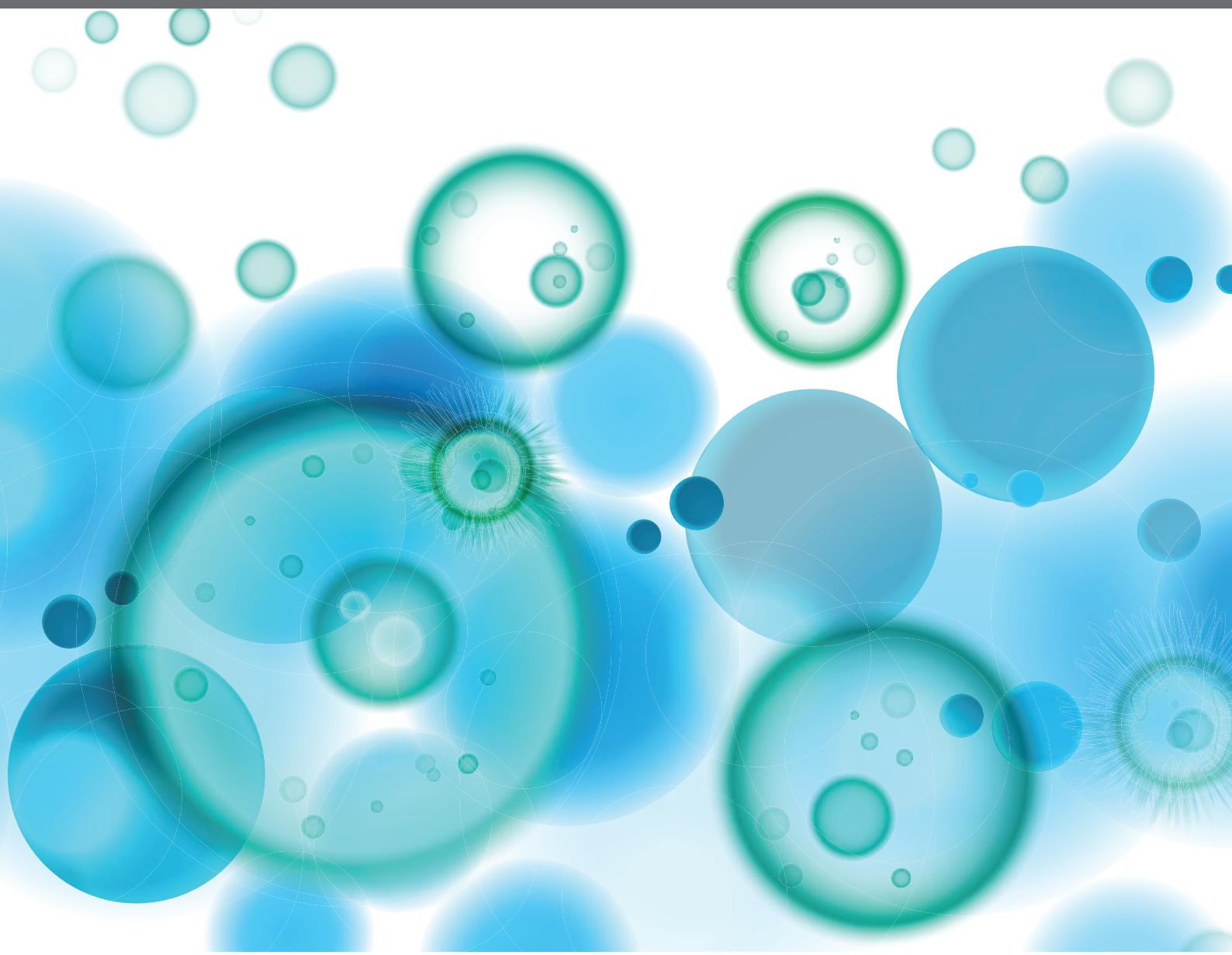
PATHOGENESIS AND THERAPY OF GRAFT-VERSUS-HOST DISEASE

EDITED BY: Brian C. Betts and Xue-Zhong Yu
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-174-2
DOI 10.3389/978-2-88963-174-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

PATHOGENESIS AND THERAPY OF GRAFT-VERSUS-HOST DISEASE

Topic Editors:
Brian C. Betts, University of Minnesota Twin Cities, United States
Xue-Zhong Yu, Medical University of South Carolina, United States

Citation: Betts, B. C., Yu, X.-Z., eds. (2019). Pathogenesis and Therapy of Graft-versus-Host Disease. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88963-174-2

Table of Contents

frontiers in Immunology

EDITORIAL
published: 31 July 2019
doi: 10.3389/fimmu.2019.01797

Editorial: Pathogenesis and Therapy of Graft-versus-Host Disease

Brian C. Betts[1]* *and Xue-Zhong Yu*[2]*

[1] Department of Medicine, University of Minnesota Twin Cities, St. Paul, MN, United States, [2] Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, United States

Keywords: All-HCT, GvHD, GvL, T cells, DCs, co-stimulation, metabolism

Editorial on the Research Topic

Pathogenesis and Therapy of Graft-versus-Host Disease

The therapeutic potential of allogeneic hematopoietic cell transplantation (allo-HCT) for the treatment of malignant diseases relies on the graft-vs.-leukemia (GVL) or graft- vs.-tumor (GVT) responses to eradicate residual tumor cells through immunologic mechanisms. Graft- vs.-host disease (GVHD) is a major cause of transplant-related morbidity and mortality following allo-HCT (allo-HCT). GVHD is clinically described in two forms: acute (aGVHD) and chronic (cGVHD). aGVHD is primarily induced by T cells commonly characterized by a type I T-cell response; whereas cGVHD is induced by both T and B cells, similar in nature to that of autoimmune disorders. Additionally, late acute GVHD, defined as occurring beyond 3 months post-transplant, is associated with high lethality. Despite advances in patient care and pharmacologic prophylaxis strategies, the incidence of GVHD, particularly cGVHD, has not greatly declined over time. In fact, effective treatment options are very limited beyond steroids. Currently, the only FDA-approved agents for steroid-refractory acute and chronic GVHD are ruxolitinib and ibrutinib, respectively. Even with these new agents, GVHD mortality and its impact on quality of life remains a major clinical challenge. Therefore, it is urgently required to further understand GVHD pathogenesis and identify novel therapeutic targets for the prevention and treatment of this devastating disease as a major complication of allo-HCT.

Since allo-reactive donor T cells are central to aGVHD pathophysiology, research has focused on donor T-cell activation, metabolism, co-stimulatory/co-inhibitory signals, differentiation, memory, and migration. Regulation of T-cell allo-responses via protein kinases, metabolites, non-coding RNAs, and other post-transcriptional pathways has also gained substantial attention in recent years. Beyond donor T cells, antigen-presenting cells (APCs) especially dendritic cells (DCs) and other lymphoid cells including natural killer (NK), NKT and innate cells (ILCs) also contribute to aGVHD pathogenesis. In addition, microbiota, tissue injury/repair, and thymopoiesis are also critically involved in aGVHD pathogenesis. The pathophysiology of cGVHD is characterized by fibrosis with inflammation resulting in organ dysfunction. Immunological mechanisms of cGVHD involve (i) aberrant conventional T and B cell activation, differentiation and interactions; and (ii) decreased production and development of regulatory T cells (Tregs). Therefore, cGVHD research is now moving toward a better understanding of the roles of B-cell signaling, activation, germinal center formation, and plasma cell differentiation; as well as the roles of T-cell signaling, activation, and differentiation into T helper and regulatory subsets. In this Research Topic, we brought together six outstanding original research and 10 state-of-the-art Review articles on the pathogenesis and therapy of GVHD that cover the following sub-topics:

OPEN ACCESS

Edited and reviewed by:
Antoine Toubert,
Paris Diderot University, France

***Correspondence:**
Brian C. Betts
bett0121@umn.edu
Xue-Zhong Yu
yux@musc.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *10 July 2019*
Accepted: *16 July 2019*
Published: *31 July 2019*

Citation:
Betts BC and Yu X-Z (2019) Editorial: Pathogenesis and Therapy of Graft-versus-Host Disease. Front. Immunol. 10:1797. doi: 10.3389/fimmu.2019.01797

IMMUNE CELLS

Significant progress has been made in defining the dichotomous role of DCs in the development of GVHD. Host-derived DCs are important to elicit allogeneic T cell responses, whereas certain donor-types of DCs derived from newly engrafted hematopoietic stem/progenitor cells (HSPCs) can amplify this GVH reaction. In contrast, some DCs also play non-redundant roles in mediating immune tolerance. They induce apoptotic deletion of host-reactive donor T cells while promoting expansion and function of Tregs. Yu et al. focused on the opposing side of the immunologic synapse, and describe how DCs mediate T cell allo-sensitization or immune tolerance after allo-HCT. In an original research paper, the Betts Lab identified a new approach to prevent GVHD that impairs monocyte-derived DC alloactivation of T cells, yet preserves GVL effect Betts et al. They demonstrated that Inhibition of XBP-1 splicing reduces migration of human monocyte-derived DCs, allo-stimulatory potency, and curtails their ability to produce IL-1β, TGFβ, and p40 cytokines, suppressing Th1 and Th17 cell priming without interfering with Treg function or GVL effects by CTL and NK cells.

Original research from the Copsel et al. Lab demonstrates that BET inhibition prevents GVHD, particularly by supporting the expansion of highly potent Tregs. Chen and Mayne describe the limited ability of allospecific $CD44^{high}$ central memory T cells (Tmem) to exert clinical GVHD, owing to functional exhaustion Huang et al. In addition, Shao et al. characterize how ILCs influence GVHD after allo-HCT. Altogether, these primary and review papers delineate the contributions of DCs, Tmems, Tregs, and ILCs in GVHD pathogenesis.

CYTOKINE NETWORKS

With regard to inflammatory cytokine networks in GVHD pathophysiology, Piper and Drobyski explore the role of STAT3-dependent inflammatory cytokines in acute GVHD of the gut. They also highlight novel translational approaches to target such cytokines to improve outcomes after allo-HCT. The report from Bastian et al. focuses on the unique anti- and pro-inflammatory properties of the IL-12 cytokine family, and how they impact GVHD onset and severity. Their article features data from pre-clinical studies and early phase clinical trials that identify p40 cytokines and receptor signaling elements, as pathogenic, and thus, candidates for therapeutic intervention. Further, primary research from Yoshihara et al. identifies how TNFα opposes stem cell engraftment. Their work suggests cytokine blockade with etanercept could enhance engraftment and possibly prevent GVHD after allo-HCT. Though a separate clinical entity from GVHD, Senyuk et al. demonstrate that post-transplant hemophagocytic lymphohistiocytosis is similarly driven by alloreactive T cells and inflammatory macrophages.

