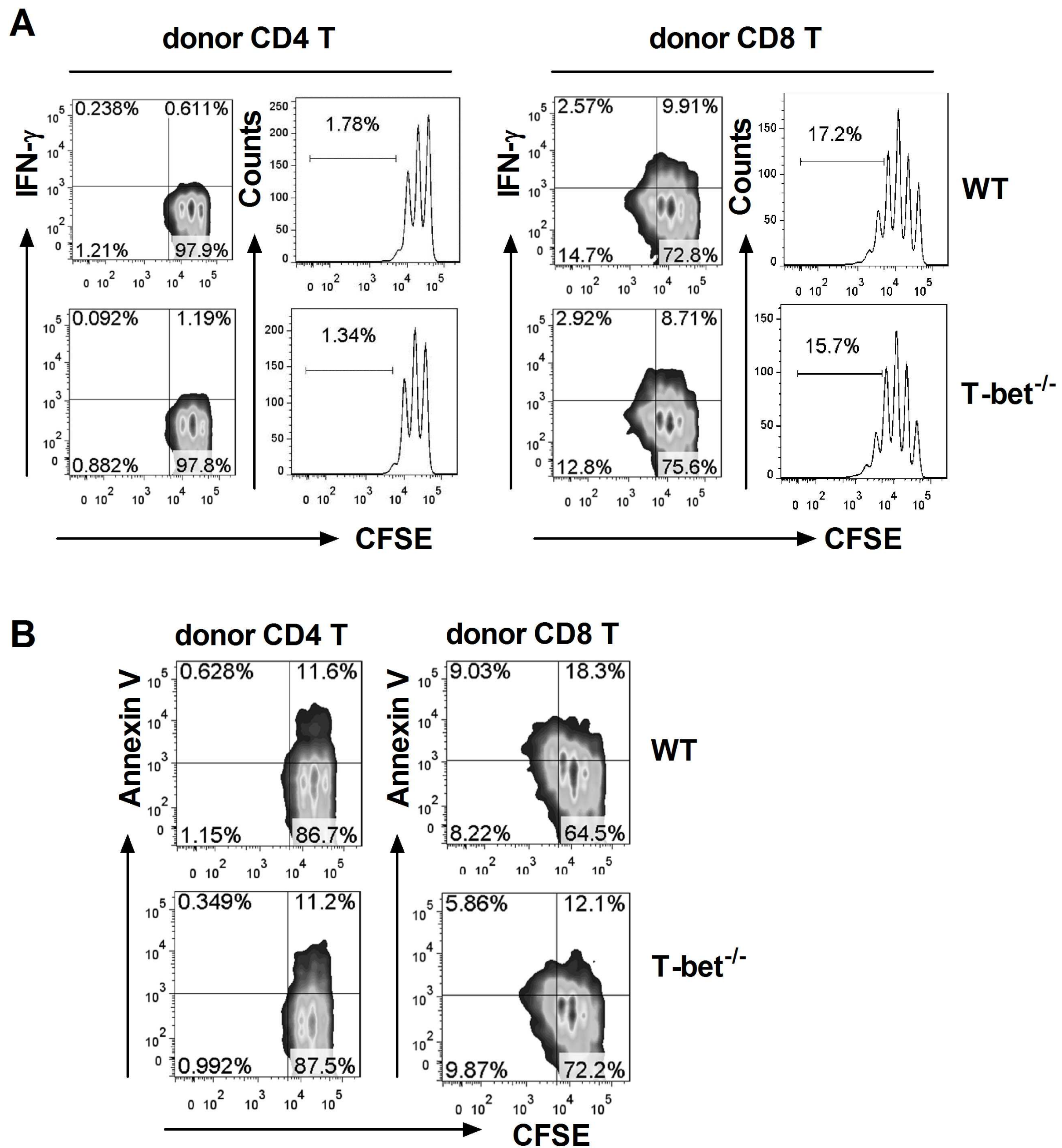


Figure 3.3 T-bet expressed on host is dispensable for activation, proliferation, and apoptosis of donor T cells in syngeneic recipients. WT or T-bet$^{-/-}$ B6 mice were lethally irradiated and transplanted with 3×10^6/mouse purified CFSE-labeled T cells from WT B6 donors (n= 3 per group). Profiles of CFSE and IFN-γ (A), and CFSE vs. Annexin V (B) in donor CD4 or CD8 T cells are shown from representative recipients in each group 4 days post-transplant.

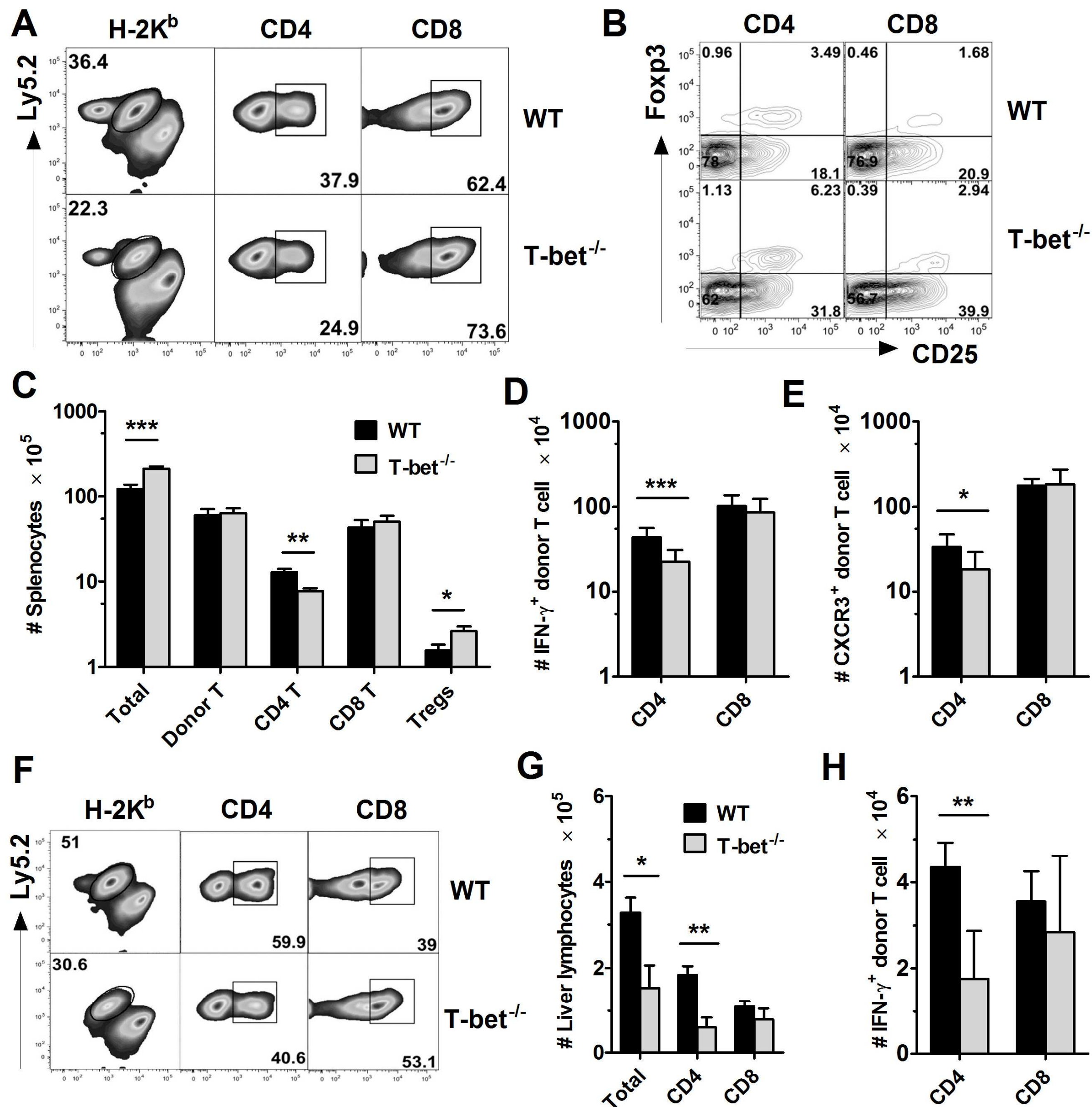


Figure 3.4 Allogeneic donor T cells significantly decrease accumulation in spleen and infiltration to liver in T-bet$^{-/-}$ recipients. Lethally irradiated WT or T-bet$^{-/-}$ mice on BALB/c background (n=9~10 per group, pooled from two experiments) were transplanted with 5×10^{6}/mouse TCD-BM from Ly5.1^{+} B6 donor alone, or plus 0.25×10^{6}/mouse purified Ly5.2^{+} T cells from B6 mice. Seven days post-transplant, mononuclear cells were isolated from recipient spleens and livers. Percentages of Ly5.2^{+} H-2K^{b+} T cells (A) or CD25^{+}Foxp3^{+} Tregs (B), and

their CD4 and CD8 subpopulation in spleen are shown. Absolute numbers of total splenocytes, total donor, CD4 or CD8 T cells and $CD25^+Foxp3^+$ Tregs in spleen are presented (C). Absolute numbers of the donor CD4 or CD8 T cells producing IFN-γ (D) or expressing CXCR3 (E) in spleen are presented. Percentages (F) and absolute number (G) of liver $Ly5.2^+$ $H\text{-}2K^{b+}$ T cells and their CD4 and CD8 subpopulation are shown. Absolute numbers of IFN-γ-producing donor CD4 or CD8 T cells in liver (H) are presented. Asterisk indicates statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.4.4 T-bet expressed on host contributes to GVHD in CD4 but not CD8-dependent miHA-mismatched model

In addition to the MHC-mismatched models, we also evaluated the effect of T-bet on APCs using more clinically relevant MHC-matched but miHA-mismatched models. Given previous findings which demonstrated that APCs derived from the host, rather than from the donor, are critical in inducing GVHD across miHA mismatch (218), we conditioned WT or T-bet$^{-/-}$ B6 recipients by TBI and transferred purified T cells from either 129S2/Sv or C3H.SW donors, representing the CD4- or CD8-dependent miHA-mismatched model, respectively (123, 218, 331). In 129S2/Sv → B6 model, WT recipients exhibited 100% lethality with severe clinical signs of GVHD, including weight loss, hunched posture, impaired mobility, ruffled fur, and skin lesions, whereas T-bet$^{-/-}$ recipients only showed mild GVHD with low mortality and morbidity (Figure 3.5 A-C). Distinct from the CD4-dependent model, in C3H.SW→ B6 model, no significant difference in survival, body weight change, or clinical scores was observed in WT versus T-bet$^{-/-}$ recipients (Figure 3.5 D-F). These data are consistent with those observed in MHC-mismatched model that T-bet on the host primarily impacts CD4 rather than CD8 donor T cells (Figure 3.2 and 3.4).

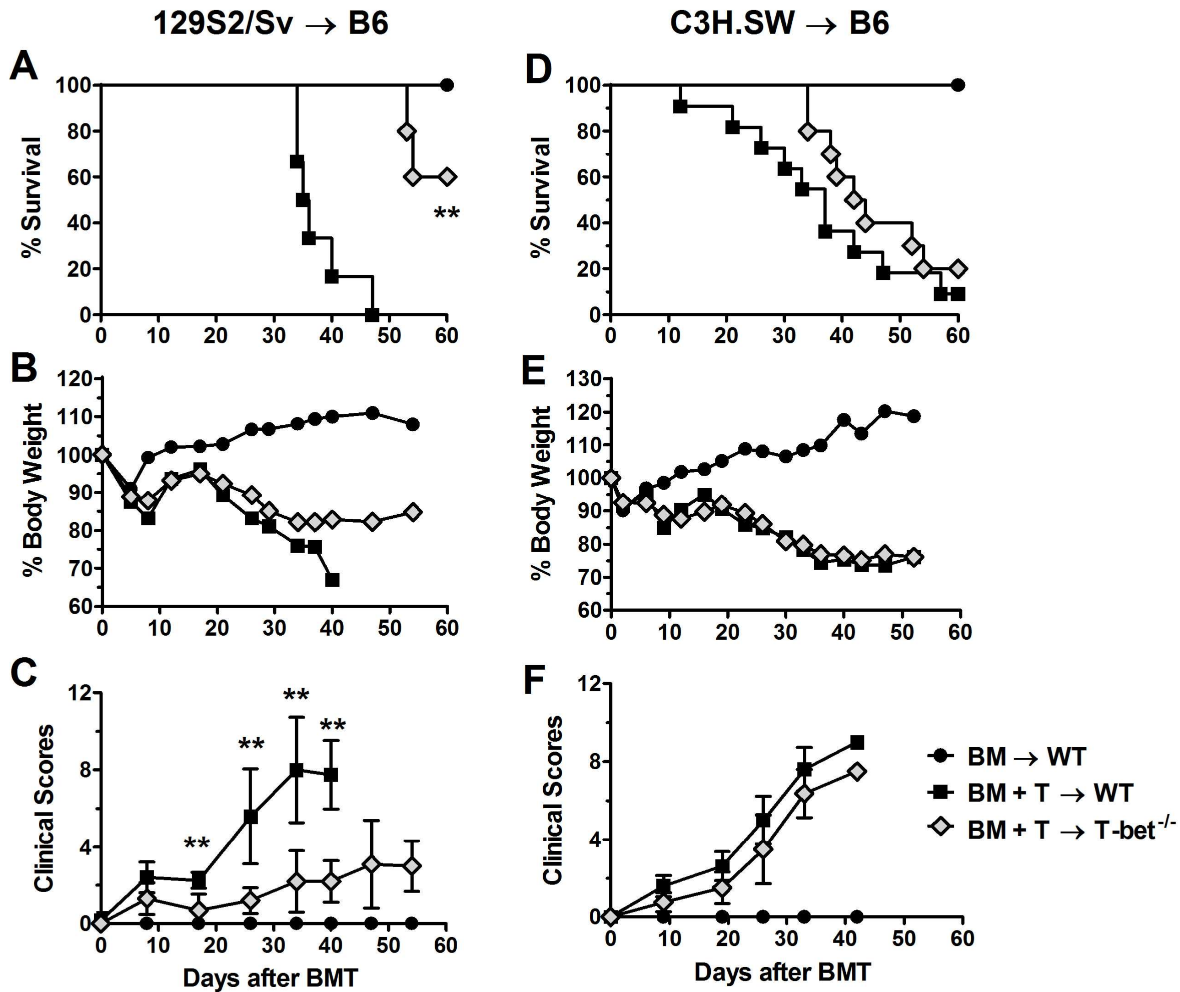


Figure 3.5 Absence of T-bet expression on host ameliorates GVHD in CD4- not CD8-depedent miHA-mismatched BMT model. Lethally irradiated WT or T-bet$^{-/-}$ mice on B6 background (n=6 per group) were transplanted with 5×10^{6}/mouse TCD-BM from 129S2/Sv donors alone, or plus purified 129S2/Sv T cells at 10×10^{6}/mouse. Recipient mice were monitored throughout the experimental period for survival (A), weight change (B), and GVHD clinical scores (C) post-transplant. Data from one representative experiment are presented. Lethally irradiated WT or T-bet$^{-/-}$ B6 mice (n=10~11 per group) were transplanted with 5×10^{6}/mouse TCD-BM from C3H.SW donors alone, or plus purified C3H.SW CD44^{-}CD25^{-} T cells at 3×10^{6}/mouse. Recipient mice were monitored throughout the experimental period for survival (D), weight change (E), and GVHD clinical scores (F) post-transplant. Data pooled from two independent experiments are presented. Asterisk indicates statistical significance between WT and T-bet$^{-/-}$ recipients given T cell transfer: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.4.5 T-bet on host hematopoietic cells is primarily responsible for promoting GVHD

Given both hematopoietic and nonhematopoietic APCs are known to contribute to the development of GVHD (233, 249), we next evaluated the role of T-bet expression on either population in mediating GVHD by using established BM chimeric mice as recipients. Severe GVHD with < 20% long-term survival was induced in [B6→ B6 Ly5.1^{+}] and [B6 Ly5.1^{+}→ B6 T-bet$^{-/-}$] chimeras in which T-bet was intact in their hematopoietic system, whereas mild GVHD with > 70% long term survival was observed in [B6 T-bet$^{-/-}$ → B6 T-bet$^{-/-}$] and [B6 T-bet$^{-/-}$ → B6 Ly5.1^{+}] chimeras in which T-bet was absent in their hematopoietic system (Figure 3.6). These results demonstrate that T-bet on recipient hematopoietic APCs plays an essential role in initiating GVHD, although the contribution of T-bet on nonhematopoietic APCs cannot be ruled out as a delayed lethality was observed in [B6 Ly5.1^{+}→ B6 T-bet$^{-/-}$] chimeras as compared to [B6→ B6 Ly5.1^{+}] chimeras (Figure 3.6).

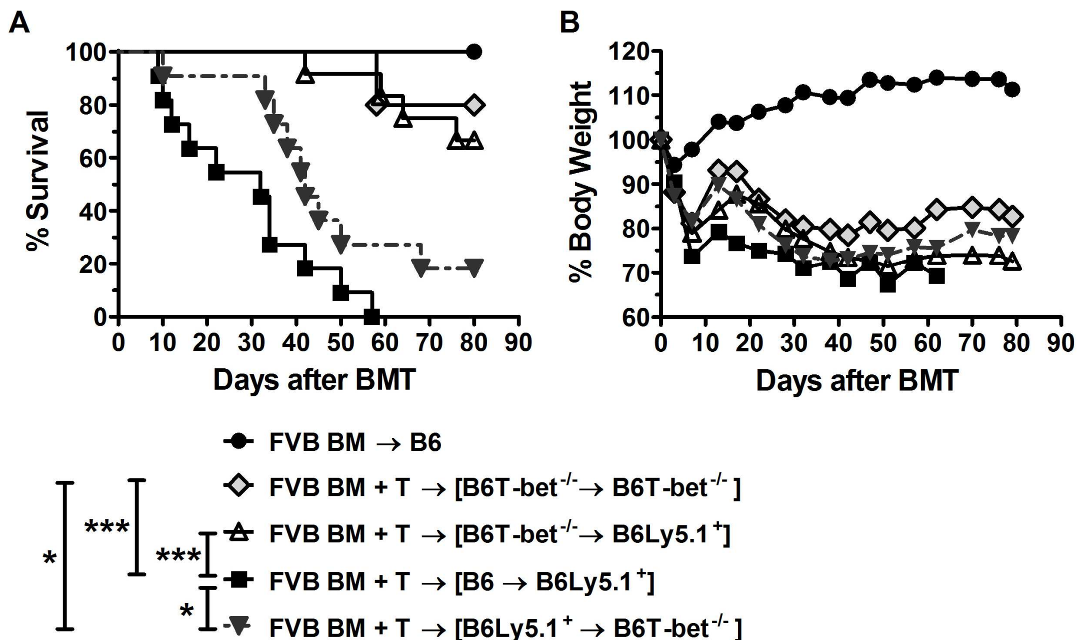


Figure 3.6 T-bet is critical for recipient hematopoietic APCs to induce GVHD. Lethally irradiated WT or four different types of chimeras ([B6→ B6 Ly5.1^{+}], [B6 T-bet$^{-/-}$ → B6 T-bet$^{-/-}$], [B6 T-bet$^{-/-}$ → B6 Ly5.1^{+}] and [B6 Ly5.1^{+} → B6 T-bet$^{-/-}$]) on B6 background (n=5~11 per group) were transplanted with 5×10^{6}/mouse TCD-BM alone or plus 2×10^{6}/mouse purified T cells from FVB donors. Recipient mice were monitored throughout the experimental period for survival (A) and weight change (B) post-transplant. Pooled data from two separate experiments are presented. Asterisk in the legend indicates statistical significance regards survival curves: *$p<0.05$, ***$p<0.001$.