CO-STIMULATION AND CO-INHIBITION

Kumar et al. characterize how co-stimulation or co-inhibitory molecules influence donor T cell allo-activation, including common co-stimulatory molecules such as CD28, ICOS, CD40, CD30, CD27, OX40, and 4-1BB and common negative regulators such as CTLA-4, PD-1, TIM-3, and LAG-3. They discuss how these co-stimulatory and co-inhibitory pathways are involved in T-cell function and contribute substantially in GVHD pathogenesis. They urge that further intensive exploration of these pathways is needed before these potential therapeutic targets could become new clinical options to control GVHD without causing severe side effects. Cassady et al. focus on how PD-L1 interactions with PD-1 and CD80 that differentially regulate auto- and allo-immunity. They discuss how these interactions can separate GVL activity from GVHD in preclinical animal models and highlight the recent clinical application and challenges of PD-L1/PD-1 blockade after HCT for augmenting GVL activity. Original research from the Zhang et al. Lab characterizes the role of ICOS in chronic GVHD pathogenesis as well as its critical influence on Treg biology. They demonstrate that ICOS promotes T- and B-cell activation and differentiation, which can promote cGVHD development; whereas ICOS is also critical for the survival and homeostasis of iTregs, which can suppress cGVHD. Hence, ICOS balances the development of cGVHD and could offer a druggable target to improve clinical outcomes after allo-HCT.

METABOLISM

Metabolism is an attractive therapeutic target to optimize cancer immunotherapy and GVHD prevention. T-cell subsets are poised to distinct metabolic pathways that can determine their function and differentiation. Because distinct T-cell subsets mediate GVH vs. GVL response, the dominant metabolic properties of these distinct subsets might serve as new therapeutic targets that can be exploited to prevent GVHD without compromising GVL activity. Tijaro-Ovalle et al. highlight the metabolic features of malignant hematopoietic cells and discuss the metabolic features that guide the function of T cells and APCs during processes involved in GVH and GVL responses. They also provide rationale for potential therapeutic interventions by targeting metabolic pathways that guide the differentiation and function of these immune cells in the context of allo-HCT. Emerging clinical and pre-clinical evidence indicates that certain micronutrients may participate in regulating GVHD risk after allo-HCT. Dietary micronutrients contribute significantly to modulating various immune responses including cell metabolisms and may influence the susceptibility to autoimmune and inflammatory diseases. Chen and Mayne summarize recent advances in our understanding with respect to the potential role of micronutrients in the pathogenesis of acute and chronic GVHD, focusing on vitamins A and D. They reveal the therapeutic benefits of vitamins A and D in controlling alloreactive T cells.

THE GVL RESPONSE

Separating pathogenic GVHD from beneficial GVL is an area of substantial interest and research among the field. In a

comprehensive review, Chang et al. summarize the biology of GVH and GVL responses in pre-clinical models and discuss potential novel therapeutic strategies to reduce the relapse rate after allo-HCT. They also review the approaches, including optimal donor selection, conditioning regimens, donor lymphocyte infusion, BCR/ABL-specific CTL, and chimeric antigen receptor-modified T cells, which have been successfully used in the clinic to enhance and preserve the GVL without aggravating GVHD. CTL plays a critical role in mediating the GVL effect. Du and Cao detail how cytotoxic pathways, particularly Fas/Fas ligand, perforin/granzyme, and cytokines, in T cells differentially contribute to GVHD vs. GVL effect.

The collection of articles in "Pathogenesis and Therapy of Graft-vs.-Host Disease" clearly provides an in depth review of our current understanding of GVHD pathophysiology. Moreover, contributions to this collection also present innovative strategies to prevent acute and chronic GVHD, preserve GVL, and support tolerizing Tregs or DCs after allo-HCT.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by W81XWH-15-RTR-IDA (to BB) and R01 HL133823 (to BB); and R01 CA169116 (to X-ZY), R21 CA192202 (to X-ZY), R01 HL 140953 (to X-ZY), R21 AI136531 (to X-ZY), R01 HL137373 (to X-ZY), and R01 AI118305 (to X-ZY).

ACKNOWLEDGMENTS

We want to sincerely thank all of the contributing authors to this collection of articles. We also want to thank BB and X-ZY lab members who contribute to the original research work and to the review article.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 06 June 2019
doi: 10.3389/fimmu.2019.01233

An Essential Role of Innate Lymphoid Cells in the Pathophysiology of Graft-vs.-Host Disease

Liang Shao[1], Shan Pan[2], Qiu-ping Zhang[2], Muhammad Jamal[2], Lu-hua Chen[3], Qian Yin[2], Ying-jie Wu[2], Jie Xiong[2], Rui-jing Xiao[2], Yok-lam Kwong[4], Fu-ling Zhou[1] and Albert K. W. Lie[4]**

[1] Department of Hematology, Zhongnan Hospital of Wuhan University, Wuhan, China, [2] Department of Immunology, School of Basic Medical Sciences, Wuhan University, Wuhan, China, [3] Department of Medicine, Li Ka Shing Faculty of Medicine, Faculty of Social Sciences, The University of Hong Kong, Hong Kong, China, [4] Division of Hematology & BMT Center, Queen Mary Hospital, Hong Kong, China

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina,
United States

Reviewed by:
Alan M. Hanash,
Memorial Sloan Kettering Cancer Center, United States
Xuefang Cao,
University of Maryland, Baltimore,
United States

***Correspondence:**
Fu-ling Zhou
zhoufuling@163.com
Albert K. W. Lie
akwlie@hku.hk

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: 02 September 2018
Accepted: 15 May 2019
Published: 06 June 2019

Citation:
Shao L, Pan S, Zhang Q, Jamal M, Chen L, Yin Q, Wu Y, Xiong J, Xiao R, Kwong Y, Zhou F and Lie AKW (2019) An Essential Role of Innate Lymphoid Cells in the Pathophysiology of Graft-vs.-Host Disease. Front. Immunol. 10:1233. doi: 10.3389/fimmu.2019.01233

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) is the only curative treatment for multiple hematologic malignancies and non-malignant hematological diseases. However, graft-vs.-host disease (GVHD), one of the main complications after allo-HSCT, remains the major reason for morbidity and non-relapse mortality. Emerging evidence has demonstrated that innate lymphoid cells (ILCs) play a non-redundant role in the pathophysiology of GVHD. In this review, we will summarize previously published data regarding the role of ILCs in the pathogenesis of GVHD.

Keywords: innate lymphoid cells, graft-vs.-host disease, NK cells, T cells, hematopoietic stem cell transplantation, ILCreg

INTRODUCTION

Definition of ILCs

Innate lymphoid cells (ILCs) encompass natural killer cells (NK) and ILC1, ILC2, and ILC3 cells (1–3). In contrast to T cells, these cells lack rearranged antigen receptors (1–3). It has been demonstrated that ILCs develop in the fetal liver and adult bone marrow, whereas mature ILCs are mainly enriched in the GI tract, lungs, liver, and skin (1–3). NK cells, which account for ∼15% of human peripheral blood (PB) lymphocytes, exert cytolytic effects, and secrete IFN-γ, granzyme B, and perforin. In mouse, NK cells are characterized by the expression of natural killer cell p46-related protein (NKp46; also known as NCR1) receptor, and expressing transcription factors T-bet and Eomes (4–6) (**Figure 1**, **Table 1**). In humans, there are two main subsets of NK cells: $CD3^{-}CD56^{bright}CD16^{-}$ and $CD3^{-}CD56^{dim}$ $CD16^{+}$ cells (4–6) (**Table 2**). ILCs exhibit a cytokine repertoire that mirrors that of T helper cells. For instance, similar to Th1 cells, ILC1 cells can respond to IL-12 and IL-15 and subsequently secrete effector cytokines, such as IFN-γ and TNF-α (4–6). However, unlike NK cells, ILC1 cells do not display cytolytic effects (15). Murine ILC1 cells express Nkp46, NK1.1, T-bet, and CD200r1, but without expression of Eomes (16).

In humans, $CD127^{+}CD161^{+}CD34^{-}$ c-Kit^{-} T-bet^{+} $Eomes^{-}IFN\text{-}\gamma^{+}$ILC1 cells are enriched in the tonsils (15–17). Interestingly, $Lin^{-}CD127^{+}CD161^{+}CD117^{-}NKp44^{-}CRTH2^{-}$ ILC1 cells have been found in the PBMCs of healthy individuals and atopic dermatitis (AD) patients (15–18).