3.4.6 T-bet regulates antigen presenting ability of donor hematopoietic APCs to induce GVHD

Because donor APCs also contribute to priming allogeneic T cells, we evaluated the effect of T-bet on donor APCs in the induction of GVHD. To reduce the contribution of recipient APCs, we lethally irradiated BALB/c mice and reconstituted recipients with TCD-BM cells from WT or T-bet$^{-/-}$ B6 donors 4 days prior to transferring allogeneic T cells. As expected, the recipients of TCD-BM cells alone from either WT or T-bet$^{-/-}$ donors survived long-term without GVHD. Additional allogeneic T cells induced more severe GVHD in the recipients that were reconstituted with WT BM than those with T-bet$^{-/-}$ BM, reflected by survival, weight loss, and clinical scores (Figure 3.7). These results indicate that T-bet also contributes to the antigen presenting ability of donor hematopoietic APCs to induce GVHD.

3.4.7 T-bet$^{-/-}$ host DCs produce less IFN-γ and induce early apoptosis of donor T cells primarily through Trail/DR5 axis

Hematopoietic APCs including DCs, B cells and macrophages that are capable of priming donor T cells, but host DCs rather than host B cells are critical in the induction of CD4 or to a lesser extent CD8 T cell-dependent GVHD (243). Given T-bet does not express on murine macrophages (5), we thus subsequently focused on host DCs, the most potent

professional APCs, and intended to understand how T-bet on host DCs modulates T-cell responses after allo-BMT. Consistent with the previous study (5), we found that T-bet$^{-/-}$ DCs expressed similar levels of MHC-II, CD86 and CD40 on day 4 post-transplant (data not shown), but they produced significantly less IFN-γ compared with WT DCs during the interaction with allogeneic, but not syngeneic T cells (Figure 3.8A). Additionally, T-bet$^{-/-}$ DCs expressed higher levels of Trail, but not FasL or TNF-α, in allogeneic recipients (Figure 3.8B), although the expression level of Trail receptor DR5 on donor T cells was comparable in either WT or T-bet$^{-/-}$ recipients (Figure 3.9A). It is worth noting that CD4 T cells expressed higher levels of DR5 than CD8 T cells especially among CFSE^{high} population in the recipients regardless of T-bet expression (Figure 3.9B).

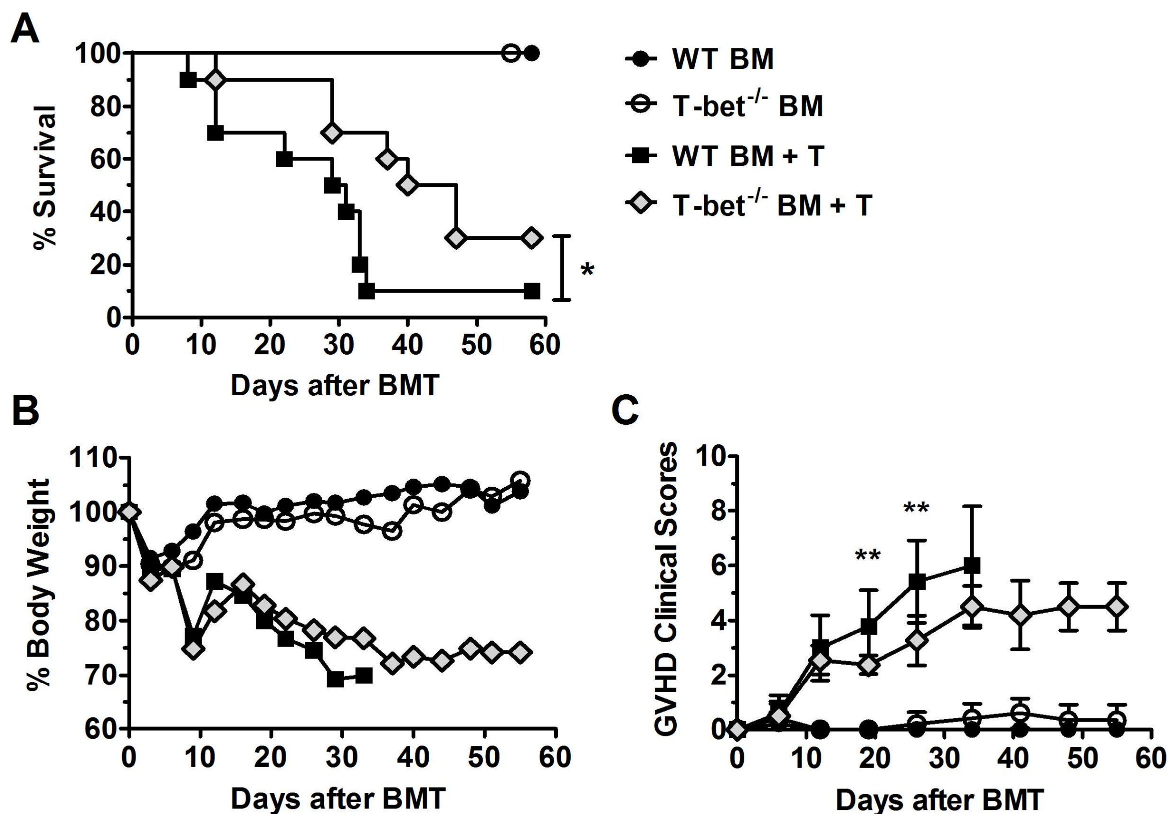


Figure 3.7 Donor hematopoietic APCs deficient for T-bet induce attenuated GVHD. Lethally irradiated BALB/c mice (n=5~10 per group, pooled from two experiments) were transplanted with 5×10^6/mouse TCD-BM alone from WT or T-bet$^{-/-}$ B6 mice on day 0, or plus additional purified T cells from WT B6 mice at 1×10^6/mouse 3 days later. Recipient mice were monitored throughout the experimental period for survival (A), weight change (B) and clinical scores (C) post-transplant. Asterisk indicates statistical significance between WT and T-bet$^{-/-}$ recipients given T cell transfer: *p<0.05, **p<0.01, ***p<0.001.

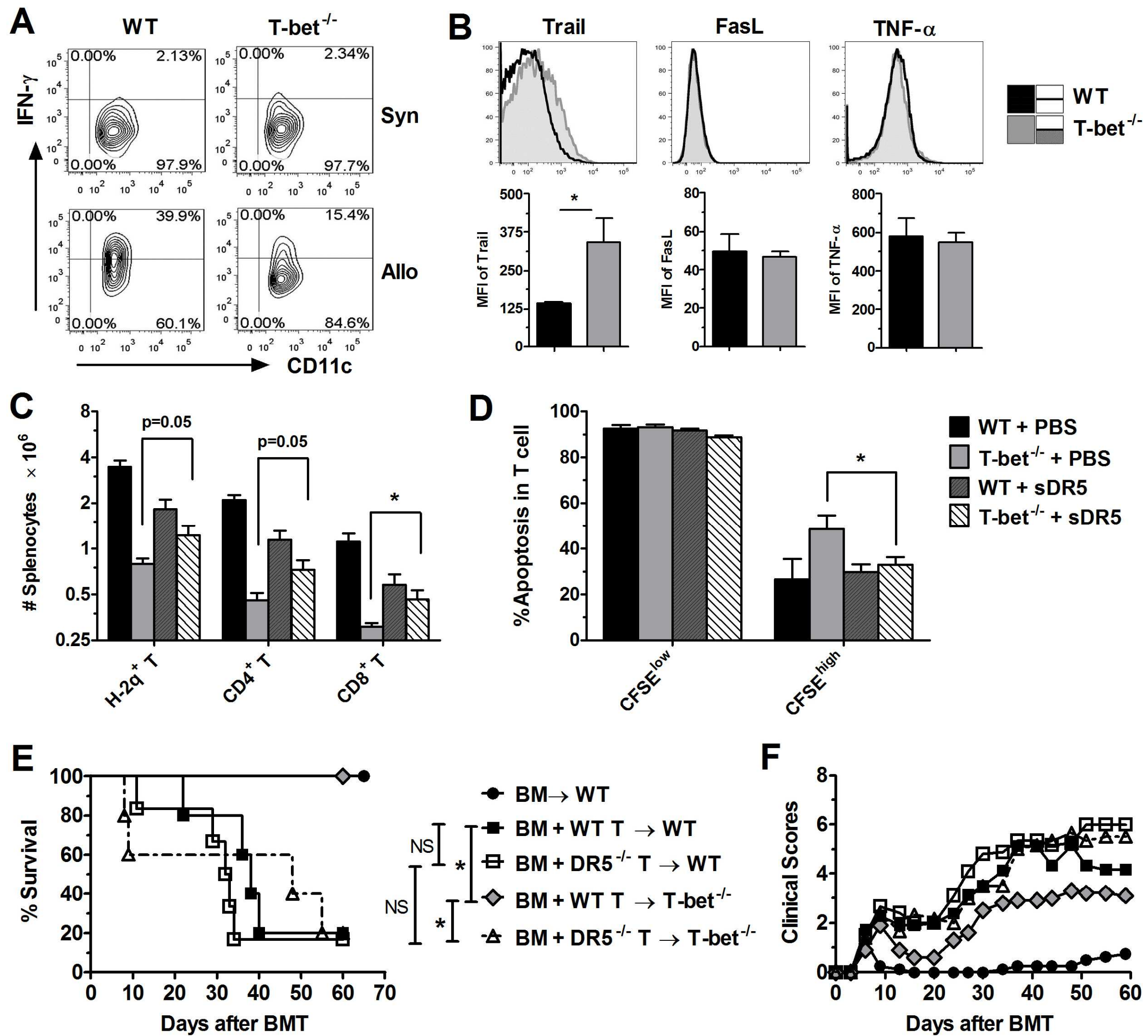


Figure 3.8 T-bet$^{-/-}$ host DCs induce early apoptosis of donor T cells primarily through Trail-DR5 pathway. WT or T-bet$^{-/-}$ B6 mice (n=3~4 per group, data from one representative experiment) were lethally irradiated and transplanted with 3×10^6/mouse purified CFSE-labeled T cells from WT B6 (syngeneic) or FVB donors (allogeneic). Production of IFN-γ (A) in host CD11c$^+$ DCs

was shown from representative recipients in each group 4 days post-transplant. In allogeneic model, histogram of Trail, FasL and TNF-α with mean fluorescent intensity (MFI) are shown (B) on gated $CD11c^+$ cells. WT or T-bet$^{-/-}$ B6 mice (n=3 per group) were lethally irradiated and transplanted with 3×10^6/mouse purified CFSE-labeled T cells from FVB donors. Recipients were i.p. injected with either PBS control or sDR5 at 100μg/mouse on day 0, and 50μg/mouse on day 1, 2, and 3. Four days post-transplant, absolute numbers (C) of total donor CD4 or CD8 T cells and percentage (D) of AnnexinV$^+$ T cells in CFSE^{high} or CFSE^{low} population in spleen were shown. Lethally irradiated WT or T-bet$^{-/-}$ BALB/c mice (n=5~6 per group) were transplanted with 5×10^6/mouse TCD-BM from WT B6 donor alone, or plus 1×10^6/mouse purified WT or DR5$^{-/-}$ T cells on B6 background. Recipient mice were monitored throughout the experimental period for survival (E), and clinical scores (F) post-transplant. Asterisk indicates statistical significance: *p<0.05, **p<0.01, ***p<0.001.

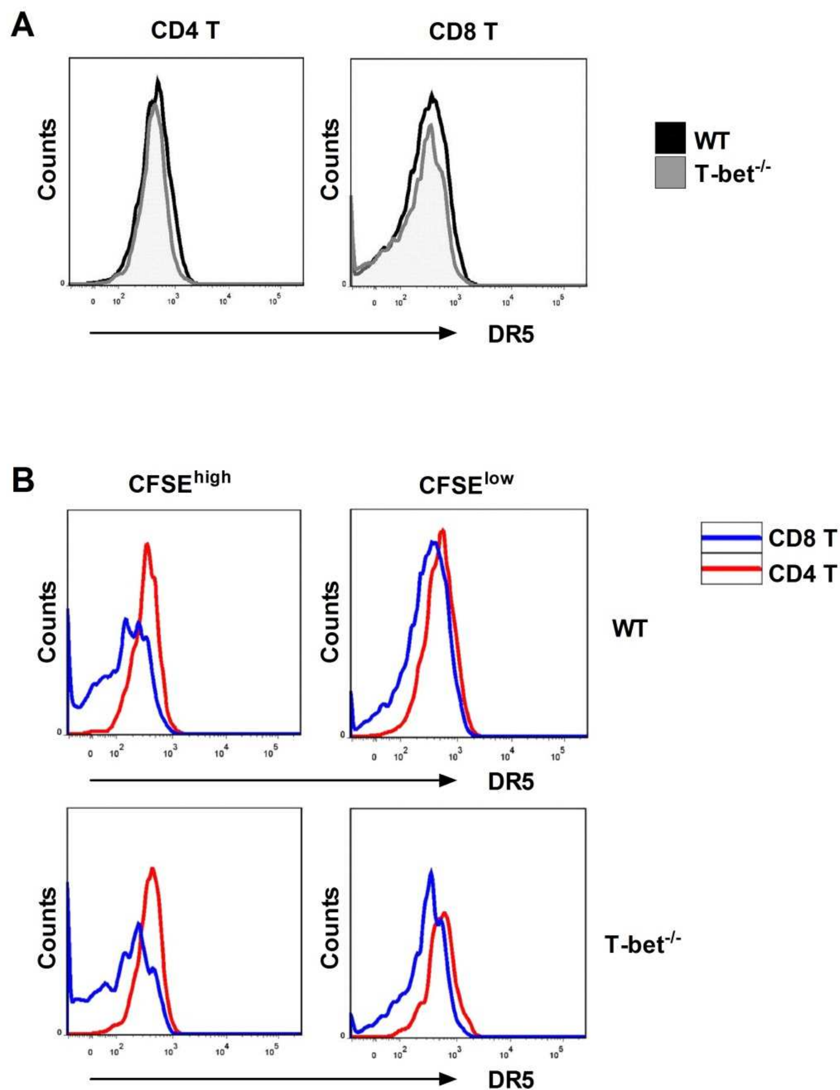


Figure 3.9 DR5 expression on donor T cells in WT or T-bet$^{-/-}$ allogeneic recipients. WT or T-bet$^{-/-}$ B6 mice were lethally irradiated and transplanted with 3×10^6/mouse purified CFSE-labeled T cells from FVB donors (n= 3 per group). Four days post-transplant, recipient spleens were harvested and stained for flow cytometry analysis. Histograms of DR5 expression on donor CD4 or CD8 T cells (A), and on CFSE^{high} or CFSE^{low} donor T cells (B) are shown from representative WT or T-bet$^{-/-}$ recipients in each group.

To test whether Trail/DR5 interaction was responsible for the induction of donor T-cell apoptosis (Figure 3.2 E and F) and subsequent reduction of GVHD (Figure 3.1) in T-bet$^{-/-}$ recipients, we utilized two strategies: blocking Trail/DR5 interaction using sDR5 or using T cells from DR5$^{-/-}$ donors. While donor T cells exhibited lower levels of expansion and higher levels of apoptosis in CFSE^{high} population in T-bet$^{-/-}$ recipients, the treatment with sDR5 largely compensated the difference in the absence of T-bet (Figure 3.8 C and D), suggesting that Trail/DR5 axis contributed to the impaired donor T-cell proliferation and increased early apoptosis in T-bet$^{-/-}$ recipients. Using DR5$^{-/-}$ mice as donors, we observed that T cells proliferated slightly faster in the absence of DR5 *in vitro* (Figure 3.10), but had a comparable ability to induce GVHD in WT recipients *in vivo* (Figure 3.8 E and F). While WT T cells induced severe GVHD in WT recipients, they failed to do in T-bet$^{-/-}$ recipients, reflected by the overall survival (Figure 3.8E) and GVHD clinical scores (Figure 3.8F). Concersely, DR5$^{-/-}$ donor T cells were able to induce severe GVHD in the recipients regardless of T-bet expression (Figure 3.8 E and F). These data strongly suggest that the Trail/DR5 interaction is a major signaling pathway responsible for donor T-cell apoptosis induced by T-bet$^{-/-}$ APCs, through which the development of GVHD is alleviated.

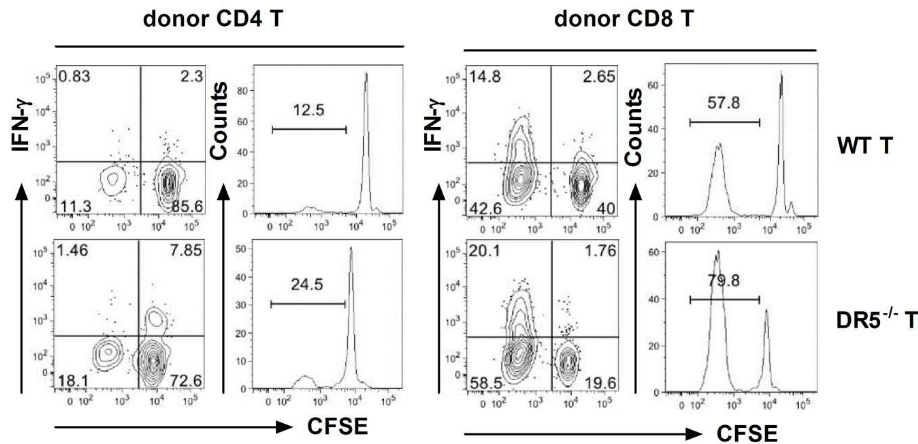


Figure 3.10 *Ex vivo* MLR profile of WT or DR5$^{-/-}$ T cells. Purified WT or DR5$^{-/-}$ splenic T cells on B6 background were labeled with CFSE, and mixed with CD11c^{high} splenic DCs from FVB donor at the ratio of 10:1 (T: DC) in a 96-well plate. Five days later, mixed cells were stimulated with PMA plus Inomycin and harvested for flow cytometry analysis. Profiles of CFSE and IFN-γ in donor CD4 or CD8 T cells were shown from representative wells in each group.

3.4.8 T-bet$^{-/-}$ recipients partially maintain GVL effect

We next evaluated whether T-bet$^{-/-}$ hosts may preserve the T-cell mediated GVL effect. Using A20 B-cell lymphoma with a relatively low dose of T cells, we observed that while donor T cells induced less severe GVHD in T-bet$^{-/-}$ recipients (Figure 3.11A) as presented above (Figure 3.1), they mediated a robust GVL effect in either WT or T-bet$^{-/-}$ recipients (Figure 3.11B, $p>0.05$). It is notable that the T-bet$^{-/-}$ recipients had delayed lymphoma relapse although somewhat higher rate in the later stage (Figure 3.11B). Using C1498 atypical myeloid leukemia with a relatively high dose of T cells, we found that T-bet$^{-/-}$ recipients had significantly better survival than WT recipients (Figure 3.11C), and most of T-bet$^{-/-}$ recipients were free from leukemia relapse (Figure 3.11D). In fact, leukemia relapse in those recipients did not occur until more than 70 days after BMT (Figure 3.11 D and E). Taken together, T-bet expression on the host is critical for GVHD induction but much less important for mediating the GVL effect. We

further observed that donor CD8 T cells expressed comparable levels of CD107a and granzyme B in T-bet$^{-/-}$ recipients compared with WT recipients (Figure 3.12), which likely contributed to preserved GVL activity.