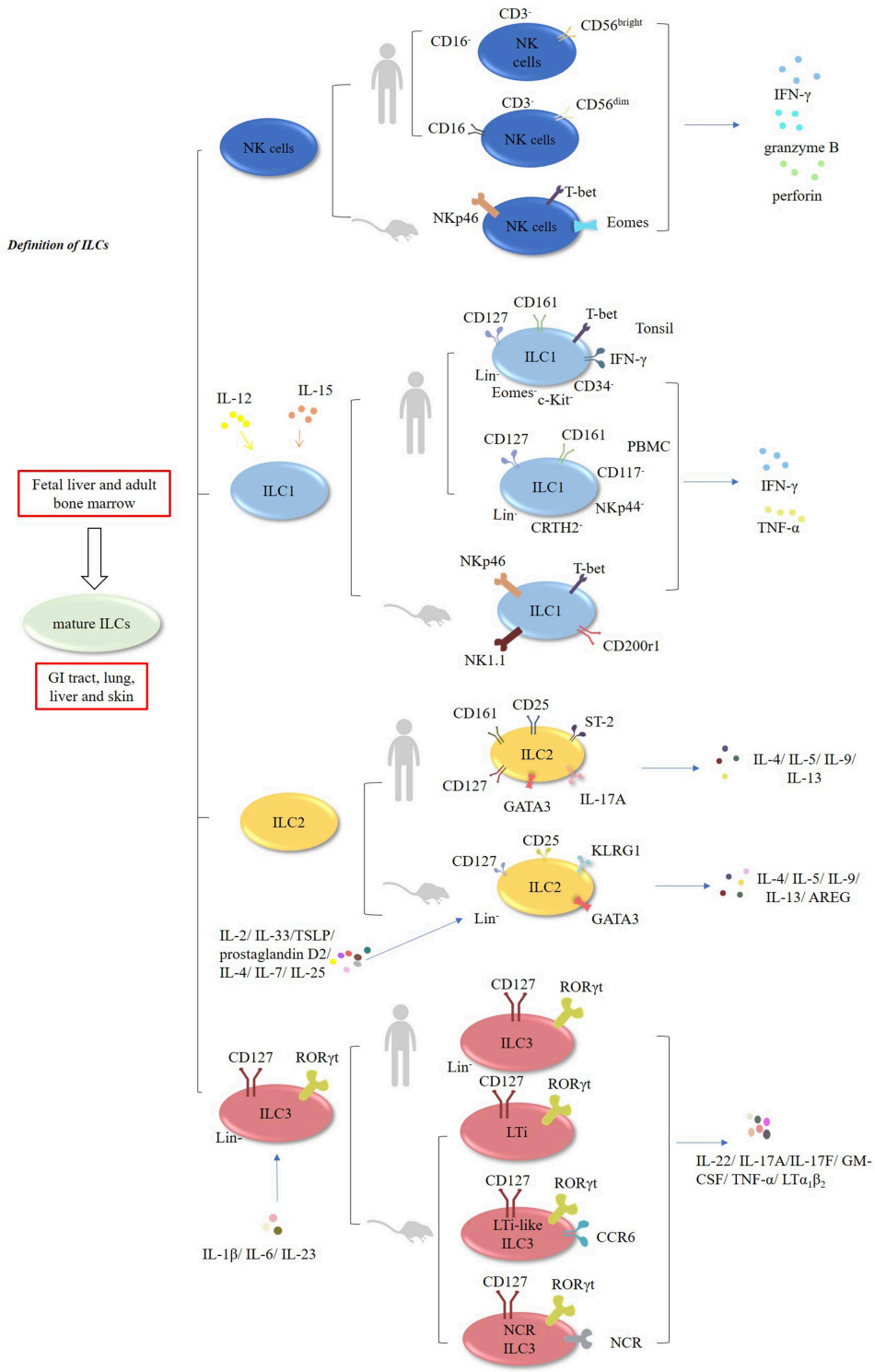


FIGURE 1 | Characteristic of ILCs. ILCs encompass NK, ILC1, ILC2, and ILC3 cells. Murine and human NK cells can secrete IFN-γ, granzyme B, and perforin. In humans, NK cells have two main subsets: $CD3^{-}CD56^{bright}CD16^{-}$ and $CD3^{-}CD56^{dim}CD16^{+}$ cells. ILC1 cells can respond to IL-12 and IL-15, and subsequently produce IFN-γ and TNF-α. In humans, $CD127^{+}CD161^{+}$ $CD34^{-}$ c-Kit^{-}T-bet^{+} $Eomes^{-}$ IFN-γ^{+} ILC1 cells are enriched in the tonsils. Additionally, $Lin^{-}CD127^{+}CD161^{+}$ $CD117^{-}$ $NKp44^{-}CRTH2^{-}$ ILC1 cells have been found in the human PBMCs. In mice, ILC2 cells are $Lin^{-}CD127^{+}CD25^{+}$ $KLRG1^{+}$ $GATA3^{high}$ cells which are responsive to IL-2, IL-4, IL-7, IL-25, IL-33, TSLP, and prostaglandin D2, and subsequently produce multiple effector cytokines. In humans, ILC2 cells express GATA3, CD127, CD161, CD25, ST-2, IL-17A, and CRTH2. Both murine and human ILC3 cells are $Lin^{-}CD127^{+}ROR\gamma t^{+}$. They are responsive to IL-1β, IL-6, and IL-23, and produce IL-22, IL-17A, IL-17F, GM-CSF, TNF-α, and $LT\alpha_1\beta_2$.

TABLE 1 | Phenotype of murine ILCs.

Marker	Mouse							
	NK		ILC1		ILC2		ILC3	
CD3	–	(7)	–	(7)	–	(7)	–	(7)
CD4	–	(8)	–	(9)	–	(9)	±	(7, 9)
CD19	–	(7)	–	(7)	–	(7)	–	(7)
CD25	±	(10)	±	(10)	+	(7, 10)	±	(10)
CD45	+	(10)	+	(7, 10)	+	(7, 10)	+	(7, 10, 11)
CD49a	±	(7, 10)	+	(7, 10)	ND	–	ND	–
CD69	±	(10)	+	(10)	ND	–	ND	–
CD90	±	(10)	+	(10)	+	(10)	+	(10)
CD94	±	(10)	ND	–	±	(10)	ND	–
CD103	±	(10)	–	(10)	ND	–	ND	–
CD117	–	(10)	±	(10)	±	(10)	+	(10)
CD122	+	(10)	+	(10)	+	(10)	–	(10)
CD127	±	(10)	±	(9, 10)	+	(9, 10)	+	(7, 9, 10)
CD160	±	(10)	+	(10)	ND	–	ND	–
CD294	–	(10)	ND	–	+	(10)	ND	–
NKp46	+	(7, 10)	+	(7, 10)	–	(10)	±	(7, 10, 11)
NK1.1	+	(7, 10)	+	(7, 10)	–	(10)	±	(10)
NKG2D	+	(10)	ND	–	–	(10)	±	(10)

ND, not determined.
+ positive; – negative; ± sometimes positive.

TABLE 2 | Phenotype of human ILCs.

Marker	Human							
	NK		ILC1		ILC2		ILC3	
CD1a	–	(5, 12)	–	(5, 12)	–	(5, 12)	–	(5, 12)
CD3	–	(12)	–	(5, 12)	–	(12)	–	(12)
CD4	–	(13)	±	(14)	–	(13)	±	(15)
CD7	+	ND	+	(9)	+	(9)	+	(9)
CD11c	–	(5, 12)	–	(5, 12)	–	(5, 12)	–	(5, 12)
CD14	–	(5, 12)	–	(5, 12)	–	(5, 12)	–	(5, 12)
CD16	±	(10, 15)	–	(10)	–	(10)	–	(10)
CD19	–	(5, 12)	–	(5, 12)	–	(5, 12)	–	(5, 12)
CD25	±	(10)	+	(10)	+	(10, 14, 15)	±	(10)
CD34	–	(5, 12)	–	(5, 12)	–	(5, 12)	–	(5, 12)
CD45	+	(5, 10)	+	(10)	+	(10)	+	(5, 10)
CD49a	±	(15)	±	(15)	ND	–	ND	–
CD56	+	(10, 15)	–	(5, 10)	–	(9, 10)	±	(9, 10)
CD69	±	(10)	±	(10)	ND	–	+	(5)
CD94	±	(5, 10)	–	(5, 10, 12)	–	(5, 10, 12)	–	(5, 10, 12)
CD103	±	(15)	±	(9, 15)	–	(9)	–	(9)
CD117	±	(10)	–	(10)	±	(10)	+	(10)
CD123	–	(5, 12)	–	(5, 12)	–	(5, 12)	–	(5, 12)
CD127	±	(10)	±	(10)	+	(5, 10, 13)	+	(5, 10)
CD294	–	(12)	–	(12)	+	(5, 12)	–	(12)
TCRαβ	–	(12)	–	(12)	–	(12)	–	(12)
TCRγδ	–	(12)	–	(12)	–	(12)	–	(12)
NKp46	+	(10)	–	(10)	–	(10)	±	(10)
NKp44	±	(10)	–	(10)	–	(10)	±	(5, 10)
NKp30	+	(10)	+	(10)	+	(10)	±	(10)
NK1.1	±	(10)	+	(5, 10)	+	(10)	±	(10)
NKG2D	+	(10)	ND	–	ND	–	±	(10)

ND, not determined.
+ positive; – negative; ± sometimes positive.

ILC2 cells are defined as $Lin^{-}CD127^{+}CD25^{+}KLRG1^{+}GATA3^{high}$ cells in mice. These cells are responsive to multiple cytokines, including IL-2, IL-4, IL-7, IL-25, IL-33, TSLP, and prostaglandin D2, and subsequently produce Th2-type cytokines, such as IL4, IL-5, IL-9, IL-13, and amphiregulin (AREG) (1, 13, 19–26). In humans, ILC2 cells express GATA3, CD127, CD161, CD25, ST-2, IL-17A, and chemo-attractant receptor-homologous molecule expressed on Th2 lymphocytes (CRTH2) (1, 13, 15).

Both murine and human ILC3 cells are identified as $Lin^{-}CD127^{+}ROR\gamma t^{+}$ cells (15). Mouse ILC3 cells consists of three subsets: lymphoid tissue-inducer cells (LTi), LTi-like CCR6-expressing ILC3 cells and NCR-expressing ILC3 cells (NCR^{+}ILC3) (1, 15). Similar to Th17 cells, they are poised to respond to the stimulation by IL-1β, IL-6, and IL-23 and subsequently produce effector cytokines, such as IL-22, IL-17A, IL-17F, GM-CSF, TNF-α, and $LT\alpha_1\beta_2$ (1, 15, 27–29).

NK cells are critical players in controlling intracellular bacterial and tumor surveillance (1, 15, 30). ILC1 cells are capable of controlling intracellular pathogens, whereas ILC2 cells have the capacity to limit extracellular parasitic worm infections, promote epithelial repair, and maintain mucosal tissue homeostasis. Notably, ILC2 cells are associated with chronic diseases such as pulmonary fibrosis, hepatic fibrosis, and atopic dermatitis (1, 2, 15, 30). NCR^{+}ILC3 cells are the most prevalent ILC3 subset in the intestine, whereas LTi-like ILC3 cells are mainly localized in the colon and lymphoid tissues (2, 30–32). ILC3 cells are key contributors to tissue repair and protect mucosal barriers against infection by extracellular bacterial and fungi (1, 2, 30–32).

Generation, Transcription, and Plasticity of ILCs

ILCs originate from common lymphoid progenitors (CLPs), which subsequently differentiate into two different lineages: the common helper-like innate lymphocyte progenitors (CHILPs) and the conventional natural killer cell progenitors (cNKps) (**Figure 2**). However, CHILPs are a heterogeneous population consisting of innate lymphoid cell precursors (ILCPs) and lymphoid tissue-inducer precursors (LTiPs) (33, 34). CHILPs are defined as $Lin^{-}IL\text{-}7R^{+}Flt\text{-}3^{-}\alpha4\beta7^{+}CD25^{-}$ $Id2^{high}PLZF^{+}$ cells and can give rise to ILC1, ILC2, ILC3, and LTi cells but not cNK cells (30, 33, 35). ILCPs are designated as $Lin^{-}CD127^{+}\alpha4\beta7^{+}PLZF^{+}$ cells and can produce all ILC lineages (33). LTiPs are the precursors of LTi cells and are defined as $Lin^{-}CD127^{+}\alpha4\beta7^{+}c\text{-}Kit^{+}$ $ROR\gamma t^{+}PLZF^{-}$ cells (33). cNKps can generate cNK cells and are unable to give rise to ILC2 and ILC3 cells. The development of cNK cells requires inhibitor of DNA binding 2 (Id2) (36–38), nuclear factor interleukin 3 (NFIL3) (39–42), thymocyte selection-associated high-mobility group box protein (TOX) (43, 44) and Eomesodermin (Eomes) (45, 46).