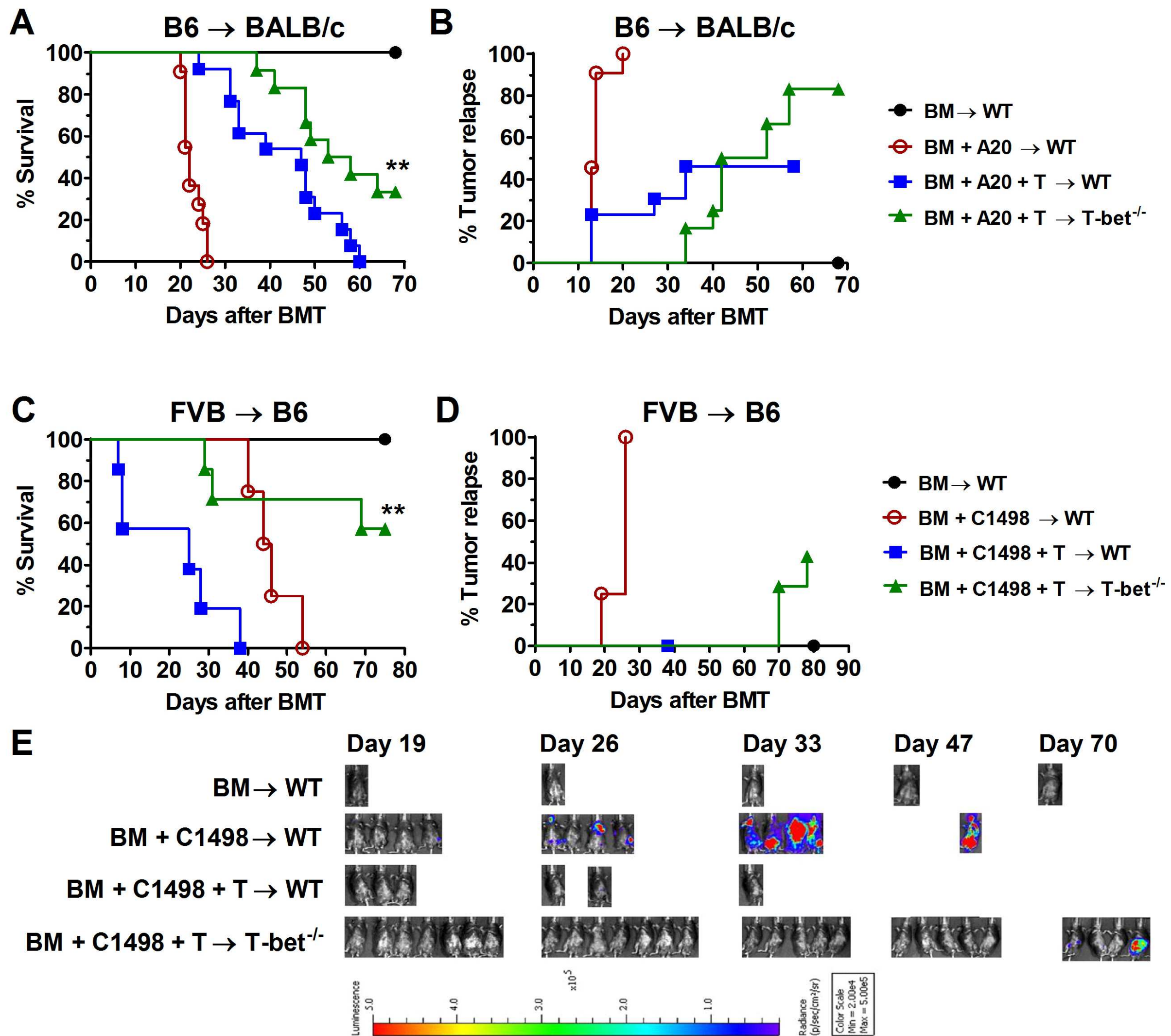


Figure 3.11 T-bet$^{-/-}$ recipients partially preserve GVL effect. WT or T-bet$^{-/-}$ BALB/c mice (n=11~13 per group) were lethally irradiated and transplanted with 5×10^6/mouse TCD-BM from WT B6 donors alone, or plus purified T cells from WT B6 donors at 0.25×10^6/mouse. Additionally, recipients were i.v. injected with 2×10^3 A20-luc on BALB/c background at the time of BMT. Recipients were monitored throughout the experimental period for survival (A), and tumor growth by luciferin i.p. injection and whole-body BLI. Percentages of tumor relapse are shown (B). Data pooled from two separate experiments. WT or T-bet$^{-/-}$ B6 mice (n=7~8 per group) were lethally irradiated and transplanted with 5×10^6/mouse TCD-BM from FVB B6

donors alone, or plus 2×10^6/mouse purified T cells from FVB donors. Additionally, recipients were i.v. injected with 1×10^5 C1498-luc generated in B6 mice at the time of BMT. Recipients were monitored throughout the experimental period for survival (C), and tumor growth. Percentages of relapse (D) and recipient BLI image (E) are shown. Tumor mortality and GVHD mortality were distinguished by BLI signal intensity and clinical manifestation of GVHD. Asterisk indicates statistical significance between WT and T-bet$^{-/-}$ recipients given T cell transfer: *p<0.05, **p<0.01, ***p<0.001.

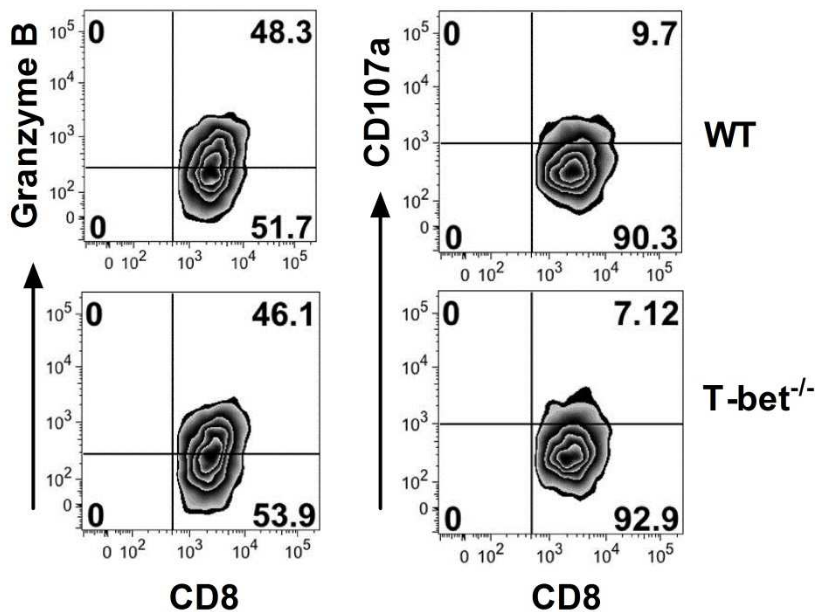


Figure 3.12 T-bet on host is not required for CD107a and granzyme B production by donor CD8 T cells. Lethally irradiated WT or T-bet$^{-/-}$ mice on BALB/c background (n=4~10 per group, pooled from two experiments) were transplanted with 5×10^6/mouse TCD-BM from Ly5.1$^+$ B6 donor alone, or plus 0.25×10^6/mouse purified Ly5.2$^+$ T cells from B6 mice. Seven days post-transplant, mononuclear cells were isolated from recipient spleens and stained for flow cytometry analysis. Percentage of donor CD8 T cell producing granzyme B and expressing CD107a was shown on gated Ly5.2$^+$ H-2K^{b+} CD8$^+$ cells in spleen from representative recipients in each group.

3.5 Discussion

As the predominant sensors of GVHD initiation, APCs become an attractive target for GVHD control. After conditioning, residual host hematopoietic APCs, nonhematopoietic APCs, and donor APCs transferred with the allograft are all capable to prime allogeneic donor T cells and contribute to GVH reactions (221, 239, 249, 250). Our data demonstrate that T-bet is critical

for the activity and function of hematopoietic APCs on both host and donor origin to induce GVHD in MHC-mismatched models (Figure 3.1, 3.6 and 3.7), through regulating donor T-cell alloresponse (Figure 3.2 A-D), apoptosis (Figure 3.2 E and F), and migration (Figure 3.4). T-bet on the host also boosts GVHD mediated by CD4 but not by CD8 T cells in miHA-mismatched BMT model. We further validate that T-bet on hematopoietic APCs, particularly DCs, promotes allogeneic T-cell response by limiting apoptosis through down-regulating Trail expression (Figure 3.8).

Either hematopoietic or nonhematopoietic APCs in the recipient are capable of activating donor T cells and inducing GVHD (219, 221, 249, 250, 261). Our chimeras BMT data demonstrate that deficiency of T-bet, particularly on host hematopoietic APCs, to a much lesser extent on nonhematopoietic APCs, attenuated the severity of GVHD (Figure 3.6). DCs are considered as the most efficacious hematopoietic APCs in T-cell priming and GVHD induction (68, 243). T-bet has been shown to regulate optimal production of IFN-γ and antigen-specific T-cell activation by DCs (5). Consistently, we also observed impaired IFN-γ production by T-bet$^{-/-}$ CD11c^{+} DCs in the allogeneic but not syngeneic recipients (Figure 3.8A) associated with compromised allogeneic T-cell activation and proliferation (Figure 3.2), suggesting that T-bet positively regulates allo-stimulatory functions of DCs.

It is clear that T-cell proliferation, activation and apoptosis in syngeneic recipients were intact regardless of T-bet expression (Figure 3.3), indicating that T-bet expression on host APCs does not impact T-cell homeostatic response. In allogeneic BMT, recipient APCs present alloantigens directly to activate donor T cells, which are essential for GVHD development (68). The fact that T-bet$^{-/-}$ recipients developed only mild GVHD (Figure 3.1) suggests the essential role of T-bet in regulating activity of recipient APCs. The first several days post-transplant are

considered to be critical in GVHD induction according to the studies of spatial and temporal dynamics of alloreactive T cell activation, proliferation, and tissue distribution (332, 333), and the most actively dividing T cells are specific for recipient alloantigens in this period of time (334, 335). Thus, the CFSE^{low} population has been referred as fast-dividing alloreactive T cells, and CFSE^{high} population as slow-dividing non-alloreactive T cells (336, 337). In MHC-mismatched BMT (Figure 3.2), the frequency of alloreactive T cells among total donor T cells should the same in WT or T-bet$^{-/-}$ recipients at the time of transplant. A large proportion of CFSE^{high} T cells undergoing apoptosis in T-bet$^{-/-}$ recipients was likely a dynamic event, in which alloreactive T cells died before they would be able to further expand. Given T-bet on the host did not affect T-cell homeostatic response, we surmise that those T cells undergoing apoptosis trigged by T-bet$^{-/-}$ APCs were alloreactive T cells. Interestingly, the percentages of apoptosis were comparable among CFSE^{low} T cells in WT or T-bet$^{-/-}$ recipients (Figure 3.2 E and F). It is possible that those fast dividing CFSE^{low} T cells were undergoing activation-induced cell death (AICD) through FasL/Fas pathway (338), and the slow-dividing CFSE^{high} T cells were instead sensitive to Trail/DR5-mediated apoptosis as discussed below. Evaluation of the expression of T cell activation markers, including CD25, CD44, CD69, CD122, and phos-ZAP70, as well as the cell cycle analysis in CFSE^{high} population, would be helpful to further understand the apoptosis mechanisms.

The apoptosis of donor T cells after allo-BMT appears to be an important process in regulating T cell homeostasis (339, 340). T-cell apoptosis generally occurrs through two pathways termed active apoptosis, mediated by death receptor signaling (341, 342), and passive apoptosis, controlled by bcl-2 family members (343). Active apoptosis has been shown to be one mechanism to limit the survival of both alloreactive and bystander donor T cells in GVHD (338).

Death receptors including Fas, TNFR, and DR5, are cell surface receptors that transmit apoptotic signals initiated by specific TNF family ligands such as FasL, TNF-α and Trail, respectively (342). Our data shows that T-bet$^{-/-}$ CD11c^{+} DCs upregulated Trail, but not FasL or TNF-α expression (Figure 3.8B), to induce allogeneic T cell apoptosis. Signal blockade by sDR5 treatment or using donor T-cell deficient for DR5 (Figure 3.8) further proved our hypothesis that, the Trail/DR5 axis plays an important role for the alleviated GVHD in T-bet$^{-/-}$ hosts. Our data is in line with a previous study, which showed that genetically modified Trail-transduced DCs protected mice from GVHD and leukemia relapse through inducing of apoptosis of both alloreactive T cells and tumor cells (285).

T-cell migration to GVHD target organs is required for GVHD development. We found less infiltrated donor CD4 but not CD8 T cells in the livers of T-bet$^{-/-}$ recipiets (Figure 3.4), which is likely due to the significantly decreased accumulation of CXCR3^{+} CD4 T cells in the spleens of those recipients, given the interaction of chemokine and chemokine receptors plays an important role in mediating T-cell migration to GVHD target organs (312, 313). Previous studies showed that CD4 and CD8 T cells have distinct characteristics and functions beyond MHC restriction, where CD8 T cells have survival advantages and lower sensitivity towards apoptosis than CD4 T cells (344). Indeed, we noticed that, CD8 T cells expressed lower levels of DR5 than CD4 T cells, particularly in CFSE^{high} population, in either WT or T-bet$^{-/-}$ allogeneic recipients (Figure 3.9B). Not only in apoptosis, but also in activation, proliferation, and migration, CD4 T cells seemed always to be more sensitive than CD8 T cells on those defects upon interacting with T-bet$^{-/-}$ APCs in MHC-mismatched BMT (Figure 3.2 and 3.4). These results are further supported in MHC-matched BMT, where T-bet expression on hosts contributes to the development of GVHD induced by CD4 but not CD8 T cells (Figure 3.5). A reduced effect of T-

bet$^{-/-}$ APCs on CD8 T cells may account for the preserved GVL effect (Figure 3.11). More specifically, we found that CD8 T cells maintained their cytolytic function reflected by preserved CD107a and granzyme B expression (Figure 3.12).

In conclusion, we demonstrated that T-bet up-regulates IFN-γ production and down-regulates Trail expression on recipient DCs, which promotes donor T-cell activation and mitigates T-cell apoptosis, respectively. T-bet plays a critical role in the development of GVHD by regulating the activity of hematopoietic APCs, particularly DCs. Taken together with our recently published findings, we propose that T-bet is a potential therapeutic target for the control of GVHD through regulating T-cell activation and differentiation as well as APC functions (46). Although currently unavailable, a small molecular inhibitor to T-bet can be developed, given other inhibitors to RORγt or c-Rel have been generated as therapeutic intervention in autoimmunity and transplantation (324, 325). Alternatively, T-bet may be silenced with siRNA or shRNA strategy as gene-based therapy (345). We are therefore optimistic that our findings will be translated into the clinic in the near future to benefit patients for controlling GVHD and leukemia relapse after allo-HSCT.

Chapter 4: Conclusions and Future Directions

Our study provides insightful mechanisms and proves the concept that targeting T-bet can largely prevent aGVHD by regulating the functions of both T cells (46) and APCs (48). Previous investigations of T-bet had mainly focused on its most well-known target IFN-γ. However, accumulating evidence indicates the existence of T-bet-dependent but IFN-γ-independent components accounting for the pathogenesis of aGVHD, as well as autoimmune diseases including EAE (6). The novelty of current study is that, we uncovered for the first time T-bet can regulate T cell function in an IFN-γ-independent manner. At gene and protein levels, we identified the molecules that uniquely regulated by T-bet are potentially critical for GVHD development, and thus may serve as valid targets for the control of GVHD after allo-BMT, which permits alternative therapeutic strategy rather than directly targeting T-bet given the transcription factor is generally difficult to be targeted pharmacologically. The novelty of our current work also lies in the negative regulation of Trail expression on host DCs by T-bet after allo-BMT, through which T-bet on DCs protects allogeneic T cells from apoptosis. Altogether, we found that T-bet positively regulates activation and function of both T cells and DCs, which sets a strong rationale to pharmacologically inhibit T-bet for the control of GVHD through both T cells and APCs synchronously.

Further studies are required to address remaining unsolved questions and translate our findings towards clinic application. The gene-based therapy (345) using siRNA or shRNA to silence T-bet could be a direct targeting strategy due to the lack of small molecular inhibitor

specific for T-bet. Mice transferred with siRNA-T-bet transfected splenocytes failed to develop EAE (49), and administration of siRNA-T-bet has been suggested could effectively alleviate established EAE in mice (346). Our pilot experiments indicate that a single dose of siRNA-T-bet (50µg/mouse) on the day of BMT failed to significantly ameliorate the overall GVHD severity (unpublished observation). Thus, the optimal dose and treatment period of siRNA-T-bet as a prophylaxis under allo-BMT settings will require further investigation.

Transcription factors generally working in pairs by forming the complex and generating chromatin modifications that associated with gene activation or repression. Same is true for T-bet to perform its translational regulation on T cell differentiation (2, 347). T-bet recruits JMJD3 (a histone H3K27 demethylase) and SET7/9 (a histone H3K4 methyltransferase) to transactivate *Ifng* gene and promote Th1 differentiation. This process is further enhanced by HLX and RUNX3, the transcription factors encoded by T-bet target genes *Hlx* and *Runx3*. T-bet binds to RUNX3 to silence *Il4* gene and it also prevents the GATA3-mediated activation of Th2-related cytokine genes *Il4*, *Il5* and *Il13*by binding to GATA3. Similarly, T-bet interacts with RUNX1 to suppress Th17 differentiation, and recruits BCL6 to block Tfh lineage commitment. These enzymes or transcription factors form complex with T-bet could also be the potential target for the prevention of aGVHD. For example, JMJD3 has recently been suggested a critical role in regulating CD4 T-cell differentiation in a Th1-dependent colitis model. Deficiency of JMJD3 inhibits Th1 differentiation while promoting Th2 and Th17 generation (348). The small molecule inhibitor GSK-J1 that is selective for the JMJ subfamily (JMJD3 and UTX) is currently available (349, 350). Inhibitors for SET7/9 were identified by the high-throughput screening (351), although the effect of SET7/9 in the development of GVHD has not been tested.

Alternatively, indirectly targeting downstream effectors of T-bet on T cells, such as those T-bet-dependent but IFN-γ-independent molecules (e.g. CXCR3, NKG2D) we identified, is presumably providing a beneficial outcome, as suggested by others (314, 323) and our unpublished work. Our preliminary data indicate that, blocking NKG2D using its mAb could effectively attenuate aGVHD in mice. One limitation is the lack of proven of those genes we identified are unique target genes directly controlled by T-bet, which could be defined and confirmed by using the chromatin immunoprecipitation (ChIP) assays. On the aspect of APCs, genetically modified DCs (e.g. over-expressing Trail) may be effective to combat aGVHD by inducing alloreactive T cell apoptosis while preventing leukemia relapse (285). This is in line with our observation in allo-BMT settings, where T-bet upregulates Trail in DCs and prevents the early apoptosis of allogeneic donor T cells, although we have not determined whether the deficiency of T-bet in DCs also induce the apoptosis of tumor cells.

Another option is to modify upstream regulators of T-bet. MicroRNAs (miRs) have the potential for novel therapeutics in GVHD (352, 353), as suggested by different studies done by us and others. MiR-17-92 (354), miR-155(355), miR-34a (356), miR-100 (357), and miR-142 (358) have all been implicated a significant role in GVHD. Further investigation on miR-29 to regulate GVHD is attractive due to inhibition of IFN-γ production by repressing both T-bet and Eomes (22). Moreover, recent studies suggest that, T-bet could be regulated by the histone metyltransferase Ezh2, at both transcriptional and posttranslational levels (359). Ezh2 can directly activate *Tbx21* transcription by binding to its promoter in Th1 cells, while Ezh2 is also required to protect T-bet degradation mediated by proteasome. Follow-up study showed that conditional loss of Ezh2 in donor T cells could attenuate GVHD while still preserve the GVL effect after allo-BMT (360). The tyrosine kinase c-Abl has been reported to catalyze the tyrosine

phosphorylation of T-bet DNA-binding domain and therefore enhance the transcriptional activation of T-bet and Th1 differentiation (361). Combinational inhibition of c-Abl and PDGF receptors using imatinib or nilotinib could protect against murine sclerodermatous cGVHD (362). The intervention of c-Abl in the prevention or treatment of aGVHD has not yet been evaluated.

Taken together, by using the genetically knockout mice, we have proved that targeting T-bet can prevent aGVHD and leukemia relapse, with additional IL-17 neutralizing Ab treatment when necessary. The next step is to achieve the translational application. As discussed above, it is challenging but promising to develop the therapeutic strategies to target T-bet or its related pathways to control GVHD and prevent leukemia relapse after allo-HSCT.

List of References

1. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655-669.
2. Lazarevic, V., L. H. Glimcher, and G. M. Lord. 2013. T-bet: a bridge between innate and adaptive immunity. *Nature reviews. Immunology* 13: 777-789.
3. Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul, and J. J. O'Shea. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proceedings of the National Academy of Sciences of the United States of America* 98: 15137-15142.
4. Lugo-Villarino, G., S. Ito, D. M. Klinman, and L. H. Glimcher. 2005. The adjuvant activity of CpG DNA requires T-bet expression in dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America* 102: 13248-13253.
5. Lugo-Villarino, G., R. Maldonado-Lopez, R. Possemato, C. Penaranda, and L. H. Glimcher. 2003. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America* 100: 7749-7754.
6. Lazarevic, V., and L. H. Glimcher. 2011. T-bet in disease. *Nature immunology* 12: 597-606.
7. Lipscomb, M. W., L. Chen, J. L. Taylor, C. Goldbach, S. C. Watkins, P. Kalinski, L. H. Butterfield, A. K. Wesa, and W. J. Storkus. 2009. Ectopic T-bet expression licenses dendritic cells for IL-12-independent priming of type 1 T cells in vitro. *Journal of immunology (Baltimore, Md. : 1950)* 183: 7250-7258.
8. Townsend, M. J., A. S. Weinmann, J. L. Matsuda, R. Salomon, P. J. Farnham, C. A. Biron, L. Gapin, and L. H. Glimcher. 2004. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20: 477-494.
9. Gordon, S. M., J. Chaix, L. J. Rupp, J. Wu, S. Madera, J. C. Sun, T. Lindsten, and S. L. Reiner. 2012. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* 36: 55-67.
10. Matsuda, J. L., Q. Zhang, R. Ndonye, S. K. Richardson, A. R. Howell, and L. Gapin. 2006. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood* 107: 2797-2805.
11. Kim, H. Y., M. Pichavant, P. Matangkasombut, Y. I. Koh, P. B. Savage, R. H. DeKruyff, and D. T. Umetsu. 2009. The development of airway hyperreactivity in T-bet-deficient mice requires CD1d-restricted NKT cells. *Journal of immunology (Baltimore, Md. : 1950)* 182: 3252-3261.

12. Spits, H., D. Artis, M. Colonna, A. Diefenbach, J. P. Di Santo, G. Eberl, S. Koyasu, R. M. Locksley, A. N. McKenzie, R. E. Mebius, F. Powrie, and E. Vivier. 2013. Innate lymphoid cells--a proposal for uniform nomenclature. *Nature reviews. Immunology* 13: 145-149.
13. Fuchs, A., W. Vermi, J. S. Lee, S. Lonardi, S. Gilfillan, R. D. Newberry, M. Cella, and M. Colonna. 2013. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity* 38: 769-781.
14. Bernink, J. H., C. P. Peters, M. Munneke, A. A. te Velde, S. L. Meijer, K. Weijer, H. S. Hreggvidsdottir, S. E. Heinsbroek, N. Legrand, C. J. Buskens, W. A. Bemelman, J. M. Mjosberg, and H. Spits. 2013. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nature immunology* 14: 221-229.
15. Powell, N., A. W. Walker, E. Stolarczyk, J. B. Canavan, M. R. Gokmen, E. Marks, I. Jackson, A. Hashim, M. A. Curtis, R. G. Jenner, J. K. Howard, J. Parkhill, T. T. MacDonald, and G. M. Lord. 2012. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. *Immunity* 37: 674-684.
16. Klose, C. S., E. A. Kiss, V. Schwierzeck, K. Ebert, T. Hoyler, Y. d'Hargues, N. Goppert, A. L. Croxford, A. Waisman, Y. Tanriver, and A. Diefenbach. 2013. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature* 494: 261-265.
17. Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nature immunology* 3: 549-557.
18. Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, and S. L. Reiner. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science (New York, N.Y.)* 292: 1907-1910.
19. Schulz, E. G., L. Mariani, A. Radbruch, and T. Hofer. 2009. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 30: 673-683.
20. Zhu, J., D. Jankovic, A. J. Oler, G. Wei, S. Sharma, G. Hu, L. Guo, R. Yagi, H. Yamane, G. Punkosdy, L. Feigenbaum, K. Zhao, and W. E. Paul. 2012. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* 37: 660-673.
21. Smith, K. M., M. Guerau-de-Arellano, S. Costinean, J. L. Williams, A. Bottoni, G. Mavrikis Cox, A. R. Satoskar, C. M. Croce, M. K. Racke, A. E. Lovett-Racke, and C. C. Whitacre. 2012. miR-29ab1 deficiency identifies a negative feedback loop controlling Th1 bias that is dysregulated in multiple sclerosis. *Journal of immunology (Baltimore, Md. : 1950)* 189: 1567-1576.
22. Steiner, D. F., M. F. Thomas, J. K. Hu, Z. Yang, J. E. Babiarz, C. D. Allen, M. Matloubian, R. Blelloch, and K. M. Ansel. 2011. MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells. *Immunity* 35: 169-181.
23. Jang, E. J., H. R. Park, J. H. Hong, and E. S. Hwang. 2013. Lysine 313 of T-box is crucial for modulation of protein stability, DNA binding, and threonine phosphorylation of T-bet. *Journal of immunology (Baltimore, Md. : 1950)* 190: 5764-5770.

24. Lazarevic, V., and L. H. Glimcher. 2011. T-bet in disease. *Nat Immunol* 12: 597-606.
25. Wilson, C. B., E. Rowell, and M. Sekimata. 2009. Epigenetic control of T-helper-cell differentiation. *Nature reviews. Immunology* 9: 91-105.
26. Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T. Y. Roh, W. T. Watford, D. E. Schones, W. Peng, H. W. Sun, W. E. Paul, J. J. O'Shea, and K. Zhao. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30: 155-167.
27. Miller, S. A., A. C. Huang, M. M. Miazgowicz, M. M. Brassil, and A. S. Weinmann. 2008. Coordinated but physically separable interaction with H3K27-demethylase and H3K4-methyltransferase activities are required for T-box protein-mediated activation of developmental gene expression. *Genes & development* 22: 2980-2993.
28. Hwang, E. S., S. J. Szabo, P. L. Schwartzberg, and L. H. Glimcher. 2005. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science (New York, N.Y.)* 307: 430-433.
29. Djuretic, I. M., D. Levanon, V. Negreanu, Y. Groner, A. Rao, and K. M. Ansel. 2007. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nature immunology* 8: 145-153.
30. Lazarevic, V., X. Chen, J. H. Shim, E. S. Hwang, E. Jang, A. N. Bolm, M. Oukka, V. K. Kuchroo, and L. H. Glimcher. 2011. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nature immunology* 12: 96-104.
31. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science (New York, N.Y.)* 295: 338-342.
32. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, H. Shen, N. Cereb, S. Y. Yang, T. Lindsten, J. Rossant, C. A. Hunter, and S. L. Reiner. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302: 1041-1043.
33. Intlekofer, A. M., N. Takemoto, E. J. Wherry, S. A. Longworth, J. T. Northrup, V. R. Palanivel, A. C. Mullen, C. R. Gasink, S. M. Kaech, J. D. Miller, L. Gapin, K. Ryan, A. P. Russ, T. Lindsten, J. S. Orange, A. W. Goldrath, R. Ahmed, and S. L. Reiner. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nature immunology* 6: 1236-1244.
34. Intlekofer, A. M., A. Banerjee, N. Takemoto, S. M. Gordon, C. S. Dejong, H. Shin, C. A. Hunter, E. J. Wherry, T. Lindsten, and S. L. Reiner. 2008. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science (New York, N.Y.)* 321: 408-411.
35. Cruz-Guilloty, F., M. E. Pipkin, I. M. Djuretic, D. Levanon, J. Lotem, M. G. Lichtenheld, Y. Groner, and A. Rao. 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *The Journal of experimental medicine* 206: 51-59.
36. Takemoto, N., A. M. Intlekofer, J. T. Northrup, E. J. Wherry, and S. L. Reiner. 2006. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *Journal of immunology (Baltimore, Md. : 1950)* 177: 7515-7519.

37. Banerjee, A., S. M. Gordon, A. M. Intlekofer, M. A. Paley, E. C. Mooney, T. Lindsten, E. J. Wherry, and S. L. Reiner. 2010. Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *Journal of immunology (Baltimore, Md. : 1950)* 185: 4988-4992.
38. Joshi, N. S., W. Cui, C. X. Dominguez, J. H. Chen, T. W. Hand, and S. M. Kaech. 2011. Increased numbers of preexisting memory CD8 T cells and decreased T-bet expression can restrain terminal differentiation of secondary effector and memory CD8 T cells. *Journal of immunology (Baltimore, Md. : 1950)* 187: 4068-4076.
39. Peng, S. L., S. J. Szabo, and L. H. Glimcher. 2002. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proceedings of the National Academy of Sciences of the United States of America* 99: 5545-5550.
40. Gerth, A. J., L. Lin, and S. L. Peng. 2003. T-bet regulates T-independent IgG2a class switching. *International immunology* 15: 937-944.
41. Liu, N., N. Ohnishi, L. Ni, S. Akira, and K. B. Bacon. 2003. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nature immunology* 4: 687-693.
42. Wang, N. S., L. J. McHeyzer-Williams, S. L. Okitsu, T. P. Burris, S. L. Reiner, and M. G. McHeyzer-Williams. 2012. Divergent transcriptional programming of class-specific B cell memory by T-bet and RORalpha. *Nature immunology* 13: 604-611.
43. Serre, K., A. F. Cunningham, R. E. Coughlan, A. C. Lino, A. Rot, E. Hub, K. Moser, R. Manz, A. Ferraro, R. Bird, K. M. Toellner, J. Demengeot, I. C. MacLennan, and E. Mohr. 2012. CD8 T cells induce T-bet-dependent migration toward CXCR3 ligands by differentiated B cells produced during responses to alum-protein vaccines. *Blood* 120: 4552-4559.
44. Yin, Z., C. Chen, S. J. Szabo, L. H. Glimcher, A. Ray, and J. Craft. 2002. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-gamma by gammadelta T cells. *Journal of immunology (Baltimore, Md. : 1950)* 168: 1566-1571.
45. Chen, L., W. He, S. T. Kim, J. Tao, Y. Gao, H. Chi, A. M. Intlekofer, B. Harvey, S. L. Reiner, Z. Yin, R. A. Flavell, and J. Craft. 2007. Epigenetic and transcriptional programs lead to default IFN-gamma production by gammadelta T cells. *Journal of immunology (Baltimore, Md. : 1950)* 178: 2730-2736.
46. Fu, J., D. Wang, Y. Yu, J. Heinrichs, Y. Wu, S. Schutt, K. Kaosaard, C. Liu, K. Haarberg, D. Bastian, D. G. McDonald, C. Anasetti, and X. Z. Yu. 2015. T-bet Is Critical for the Development of Acute Graft-versus-Host Disease through Controlling T Cell Differentiation and Function. *Journal of immunology (Baltimore, Md. : 1950)* 194: 388-397.
47. Yu, Y., D. Wang, C. Liu, K. Kaosaard, K. Semple, C. Anasetti, and X. Z. Yu. 2011. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. *Blood* 118: 5011-5020.
48. Fu, J., Y. Wu, H. Nguyen, J. L. Heinrichs, S. Schutt, C. Liu, C. Anasetti, and X.-Z. Yu. 2014. T-Bet Is Critical for the Development of Acute Graft-Versus-Host Disease By Regulating Hematopoietic Antigen Presenting Cells. In *56th ASH Annual Meeting*. American Society of Hematology. 846-846.

49. Yang, Y., J. Weiner, Y. Liu, A. J. Smith, D. J. Huss, R. Winger, H. Peng, P. D. Cravens, M. K. Racke, and A. E. Lovett-Racke. 2009. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *The Journal of experimental medicine* 206: 1549-1564.
50. Bettelli, E., B. Sullivan, S. J. Szabo, R. A. Sobel, L. H. Glimcher, and V. K. Kuchroo. 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 200: 79-87.
51. Nath, N., R. Prasad, S. Giri, A. K. Singh, and I. Singh. 2006. T-bet is essential for the progression of experimental autoimmune encephalomyelitis. *Immunology* 118: 384-391.
52. Grifka-Walk, H. M., S. J. Lalor, and B. M. Segal. 2013. Highly polarized Th17 cells induce EAE via a T-bet independent mechanism. *European journal of immunology* 43: 2824-2831.
53. O'Connor, R. A., H. Cambrook, K. Huettner, and S. M. Anderton. 2013. T-bet is essential for Th1-mediated, but not Th17-mediated, CNS autoimmune disease. *European journal of immunology* 43: 2818-2823.
54. Neurath, M. F., B. Weigmann, S. Finotto, J. Glickman, E. Nieuwenhuis, H. Iijima, A. Mizoguchi, E. Mizoguchi, J. Mudter, P. R. Galle, A. Bhan, F. Autschbach, B. M. Sullivan, S. J. Szabo, L. H. Glimcher, and R. S. Blumberg. 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *The Journal of experimental medicine* 195: 1129-1143.
55. Matsuoka, K., N. Inoue, T. Sato, S. Okamoto, T. Hisamatsu, Y. Kishi, A. Sakuraba, O. Hitotsumatsu, H. Ogata, K. Koganei, T. Fukushima, T. Kanai, M. Watanabe, H. Ishii, and T. Hibi. 2004. T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated immunopathology in Crohn's disease. *Gut* 53: 1303-1308.
56. Sasaki, Y., K. Ihara, N. Matsuura, H. Kohno, S. Nagafuchi, R. Kuromaru, K. Kusuhara, R. Takeya, T. Hoey, H. Sumimoto, and T. Hara. 2004. Identification of a novel type 1 diabetes susceptibility gene, T-bet. *Human genetics* 115: 177-184.
57. Esensten, J. H., M. R. Lee, L. H. Glimcher, and J. A. Bluestone. 2009. T-bet-deficient NOD mice are protected from diabetes due to defects in both T cell and innate immune system function. *Journal of immunology (Baltimore, Md. : 1950)* 183: 75-82.
58. Wang, J., J. W. Fathman, G. Lugo-Villarino, L. Scimone, U. von Andrian, D. M. Dorfman, and L. H. Glimcher. 2006. Transcription factor T-bet regulates inflammatory arthritis through its function in dendritic cells. *The Journal of clinical investigation* 116: 414-421.
59. Finotto, S., M. F. Neurath, J. N. Glickman, S. Qin, H. A. Lehr, F. H. Green, K. Ackerman, K. Haley, P. R. Galle, S. J. Szabo, J. M. Drazen, G. T. De Sanctis, and L. H. Glimcher. 2002. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science (New York, N.Y.)* 295: 336-338.
60. Raby, B. A., E. S. Hwang, K. Van Steen, K. Tantisira, S. Peng, A. Litonjua, R. Lazarus, C. Giallourakis, J. D. Rioux, D. Sparrow, E. K. Silverman, L. H. Glimcher, and S. T. Weiss. 2006. T-bet polymorphisms are associated with asthma and airway hyperresponsiveness. *American journal of respiratory and critical care medicine* 173: 64-70.
61. Yuan, X., J. Paez-Cortez, I. Schmitt-Knosalla, F. D'Addio, B. Mfarrej, M. Donnarumma, A. Habicht, M. R. Clarkson, J. Iacomini, L. H. Glimcher, M. H. Sayegh, and M. J. Ansari. 2008. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *The Journal of experimental medicine* 205: 3133-3144.

62. Sabet-Baktach, M., E. Eggenhofer, J. Rovira, P. Renner, M. Lantow, S. A. Farkas, M. Malaise, K. Edtinger, Z. Shaotang, G. E. Koehl, M. H. Dahlke, H. J. Schlitt, E. K. Geissler, and A. Kroemer. 2013. Double deficiency for RORgammat and T-bet drives Th2-mediated allograft rejection in mice. *Journal of immunology (Baltimore, Md. : 1950)* 191: 4440-4446.
63. Ferrara, J. L., J. E. Levine, P. Reddy, and E. Holler. 2009. Graft-versus-host disease. *Lancet* 373: 1550-1561.
64. Goker, H., I. C. Haznedaroglu, and N. J. Chao. 2001. Acute graft-vs-host disease: pathobiology and management. *Experimental hematology* 29: 259-277.
65. Appelbaum, F. R. 2001. Haematopoietic cell transplantation as immunotherapy. *Nature* 411: 385-389.
66. Blazar, B. R., and W. J. Murphy. 2005. Bone marrow transplantation and approaches to avoid graft-versus-host disease (GVHD). *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 360: 1747-1767.
67. Reddy, P., R. Negrin, and G. R. Hill. 2008. Mouse Models of Bone Marrow Transplantation. *Biology of Blood and Marrow Transplantation* 14: 129-135.
68. Shlomchik, W. D. 2007. Graft-versus-host disease. *Nature reviews. Immunology* 7: 340-352.
69. Socie, G., and B. R. Blazar. 2009. Acute graft-versus-host disease: from the bench to the bedside. *Blood* 114: 4327-4336.
70. Vincent, K., D. C. Roy, and C. Perreault. 2011. Next-generation leukemia immunotherapy. *Blood* 118: 2951-2959.
71. Kolb, H. J. 2008. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood* 112: 4371-4383.
72. Jenq, R. R., and M. R. van den Brink. 2010. Allogeneic haematopoietic stem cell transplantation: individualized stem cell and immune therapy of cancer. *Nature reviews. Cancer* 10: 213-221.
73. Ghosh, A., Y. Dogan, M. Moroz, A. M. Holland, N. L. Yim, U. K. Rao, L. F. Young, D. Tannenbaum, D. Masih, E. Velardi, J. J. Tsai, R. R. Jenq, O. Penack, A. M. Hanash, O. M. Smith, K. Piersanti, C. Lezcano, G. F. Murphy, C. Liu, M. L. Palomba, M. G. Sauer, M. Sadelain, V. Ponomarev, and M. R. M. van den Brink. 2013. Adoptively transferred TRAIL+ T cells suppress GVHD and augment antitumor activity. *The Journal of clinical investigation* 123: 2654-2662.
74. Tato, C. M. O. S., John J. 2006. Immunology: What does it mean to be just 17? *Nature* 441: 166-168.
75. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature immunology* 9: 1341-1346.
76. Duhen, T., R. Geiger, D. Jarrossay, A. Lanzavecchia, and F. Sallusto. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature immunology* 10: 857-863.
77. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29: 138-149.