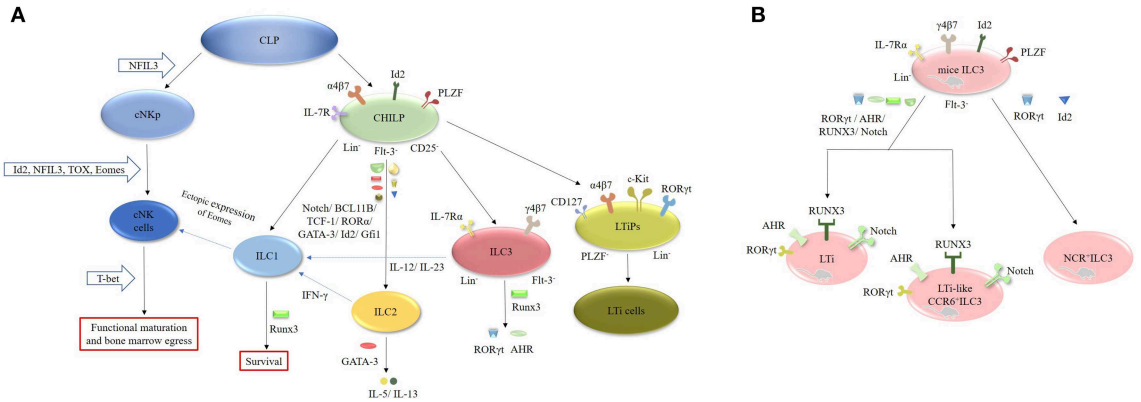


FIGURE 2 | Generation, transcription and plasticity of ILCs. **(A)** ILCs originate from CLPs, which subsequently differentiate into CHILPs and cNKps. cNKps can generate cNK cells. The development of cNK cells requires Id2, NFIL3, TOX, and Eomes. Its functional maturation and bone marrow egress of these cells requires T-bet. ILC1 cells arise from Id2$^+$PLZF$^+$CHILP progenitor cells. The development of ILC2 cells requires Id2, GATA-3, RORα, TCF-1, BCL11B, and Notch. ILC1 cells can be converted into NK cells after ectopic expression of Eomes. IL-12 can endow ILC2 cells with ILC1 features by secreting IFN-γ, whereas IL-12 and IL-23 can induce the transition of ILC3 cells into ILC1 cells. The development of ILC2 cells requires Id2, GATA-3, RORα, TCF-1, BCL11B, and Notch. RUNX3 is necessary for the expression of RORγt and AHR in ILC3 cells. **(B)** The development of murine LTi and LTi-like ILC3 cells requires the expression of RORγt, AHR, RUNX3, and Notch, while the development of NCR$^+$ILC3 cells need RORγt and Id2.

However, the functional maturation and bone marrow egress of these cells requires T-bet (45–48). NFIL3 is involved in the development of bone marrow-derived NK cells from CLPs under homeostatic conditions and is necessary for the formation of splenic and thymic NK cells (39–42). Unlike cNK cells, ILC1 cells arise from Id2$^+$PLZF$^+$CHILP progenitor cells (49). Interestingly, the development of ILC2 cells requires Id2 (36, 37), GATA-binding protein 3 (GATA-3) (50–52), RORα (53), transcription factor 1 (TCF-1) (54–56), BCL11B (57, 58), and Notch (59, 60). GATA-3 is crucial for the secretion of effector cytokines, such as IL-5 and IL-13, by mature ILC2 cells (50–52, 61). In addition, Gfi1 can promote the development of ILC2 cells and control their responsiveness during infection by *Nippostrongylus brasiliensis* and protect against allergen-induced lung inflammation (62). Runx3 is another key factor in the differentiation of ILC1 and ILC3 cells. It controls the survival of ILC1 cells and is necessary for the expression of RORγt and AHR in ILC3 cells (7, 63).

ILC3 cells differentiate from Lin$^-$IL-7Rα$^+$Flt3$^-$γ4β7$^+$ fetal liver progenitors and express Id2 and RORγt in mice (1, 37). The development of murine LTi cells and LTi-like ILC3 cells requires the expression of RORγt, the aryl hydrocarbon receptor (AHR), RUNX3 and Notch (1, 2, 37, 64). The AHR seems to be involved in the expansion of CCR6$^{-/low}$ILC3 cells (65–68). AHR$^{-/-}$ mice exhibit a decrease in CCR6$^{-/low}$ILC3 cells without alteration in the CCR6$^+$ILC3 population. Furthermore, T-bet controls the fate and function of CCR6$^-$RORγt$^+$ILCs. Postnatal CCR6$^-$RORγt$^+$ILCs upregulate T-bet, which is modulated by the commensal microbiota. Tbx21$^{-/-}$ mice exhibit normal development of CCR6$^-$RORγt$^+$ cells, but they fail to differentiate into NKp46$^+$RORγt$^+$ ILCs, suggesting that T-bet is necessary for the differentiation of NKp46$^+$RORγt$^+$ ILCs in mice (8, 69). Additionally, the IL-1β/IL-1R/MyD88 pathway controls the production of IL-22 by NKp46$^+$RORγt$^+$ILCs in the small intestine (SI) of mice (70). In contrast to mice, both human Lin$^-$CD34$^+$CD45RA$^+$CD117$^+$IL-1R$^+$RORγt$^+$ cells and stage 2 IL-1R$^+$ cells in secondary lymphoid tissues (SLT) can differentiate into nearly all ILC populations including NK cells (71). Collectively, these results demonstrate that the development of ILCs is not dependent on a single "master regulator" but on a complex network of transcription factors (TFs) (1, 15, 31). Interestingly, recent studies have focused on the plasticity of ILCs. For instance, ILC1 cells can be converted into NK cells after ectopic expression of Eomes (31, 48). IL-12 can endow ILC2 cells with ILC1 features by secreting IFN-γ (60, 72), whereas IL-12 and IL-23 can induce the transition of ILC3 cells into ILC1 cells (60, 73, 74). Furthermore, dermal NCR$^-$ILC3 cells can be transformed into NCR$^+$ ILC3 cells in the presence of IL-1β and IL-23 *in vitro* (42, 75–77).

Localization and Migration of ILCs

NK cells are mainly located in the bone marrow, lymph nodes, spleen, lungs, and liver, whereas ILC1 cells mainly reside in the intestinal intraepithelia (IE) (2, 78, 79). ILC2 cells are located in the lungs and lamina propria of the small intestine (SI) and skin, whereas ILC3 cells are predominantly located in the lamina propria, Peyer's patches and lymphoid follicles of the small intestine (78, 79).

It is generally considered that fetal liver and bone marrow are the "factories" where ILC subsets are generated (1, 2). However, a report by Gasteiger et al. have indicated that the vast majority of ILCs in both lymphoid and non-mymphoid organs are long-lived tissue-resident under steady state (80). Another elegant study by Di Santo JP's lab has proposed a model of "ILC-poiesis" and provided a mechanism by which tissue ILCs could be replenished from blood ILCPs in response to steady-state losses and under the circumstance of infection and inflammation (81–83).

Recently, increasing evidence has indicated that ILC1 and ILC3 cells can migrate into SLTs, depending on integrins and

chemo-attractant receptors, whereas the migration of ILC2 cells from hematopoietic sites to target tissues is independent of the aforementioned receptors.

It has been indicated that the migration of NK cells to LNs via high endothelial cells (HEVs) might be mediated by CCR7 or CXCR3. The migration of ILC1 and ILC3 cells to SLTs occurs in a CCR7-dependent manner (84, 85). ILC2 cells, located in the bone marrow, spleen as well as mesenteric lymph nodes, constitutively express CCR9 and α4β7, rather than the RA-dependent homing receptor (79, 84). The migration of LTi-ILC3 cells to lymphoid follicles and the spleen marginal zone is regulated by the CXCL13-CXCR5 axis (86). Notably, trafficking receptor switches play a crucial role in the migration of ILCs. For instance, activation of spleen ILC3 cells induces upregulation of CCR9 and α4β7 with concomitant downregulation of CCR7 in the presence of IL-7 and *all-trans* retinoic acid (RA) and prompts the migration of these cells to the intestine (84, 87, 88).

ILCs and GVHD

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) is the most powerful therapy for hematologic malignancies and a majority of non-malignant hematological diseases. One of the major barriers to the efficacy of allo-HSCT is the occurrence of GVHD. Radiotherapy/chemotherapy induction regimens damage epithelia, especially the intestinal mucosa, in recipients, followed by the translocation of commensal microbiotas from the GI tract into the peripheral blood. Subsequent activation of adaptive immunity promotes the occurrence of aGVHD (89–94).

The Role of Donor-Derived ILCs in GVHD

The role of NK cells in the pathogenesis of GVHD seems to be controversial (95, 96). Early studies indicated that target organs, such as the skin, liver, and GI tract, in HSCT recipients with aGVHD were infiltrated with NK cells, suggesting that NK cells might promote the development of GVHD (97–99). In accordance, administration of NK cell depleting antibodies against GM1 or NK1.1 significantly mitigated GVHD in murine models (100, 101). Cooley et al. have demonstrated that, in unrelated HSCT, increased production of IFN-γ by NK cells has correlated with more aGVHD, and decreased KIR expression has associated with inferior survival of patients, suggesting that NK cells might promote GVHD via secretion of inflammatory cytokines such as IFN-γ and TNF-α (102).

Recently, a first-in-human trial of adoptive transfer of donor-derived IL-15/4-1BBL -activated NK cells was conducted in an HLA-matched, T-cell-depleted non-myeloablative peripheral blood stem cell transplantation (103). In this clinical trial, five of nine transplant recipients experienced acute GVHD, with grade 4 GVHD in three patients. Together, the aforementioned studies seem to support the notion that NK cells promote GVHD. However, contradictory results were obtained from other studies where adoptive transfer of donor-derived NK cells into HSCT recipients can prevent the occurrence of GVHD in mouse and humans (104–107). In an MHC mismatched murine model (BALB/c→ C57BL6), IL-2–activated donor-derived NK cells were administered with allogeneic bone marrow cells and splenocytes (104). Mice receiving pre-activated donor-derived NK cells significantly delayed the onset of GVHD and prolonged the survival of mice. Consistently, these mice exhibited no infiltration of inflammatory cells with normal structure of gut (104). In accordance, another animal study by Song et al. has shown that single infusion of IL-12/IL-18- pre-activated donor NK cells one day 0 after HSCT has mitigated severe or mild aGVHD, and enhanced GVL effects (108).

In line with animal data, clinical results from a phase 1 clinical trial have shown that the infusion of high doses of *ex vivo*-membrane-bound interleukin 21(mbIL-21) expanded donor-derived NK cells is safe without adverse effects, without increased GVHD or high mortality (109). Therefore, early infusion of pre-activated donor-derived NK cells has the potential of prevention of GVHD. However, it should be taken into account that different strategy for the activation of donor-derived NK cells might bring different outcomes. Other important issues that should be considered are the infusion timing of NK cells, MHC/HLA matching degree between donors and recipients as well as the pretreatment strategy before HSCT.

Interestingly, NK cells can alleviate cGVHD by directly constraining recipient minor histocompatibility Ag (mHA)-triggered proliferation of donor-derived $CD4^+$ T cells in a Fas-dependent manner (110). Evidence from Ruggeri L's report has indicated that the KIR ligand incompatibility between donor and recipient might endow donor-derived NK cells to prevent the occurrence of GVHD, via direct depletion of recipient-derived antigen-presenting cells (APCs) (107). Clinical investigation on the early NK cell reconsitution in 82 patients following T cell-depleted allo-SCT have shown that NK cell number at day 14 after HSCT was inversely correlated with the incidence of grade II-IV aGVHD (111). Mechanistically, NK cells at day 14 produced high levels of IL-10 and showed upregulation of gene transcript of IL-10 compared with healthy individuals, suggesting that the regulatory phenotype might enable NK cells to suppress the development of GVHD (111).

Together, NK cells could prevent GVHD via (1) direct lysing of activated T cells; (2) indirect inhibition of T cell proliferation through depleting host APCs; (3) production of suppressive cytokines, such as IL-10 (**Figure 3**).

Only one clinical study by Munneke et al. have tried to elucidate the role of ILC1s in GVHD after HSCT (12). In the study, patients without developing aGVHD diplayed increased proportions of skin-homing donor-derived ILC1s. Notably, following transplantation, patients with more severe GVHD exhibited fewer circulating ILC1s in PB, compared with healthy controls. Mobilization of ILC1s seemed to be associated with increased expression of CD69, CLA, and CCR10 which correlated with less severe progression of GVHD (12). However, the functionality of these aforementioned ILC1s was not determined in this study. Further question is whether skin-homing ILC1s alone can prevent the occurrence of GVHD? As we know, multiple organs, including GI tract, skin, lung, liver, and mouth, in recipients are targeted in GVHD, while ILC1s-expressing CLA and CCR10, which are skin homing markers, might only traffic to the skin. Therefore, further experiments where direct infusion of ILC1s into recipients with GVHD need to be taken and will

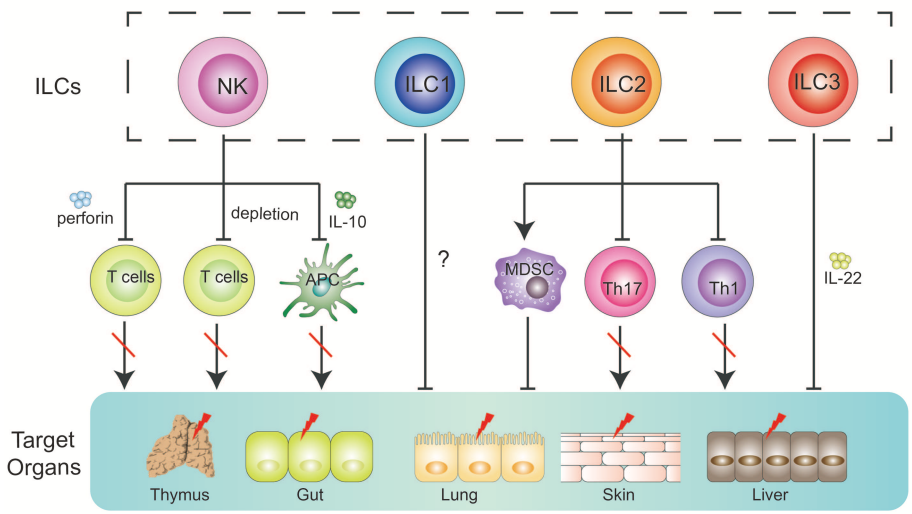


FIGURE 3 | Role of ILCs in GVHD. NK cells can suppress GVHD via three main mechanisms, including direct lysing of activated T cells, indirect inhibition of T cell proliferation through depleting host APCs and production of suppressive cytokines, such as IL-10. ILC1 cells might migrate to the skin and alleviate cutaneous GVHD. Intravenous infusion of donor-derived ILC2 cells into ongoing GVHD mice can reduce the production of Th1 and Th17 cells while increasing the number of MDSCs via secreting IL-13. ILC3 cells play a protective role in GVHD. Recipient-derived ILC3 cells can alleviate pretreatment regimen-induced GI tract lesion via secretion of IL-22. Furthermore, these ILC3 cells can improve thymopoiesis in the hosts after HSCT.

be beneficial to the understanding of the role of ILC1s in the prevention of GVHD.

It has been shown that ILC2 cells in the lower GI tract but not in the lung are sensitive to conditioning treatment and exhibit a limited repopulation ability from donor bone marrow (112). Remarkably, a single infusion of donor-derived ILC2 cells at day 7 post-HSCT was shown to remain effective at reducing the severity and mortality of ongoing aGVHD in murine model. Intravenously infused ILC2 cells migrated to the GI tract, produced Th2 cytokines, limited inflammatory Th1 and Th17 cells, and induced myeloid-derived suppressor cells (MDSCs). IL-13 produced by ILC2 cells seemed to be involved in this process. Importantly, infusion of donor ILC2 cells did not affect the beneficial graft-vs.-leukemia (GVL) effect (106). Collectively, these data indicate that intravenously infused donor-derived ILC2 cells have the capacity to alleviate ongoing aGVHD without affecting the beneficial GVL effect in murine models (112). However, several questions still require further elucidation. For instance, how do intravenously infused donor-derived ILC2 cells migrate to the GI tract in the context of GVHD? Why do these cells not migrate to the lungs of recipients? Furthermore, how do these cells survive during the migration process? All these questions require further investigation.

The Role of Recipient-Derived ILCs in GVHD

An increasing body of evidence has indicated that ILC3 cells have the capacity to promote tissue repair. Under homeostatic circumstances, ILC3 cells can respond to environmental signals and maintain tissue homeostasis. In contrast, abnormal signals from infection or tissue damage can activate the ILC3 response (9, 113, 114). Therefore, in GVHD conditions, induction of regimen-induced tissue damage might cause a dysregulated ILC3 response.

In an animal model, a deficiency in recipient-derived IL-22 was shown to significantly increase the severity and mortality of GVHD (113). Furthermore, pretransplantation conditions increased the intestinal expression of IL-22 in recipients, which was mainly produced by recipient-derived $CD45^{+}CD3^{-}ROR\gamma t^{+}NKp46^{-}IL\text{-}7R\alpha^{+}$ $CCR6^{+}$ ILCs. In accordance, IL-22 deficiency resulted in more severe epithelial damage during aGVHD and significant loss of intestinal stem cells. Taken together, these data suggest that loss of tissue-protective IL-22-producing ILCs in the intestines of recipients might be a pathological factor responsible for the GI tract lesions observed in aGVHD (113).

Recent work has shed light on the correlation between thymopoiesis and GVHD. Mice with GVHD after allo-HSCT exhibited a loss of intrathymic ILC3s, decreased intrathymic levels of IL-22 and impaired recovery of thymopoiesis. Not surprisingly, $IL\text{-}22^{-/-}$ mice that underwent transplantation showed an increased severity of GVHD-associated thymic injury. IL-22 $receptor^{-/-}$ recipient mice that underwent transplantation displayed increased numbers of cortical and medullary thymic epithelial cells (TECs). In accordance, administration of exogenous IL-22 after transplantation improved thymopoiesis and promoted the development of new thymus-derived peripheral T cells (115, 116). These findings encourage researchers to uncover what actually occurs after loss of ILC3s in the hosts induced by an induction regimen.

CONCLUDING REMARKS

Although studies on ILCs have become a focus of research in recent years, the precise role of ILCs in the pathogenesis of GVHD remains elusive. Many questions remain to be answered in the future. For instance, what is the precise role of ILC1 cells in the pathology of GVHD? Can intravenous infusion of ILC3 cells alleviate ongoing GVHD? Lastly, how do these cells migrate to the GI tract in recipients after intravenous transfer? How about the clinical application of ILC2 for the treatment of GVHD? A recent study identified a cell population–ILCregs (117). Like Tregs, ILCregs have the suppressive ability to curb ILCs. Therefore, the question remains whether ILCregs play a role in the pathogenesis of GVHD? Additionally, what is the interaction between ILCs and ILCregs at the onset of GVHD? These questions require further elucidation in future work.

AUTHOR CONTRIBUTIONS

LS drafted the manuscript. AL, FZ, and QZ revised the manuscript. SP completed the figures. MJ and LC did the language editing. All authors read this manuscript and approved its submission.

FUNDING

This work was supported by the National Natural Science Foundation of China (No.81500151 and 81770179), the Natural Foundation of Hubei Province (China) (No.2017CFB631), and The Innovative Foundation of Zhongnan Hospital of Wuhan University (No. znpy2018025).