78. Antin, J. H., and J. L. Ferrara. 1992. Cytokine dysregulation and acute graft-versus-host disease. *Blood* 80: 2964-2968.
79. Blazar, B. R., R. Korngold, and D. A. Vallera. 1997. Recent advances in graft-versus-host disease (GVHD) prevention. *Immunological reviews* 157: 79-109.
80. Iclozan, C., Y. Yu, C. Liu, Y. Liang, T. Yi, C. Anasetti, and X. Z. Yu. 2010. T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 16: 170-178.
81. Carlson, M. J., M. L. West, J. M. Coghill, A. Panoskaltsis-Mortari, B. R. Blazar, and J. S. Serody. 2009. In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. *Blood* 113: 1365-1374.
82. Romagnani, S. 1992. Type 1 T helper and type 2 T helper cells: Functions, regulation and role in protection and disease. *Int J Clin Lab Res* 21: 152-158.
83. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of immunology (Baltimore, Md. : 1950)* 136: 2348-2357.
84. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *The Journal of experimental medicine* 166: 1229-1244.
85. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology* 7: 145-173.
86. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587-596.
87. Grogan, J. L., M. Mohrs, B. Harmon, D. A. Lacy, J. W. Sedat, and R. M. Locksley. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 14: 205-215.
88. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383: 787-793.
89. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nature immunology* 8: 345-350.
90. Laurence, A., and J. J. O'Shea. 2007. T(H)-17 differentiation: of mice and men. *Nature immunology* 8: 903-905.
91. Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *The Journal of biological chemistry* 278: 1910-1914.
92. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441: 231-234.

93. McGeachy, M. J., Y. Chen, C. M. Tato, A. Laurence, B. Joyce-Shaikh, W. M. Blumenschein, T. K. McClanahan, J. J. O'Shea, and D. J. Cua. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nature immunology* 10: 314-324.
94. Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard, and D. R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nature immunology* 8: 967-974.
95. Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T. B. Strom, M. Oukka, and V. K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448: 484-487.
96. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179-189.
97. Mathur, A. N., H. C. Chang, D. G. Zisoulis, G. L. Stritesky, Q. Yu, J. T. O'Malley, R. Kapur, D. E. Levy, G. S. Kansas, and M. H. Kaplan. 2007. Stat3 and Stat4 direct development of IL-17-secreting Th cells. *Journal of immunology (Baltimore, Md. : 1950)* 178: 4901-4907.
98. Ivanov, II, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121-1133.
99. Samoilova, E. B., J. L. Horton, B. Hilliard, T. S. Liu, and Y. Chen. 1998. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. *Journal of immunology (Baltimore, Md. : 1950)* 161: 6480-6486.
100. Kidoya, H., M. Umemura, T. Kawabe, G. Matsuzaki, A. Yahagi, R. Imamura, and T. Suda. 2005. Fas ligand induces cell-autonomous IL-23 production in dendritic cells, a mechanism for Fas ligand-induced IL-17 production. *Journal of immunology (Baltimore, Md. : 1950)* 175: 8024-8031.
101. Sutton, C., C. Brereton, B. Keogh, K. H. Mills, and E. C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *The Journal of experimental medicine* 203: 1685-1691.
102. Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28: 454-467.
103. Dardalhon, V., T. Korn, V. K. Kuchroo, and A. C. Anderson. 2008. Role of Th1 and Th17 cells in organ-specific autoimmunity. *Journal of autoimmunity* 31: 252-256.
104. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8: 275-283.
105. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nature reviews. Immunology* 2: 933-944.
106. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature immunology* 6: 1123-1132.

107. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature immunology* 6: 1133-1141.
108. Luger, D., P. B. Silver, J. Tang, D. Cua, Z. Chen, Y. Iwakura, E. P. Bowman, N. M. Sgambellone, C. C. Chan, and R. R. Caspi. 2008. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *The Journal of experimental medicine* 205: 799-810.
109. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunological reviews* 182: 18-32.
110. Abbas, A. K., C. Benoist, J. A. Bluestone, D. J. Campbell, S. Ghosh, S. Hori, S. Jiang, V. K. Kuchroo, D. Mathis, M. G. Roncarolo, A. Rudensky, S. Sakaguchi, E. M. Shevach, D. A. Vignali, and S. F. Ziegler. 2013. Regulatory T cells: recommendations to simplify the nomenclature. *Nature immunology* 14: 307-308.
111. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature immunology* 6: 345-352.
112. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329-341.
113. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-238.
114. Kimura, A., and T. Kishimoto. 2010. IL-6: regulator of Treg/Th17 balance. *European journal of immunology* 40: 1830-1835.
115. Zhou, L., J. E. Lopes, M. M. Chong, Ivanov, II, R. Min, G. D. Victora, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, S. F. Ziegler, and D. R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453: 236-240.
116. Ho, I. C., and L. H. Glimcher. 2002. Transcription: tantalizing times for T cells. *Cell* 109 Suppl: S109-120.
117. Jenner, R. G., M. J. Townsend, I. Jackson, K. Sun, R. D. Bouwman, R. A. Young, L. H. Glimcher, and G. M. Lord. 2009. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proceedings of the National Academy of Sciences of the United States of America* 106: 17876-17881.
118. Lord, G. M., R. M. Rao, H. Choe, B. M. Sullivan, A. H. Lichtman, F. W. Luscinskas, and L. H. Glimcher. 2005. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood* 106: 3432-3439.
119. Oestreich, K. J., A. C. Huang, and A. S. Weinmann. 2011. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *The Journal of experimental medicine* 208: 1001-1013.

120. Komine, O., K. Hayashi, W. Natsume, T. Watanabe, Y. Seki, N. Seki, R. Yagi, W. Sukzuki, H. Tamauchi, K. Hozumi, S. Habu, M. Kubo, and M. Satake. 2003. The Runx1 transcription factor inhibits the differentiation of naive CD4+ T cells into the Th2 lineage by repressing GATA3 expression. *The Journal of experimental medicine* 198: 51-61.
121. Schindler, C., and C. Plumlee. 2008. Inteferons pen the JAK-STAT pathway. *Seminars in cell & developmental biology* 19: 311-318.
122. Leng, C., M. Gries, J. Ziegler, A. Lokshin, P. Mascagni, S. Lentzsch, and M. Y. Mapara. 2006. Reduction of graft-versus-host disease by histone deacetylase inhibitor suberonylanilide hydroxamic acid is associated with modulation of inflammatory cytokine milieu and involves inhibition of STAT1. *Experimental hematology* 34: 776-787.
123. Ma, H., C. Lu, J. Ziegler, A. Liu, A. Sepulveda, H. Okada, S. Lentzsch, and M. Y. Mapara. 2011. Absence of Stat1 in donor CD4(+) T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice. *The Journal of clinical investigation* 121: 2554-2569.
124. Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld, and J. N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382: 171-174.
125. Thieu, V. T., Q. Yu, H. C. Chang, N. Yeh, E. T. Nguyen, S. Sehra, and M. H. Kaplan. 2008. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. *Immunity* 29: 679-690.
126. Nikolic, B., S. Lee, R. T. Bronson, M. J. Grusby, and M. Sykes. 2000. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. *The Journal of clinical investigation* 105: 1289-1298.
127. Gerondakis, S., R. Grumont, R. Gugasyan, L. Wong, I. Isomura, W. Ho, and A. Banerjee. 2006. Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models. *Oncogene* 25: 6781-6799.
128. Mullen, A. C., A. S. Hutchins, F. A. High, H. W. Lee, K. J. Sykes, L. A. Chodosh, and S. L. Reiner. 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nature immunology* 3: 652-658.
129. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 9: 765-775.
130. Yu, Y., D. Wang, K. Kaosaard, C. Liu, J. Fu, K. Haarberg, C. Anasetti, A. A. Beg, and X. Z. Yu. 2013. c-Rel is an essential transcription factor for the development of acute graft-versus-host disease in mice. *Eur J Immunol*.
131. Yang, X. O., B. P. Pappu, R. Nurieva, A. Akimzhanov, H. S. Kang, Y. Chung, L. Ma, B. Shah, A. D. Panopoulos, K. S. Schluns, S. S. Watowich, Q. Tian, A. M. Jetten, and C. Dong. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28: 29-39.
132. Fulton, L. M., M. J. Carlson, J. M. Coghill, L. E. Ott, M. L. West, A. Panoskaltsis-Mortari, D. R. Littman, B. R. Blazar, and J. S. Serody. 2012. Attenuation of acute graft-versus-host disease in the absence of the transcription factor RORgammat. *Journal of immunology (Baltimore, Md. : 1950)* 189: 1765-1772.

133. Chen, Z., A. Laurence, Y. Kanno, M. Pacher-Zavisin, B. M. Zhu, C. Tato, A. Yoshimura, L. Hennighausen, and J. J. O'Shea. 2006. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proceedings of the National Academy of Sciences of the United States of America* 103: 8137-8142.
134. Yang, X. O., A. D. Panopoulos, R. Nurieva, S. H. Chang, D. Wang, S. S. Watowich, and C. Dong. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *The Journal of biological chemistry* 282: 9358-9363.
135. Ma, H. H., J. Ziegler, C. Li, A. Sepulveda, A. Bedeir, J. Grandis, S. Lentzsch, and M. Y. Mapara. 2011. Sequential activation of inflammatory signaling pathways during graft-versus-host disease (GVHD): early role for STAT1 and STAT3. *Cellular immunology* 268: 37-46.
136. Lu, S. X., O. Alpdogan, J. Lin, R. Balderas, R. Campos-Gonzalez, X. Wang, G. J. Gao, D. Suh, C. King, M. Chow, O. M. Smith, V. M. Hubbard, J. L. Bautista, J. Cabrera-Perez, J. L. Zakrzewski, A. A. Kochman, A. Chow, G. Altan-Bonnet, and M. R. van den Brink. 2008. STAT-3 and ERK 1/2 phosphorylation are critical for T-cell alloactivation and graft-versus-host disease. *Blood* 112: 5254-5258.
137. Radojcic, V., M. A. Pletneva, H. R. Yen, S. Ivcevic, A. Panoskaltsis-Mortari, A. C. Gilliam, C. G. Drake, B. R. Blazar, and L. Luznik. 2010. STAT3 signaling in CD4+ T cells is critical for the pathogenesis of chronic sclerodermatous graft-versus-host disease in a murine model. *Journal of immunology (Baltimore, Md. : 1950)* 184: 764-774.
138. Veldhoen, M., K. Hirota, A. M. Westendorf, J. Buer, L. Dumoutier, J. C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453: 106-109.
139. Kimura, A., T. Naka, K. Nohara, Y. Fujii-Kuriyama, and T. Kishimoto. 2008. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proceedings of the National Academy of Sciences of the United States of America* 105: 9721-9726.
140. Rohlman, D., S. Punj, J. Pennington, S. Bradford, and N. I. Kerkvliet. 2013. Suppression of Acute Graft-Versus-Host Response by TCDD Is Independent of the CTLA-4-IFN-gamma-IDO pathway. *Toxicological sciences : an official journal of the Society of Toxicology*.
141. Ciofani, M., A. Madar, C. Galan, M. Sellars, K. Mace, F. Pauli, A. Agarwal, W. Huang, C. N. Parkurst, M. Muratet, K. M. Newberry, S. Meadows, A. Greenfield, Y. Yang, P. Jain, F. K. Kirigin, C. Birchmeier, E. F. Wagner, K. M. Murphy, R. M. Myers, R. Bonneau, and D. R. Littman. 2012. A validated regulatory network for Th17 cell specification. *Cell* 151: 289-303.
142. Brustle, A., S. Heink, M. Huber, C. Rosenplanter, C. Stadelmann, P. Yu, E. Arpaia, T. W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nature immunology* 8: 958-966.
143. Brunstein, C. G., J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, P. B. McGlave, B. R. Blazar, and J. E. Wagner. 2011. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117: 1061-1070.

144. Hippen, K. L., S. C. Merkel, D. K. Schirm, C. M. Sieben, D. Sumstad, D. M. Kadidlo, D. H. McKenna, J. S. Bromberg, B. L. Levine, J. L. Riley, C. H. June, P. Scheinberg, D. C. Douek, J. S. Miller, J. E. Wagner, and B. R. Blazar. 2011. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Science translational medicine* 3: 83ra41.
145. Semple, K., Y. Yu, D. Wang, C. Anasetti, and X. Z. Yu. 2011. Efficient and selective prevention of GVHD by antigen-specific induced Tregs via linked-suppression in mice. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 17: 309-318.
146. Veerapathran, A., J. Pidala, F. Beato, B. Betts, J. Kim, J. G. Turner, M. K. Hellerstein, X. Z. Yu, W. Janssen, and C. Anasetti. 2013. Human regulatory T cells against minor histocompatibility antigens: ex vivo expansion for prevention of graft-versus-host disease. *Blood*.
147. Pidala, J., and C. Anasetti. 2010. Can antigen-specific regulatory T cells protect against graft versus host disease and spare anti-malignancy alloresponse? *Haematologica* 95: 660-665.
148. Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annual review of immunology* 30: 531-564.
149. Blazar, B. R., W. J. Murphy, and M. Abedi. 2012. Advances in graft-versus-host disease biology and therapy. *Nature reviews. Immunology* 12: 443-458.
150. Miura, Y., C. J. Thoburn, E. C. Bright, M. L. Phelps, T. Shin, E. C. Matsui, W. H. Matsui, S. Arai, E. J. Fuchs, G. B. Vogelsang, R. J. Jones, and A. D. Hess. 2004. Association of Foxp3 regulatory gene expression with graft-versus-host disease. *Blood* 104: 2187-2193.
151. Hess, A. D. 2006. Modulation of graft-versus-host disease: role of regulatory T lymphocytes. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 12: 13-21.
152. Ferrara, J. L., and W. Krenger. 1998. Graft-versus-host disease: the influence of type 1 and type 2 T cell cytokines. *Transfusion medicine reviews* 12: 1-17.
153. Lu, Y., S. Sakamaki, H. Kuroda, T. Kusakabe, Y. Konuma, T. Akiyama, A. Fujimi, N. Takemoto, K. Nishiie, T. Matsunaga, Y. Hirayama, J. Kato, S. Kon, K. Kogawa, and Y. Niitsu. 2001. Prevention of lethal acute graft-versus-host disease in mice by oral administration of T helper 1 inhibitor, TAK-603. *Blood* 97: 1123-1130.
154. Brok, H. P., P. J. Heidt, P. H. van der Meide, C. Zurcher, and J. M. Vossen. 1993. Interferon-gamma prevents graft-versus-host disease after allogeneic bone marrow transplantation in mice. *Journal of immunology (Baltimore, Md. : 1950)* 151: 6451-6459.
155. Welniak, L. A., B. R. Blazar, M. R. Anver, R. H. Wiltrout, and W. J. Murphy. 2000. Opposing roles of interferon-gamma on CD4+ T cell-mediated graft-versus-host disease: effects of conditioning. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 6: 604-612.
156. Robb, R. J., and G. R. Hill. 2012. The interferon-dependent orchestration of innate and adaptive immunity after transplantation. *Blood* 119: 5351-5358.
157. Yang, Y. G., J. Qi, M. G. Wang, and M. Sykes. 2002. Donor-derived interferon gamma separates graft-versus-leukemia effects and graft-versus-host disease induced by donor CD8 T cells. *Blood* 99: 4207-4215.

158. Sun, K., H. H. Hsiao, M. Li, E. Ames, M. Bouchlaka, L. A. Welniak, T. Hagino, J. Jagdeo, C. C. Pai, M. Chen, B. R. Blazar, M. Abedi, and W. J. Murphy. 2012. IFN-gamma receptor-deficient donor T cells mediate protection from graft-versus-host disease and preserve graft-versus-tumor responses after allogeneic bone marrow transplantation. *Journal of immunology (Baltimore, Md. : 1950)* 189: 2033-2042.
159. Yi, T., Y. Chen, L. Wang, G. Du, D. Huang, D. Zhao, H. Johnston, J. Young, I. Todorov, D. T. Umetsu, L. Chen, Y. Iwakura, F. Kandeel, S. Forman, and D. Zeng. 2009. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood* 114: 3101-3112.
160. Mauermann, N., J. Burian, C. von Garnier, S. Dirnhofer, D. Germano, C. Schuett, M. Tamm, R. Bingisser, U. Eriksson, and L. Hunziker. 2008. Interferon-gamma regulates idiopathic pneumonia syndrome, a Th17+CD4+ T-cell-mediated graft-versus-host disease. *American journal of respiratory and critical care medicine* 178: 379-388.
161. Burman, A. C., T. Banovic, R. D. Kuns, A. D. Clouston, A. C. Stanley, E. S. Morris, V. Rowe, H. Bofinger, R. Skoczylas, N. Raffelt, O. Fahy, S. R. McColl, C. R. Engwerda, K. P. McDonald, and G. R. Hill. 2007. IFNgamma differentially controls the development of idiopathic pneumonia syndrome and GVHD of the gastrointestinal tract. *Blood* 110: 1064-1072.
162. Levine, J. E. 2011. Implications of TNF-alpha in the pathogenesis and management of GVHD. *International journal of hematology* 93: 571-577.
163. Cooke, K. R., G. R. Hill, J. M. Crawford, D. Bungard, Y. S. Brinson, J. Delmonte, Jr., and J. L. Ferrara. 1998. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *The Journal of clinical investigation* 102: 1882-1891.
164. Korngold, R., J. C. Marini, M. E. de Baca, G. F. Murphy, and J. Giles-Komar. 2003. Role of tumor necrosis factor-alpha in graft-versus-host disease and graft-versus-leukemia responses. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 9: 292-303.
165. Couriel, D., R. Saliba, K. Hicks, C. Ippoliti, M. de Lima, C. Hosing, I. Khouri, B. Andersson, J. Gajewski, M. Donato, P. Anderlini, D. P. Kontoyiannis, A. Cohen, T. Martin, S. Giralt, and R. Champlin. 2004. Tumor necrosis factor-alpha blockade for the treatment of acute GVHD. *Blood* 104: 649-654.
166. Atkins, M. B., M. T. Lotze, J. P. Dutcher, R. I. Fisher, G. Weiss, K. Margolin, J. Abrams, M. Sznol, D. Parkinson, M. Hawkins, C. Paradise, L. Kunkel, and S. A. Rosenberg. 1999. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 17: 2105-2116.
167. Sykes, M., M. W. Harty, G. L. Szot, and D. A. Pearson. 1994. Interleukin-2 inhibits graft-versus-host disease-promoting activity of CD4+ cells while preserving CD4- and CD8-mediated graft-versus-leukemia effects. *Blood* 83: 2560-2569.
168. Koreth, J., K. Matsuoka, H. T. Kim, S. M. McDonough, B. Bindra, E. P. Alyea, 3rd, P. Armand, C. Cutler, V. T. Ho, N. S. Treister, D. C. Bienfang, S. Prasad, D. Tzachanis, R. M. Joyce, D. E. Avigan, J. H. Antin, J. Ritz, and R. J. Soiffer. 2011. Interleukin-2 and regulatory T cells in graft-versus-host disease. *The New England journal of medicine* 365: 2055-2066.