REFERENCES

1. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol*. (2012) 30:647–75. doi: 10.1146/annurev-immunol-020711-075053
2. Kim CH, Hashimoto-Hill S, Kim M. Migration and Tissue Tropism of Innate Lymphoid Cells. *Trends Immunol*. (2016) 37:68–79. doi: 10.1016/j.it.2015.11.003
3. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol*. (2011) 12:21–7. doi: 10.1038/ni.1962
4. Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-γ-producing cells. *Immunity*. (2013) 38:769–81. doi: 10.1016/j.immuni.2013.02.010
5. Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol*. (2013) 14, 221–9. doi: 10.1038/ni.2534
6. Mortha A, Burrows K. Cytokine networks between innate lymphoid cells and myeloid cells. *Front Immunol*. (2018) 9:191. doi: 10.3389/fimmu.2018.00191
7. Ebihara T, Song C, Ryu SH, Plougastel-Douglas B, Yang L, Levanon D, et al. Runx3 specifies lineage commitment of innate lymphoid cells. *Nat Immunol*. (2015) 16:1124–33. doi: 10.1038/ni.3272
8. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. *Nature*. (2013) 494:261–5. doi: 10.1038/nature11813
9. Konya V, Mjösberg J. Innate lymphoid cells in graft-versus-host disease. *Am J Transplant*. (2015) 15:2795–801. doi: 10.1111/ajt.13394
10. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell*. (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
11. Liu B, Ye B, Zhu X, Huang G, Yang L, Zhu P, et al. IL-7Rα glutamylation and activation of transcription factor Sall3 promote group 3 ILC development. *Nat Commun*. (2017) 8:231. doi: 10.1038/s41467-017-00235-x
12. Munneke JM, Björklund AT, Mjösberg JM, Garming-Legert K, Bernink JH, Blom B, et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. *Blood*. (2014) 124:812–21. doi: 10.1182/blood-2013-11-536888
13. Mjösberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol*. (2011) 12:1055–62. doi: 10.1038/ni.2104
14. Tait Wojno ED, Artis D. Emerging concepts and future challenges in innate lymphoid cell biology. *J Exp Med*. (2016) 213:2229–48. doi: 10.1084/jem.20160525
15. Montaldo E, Vacca P, Vitale C, Moretta F, Locatelli F, Mingari MC, et al. Human innate lymphoid cells. *Immunol Lett*. (2016).179:2–8. doi: 10.1016/j.imlet.2016.01.007
16. Adams NM, Sun JC. Spatial and temporal coordination of antiviral responses by group 1 ILCs. *Immunol Rev*. (2018) 286:23–36. doi: 10.1111/imr.12710
17. Colonna M. Innate lymphoid cells: diversity, plasticity, and unique functions in immunity. *Immunity*. (2018) 48:1104–17. doi: 10.1016/j.immuni2018.05.013
18. Villanova F, Flutter B, Tosi I, Grys K, Sreeneebus H, Perera GK, et al. Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis. *J Invest Dermatol*. (2014) 134:1–8. doi: 10.1038/jid.2013.477
19. Kim BS, Siracusa MC, Saenz SA, Noti M, Monticelli LA, Sonnenberg GF, et al. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med*. (2013) 5:170ra16. doi: 10.1126/scitranslmed.3005374
20. Imai Y, Yasuda K, Sakaguchi Y, Haneda T, Mizutani H, Yoshimoto T, et al. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci USA*. (2013) 110:13921–6. doi: 10.1073/pnas.1307321110
21. Xue L, Salimi M, Panse I, Mjosberg JM, McKenzie AN, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol*. (2014) 133:1184–94. doi: 10.1016/j.jaci.2013.10.056
22. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol*. (2013) 132:205–13. doi: 10.1016/j.jaci.2013.03.048
23. Karta MR, Broide DH, Doherty TA. Insights into group 2 innate lymphoid cells in human airway disease. *Curr Allergy Asthma Rep*. (2016) 16:8. doi: 10.1007/s11882-015-0581-6
24. Monticelli LA, Osborne LC, Noti M, Tran SV, Zaiss DM, Artis D. IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions. *Proc Natl Acad Sci USA*. (2015) 112:10762–7. doi: 10.1073/pnas.1509070112
25. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol*. (2011) 12:1045–54. doi: 10.1031/ni.2131
26. Zaiss DMW, Gause WC, Osborne LC, Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity*. (2015) 42:216–26. doi: 10.1016/j.immuni.2015.01.020
27. Kruglov AA, Grivennikov SI, Kuprash DV, Winsauer C, Prepens S, Seleznik GM, et al. Nonredundant function of soluble LTα3 produced by innate lymphoid cells in intestinal homeostasis. *Science*. (2013) 342:1243–6. doi: 10.1126/science.1243364

28. Cording S, Medvedovic J, Cherrier M, Eberl G. Development and regulation of RORγt(+) innate lymphoid cells. *FEBS Lett*. (2014) 588:4176–81. doi: 10.1016/j.febslet.2014.03.034
29. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science*. (2014) 343:1249288. doi: 10.1126/science.1249288
30. Zook EC, Kee BL. Development of innate lymphoid cells. *Nat Immunol*. (2016) 17:775–82. doi: 10.1038/ni.3481
31. Serafini N, Vosshenrich CA, Di Santo JP. Transcriptional regulation of innate lymphoid cell fate. *Nat Rev Immunol*. (2015) 15:415–28. doi: 10.1038/nri3855
32. Lim AI, Verrier T, Vosshenrich CA, Di Santo JP. Developmental options and functional plasticity of innate lymphoid cells. *Curr Opin Immunol*. (2017) 44:61–8. doi: 10.1016/j.coi.2017.03.010
33. Ishizuka IE, Constantinides MG, Gudjonson H, Bendelac A. The innate lymphoid cell precursor. *Annu Rev Immunol*. (2016) 34:299–316. doi: 10.1146/annurev-immunol-041015-055549
34. Klose CSN, Flach M, Möhle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*. (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030
35. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature*. (2014) 508:397–401. doi: 10.1038/nature13047
36. Guo X, Liang Y, Zhang Y, Lasorella A, Kee BL, Fu YX. Innate lymphoid cells control early colonization resistance against intestinal pathogens through ID2-dependent regulation of the microbiota. *Immunity*. (2015) 42:731–43. doi: 10.1016/j.immuni.2015.03.012
37. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORgammat sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *J Exp Med*. (2012) 209:729–40. doi: 10.1084/jem.20111594
38. Geiger TL, Sun JC. Development and maturation of natural killer cells. *Curr Opin Immunol*. (2016) 39:82–9. doi: 10.1016/j.coi.2016.01.007
39. Kamizono S, Duncan GS, Seidel MG, Morimoto A, Hamada K, Grosveld G, et al. Nfil3/E4bp4 is required for the development and maturation of NK cells *in vivo*. *J Exp Med*. (2009) 206:2977–86. doi: 10.1084/jem.20092176
40. Xu W, Domingues RG, Fonseca-Pereira D, Ferreira M, Ribeiro H, Lopez-Lastra S, et al. NFIL3 orchestrates the emergence of common helper innate lymphoid cell precursors. *Cell Rep*. (2015) 10:2043–54. doi: 10.1016/j.celrep.2015.02.057
41. Kostrzewski T, Borg AJ, Meng Y, Filipovic I, Male V, Wack A, et al. Multiple levels of control determine how E4bp4/Nfil3 regulates NK cell development. *J Immunol*. (2018) 200:1370–81. doi: 10.4049/jimmunol.1700981
42. Sciumè G, Shih HY, Mikami Y, O'Shea JJ. Epigenomic views of innate lymphoid cells. *Front Immunol*. (2017) 8:1579. doi: 10.3389/fimmu.2017.01579
43. Vong QP, Leung WH, Houston J, Li Y, Rooney B, Holladay M, et al. TOX2 regulates human natural killer cell development by controlling T-BET expression. *Blood*. (2014) 124:3905–13. doi: 10.1182/blood-2014-06-582965
44. Mora-Velandia LM, Castro-Escamilla O, Méndez AG, Aguilar-Flores C, Velázquez-Avila M, Tussié-Luna MI, et al. A human Lin-CD123+ CD127low population endowed with ILC features and migratory capabilities contributes to immunopathological hallmarks of psoriasis. *Front Immunol*. (2017) 8:176. doi: 10.3389/fimmu.2017.00176
45. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J Exp Med*. (2014) 211:563–77. doi: 10.1084/jem.20131560
46. Zhang J, Marotel M, Fauteux-Daniel S, Mathieu AL, Viel S, Marçais A, et al. T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1. *Eur J Immunol*. (2018) 48:738–50. doi: 10.1002/eji.201747299
47. Simonetta F, Pradier A, Roosnek E. T-bet and eomesodermin in NK cell development, maturation, and function. *Front Immunol*. (2016) 7:241. doi: 10.3389/fimmu.2016.00241
48. Pikovskaya O, Chaix J, Rothman NJ, Collins A, Chen YH, Scipi-oni A M, et al. Cutting edge: eomesodermin is sufficient to direct type 1 innate lymphocyte development into the conventional NK lineage. *J Immunol*. (2016) 196:1449–54. doi: 10.4049/jimmunol.1502396
49. Constantinides MG, Gudjonson H, McDonald BD, Ishizuka IE, Verhoef PA, Dinner AR, et al. PLZF expression maps the early stages of ILC1 lineage development. *Proc Natl Acad Sci USA*. (2015) 112:5123–8. doi: 10.1073/pnas.1423244112
50. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity*. (2012) 37:649–59. doi: 10.1016/j.immuni.2012.08.015
51. Klein Wolterink RG, Serafini N, van Nimwegen M, Vosshenrich CA, de Bruijn MJ, Fonseca Pereira D, et al. Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5+ and IL-13+ type 2 innate lymphoid cells. *Proc Natl Acad Sci USA*. (2013) 110:10240–5. doi: 10.1073/pnas.1217158110
52. Walker JA, McKenzie AN. Development and function of group 2 innate lymphoid cells. *Curr Opin Immunol*. (2013) 25:148–55. doi: 10.1016/j.coi.2013.02.010
53. Wong SH, Walker JA, Jolin HE, Drynan LF, Hams E, Camelo A, et al. Transcription factor RORalpha is critical for nuocyte development. *Nat Immunol*. (2012) 13:229–36. doi: 10.1038/ni.2208
54. Mielke LA, Groom JR, Rankin LC, Seillet C, Masson F, Putoczki T, et al. TCF-1 controls ILC2 and NKp46+RORγt+ innate lymphocyte differentiation and protection in intestinal inflammation. *J Immunol*. (2013) 191:4383–91. doi: 10.4049/jimmunol.1301228
55. Ishizuka IE, Chea S, Gudjonson H, Constantinides MG, Dinner AR, Bendelac A, et al. Single-cell analysis defines the divergence between the innate lymphoid cell lineage and lymphoid tissue-inducer cell lineage. *Nat Immunol*. (2016) 17:269–76. doi: 10.1038/ni.3344
56. Seillet C, Mielke LA, Amann-Zalcenstein DB, Su S, Gao J, Almeida FF, et al. Deciphering the innate lymphoid cell transcriptional program. *Cell Rep*. (2016) 17:436–47. doi: 10.1016/j.celrep.09.025
57. Yu Y, Wang C, Clare S, Wang J, Lee SC, Brandt C, et al. The transcription factor Bcl11b is specifically expressed in group 2 innate lymphoid cells and is essential for their development. *J Exp Med*. (2015) 212:865–74. doi: 10.1084/jem.20142318
58. Califano D, Cho JJ, Uddin MN, Lorentsen KJ, Yang Q, Bhandoola A, et al. Transcription factor Bcl11b controls identity and function of mature type 2 innate lymphoid cells. *Immunity*. (2015) 43:354–68. doi: 10.1016/j.immuni.2015.07.005
59. Zhang K, Xu X, Pasha MA, Siebel CW, Costello A, Haczku A, et al. Cutting edge: notch signaling promotes the plasticity of group-2 innate lymphoid cells. *J Immunol*. (2017) 198:1798–803. doi: 10.4049/jimmunol.1601421
60. Lim AW, McKenzie AN. Deciphering the transcriptional switches of innate lymphoid cell programming: the right factors at the right time. *Genes Immun*. (2015) 16:177–86. doi: 10.1038/gene.2014.83
61. Tindemans I, Serafini N, Di Santo JP, Hendriks RW. GATA-3 function in innate and adaptive immunity. *Immunity*. (2014) 41:191–206. doi: 10.1016/j.immuni.2014.06.006
62. Spooner CJ, Lesch J, Yan D, Khan AA, Abbas A, Ramirez-Carrozzi V, et al. Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nat Immunol*. (2013) 14:1229–36. doi: 10.1038/ni.2743
63. Harly C, Cam M, Kaye J, Bhandoola A. Development and differentiation of early innate lymphoid progenitors. *J Exp Med*. (2018) 215:249–62. doi: 10.1084/jem.20170832
64. Rankin LC, Groom JR, Chopin M, Herold MJ, Walker JA, Mielke LA, et al. The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. *Nat Immunol*. (2013) 14:389–95. doi: 10.1038/ni.2545
65. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, Esser C, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science*. (2011) 334:1561–5. doi: 10.1126/science.1214914
66. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity*. (2012) 36:92–104. doi: 10.1016/j.immuni.2011.11.011