169. Saadoun, D., M. Rosenzwajg, F. Joly, A. Six, F. Carrat, V. Thibault, D. Sene, P. Cacoub, and D. Klatzmann. 2011. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *The New England journal of medicine* 365: 2067-2077.
170. Shin, H. J., J. Baker, D. B. Leveson-Gower, A. T. Smith, E. I. Sega, and R. S. Negrin. 2011. Rapamycin and IL-2 reduce lethal acute graft-versus-host disease associated with increased expansion of donor type CD4+CD25+Foxp3+ regulatory T cells. *Blood* 118: 2342-2350.
171. Kappel, L. W., G. L. Goldberg, C. G. King, D. Y. Suh, O. M. Smith, C. Ligh, A. M. Holland, J. Grubin, N. M. Mark, C. Liu, Y. Iwakura, G. Heller, and M. R. van den Brink. 2009. IL-17 contributes to CD4-mediated graft-versus-host disease. *Blood* 113: 945-952.
172. Yi, T., D. Zhao, C. L. Lin, C. Zhang, Y. Chen, I. Todorov, T. LeBon, F. Kandeel, S. Forman, and D. Zeng. 2008. Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. *Blood* 112: 2101-2110.
173. Banovic, T., K. P. MacDonald, E. S. Morris, V. Rowe, R. Kuns, A. Don, J. Kelly, S. Ledbetter, A. D. Clouston, and G. R. Hill. 2005. TGF-beta in allogeneic stem cell transplantation: friend or foe? *Blood* 106: 2206-2214.
174. Coghill, J. M., S. Sarantopoulos, T. P. Moran, W. J. Murphy, B. R. Blazar, and J. S. Serody. 2011. Effector CD4+ T cells, the cytokines they generate, and GVHD: something old and something new. *Blood* 117: 3268-3276.
175. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, S. Schrader, S. Burkhead, M. Heipel, C. Brandt, J. L. Kuijper, J. Kramer, D. Conklin, S. R. Presnell, J. Berry, F. Shiota, S. Bort, K. Hambly, S. Mudri, C. Clegg, M. Moore, F. J. Grant, C. Lofton-Day, T. Gilbert, F. Rayond, A. Ching, L. Yao, D. Smith, P. Webster, T. Whitmore, M. Maurer, K. Kaushansky, R. D. Holly, and D. Foster. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408: 57-63.
176. Bucher, C., L. Koch, C. Vogtenhuber, E. Goren, M. Munger, A. Panoskaltsis-Mortari, P. Sivakumar, and B. R. Blazar. 2009. IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. *Blood* 114: 5375-5384.
177. Hanash, A. M., L. W. Kappel, N. L. Yim, R. A. Nejat, G. L. Goldberg, O. M. Smith, U. K. Rao, L. Dykstra, I. K. Na, A. M. Holland, J. A. Dudakov, C. Liu, G. F. Murphy, W. J. Leonard, G. Heller, and M. R. van den Brink. 2011. Abrogation of donor T-cell IL-21 signaling leads to tissue-specific modulation of immunity and separation of GVHD from GVL. *Blood* 118: 446-455.
178. Yang, L., D. E. Anderson, C. Baecher-Allan, W. D. Hastings, E. Bettelli, M. Oukka, V. K. Kuchroo, and D. A. Hafler. 2008. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* 454: 350-352.
179. Hippen, K. L., C. Bucher, D. K. Schirm, A. M. Bearl, T. Brender, K. A. Mink, K. S. Waggie, R. Peffault de Latour, A. Janin, J. M. Curtsinger, S. R. Dillon, J. S. Miller, G. Socie, and B. R. Blazar. 2012. Blocking IL-21 signaling ameliorates xenogeneic GVHD induced by human lymphocytes. *Blood* 119: 619-628.
180. Das, R., R. Komorowski, M. J. Hessner, H. Subramanian, C. S. Huettner, D. Cua, and W. R. Drobyski. 2010. Blockade of interleukin-23 signaling results in targeted protection of the colon and allows for separation of graft-versus-host and graft-versus-leukemia responses. *Blood* 115: 5249-5258.

181. Chen, X., R. Das, R. Komorowski, A. Beres, M. J. Hessner, M. Mihara, and W. R. Drobyski. 2009. Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood* 114: 891-900.
182. Tawara, I., M. Koyama, C. Liu, T. Toubai, D. Thomas, R. Evers, P. Chockley, E. Nieves, Y. Sun, K. P. Lowler, C. Malter, N. Nishimoto, G. R. Hill, and P. Reddy. 2011. Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clinical cancer research : an official journal of the American Association for Cancer Research* 17: 77-88.
183. New, J. Y., B. Li, W. P. Koh, H. K. Ng, S. Y. Tan, E. H. Yap, S. H. Chan, and H. Z. Hu. 2002. T cell infiltration and chemokine expression: relevance to the disease localization in murine graft-versus-host disease. *Bone marrow transplantation* 29: 979-986.
184. Mapara, M. Y., C. Leng, Y. M. Kim, R. Bronson, A. Lokshin, A. Luster, and M. Sykes. 2006. Expression of chemokines in GVHD target organs is influenced by conditioning and genetic factors and amplified by GVHR. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 12: 623-634.
185. Cavet, J., A. M. Dickinson, J. Norden, P. R. Taylor, G. H. Jackson, and P. G. Middleton. 2001. Interferon-gamma and interleukin-6 gene polymorphisms associate with graft-versus-host disease in HLA-matched sibling bone marrow transplantation. *Blood* 98: 1594-1600.
186. Ritchie, D., J. Seconi, C. Wood, J. Walton, and V. Watt. 2005. Prospective monitoring of tumor necrosis factor alpha and interferon gamma to predict the onset of acute and chronic graft-versus-host disease after allogeneic stem cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 11: 706-712.
187. Roy, J., B. R. Blazar, L. Ochs, and D. J. Weisdorf. 1995. The tissue expression of cytokines in human acute cutaneous graft-versus-host disease. *Transplantation* 60: 343-348.
188. Bonnotte, B., A. M. Burdiles, J. Chehimi, G. Carayol, C. Pardoux, P. Y. Dietrich, M. Kubin, J. Y. Blay, A. Caignard, A. Ibrahim, E. Robinet, M. Hayat, J. L. Pico, J. H. Bourhis, and S. Chouaib. 1996. Serum interleukin-12 levels in patients undergoing allogeneic or autologous bone marrow transplantation. *European cytokine network* 7: 389-394.
189. Tanaka, J., M. Imamura, M. Kasai, N. Masauzi, A. Matsuura, H. Ohizumi, K. Morii, Y. Kiyama, T. Naohara, M. Saitho, and et al. 1993. Cytokine gene expression in peripheral blood mononuclear cells during graft-versus-host disease after allogeneic bone marrow transplantation. *British journal of haematology* 85: 558-565.
190. Carayol, G., J. H. Bourhis, M. Guillard, J. Bosq, C. Pailler, L. Castagna, J. P. Vernant, J. L. Pico, M. Hayat, S. Chouaib, and A. Caignard. 1997. Quantitative analysis of T helper 1, T helper 2, and inflammatory cytokine expression in patients after allogeneic bone marrow transplantation: relationship with the occurrence of acute graft-versus-host disease. *Transplantation* 63: 1307-1313.
191. Zhao, X. Y., L. L. Xu, S. Y. Lu, and X. J. Huang. 2011. IL-17-producing T cells contribute to acute graft-versus-host disease in patients undergoing unmanipulated blood and marrow transplantation. *European journal of immunology* 41: 514-526.

192. Espinoza, J. L., A. Takami, K. Nakata, M. Onizuka, T. Kawase, H. Akiyama, K. Miyamura, Y. Morishima, T. Fukuda, Y. Kodera, and S. Nakao. 2011. A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation. *PloS one* 6: e26229.
193. Dander, E., A. Balduzzi, G. Zappa, G. Lucchini, P. Perseghin, V. Andre, E. Todisco, D. Rahal, M. Migliavacca, D. Longoni, G. Solinas, A. Villa, E. Berti, P. D. Mina, M. Parma, P. Allavena, E. Biagi, A. Rovelli, A. Biondi, and G. D'Amico. 2009. Interleukin-17-producing T-helper cells as new potential player mediating graft-versus-host disease in patients undergoing allogeneic stem-cell transplantation. *Transplantation* 88: 1261-1272.
194. Ratajczak, P., A. Janin, R. Peffault de Latour, C. Leboeuf, A. Desveaux, K. Keyvanfar, M. Robin, E. Clave, C. Douay, A. Quinquenel, C. Pichereau, P. Bertheau, J. Y. Mary, and G. Socie. 2010. Th17/Treg ratio in human graft-versus-host disease. *Blood* 116: 1165-1171.
195. Gergis, U., J. Arnason, R. Yantiss, T. Shore, U. Wissa, E. Feldman, and T. Woodworth. 2010. Effectiveness and safety of tocilizumab, an anti-interleukin-6 receptor monoclonal antibody, in a patient with refractory GI graft-versus-host disease. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 28: e602-604.
196. Drobyski, W. R., M. Pasquini, K. Kovatovic, J. Palmer, J. Douglas Rizzo, A. Saad, W. Saber, and P. Hari. 2011. Tocilizumab for the treatment of steroid refractory graft-versus-host disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 17: 1862-1868.
197. Betts, B. C., O. Abdel-Wahab, S. A. Curran, E. T. St Angelo, P. Koppikar, G. Heller, R. L. Levine, and J. W. Young. 2011. Janus kinase-2 inhibition induces durable tolerance to alloantigen by human dendritic cell-stimulated T cells yet preserves immunity to recall antigen. *Blood* 118: 5330-5339.
198. Pidala, J., L. Perez, F. Beato, and C. Anasetti. 2012. Ustekinumab demonstrates activity in glucocorticoid-refractory acute GVHD. *Bone marrow transplantation* 47: 747-748.
199. Williamson, E., P. Garside, J. A. Bradley, I. A. More, and A. M. Mowat. 1997. Neutralizing IL-12 during induction of murine acute graft-versus-host disease polarizes the cytokine profile toward a Th2-type alloimmune response and confers long term protection from disease. *Journal of immunology (Baltimore, Md. : 1950)* 159: 1208-1215.
200. Das, R., X. Chen, R. Komorowski, M. J. Hessner, and W. R. Drobyski. 2009. Interleukin-23 secretion by donor antigen-presenting cells is critical for organ-specific pathology in graft-versus-host disease. *Blood* 113: 2352-2362.
201. Johnston, L. J., J. Brown, J. A. Shizuru, K. E. Stockerl-Goldstein, M. J. Stuart, K. G. Blume, R. S. Negrin, and N. J. Chao. 2005. Rapamycin (sirolimus) for treatment of chronic graft-versus-host disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 11: 47-55.
202. Antin, J. H., H. T. Kim, C. Cutler, V. T. Ho, S. J. Lee, D. B. Miklos, E. P. Hochberg, C. J. Wu, E. P. Alyea, and R. J. Soiffer. 2003. Sirolimus, tacrolimus, and low-dose methotrexate for graft-versus-host disease prophylaxis in mismatched related donor or unrelated donor transplantation. *Blood* 102: 1601-1605.
203. Pidala, J., J. Kim, and C. Anasetti. 2009. Sirolimus as primary treatment of acute graft-versus-host disease following allogeneic hematopoietic cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 15: 881-885.

204. Peter, C., H. Waldmann, and S. P. Cobbold. 2010. mTOR signalling and metabolic regulation of T cell differentiation. *Current opinion in immunology* 22: 655-661.
205. Delgoffe, G. M., T. P. Kole, Y. Zheng, P. E. Zarek, K. L. Matthews, B. Xiao, P. F. Worley, S. C. Kozma, and J. D. Powell. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30: 832-844.
206. Lee, K., P. Gudapati, S. Dragovic, C. Spencer, S. Joyce, N. Killeen, M. A. Magnuson, and M. Boothby. 2010. Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity* 32: 743-753.
207. Battaglia, M., A. Stabilini, and M. G. Roncarolo. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105: 4743-4748.
208. Tresoldi, E., I. Dell'Albani, A. Stabilini, T. Jofra, A. Valle, N. Gagliani, A. Bondanza, M. G. Roncarolo, and M. Battaglia. 2011. Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells. *Haematologica* 96: 1357-1365.
209. 2012. Abstracts of the 38th Annual Meeting of the European Group for Blood and Marrow Transplantation. April 1-4, 2012. Geneva, Switzerland. *Bone marrow transplantation* 47 Suppl 1: S1-527.
210. Liao, J. K., and U. Laufs. 2005. Pleiotropic effects of statins. *Annual review of pharmacology and toxicology* 45: 89-118.
211. Bensinger, S. J., M. N. Bradley, S. B. Joseph, N. Zelcer, E. M. Janssen, M. A. Hausner, R. Shih, J. S. Parks, P. A. Edwards, B. D. Jamieson, and P. Tontonoz. 2008. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell* 134: 97-111.
212. Cui, G., X. Qin, L. Wu, Y. Zhang, X. Sheng, Q. Yu, H. Sheng, B. Xi, J. Z. Zhang, and Y. Q. Zang. 2011. Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation. *The Journal of clinical investigation* 121: 658-670.
213. Greenwood, J., L. Steinman, and S. S. Zamvil. 2006. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nature reviews. Immunology* 6: 358-370.
214. Wenger, N. K., S. J. Lewis, D. M. Herrington, V. Bittner, and F. K. Welty. 2007. Outcomes of using high- or low-dose atorvastatin in patients 65 years of age or older with stable coronary heart disease. *Annals of internal medicine* 147: 1-9.
215. Mausner-Fainberg, K., G. Luboshits, A. Mor, S. Maysel-Auslender, A. Rubinstein, G. Keren, and J. George. 2008. The effect of HMG-CoA reductase inhibitors on naturally occurring CD4+CD25+ T cells. *Atherosclerosis* 197: 829-839.
216. Hamadani, M., F. T. Awan, and S. M. Devine. 2008. The impact of HMG-CoA reductase inhibition on the incidence and severity of graft-versus-host disease in patients with acute leukemia undergoing allogeneic transplantation. *Blood* 111: 3901-3902.
217. Hamadani, M., M. D. Craig, L. F. Gibson, and S. C. Remick. 2011. The evolving role of statins in hematopoietic stem and progenitor cell transplantation. *American journal of blood research* 1: 57-64.
218. Reddy, P., and J. L. M. Ferrara. 2008. Mouse models of graft-versus-host disease. In *StemBook*. Harvard Stem Cell Institute, Cambridge (MA).
219. Teshima, T., R. Ordemann, P. Reddy, S. Gagin, C. Liu, K. R. Cooke, and J. L. Ferrara. 2002. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nature medicine* 8: 575-581.

220. Matte-Martone, C., J. Liu, D. Jain, J. McNiff, and W. D. Shlomchik. 2008. CD8+ but not CD4+ T cells require cognate interactions with target tissues to mediate GVHD across only minor H antigens, whereas both CD4+ and CD8+ T cells require direct leukemic contact to mediate GVL. *Blood* 111: 3884-3892.
221. Shlomchik, W. D., M. S. Couzens, C. B. Tang, J. McNiff, M. E. Robert, J. Liu, M. J. Shlomchik, and S. G. Emerson. 1999. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science (New York, N.Y.)* 285: 412-415.
222. Graubert, T. A., J. F. DiPersio, J. H. Russell, and T. J. Ley. 1997. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *The Journal of clinical investigation* 100: 904-911.
223. Maeda, Y., R. B. Levy, P. Reddy, C. Liu, S. G. Clouthier, T. Teshima, and J. L. Ferrara. 2005. Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. *Blood* 105: 2023-2027.
224. Hartmann, N., F. Leithauser, C. Albers, J. Duyster, P. Moller, K. M. Debatin, and G. Strauss. 2011. In vitro-established alloantigen-specific CD8+ CTLs mediate graft-versus-tumor activity in the absence of graft-versus-host disease. *Leukemia* 25: 848-855.
225. Flutter, B., N. Edwards, F. Fallah-Arani, S. Henderson, J. G. Chai, S. Sivakumaran, S. Ghorashian, C. L. Bennett, G. J. Freeman, M. Sykes, and R. Chakraverty. 2010. Nonhematopoietic antigen blocks memory programming of alloreactive CD8+ T cells and drives their eventual exhaustion in mouse models of bone marrow transplantation. *The Journal of clinical investigation* 120: 3855-3868.
226. Clark, W. R., C. M. Walsh, A. A. Glass, F. Hayashi, M. Matloubian, and R. Ahmed. 1995. Molecular pathways of CTL-mediated cytotoxicity. *Immunological reviews* 146: 33-44.
227. Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1995. Lymphocyte-mediated cytotoxicity in vitro and in vivo: mechanisms and significance. *Immunological reviews* 146: 95-115.
228. Barrett, J., and Y.-Z. Jiang. 2000. *Allogeneic Immunotherapy Malignant Diseases*. Marcel Dekker, New York, NY, USA.
229. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *Journal of immunological methods* 281: 65-78.
230. Aktas, E., U. C. Kucuksezer, S. Bilgic, G. Erten, and G. Deniz. 2009. Relationship between CD107a expression and cytotoxic activity. *Cellular immunology* 254: 149-154.
231. Marelli-Berg, F. M., and R. I. Lechler. 1999. Antigen presentation by parenchymal cells: a route to peripheral tolerance? *Immunological reviews* 172: 297-314.
232. Unanue, E. R. 2002. Perspective on antigen processing and presentation. *Immunological reviews* 185: 86-102.
233. Stenger, E. O., H. R. Turnquist, M. Y. Mapara, and A. W. Thomson. 2012. Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity. *Blood* 119: 5088-5103.
234. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annual review of immunology* 18: 767-811.

235. Rodriguez-Pinto, D. 2005. B cells as antigen presenting cells. *Cellular immunology* 238: 67-75.
236. Murray, P. J., and T. A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology* 11: 723-737.
237. Duraes, F. V., C. Thelemann, K. Sarter, H. Acha-Orbea, S. Hugues, and W. Reith. 2013. Role of major histocompatibility complex class II expression by non-hematopoietic cells in autoimmune and inflammatory disorders: facts and fiction. *Tissue antigens* 82: 1-15.
238. Chakraverty, R., and M. Sykes. 2007. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. *Blood* 110: 9-17.
239. Matte, C. C., J. Liu, J. Cormier, B. E. Anderson, I. Athanasiadis, D. Jain, J. McNiff, and W. D. Shlomchik. 2004. Donor APCs are required for maximal GVHD but not for GVL. *Nature medicine* 10: 987-992.
240. Markey, K. A., K. P. MacDonald, and G. R. Hill. 2014. The biology of graft-versus-host disease: experimental systems instructing clinical practice. *Blood* 124: 354-362.
241. Anderson, B. E., J. M. McNiff, D. Jain, B. R. Blazar, W. D. Shlomchik, and M. J. Shlomchik. 2005. Distinct roles for donor- and host-derived antigen-presenting cells and costimulatory molecules in murine chronic graft-versus-host disease: requirements depend on target organ. *Blood* 105: 2227-2234.
242. Zhang, Y., J. P. Louboutin, J. Zhu, A. J. Rivera, and S. G. Emerson. 2002. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. *The Journal of clinical investigation* 109: 1335-1344.
243. Duffner, U. A., Y. Maeda, K. R. Cooke, P. Reddy, R. Ordemann, C. Liu, J. L. Ferrara, and T. Teshima. 2004. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. *Journal of immunology (Baltimore, Md. : 1950)* 172: 7393-7398.
244. Mapara, M. Y., Y. M. Kim, S. P. Wang, R. Bronson, D. H. Sachs, and M. Sykes. 2002. Donor lymphocyte infusions mediate superior graft-versus-leukemia effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. *Blood* 100: 1903-1909.
245. Chan, G. W., G. Gorgun, K. B. Miller, and F. M. Foss. 2003. Persistence of host dendritic cells after transplantation is associated with graft-versus-host disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 9: 170-176.
246. Lenarsky, C. 1995. Immune recovery after bone marrow transplantation. *Current opinion in hematology* 2: 409-412.
247. Markey, K. A., T. Banovic, R. D. Kuns, S. D. Olver, A. L. Don, N. C. Raffelt, Y. A. Wilson, L. J. Raggatt, A. R. Pettit, J. S. Bromberg, G. R. Hill, and K. P. MacDonald. 2009. Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. *Blood* 113: 5644-5649.
248. Reddy, V., J. A. Iturraspe, A. C. Tzolas, H. U. Meier-Kriesche, J. Schold, and J. R. Wingard. 2004. Low dendritic cell count after allogeneic hematopoietic stem cell transplantation predicts relapse, death, and acute graft-versus-host disease. *Blood* 103: 4330-4335.

249. Koyama, M., R. D. Kuns, S. D. Olver, N. C. Raffelt, Y. A. Wilson, A. L. Don, K. E. Lineburg, M. Cheong, R. J. Robb, K. A. Markey, A. Varelias, B. Malissen, G. J. Hammerling, A. D. Clouston, C. R. Engwerda, P. Bhat, K. P. MacDonald, and G. R. Hill. 2012. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nature medicine* 18: 135-142.
250. Li, H., A. J. Demetris, J. McNiff, C. Matte-Martone, H. S. Tan, D. M. Rothstein, F. G. Lakkis, and W. D. Shlomchik. 2012. Profound depletion of host conventional dendritic cells, plasmacytoid dendritic cells, and B cells does not prevent graft-versus-host disease induction. *Journal of immunology (Baltimore, Md. : 1950)* 188: 3804-3811.
251. Wilson, J., H. Cullup, R. Lourie, Y. Sheng, A. Palkova, K. J. Radford, A. M. Dickinson, A. M. Rice, D. N. Hart, and D. J. Munster. 2009. Antibody to the dendritic cell surface activation antigen CD83 prevents acute graft-versus-host disease. *The Journal of experimental medicine* 206: 387-398.
252. Schultz, K. R., J. Paquet, S. Bader, and K. T. HayGlass. 1995. Requirement for B cells in T cell priming to minor histocompatibility antigens and development of graft-versus-host disease. *Bone marrow transplantation* 16: 289-295.
253. Kamble, R., M. Oholendt, and G. Carrum. 2006. Rituximab responsive refractory acute graft-versus-host disease. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 12: 1201-1202.
254. Ratanatharathorn, V., B. Logan, D. Wang, M. Horowitz, J. P. Uberti, O. Ringden, R. P. Gale, H. Khoury, M. Arora, S. Spellman, C. Cutler, J. Antin, M. Bornhauser, G. Hale, L. Verdonck, M. Cairo, V. Gupta, and S. Pavletic. 2009. Prior rituximab correlates with less acute graft-versus-host disease and better survival in B-cell lymphoma patients who received allogeneic peripheral blood stem cell transplantation. *British journal of haematology* 145: 816-824.
255. Christopeit, M., V. Schutte, S. Theurich, T. Weber, W. Grothe, and G. Behre. 2009. Rituximab reduces the incidence of acute graft-versus-host disease. *Blood* 113: 3130-3131.
256. Kharfan-Dabaja, M. A., and C. S. Cutler. 2011. Rituximab for prevention and treatment of graft-versus-host disease. *International journal of hematology* 93: 578-585.
257. Rowe, V., T. Banovic, K. P. MacDonald, R. Kuns, A. L. Don, E. S. Morris, A. C. Burman, H. M. Bofinger, A. D. Clouston, and G. R. Hill. 2006. Host B cells produce IL-10 following TBI and attenuate acute GVHD after allogeneic bone marrow transplantation. *Blood* 108: 2485-2492.
258. Chen, L. C., J. C. Delgado, P. E. Jensen, and X. Chen. 2009. Direct expansion of human allospecific FoxP3+CD4+ regulatory T cells with allogeneic B cells for therapeutic application. *Journal of immunology (Baltimore, Md. : 1950)* 183: 4094-4102.
259. Haniffa, M., F. Ginhoux, X. N. Wang, V. Bigley, M. Abel, I. Dimmick, S. Bullock, M. Grisotto, T. Booth, P. Taub, C. Hilkens, M. Merad, and M. Collin. 2009. Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation. *The Journal of experimental medicine* 206: 371-385.

260. Hashimoto, D., A. Chow, M. Greter, Y. Saenger, W. H. Kwan, M. Leboeuf, F. Ginhoux, J. C. Ochando, Y. Kunisaki, N. van Rooijen, C. Liu, T. Teshima, P. S. Heeger, E. R. Stanley, P. S. Frenette, and M. Merad. 2011. Pretransplant CSF-1 therapy expands recipient macrophages and ameliorates GVHD after allogeneic hematopoietic cell transplantation. *The Journal of experimental medicine* 208: 1069-1082.
261. Toubai, T., I. Tawara, Y. Sun, C. Liu, E. Nieves, R. Evers, T. Friedman, R. Korngold, and P. Reddy. 2012. Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radiosensitive host hematopoietic-derived antigen-presenting cells. *Blood* 119: 3844-3853.
262. Bland, P. 1988. MHC class II expression by the gut epithelium. *Immunology today* 9: 174-178.
263. Gray, D. H., N. Seach, T. Ueno, M. K. Milton, A. Liston, A. M. Lew, C. C. Goodnow, and R. L. Boyd. 2006. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* 108: 3777-3785.
264. Wang, X., H. Li, C. Matte-Martone, W. Cui, N. Li, H. S. Tan, D. Roopenian, and W. D. Shlomchik. 2011. Mechanisms of antigen presentation to T cells in murine graft-versus-host disease: cross-presentation and the appearance of cross-presentation. *Blood* 118: 6426-6437.
265. Bleakley, M., and S. R. Riddell. 2004. Molecules and mechanisms of the graft-versus-leukaemia effect. *Nature reviews. Cancer* 4: 371-380.
266. Reddy, P., Y. Maeda, C. Liu, O. I. Krijanovski, R. Korngold, and J. L. Ferrara. 2005. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nature medicine* 11: 1244-1249.
267. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21: 137-148.
268. Toubai, T., Y. Sun, G. Luker, J. Liu, K. E. Luker, I. Tawara, R. Evers, C. Liu, N. Mathewson, C. Malter, E. Nieves, S. Choi, K. M. Murphy, and P. Reddy. 2013. Host-derived CD8+ dendritic cells are required for induction of optimal graft-versus-tumor responses after experimental allogeneic bone marrow transplantation. *Blood* 121: 4231-4241.
269. Mackinnon, S., E. B. Papadopoulos, M. H. Carabasi, L. Reich, N. H. Collins, F. Boulad, H. Castro-Malaspina, B. H. Childs, A. P. Gillio, N. A. Kernan, and et al. 1995. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood* 86: 1261-1268.
270. Plantinga, M., C. de Haar, S. Nierkens, and J. J. Boelens. 2014. Dendritic Cell Therapy in an Allogeneic-Hematopoietic Cell Transplantation Setting: An Effective Strategy toward Better Disease Control? *Frontiers in immunology* 5: 218.
271. Buggins, A. G., G. J. Mufti, J. Salisbury, J. Codd, N. Westwood, M. Arno, K. Fishlock, A. Pagliuca, and S. Devereux. 2002. Peripheral blood but not tissue dendritic cells express CD52 and are depleted by treatment with alemtuzumab. *Blood* 100: 1715-1720.
272. Klangsinsirikul, P., G. I. Carter, J. L. Byrne, G. Hale, and N. H. Russell. 2002. Campath-1G causes rapid depletion of circulating host dendritic cells (DCs) before allogeneic transplantation but does not delay donor DC reconstitution. *Blood* 99: 2586-2591.

273. Schnitzler, M., J. Hasskarl, M. Egger, H. Bertz, and J. Finke. 2009. Successful treatment of severe acute intestinal graft-versus-host resistant to systemic and topical steroids with alemtuzumab. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 15: 910-918.
274. Schub, N., A. Gunther, A. Schrauder, A. Claviez, C. Ehlert, M. Gramatzki, and R. Repp. 2011. Therapy of steroid-refractory acute GVHD with CD52 antibody alemtuzumab is effective. *Bone marrow transplantation* 46: 143-147.
275. Jardine, L., A. Publicover, V. Bigley, G. Hale, K. Pearce, A. Dickinson, G. Jackson, and M. Collin. 2015. A comparative study of reduced dose alemtuzumab in matched unrelated donor and related donor reduced intensity transplants. *British journal of haematology*.
276. Collin, M. P., D. Munster, G. Clark, X. N. Wang, A. M. Dickinson, and D. N. Hart. 2005. In vitro depletion of tissue-derived dendritic cells by CMRF-44 antibody and alemtuzumab: implications for the control of Graft-versus-host disease. *Transplantation* 79: 722-725.
277. Chen, X., C. H. Chang, R. Stein, and D. M. Goldenberg. 2012. The humanized anti-HLA-DR moAb, IMMU-114, depletes APCs and reduces alloreactive T cells: implications for preventing GVHD. *Bone marrow transplantation* 47: 967-980.
278. Reddy, P., Y. Sun, T. Toubai, R. Duran-Struuck, S. G. Clouthier, E. Weisiger, Y. Maeda, I. Tawara, O. Krijanovski, E. Gatza, C. Liu, C. Malter, P. Mascagni, C. A. Dinarello, and J. L. Ferrara. 2008. Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. *The Journal of clinical investigation* 118: 2562-2573.
279. Tao, Y., W. Zhang, Y. Fang, D. Yang, L. Wang, H. Zhou, and J. Wang. 2011. Bortezomib attenuates acute graft-vs.-host disease through interfering with host immature dendritic cells. *Experimental hematology* 39: 710-720.
280. Sun, K., L. A. Welniak, A. Panoskaltsis-Mortari, M. J. O'Shaughnessy, H. Liu, I. Barao, W. Riordan, R. Sitcheran, C. Wysocki, J. S. Serody, B. R. Blazar, T. J. Sayers, and W. J. Murphy. 2004. Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. *Proceedings of the National Academy of Sciences of the United States of America* 101: 8120-8125.
281. Koreth, J., K. E. Stevenson, H. T. Kim, M. Garcia, V. T. Ho, P. Armand, C. Cutler, J. Ritz, J. H. Antin, R. J. Soiffer, and E. P. Alyea, 3rd. 2009. Bortezomib, tacrolimus, and methotrexate for prophylaxis of graft-versus-host disease after reduced-intensity conditioning allogeneic stem cell transplantation from HLA-mismatched unrelated donors. *Blood* 114: 3956-3959.
282. Sato, K., N. Yamashita, N. Yamashita, M. Baba, and T. Matsuyama. 2003. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity* 18: 367-379.
283. Sato, K., N. Yamashita, M. Baba, and T. Matsuyama. 2003. Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* 101: 3581-3589.
284. Fujita, S., Y. Sato, K. Sato, K. Eizumi, T. Fukaya, M. Kubo, N. Yamashita, and K. Sato. 2007. Regulatory dendritic cells protect against cutaneous chronic graft-versus-host disease mediated through CD4+CD25+Foxp3+ regulatory T cells. *Blood* 110: 3793-3803.

285. Sato, K., T. Nakaoka, N. Yamashita, H. Yagita, H. Kawasaki, C. Morimoto, M. Baba, and T. Matsuyama. 2005. TRAIL-transduced dendritic cells protect mice from acute graft-versus-host disease and leukemia relapse. *Journal of immunology (Baltimore, Md. : 1950)* 174: 4025-4033.
286. Fu, J., J. Heinrichs, and X. Z. Yu. 2014. Helper T-Cell Differentiation in Graft-Versus-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation. *Archivum immunologiae et therapiae experimentalis*.
287. Murphy, W. J., L. A. Welniak, D. D. Taub, R. H. Wiltrout, P. A. Taylor, D. A. Vallera, M. Kopf, H. Young, D. L. Longo, and B. R. Blazar. 1998. Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. *The Journal of clinical investigation* 102: 1742-1748.
288. Yang, Y. G., B. R. Dey, J. J. Sergio, D. A. Pearson, and M. Sykes. 1998. Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. *The Journal of clinical investigation* 102: 2126-2135.
289. Yu, Y., D. Wang, K. Kaosaard, C. Liu, J. Fu, K. Haarberg, C. Anasetti, A. A. Beg, and X. Z. Yu. 2013. c-Rel is an essential transcription factor for the development of acute graft-versus-host disease in mice. *European journal of immunology* 43: 2327-2337.
290. Cooke, K. R., L. Kobzik, T. R. Martin, J. Brewer, J. Delmonte, Jr., J. M. Crawford, and J. L. Ferrara. 1996. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood* 88: 3230-3239.
291. Liang, Y., C. Liu, J. Y. Djeu, B. Zhong, T. Peters, K. Scharffetter-Kochanek, C. Anasetti, and X. Z. Yu. 2008. Beta2 integrins separate graft-versus-host disease and graft-versus-leukemia effects. *Blood* 111: 954-962.
292. Valenzuela, J. O., C. Iclozan, M. S. Hossain, M. Prlic, E. Hopewell, C. C. Bronk, J. Wang, E. Celis, R. W. Engelman, B. R. Blazar, M. J. Bevan, E. K. Waller, X. Z. Yu, and A. A. Beg. 2009. PKCtheta is required for alloreactivity and GVHD but not for immune responses toward leukemia and infection in mice. *The Journal of clinical investigation* 119: 3774-3786.
293. Yu, X. Z., M. H. Albert, and C. Anasetti. 2006. Alloantigen affinity and CD4 help determine severity of graft-versus-host disease mediated by CD8 donor T cells. *Journal of immunology (Baltimore, Md. : 1950)* 176: 3383-3390.
294. Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic acids research* 31: e15.
295. Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics (Oxford, England)* 4: 249-264.
296. Zhu, J., H. Yamane, and W. E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28: 445-489.
297. Gowdy, K. M., J. L. Nugent, T. Martinu, E. Potts, L. D. Snyder, W. M. Foster, and S. M. Palmer. 2012. Protective role of T-bet and Th1 cytokines in pulmonary graft-versus-host disease and peribronchiolar fibrosis. *American journal of respiratory cell and molecular biology* 46: 249-256.
298. Goulmy, E. 2006. Minor histocompatibility antigens: from transplantation problems to therapy of cancer. *Human immunology* 67: 433-438.

299. Choi, J., E. D. Ziga, J. Ritchey, L. Collins, J. L. Prior, M. L. Cooper, D. Piwnica-Worms, and J. F. DiPersio. 2012. IFNgammaR signaling mediates alloreactive T-cell trafficking and GVHD. *Blood* 120: 4093-4103.
300. Guo, S., D. Cobb, and R. B. Smeltz. 2009. T-bet inhibits the in vivo differentiation of parasite-specific CD4+ Th17 cells in a T cell-intrinsic manner. *Journal of immunology (Baltimore, Md. : 1950)* 182: 6179-6186.
301. Shono, Y., S. Ueha, Y. Wang, J. Abe, M. Kurachi, Y. Matsuno, T. Sugiyama, T. Nagasawa, M. Imamura, and K. Matsushima. 2010. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood* 115: 5401-5411.
302. Lee, Y., A. Awasthi, N. Yosef, F. J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D. A. Hafler, R. A. Sobel, A. Regev, and V. K. Kuchroo. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nature immunology* 13: 991-999.
303. Brustle, A., D. Brenner, C. B. Knobbe, P. A. Lang, C. Virtanen, B. M. Hershenfield, C. Reardon, S. M. Lacher, J. Ruland, P. S. Ohashi, and T. W. Mak. 2012. The NF-kappaB regulator MALT1 determines the encephalitogenic potential of Th17 cells. *The Journal of clinical investigation* 122: 4698-4709.
304. McGeachy, M. J. 2011. GM-CSF: the secret weapon in the T(H)17 arsenal. *Nature immunology* 12: 521-522.
305. Orsini, E., R. Bellucci, E. P. Alyea, R. Schlossman, C. Canning, S. McLaughlin, P. Ghia, K. C. Anderson, and J. Ritz. 2003. Expansion of tumor-specific CD8+ T cell clones in patients with relapsed myeloma after donor lymphocyte infusion. *Cancer research* 63: 2561-2568.
306. Chung, A. S., X. Wu, G. Zhuang, H. Ngu, I. Kasman, J. Zhang, J. M. Vernes, Z. Jiang, Y. G. Meng, F. V. Peale, W. Ouyang, and N. Ferrara. 2013. An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. *Nature medicine* 19: 1114-1123.
307. Wang, L., T. Yi, M. Kortylewski, D. M. Pardoll, D. Zeng, and H. Yu. 2009. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *The Journal of experimental medicine* 206: 1457-1464.
308. Gu, F. M., Q. L. Li, Q. Gao, J. H. Jiang, K. Zhu, X. Y. Huang, J. F. Pan, J. Yan, J. H. Hu, Z. Wang, Z. Dai, J. Fan, and J. Zhou. 2011. IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma. *Molecular cancer* 10: 150.
309. Tsang, J. Y., J. G. Chai, and R. Lechler. 2003. Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion? *Blood* 101: 2704-2710.
310. Davis, D. M. 2007. Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. *Nature reviews. Immunology* 7: 238-243.
311. Hepworth, M. R., L. A. Monticelli, T. C. Fung, C. G. Ziegler, S. Grunberg, R. Sinha, A. R. Mantegazza, H. L. Ma, A. Crawford, J. M. Angelosanto, E. J. Wherry, P. A. Koni, F. D. Bushman, C. O. Elson, G. Eberl, D. Artis, and G. F. Sonnenberg. 2013. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature* 498: 113-117.

312. Wysocki, C. A., A. Panoskaltsis-Mortari, B. R. Blazar, and J. S. Serody. 2005. Leukocyte migration and graft-versus-host disease. *Blood* 105: 4191-4199.
313. Castor, M. G., V. Pinho, and M. M. Teixeira. 2012. The role of chemokines in mediating graft versus host disease: opportunities for novel therapeutics. *Frontiers in pharmacology* 3: 23.
314. He, S., Q. Cao, Y. Qiu, J. Mi, J. Z. Zhang, M. Jin, H. Ge, S. G. Emerson, Y. Zhang, and Y. Zhang. 2008. A new approach to the blocking of alloreactive T cell-mediated graft-versus-host disease by in vivo administration of anti-CXCR3 neutralizing antibody. *Journal of immunology (Baltimore, Md. : 1950)* 181: 7581-7592.
315. Reshef, R., S. M. Luger, E. O. Hexner, A. W. Loren, N. V. Frey, S. D. Nasta, S. C. Goldstein, E. A. Stadtmauer, J. Smith, S. Bailey, R. Mick, D. F. Heitjan, S. G. Emerson, J. A. Hoxie, R. H. Vonderheide, and D. L. Porter. 2012. Blockade of lymphocyte chemotaxis in visceral graft-versus-host disease. *The New England journal of medicine* 367: 135-145.
316. Rollins, B. J. 1997. Chemokines. *Blood* 90: 909-928.
317. Kawamura, H., H. Yagita, T. Nisizawa, N. Izumi, C. Miyaji, R. E. Vance, D. H. Raulet, K. Okumura, and T. Abo. 2005. Amelioration of acute graft-versus-host disease by NKG2A engagement on donor T cells. *European journal of immunology* 35: 2358-2366.
318. Turman, M. A., T. Yabe, C. McSherry, F. H. Bach, and J. P. Houchins. 1993. Characterization of a novel gene (NKG7) on human chromosome 19 that is expressed in natural killer cells and T cells. *Human immunology* 36: 34-40.
319. Sale, G. E., P. Anderson, M. Browne, and D. Myerson. 1992. Evidence of cytotoxic T-cell destruction of epidermal cells in human graft-vs-host disease. Immunohistology with monoclonal antibody TIA-1. *Archives of pathology & laboratory medicine* 116: 622-625.
320. Ferber, I. A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C. G. Fathman. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *Journal of immunology (Baltimore, Md. : 1950)* 156: 5-7.
321. Willenborg, D. O., S. Fordham, C. C. Bernard, W. B. Cowden, and I. A. Ramshaw. 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *Journal of immunology (Baltimore, Md. : 1950)* 157: 3223-3227.
322. Markiewicz, M. A., L. N. Carayannopoulos, O. V. Naidenko, K. Matsui, W. R. Burack, E. L. Wise, D. H. Fremont, P. M. Allen, W. M. Yokoyama, M. Colonna, and A. S. Shaw. 2005. Costimulation through NKG2D enhances murine CD8+ CTL function: similarities and differences between NKG2D and CD28 costimulation. *Journal of immunology (Baltimore, Md. : 1950)* 175: 2825-2833.
323. Karimi, M., T. M. Leichner, A. Satake, D. Raulet, and T. Kambayashi. 2013. Transient NKG2D Blockade Attenuates Graft-Versus-Host Disease While Preserving Graft-Versus-Leukemia Effects. *Blood* 122: 3242-3242.
324. Shono, Y., A. Z. Tuckett, S. Ouk, H. C. Liou, G. Altan-Bonnet, J. J. Tsai, J. E. Oyler, O. M. Smith, M. L. West, N. V. Singer, E. Doubrovina, D. Pankov, C. V. Undhad, G. F. Murphy, C. Lezcano, C. Liu, R. J. O'Reilly, M. R. van den Brink, and J. L. Zakrzewski. 2014. A small-molecule c-Rel inhibitor reduces alloactivation of T cells without compromising antitumor activity. *Cancer discovery* 4: 578-591.