67. Qiu J, Zhou L. Aryl hydrocarbon receptor promotes RORγt+ group 3 ILCs and controls intestinal immunity and inflammation. *Semin Immunopathol*. (2013) 35:657–70. doi: 10.1007/s00281-013-0393-5
68. Li S, Bostick JW, Zhou L. Regulation of innate lymphoid cells by Aryl hydrocarbon receptor. *Front Immunol*. (2018) 8:1909. doi: 10.3389/fimmu.2017.01909
69. Powell N, Walker AW, Stolarczyk E, Canavan JB, Gökmen MR, Marks E, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. *Immunity*. (2012) 37:674–84. doi: 10.1016/j.immuni.2012.09.008
70. Reynders A, Yessaad N, Vu Manh TP, Dalod M, Fenis A, Aubry C, et al. Identity, regulation and *in vivo* function of gut NKp46+RORγt+ and NKp46+RORγt- lymphoid cells. *EMBO J*. (2011) 30:2934–47. doi: 10.1038/emboj.2011.201
71. Scoville SD, Mundy-Bosse BL, Zhang MH, Chen L, Zhang X, Keller KA, et al. A progenitor cell expressing transcription factor RORγt generates all human innate lymphoid cell subsets. *Immunity*. (2016) 44:1140–50. doi: 10.1016/j.immuni.2016.04.007
72. Lim AI, Menegatti S, Bustamante J, Le Bourhis L, Allez M, Rogge L, et al. IL-12 drives functional plasticity of human group 2 innate lymphoid cells. *J Exp Med*. (2016) 213:569–83. doi: 10.1084/jem.20151750
73. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and−23 control plasticity of CD127(+) Group 1 and Group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity*. (2015) 43:146–60. doi: 10.1016/j.immuni.2015.06.019
74. Montaldo E, Juelke K, Romagnani C. Group 3 innate lymphoid cells (ILC3s): Origin, differentiation, and plasticity in humans and mice. *Eur J Immunol*. (2015) 45:2171–82. doi: 10.1002/eji.201545598
75. Teunissen MBM, Munneke JM, Bernink JH, Spuls PI, Res PCM, Te Velde A, et al. Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR(+) ILC3 in lesional skin and blood of psoriasis patients. *J Invest Dermatol*. (2014) 134:2351–60. doi: 10.1038/jid.2014.146
76. Zhong C, Zhu J. Transcriptional regulators dictate innate lymphoid cell fates. *Protein Cell*. (2017) 8:242–54. doi: 10.1007/s13238-017-0369-7
77. Simoni Y, Newell EW. Dissecting human ILC heterogeneity: more than just three subsets. *Immunology*. (2018) 153:297–303. doi: 10.1111/imm.12862
78. Soriani A, Stabile H, Gismondi A, Santoni A, Bernardini G. Chemokine regulation of innate lymphoid cell tissue distribution and function. *Cytokine Growth Factor Rev*. (2018). doi: 10.1016/j.cytogfr.2018.02.003
79. Ignacio A, Breda CNS, Camara NOS. Innate lymphoid cells in tissue homeostasis and diseases. *World J Hepatol*. (2017) 9:979–89. doi: 10.4254/wjh.v9.i23.979
80. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science*. (2015) 350:981–5. doi: 10.1126/science.aac9593
81. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, Serafini N, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell*. (2017) 168:1086–100.e10. doi: 10.1016/j.cell.2017.02.021
82. Lim AI, Di Santo JP. ILC-poiesis: ensuring tissue ILC differentiation at the right place and time. *Eur J Immunol*. (2018) 49:11–8. doi: 10.1002/eji.201747294
83. Mjösberg J, Mazzurana L. ILC-poiesis: making tissue ILCs from blood. *Immunity*. (2017) 46:344–6. doi: 10.1016/j.immuni.2017.03.002
84. Kim MH, Taparowsky EJ, Kim CH. Retinoic acid differentially regulates the migration of innate lymphoid cell subsets to the gut. *Immunity*. (2015) 43:107–19. doi: 10.1016/j.immuni.2015.06.009
85. Mackley EC, Houston S, Marriott CL, Halford EE, Lucas B, Cerovic V, et al. CCR7-dependent trafficking of RORγ+ ILCs creates a unique microenvironment within mucosal draining lymph nodes. *Nat Commun*. (2015) 6:5862. doi: 10.1038/ncomms6862
86. Marchesi F, Martin AP, Thirunarayanan N, Devany E, Mayer L, Grisotto MG, et al. CXCL13 expression in the gut promotes accumulation of IL-22-producing lymphoid tissue-inducer cells, and formation of isolated lymphoid follicles. *Mucosal Immunol*. (2009) 2:486–94. doi: 10.1038/mi.2009.113
87. van de Pavert SA, Ferreira M, Domingues RG, Ribeiro H, Molenaar R, Moreira-Santos L, et al. Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. *Nature*. (2014) 508:123–7. doi: 10.1038/nature13158
88. Goverse G, Labao-Almeida C, Ferreira M, Molenaar R, Wahlen S, Konijn T, et al. Vitamin A controls the presence of RORγ+ innate lymphoid cells and lymphoid tissue in the small intestine. *J Immunol*. (2016) 196:5148–55. doi: 10.4049/jimmunol.1501106
89. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. *N Engl J Med*. (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
90. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
91. Hu Y, Cui Q, Luo C, Luo Y, Shi J, Huang H. A promising sword of tomorrow: human γδ T cell strategies reconcile allo-HSCT complications. *Blood Rev*. (2016) 30:179–88. doi: 10.1016/j.blre.2015.11.002
92. Zeng D, Lan F, Hoffmann P, Strober S. Suppression of graft-versus-host disease by naturally occurring regulatory T cells. *Transplantation*. (2004) 77(1 Suppl.): S9–11. doi: 10.1097/01.TP.0000106475.38978.11
93. Pessach I, Tsirigotis P, Nagler A. The gastrointestinal tract: properties and role in allogeneic hematopoietic stem cell transplantation. *Expert Rev Hematol*. (2017) 10:315–26. doi: 10.1080/17474086.2017.1288566
94. Zeiser R, Socié G, Blazar BR. Pathogenesis of acute graft-versus-host disease: from intestinal microbiota alterations to donor T cell activation. *Br J Haematol*. (2016) 175:191–207. doi: 10.1111/bjh.14295
95. Blazar BR, MacDonald KPA, Hill GR. Immune regulatory cell infusion for graft-versus-host disease prevention and therapy. *Blood*. (2018) 131:2651–60. doi: 10.1182/blood-2017-11-785865
96. Simonetta F, Alvarez M, Negrin RS. Natural killer cells in graft-versus-host-disease after allogeneic hematopoietic cell transplantation. *Front Immunol*. (2017) 8:465. doi: 10.3389/fimmu. 2017.00465
97. Horn TD, Haskell J. The lymphocytic infiltrate in acute cutaneous allogeneic graft-versus-host reactions lacks evidence for phenotypic restriction in donor-derived cells. *J Cutan Pathol*. (1998) 25:210–4.
98. Dilly SA, Sloane JP. An immunohistological study of human hepatic graft-versus-host disease. *Clin Exp Immunol*. (1985) 62:545–53.
99. Roy J, Platt JL, Weisdorf DJ. The immunopathology of upper gastrointestinal acute graft-versus-host disease. Lymphoid cells and endothelial adhesion molecules. *Transplantation*. (1993) 55:572–8.
100. Charley MR, Mikhael A, Hackett J, Kumar V, Bennett M. Mechanism of anti-asialo GM1 prevention of graft-vs-host disease: identification of allo-antigen activated T cells. *J Invest Dermatol*. (1988) 91:202–6.
101. Zeng D, Lewis D, Dejbakhsh-Jones S, Lan F, García-Ojeda M, Sibley R, et al. Bone marrow NK1.1(-) and NK1.1(+) T cells reciprocally regulate acute graft versus host disease. *J Exp Med*. (1999) 189:1073–81.
102. Cooley S, McCullar V, Wangen R, Bergemann TL, Spellman S, Weisdorf DJ, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood*. (2005) 106:4370–6. doi: 10.1182/blood-2005-04-1644
103. Shah NN, Baird K, Delbrook CP, Fleisher TA, Kohler ME, Rampertaap S, et al. Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. *Blood*. (2015) 125:784–92. doi: 10.1182/blood-2014-07-592881
104. Tanaka M, Kobayashi S, Numata A, Tachibana T, Takasaki H, Maruta A, et al. The impact of the dose of natural killer cells in the graft on severe acute graft-versus-host disease after unrelated bone marrow transplantation. *Leuk Res*. (2012) 36:699–703. doi: 10.1016/j.leukres.2011.11.009
105. Kim SY, Lee H, Han MS, Shim H, Eom HS, Park B, et al. Post-transplantation natural killer cell count: a predictor of acute graft-versus-host disease and survival outcomes after allogeneic hematopoietic stem cell transplantation. *Clin Lymphoma Myeloma Leuk*. (2016) 16:527–35.e2. doi: 10.1016/j.clml.2016.06.013
106. Wong PPC, Kariminia A, Jones D, Eaves CJ, Foley R, Ivison S, et al. Plerixafor effectively mobilizes CD56bright NK cells in blood, providing an allograft predicted to protect against GVHD. *Blood*. (2018) 131:2863–6. doi: 10.1182/blood-2018-03-836700
107. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. (2002) 295:2097–100. doi: 10.1126/science.1068440