325. Skepner, J., R. Ramesh, M. Trocha, D. Schmidt, E. Baloglu, M. Lobera, T. Carlson, J. Hill, L. A. Orband-Miller, A. Barnes, M. Boudjelal, M. Sundrud, S. Ghosh, and J. Yang. 2014. Pharmacologic inhibition of RORgammat regulates Th17 signature gene expression and suppresses cutaneous inflammation in vivo. *Journal of immunology (Baltimore, Md. : 1950)* 192: 2564-2575.
326. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-252.
327. Buono, C., C. J. Binder, G. Stavrakis, J. L. Witztum, L. H. Glimcher, and A. H. Lichtman. 2005. T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proceedings of the National Academy of Sciences of the United States of America* 102: 1596-1601.
328. Rubtsova, K., A. V. Rubtsov, L. F. van Dyk, J. W. Kappler, and P. Marrack. 2013. T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance. *Proceedings of the National Academy of Sciences of the United States of America* 110: E3216-3224.
329. Garrett, W. S., G. M. Lord, S. Punit, G. Lugo-Villarino, S. K. Mazmanian, S. Ito, J. N. Glickman, and L. H. Glimcher. 2007. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell* 131: 33-45.
330. Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke, B. Hilliard, and Y. Chen. 2000. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. *The Journal of experimental medicine* 191: 1095-1104.
331. Anderson, B. E., P. A. Taylor, J. M. McNiff, D. Jain, A. J. Demetris, A. Panoskaltsis-Mortari, A. Ager, B. R. Blazar, W. D. Shlomchik, and M. J. Shlomchik. 2008. Effects of donor T-cell trafficking and priming site on graft-versus-host disease induction by naive and memory phenotype CD4 T cells. *Blood* 111: 5242-5251.
332. Beilhack, A., S. Schulz, J. Baker, G. F. Beilhack, C. B. Wieland, E. I. Herman, E. M. Baker, Y. A. Cao, C. H. Contag, and R. S. Negrin. 2005. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood* 106: 1113-1122.
333. Panoskaltsis-Mortari, A., A. Price, J. R. Hermanson, E. Taras, C. Lees, J. S. Serody, and B. R. Blazar. 2004. In vivo imaging of graft-versus-host-disease in mice. *Blood* 103: 3590-3598.
334. Leibnitz, R. R., P. E. Lipsky, and D. L. Thiele. 1994. Reactivity of hybridomas derived from T cells activated in vivo during graft-versus-host disease. *Journal of immunology (Baltimore, Md. : 1950)* 153: 4959-4968.
335. Cohen, J. L., O. Boyer, B. Salomon, R. Onclercq, F. Charlotte, S. Bruel, G. Boisserie, and D. Klatzmann. 1997. Prevention of graft-versus-host disease in mice using a suicide gene expressed in T lymphocytes. *Blood* 89: 4636-4645.
336. Alpdogan, O., S. J. Muriglan, J. M. Eng, L. M. Willis, A. S. Greenberg, B. J. Kappel, and M. R. van den Brink. 2003. IL-7 enhances peripheral T cell reconstitution after allogeneic hematopoietic stem cell transplantation. *The Journal of clinical investigation* 112: 1095-1107.
337. Suchin, E. J., P. B. Langmuir, E. Palmer, M. H. Sayegh, A. D. Wells, and L. A. Turka. 2001. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *Journal of immunology (Baltimore, Md. : 1950)* 166: 973-981.

338. Brochu, S., B. Rioux-Masse, J. Roy, D. C. Roy, and C. Perreault. 1999. Massive activation-induced cell death of alloreactive T cells with apoptosis of bystander postthymic T cells prevents immune reconstitution in mice with graft-versus-host disease. *Blood* 94: 390-400.
339. Hebib, N. C., O. Deas, M. Rouleau, A. Durrbach, B. Charpentier, F. Beaujean, J. P. Vernant, and A. Senik. 1999. Peripheral blood T cells generated after allogeneic bone marrow transplantation: lower levels of bcl-2 protein and enhanced sensitivity to spontaneous and CD95-mediated apoptosis in vitro. Abrogation of the apoptotic phenotype coincides with the recovery of normal naive/primed T-cell profiles. *Blood* 94: 1803-1813.
340. Lin, M. T., L. H. Tseng, H. Frangoul, T. Gooley, J. Pei, A. Barsoukov, Y. Akatsuka, and J. A. Hansen. 2000. Increased apoptosis of peripheral blood T cells following allogeneic hematopoietic cell transplantation. *Blood* 95: 3832-3839.
341. Russell, J. H. 1995. Activation-induced death of mature T cells in the regulation of immune responses. *Current opinion in immunology* 7: 382-388.
342. Guicciardi, M. E., and G. J. Gores. 2009. Life and death by death receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23: 1625-1637.
343. Gross, A., J. M. McDonnell, and S. J. Korsmeyer. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes & development* 13: 1899-1911.
344. Csencsits, K. L., and D. K. Bishop. 2003. Contrasting alloreactive CD4+ and CD8+ T cells: there's more to it than MHC restriction. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 3: 107-115.
345. Yin, H., R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin, and D. G. Anderson. 2014. Non-viral vectors for gene-based therapy. *Nature reviews. Genetics* 15: 541-555.
346. Gocke, A. R., P. D. Cravens, L. H. Ben, R. Z. Hussain, S. C. Northrop, M. K. Racke, and A. E. Lovett-Racke. 2007. T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. *Journal of immunology (Baltimore, Md. : 1950)* 178: 1341-1348.
347. He, S., Q. Tong, D. K. Bishop, and Y. Zhang. 2013. Histone methyltransferase and histone methylation in inflammatory T-cell responses. *Immunotherapy* 5: 989-1004.
348. Li, Q., J. Zou, M. Wang, X. Ding, I. Chepelev, X. Zhou, W. Zhao, G. Wei, J. Cui, K. Zhao, H. Y. Wang, and R. F. Wang. 2014. Critical role of histone demethylase Jmjd3 in the regulation of CD4+ T-cell differentiation. *Nature communications* 5: 5780.
349. Kruidenier, L., C. W. Chung, Z. Cheng, J. Liddle, K. Che, G. Joberty, M. Bantscheff, C. Bountra, A. Bridges, H. Diallo, D. Eberhard, S. Hutchinson, E. Jones, R. Katso, M. Leveridge, P. K. Mander, J. Mosley, C. Ramirez-Molina, P. Rowland, C. J. Schofield, R. J. Sheppard, J. E. Smith, C. Swales, R. Tanner, P. Thomas, A. Tumber, G. Drewes, U. Oppermann, D. J. Patel, K. Lee, and D. M. Wilson. 2012. A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature* 488: 404-408.

350. Kruidenier, L., C. W. Chung, Z. Cheng, J. Liddle, K. Che, G. Joberty, M. Bantscheff, C. Bountra, A. Bridges, H. Diallo, D. Eberhard, S. Hutchinson, E. Jones, R. Katso, M. Leveridge, P. K. Mander, J. Mosley, C. Ramirez-Molina, P. Rowland, C. J. Schofield, R. J. Sheppard, J. E. Smith, C. Swales, R. Tanner, P. Thomas, A. Tumber, G. Drewes, U. Oppermann, D. J. Patel, K. Lee, and D. M. Wilson. 2014. Kruidenier et al. reply. *Nature* 514: E2.
351. Francis, N. J., M. Rowlands, P. Workman, K. Jones, and W. Aherne. 2012. Small-molecule inhibitors of the protein methyltransferase SET7/9 identified in a high-throughput screen. *Journal of biomolecular screening* 17: 1102-1109.
352. Sun, Y., I. Tawara, M. Zhao, Z. S. Qin, T. Toubai, N. Mathewson, H. Tamaki, E. Nieves, A. M. Chinnaiyan, and P. Reddy. 2013. Allogeneic T cell responses are regulated by a specific miRNA-mRNA network. *The Journal of clinical investigation* 123: 4739-4754.
353. Atarod, S., and A. M. Dickinson. 2013. MicroRNAs: The Missing Link in the Biology of Graft-Versus-Host Disease? *Frontiers in immunology* 4: 420.
354. Wu, Y., D. Bastian, J. L. Heinrichs, J. Fu, H. Nguyen, Q.-J. Li, C. Xia, and X.-Z. Yu. 2014. *Microrna-17-92 Cluster: Novel Target for Controlling Gvhd While Preserving GVL Effect*.
355. Ranganathan, P., C. E. Heaphy, S. Costinean, N. Stauffer, C. Na, M. Hamadani, R. Santhanam, C. Mao, P. A. Taylor, S. Sandhu, G. He, A. Shana'ah, G. J. Nuovo, A. Lagana, L. Cascione, S. Obad, O. Broom, S. Kauppinen, J. C. Byrd, M. Caligiuri, D. Perrotti, G. A. Hadley, G. Marcucci, S. M. Devine, B. R. Blazar, C. M. Croce, and R. Garzon. 2012. Regulation of acute graft-versus-host disease by microRNA-155. *Blood* 119: 4786-4797.
356. Wang, L., M. Romero, P. Ratajczak, C. Leboeuf, S. Belhadj, R. Peffault de Latour, W. L. Zhao, G. Socie, and A. Janin. 2013. Increased apoptosis is linked to severe acute GVHD in patients with Fanconi anemia. *Bone marrow transplantation* 48: 849-853.
357. Leonhardt, F., S. Grundmann, M. Behe, F. Bluhm, R. A. Dumont, F. Braun, M. Fani, K. Riesner, G. Prinz, A. K. Hechinger, U. V. Gerlach, H. Dierbach, O. Penack, A. Schmitt-Graff, J. Finke, W. A. Weber, and R. Zeiser. 2013. Inflammatory neovascularization during graft-versus-host disease is regulated by alphav integrin and miR-100. *Blood* 121: 3307-3318.
358. Oravecz-Wilson, K., T. Saunders, Y. Wang, T. Toubai, N. Mathewson, C. Liu, G. Hou, E. Cummings, C. Ross, and P. Reddy. 2013. *Atypical E2F Dependent Dysregulation Of Cell Cycling By Microrna-142 Regulates T-Cell Responses and Experimental Graft-Versus-Host Disease*.
359. Tong, Q., S. He, F. Xie, K. Mochizuki, Y. Liu, I. Mochizuki, L. Meng, H. Sun, Y. Zhang, Y. Guo, E. Hexner, and Y. Zhang. 2014. Ezh2 regulates transcriptional and posttranslational expression of T-bet and promotes Th1 cell responses mediating aplastic anemia in mice. *Journal of immunology (Baltimore, Md. : 1950)* 192: 5012-5022.
360. He, S., F. Xie, Y. Liu, Q. Tong, K. Mochizuki, P. E. Lapinski, R. S. Mani, P. Reddy, I. Mochizuki, A. M. Chinnaiyan, S. Mineishi, P. D. King, and Y. Zhang. 2013. The histone methyltransferase Ezh2 is a crucial epigenetic regulator of allogeneic T-cell responses mediating graft-versus-host disease. *Blood* 122: 4119-4128.

361. Chen, A., S. M. Lee, B. Gao, S. Shannon, Z. Zhu, and D. Fang. 2011. c-Abl-mediated tyrosine phosphorylation of the T-bet DNA-binding domain regulates CD4+ T-cell differentiation and allergic lung inflammation. *Molecular and cellular biology* 31: 3445-3456.
362. Zerr, P., A. Distler, K. Palumbo-Zerr, M. Tomcik, S. Vollath, C. Dees, F. Egberts, I. Tinazzi, F. Del Galdo, O. Distler, G. Schett, B. M. Spriewald, and J. H. Distler. 2012. Combined inhibition of c-Abl and PDGF receptors for prevention and treatment of murine sclerodermatous chronic graft-versus-host disease. *The American journal of pathology* 181: 1672-1680.

Appendices

Appendix A

SPRINGER LICENSE
TERMS AND CONDITIONS

Feb 17, 2015

This is a License Agreement between Jianing Fu ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3571710228475
License date	Feb 17, 2015
Licensed content publisher	Springer
Licensed content publication	Archivum Immunologiae et Therapiae Experimentalis
Licensed content title	Helper T-Cell Differentiation in Graft-Versus-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation
Licensed content author	Jianing Fu
Licensed content date	Jan 1, 2014
Volume number	62
Issue number	4
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	5
Author of this Springer article	Yes and you are the sole author of the new work
Order reference number	None
Title of your thesis / dissertation	Targeting T-bet for Prevention of Graft-Versus-Host Disease and Leukemia Relapse after Allogeneic Hematopoietic Stem Cell Transplantation
Expected completion date	Mar 2015
Estimated size(pages)	140

Appendix B

Copyright Transfer

All manuscripts are considered to be the property of AAI from the time of submission. Should AAI not publish the paper, AAI releases its rights therein at the time the manuscript is returned to the corresponding author.

Manuscripts published in *The Journal of Immunology* become the sole property of, with all rights in copyright reserved to, The American Association of Immunologists, Inc. (AAI).

The corresponding author, on behalf of all authors, signs a copyright transfer form. Authors of articles written as part of their official duties as employees of the U.S. government are exempt from this requirement for transfer of copyright.

Authors retain the following permissions as long as proper attribution (Originally published in *The Journal of Immunology*. Author(s). Year. Title. J. Immunol. Vol: pp-pp. Copyright © [year] The American Association of Immunologists, Inc.) is included:

1. If mandated by the funder of the research, authors may deposit the accepted, uncopyedited version of the manuscript into PubMed Central, with an embargo period (time at which the manuscript is available to the public) of 12 months after publication. This right applies solely to deposition into PubMed Central; the authors agree not to deposit the manuscript to any other repository (except a thesis repository if required - see item 3), agency, or entity. Authors whose funder mandates a PubMed Central embargo of 6 months have permission to make the manuscript available at 6 months after publication. This right applies only to articles under a funder mandate to submit to PubMed Central (for research funded by NIH, this means articles accepted for publication on or after April 7, 2008).
2. To reuse original figures or tables in the author's own work. This permission extends to the author only, not to the institution. The author may not permit others to reproduce their figures or tables in works not written by the author.
3. To include the final, published version of the article in a thesis and/or dissertation in print. If required by the degree-conferring institution, an electronic version of the final, published version may be deposited into a thesis repository as long as a link to the article on *The Journal of Immunology* website is included.
4. To reprint the article in print collections composed solely of the author's own writings. Permission must be obtained from AAI to reprint the article in any publication that includes the work of others.
5. To post a copy of the accepted, uncopyedited version on the author's personal website with a notice that "This is the accepted, uncopyedited version of the manuscript. The definitive version was published in *The Journal of Immunology* Vol: pp-pp. DOI." A hyperlink to the published article on *The Journal of Immunology* website must be included; the published PDF may not be displayed. For posting on any other website, including any social media, corporate or government (other than PubMed Central according to the terms in item 1 above) website, permission must be requested from AAI. POSTING TO INSTITUTIONAL REPOSITORIES IS NOT PERMITTED.
6. To present the work orally in part or in its entirety.
7. If the work was prepared as a work-for-hire, the author's employer may print out PDFs or make photocopies for internal use only. Distribution of the article by the author's employer in other formats or for any other purpose requires permission from AAI.

Authors reusing their own material as described in items 1-7 do not need to contact AAI for permission, except where noted. For all other reuse, the authors should contact AAI.

Appendix C

American Society of Hematology LICENSE
TERMS AND CONDITIONS

Mar 17, 2015

This is a License Agreement between Jianing Fu ("You") and American Society of Hematology ("American Society of Hematology") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Society of Hematology, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3580990113432
License date	Mar 02, 2015
Licensed content publisher	American Society of Hematology
Licensed content title	Blood : journal of the American Society of Hematology
Licensed content date	Jan 1, 1946
Type of Use	Thesis/Dissertation
Requestor type	Author of requested content
Format	Electronic
Portion	abstract
Title or numeric reference of the portion(s)	Chapter 3 3.1 Abstract
Title of the article or chapter the portion is from	Chapter 3: T-bet Promotes Acute Graft-versus-Host Disease through Hematopoietic Antigen Presenting Cells in Mice
Editor of portion(s)	N/A
Author of portion(s)	N/A
Volume of serial or monograph.	N/A
Page range of the portion	70-71
Publication date of portion	04/11/2016
Rights for	Main product
Duration of use	Current edition and up to 5 years
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no

Appendix D

Institutional Animal Care and Use Committee

Medical University of South Carolina
171 Ashley Avenue - Charleston, SC - 29425
DHHS Assurance #A3428-01
AAALAC Full Accreditation: 11/5/1987

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

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

Initial Approval Date: February 28, 2013

All Species Approved:
MICE

==

Statement of Investigator:

The Principal Investigator assures the following:

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

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

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

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

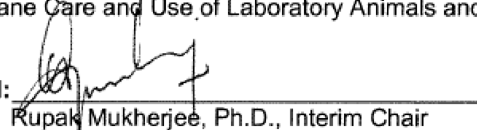
5. Personnel have been adequately trained for the procedures which they will be performing.

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

==

Statement of the IACUC:

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

Signature of Approval: 

Rupak Mukherjee, Ph.D., Interim Chair

About the author

Jianing Fu was born in Jilin City, Jilin Province, China, and graduated from Jiangcheng High School in Jilin City in 2004. Jianing went to Peking University, Beijing, China, and earned a bachelor of science in Pharmaceutical Sciences in 2008, and a master of science in Chemical Biology in 2010. During the fall of 2010, Jianing enrolled in the Cancer Biology PhD Program at H. Lee Moffitt Cancer Center and University of South Florida (USF) in Tampa, Florida, USA. Jianing worked for four years in the laboratory of Dr. Xue-Zhong Yu, including two years in Tampa, and two years curricular practical training in Charleston, Medical University of South Carolina. Her project is to determine the mechanism of targeting T-bet to prevent graft-versus-host disease after allogeneic bone marrow transplantation. During her tenure in Dr. Yu's lab, Jianing was first author on an original research article and a review paper, and first author of another research manuscript under review. Jianing attended the American Society of Hematology (ASH) meeting in 2012 and 2014, and the American Association of Immunologists (AAI) meeting in 2013, where she gave three oral presentations about her research work. Jianing also received two Abstract Achievement Awards from ASH, and two Travel Awards and one Community Service Award from Cancer Biology PhD Program at USF.

During her PhD work, Jianing took classes towards a certificate in Clinical Investigation from USF. Upon graduation, Jianing has accepted a postdoctoral position in the Columbia Center of Translational Immunology (CCTI) at the Columbia University in New York.