108. Song Y, Hu B, Liu Y, Jin Z, Zhang Y, Lin D, et al. IL-12/IL-18- preactivated donor NK cells enhance GVL effects and mitigate GvHD after allogeneic hematopoietic stem cell transplantation. *Eur J Immunol*. (2018) 48:670–82. doi: 10.1002/eji.201747177
109. Ciurea SO, Schafer JR, Bassett R, Denman CJ, Cao K, Willis D, et al. Phase 1 clinical trial using mbIL21 *ex vivo*-expanded donor-derived NK cells after haploidentical transplantation. *Blood*. (2017) 130:1857–68. doi: 10.1182/blood-2017-05-785659
110. Noval Rivas M, Hazzan M, Weatherly K, Gaudray F, Salmon I, Braun MY. NK cell regulation of CD4 T cell-mediated graft-versus-host disease. *J Immunol*. (2010) 184:6790–8. doi: 10.4049/jimmunol.0902598
111. Chan YLT, Zuo J, Inman C, Croft W, Begum J, Croudace J, et al. NK cells produce high levels of IL-10 early after allogeneic stem cell transplantation and suppress development of acute GVHD. *Eur J Immunol*. 48:316–29. doi: 10.1002/eji.201747134
112. Bruce DW, Stefanski HE, Vincent BG, Dant TA, Reisdorf S, Bommiasamy H, et al. Type 2 innate lymphoid cells treat and prevent acute gastrointestinal graft-versus-host disease. *J Clin Invest*. (2017) 127:1813–25. doi: 10.1172/JCI91816
113. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. Immunity. (2012) 37:339–50. doi: 10.1016/j.immuni.2012.05.028
114. Karrich JJ, Cupedo T. Group 3 innate lymphoid cells in tissue damage and graft-versus-host disease pathogenesis. *Curr Opin Hematol*. (2016) 23:410–5. doi: 10.1097/MOH.0000000000000262
115. Dudakov JA, Mertelsmann AM, O'Connor MH, Jenq RR, Velardi E, Young LF, et al. Loss of thymic innate lymphoid cells leads to impaired thymopoiesis in experimental graft-versus-host disease. *Blood*. (2017) 130:933–42. doi: 10.1182/blood-2017-01-762658
116. Komanduri KV. Innately interesting interactions. *Blood*. (2017) 130:844–5. doi: 10.1182/blood-2017-06-791848
117. Wang S, Xia P, Chen Y, Qu Y, Xiong Z, Ye B, et al. Regulatory innate lymphoid cells control innate intestinal inflammation. *Cell*. (2017) 171:201–16. doi: 10.1016/j.cell.2017.07.027

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 08 May 2019
doi: 10.3389/fimmu.2019.00988

The IL-12 Cytokine and Receptor Family in Graft-vs.-Host Disease

David Bastian[1], *Yongxia Wu*[1], *Brian C. Betts*[2] *and Xue-Zhong Yu*[1,3]*

[1] Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, United States, [2] Department of Medicine, University of Minnesota, Minneapolis, MN, United States, [3] Department of Medicine, Medical University of South Carolina, Charleston, SC, United States

Allogeneic hematopoietic cell transplantation (allo-HCT) is performed with curative intent for high- risk blood cancers and bone marrow failure syndromes; yet the development of acute and chronic graft-vs.-host disease (GVHD) remain preeminent causes of death and morbidity. The IL-12 family of cytokines is comprised of IL-12, IL-23, IL-27, IL-35, and IL-39. This family of cytokines is biologically distinct in that they are composed of functional heterodimers, which bind to cognate heterodimeric receptor chains expressed on T cells. Of these, IL-12 and IL-23 share a common β cytokine subunit, p40, as well as a receptor chain: IL-12Rβ1. IL-12 and IL-23 have been documented as proinflammatory mediators of GVHD, responsible for T helper 1 (Th1) differentiation and T helper 17 (Th17) stabilization, respectively. The role of IL-27 is less defined, seemingly immune suppressive via IL-10 secretion by Type 1 regulatory (Tr1) cells yet promoting inflammation through impairing $CD4^+$ T regulatory (Treg) development and/or enhancing Th1 differentiation. More recently, IL-35 was described as a potent anti-inflammatory agent produced by regulatory B and T cells. The role of the newest member, IL-39, has been implicated in proinflammatory B cell responses but has not been explored in the context of allo-HCT. This review is directed at discussing the current literature relevant to each IL-12-family cytokine and cognate receptor engagement, as well as the consequential downstream signaling implications, during GVHD pathogenesis. Additionally, we will provide an overview of translational strategies targeting the IL-12 family cytokines, their receptors, and subsequent signal transduction to control GVHD.

Keywords: GVHD, signal transduction, GVT, cytokine receptor, cytokine, IL-12 cytokines, IL-12 family cytokine receptors, HCT

OPEN ACCESS

Edited by:
Alok Srivastava, Christian Medical College and Hospital, India

Reviewed by:
Katrina Gee, Queen's University, Canada
Evren Alici, Karolinska Institute (KI), Sweden

***Correspondence:**
Xue-Zhong Yu
yux@musc.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *09 January 2019*
Accepted: *16 April 2019*
Published: *08 May 2019*

Citation:
Bastian D, Wu Y, Betts BC and Yu X-Z (2019) The IL-12 Cytokine and Receptor Family in Graft-vs.-Host Disease. Front. Immunol. 10:988. doi: 10.3389/fimmu.2019.00988

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic hematopoietic cell transplantation (allo-HCT) is performed with curative intent for high-risk blood cancers and bone marrow failure syndromes. The efficacy of allo-HCT lies in the ability of donor T cells to mediate a potent anti-tumor response in transplant recipients, known as the graft-vs.-tumor (GVT) effect, coupled with the benefit derived from pre-transplant conditioning (1, 2). The success of allo-HCT is compromised by the development of graft-vs.-host-disease (GVHD), a complication mediated by mature donor T cells present in the graft against normal host tissue. The incidence of acute GVHD (aGVHD), a significant cause of mortality after allo-HCT, has been significantly reduced over the past decade. Transplant-related mortality has declined

with the implementation of reduced intensity conditioning (RIC) regimens, new GVHD prophylaxis strategies, and the development of molecular methods aiding in early detection of viral and fungal infections in concert with modern anti-infectious agents (3–5). However, aGVHD still affects 20 to 70% of allo-HCT patients (6). Current clinical regimens for GVHD patients are primarily based on non-specific immunosuppressants for prophylaxis and treatment, such as calcineurin inhibitors or glucocorticosteroids, respectively (7). These broadly-acting agents fail to induce immune tolerance, increase susceptibility to opportunistic infections, and compromise GVT activity (8). Research in the field is focused on reducing GVHD without compromising the GVT effect. The current consensus on the initiation of GVHD pathophysiology can be divided into three primary phases:

1) ***Host tissue injury caused by conditioning regimens*** leads to the release of proinflammatory cytokines. Tissue damage from pre- transplant conditioning regimens results in a prolonged (up to 12 weeks post allo-HCT) increase of various cytokines; these include interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 21 (IL-21), interleukin 23 (IL-23), transforming growth factor β (TGFβ), and tumor necrosis factor α (TNFα) (9–11). These cytokines are primarily produced by activated dendritic cells (DCs) in response to tissue damage and microbe exposure, in concert with release of damage associated molecular patterns (DAMPs), including high mobility group protein B1 (HMGB-1) and adenosine triphosphate (ATP), as well as pathogen associated molecular patterns (PAMPs), which include lipopolysaccharide (LPS) and peptidoglycan. Both DAMPs and PAMPs can activate APCs, such as DCs and macrophages.
2) ***Donor T cell activation*** by activated APCs leads to differentiation into effector T cells, such as T helper type 1 (Th1) and T helper type 17 (Th17), both of which are pathogenic and associated with GVHD severity and mortality (12).
3) ***Effector T cell migration and target tissue destruction*** by activated donor T cells results in the initiation of GVHD (12, 13).

A myriad of cytokines, chemokines, receptors, and transcription factors are associated with T cell activation and associated inflammation, hence playing a central role in the development of GVHD. Classically, Th1 cells are believed to play a critical role in the induction of GVHD; although our group and others have demonstrated that Th17 cells also contribute (15). By targeting Th1 and Th17 specific transcription factors, T-box transcription factor TBX21 (T-bet) and Retinoic acid- related orphan receptor gamma (RORγt), respectively, it was observed that both Th1 and Th17 subsets contribute to GVHD development; yet either lineage alone is sufficient to induce GVHD (14, 15). Thus, both lineages must to be blocked in order to control GVHD. Efficacy of targeting these T cell differentiation pathways at the cytokine level are under investigation in clinical trials. Strategies for protecting/promoting prompt repair of target tissues may also reduce GVHD severity.

PATHOGENESIS OF ACUTE AND CHRONIC GVHD

Acute GVHD (aGVHD) is manifested by a strong inflammatory component resulting from robust donor T cell activation and expansion. Prior to transplant, conditioning regimens involving chemotherapy and/or irradiation cause damage to host epithelial tissues, and subsequent release of danger signals such as chemokines and cytokines. The inflammatory milieu is then amplified by an activated innate immune response, consisting of APCs, natural killer cells (NK cells), neutrophils, and macrophages (13). Donor CD4 and CD8 T cell recognition of major or minor histocompatibility antigens, directly or indirectly, by host and donor APCs in conjunction with activation of the innate immune response creates a "cytokine storm" consisting of such components as interferon gamma (IFNγ), TNFα, IL-6, IL-12, and IL-23, among others (8, 16). The aforementioned combination of inflammatory factors culminates in T cell infiltration and subsequent destruction of host tissues, namely the skin, lung, liver, and gastrointestinal tract (GI tract) (16–19).

Chronic GVHD (cGVHD) is widely systemic and can affect essentially any of the major organ systems (8, 20). While largely undefined, the origin of cGVHD pathogenesis has been linked to thymic damage caused by conditioning, resulting in aberrant selection and subsequent release of allo/autoreactive T cells (21). Older patients receiving RIC have also been observed with cGVHD, which is potentially due to reduced thymic reserve/function (22, 23). The activation of these T cells results in cytokine production and consequential activation of macrophages and fibroblasts. Chronically stimulated donor T cells interact with bone marrow-derived B cells and produce additional factors contributing to fibroblast proliferation and activation (21, 24). In particular, T follicular helper (Tfh) cells interact with B cells via CD40L-CD40 to promote B cell proliferation, differentiation, and antibody isotype switching (25). These Tfh-B cell interactions subsequently lead to germinal center formation in which antibody diversification and affinity maturation occur, ultimately leading to an adaptive immune response (21, 24, 25). The resultant autoantibody production and tissue fibrosis lead to end organ damage (26).

THE IL-12 FAMILY OF CYTOKINES AND THEIR RECEPTORS

The IL-12 family of cytokines can direct the donor immune response to execute a range of proinflammatory and immunosuppressive functions that are relevant in GVHD (**Figure 1**). They are primarily secreted by cells of myeloid origin in response to inflammatory stimuli, such as microbial products or fungal infections (49). While part of the type 1 hematopoietin family of cytokines, IL-12 family members are unique in that each member is comprised of two different subunits, or heterodimers, in which either the α or β subunit is shared among the others (46). The α-subunits include p19 (IL-23/IL-39), IL-27p28 (IL-27), and p35 (IL12/IL-35). The β-

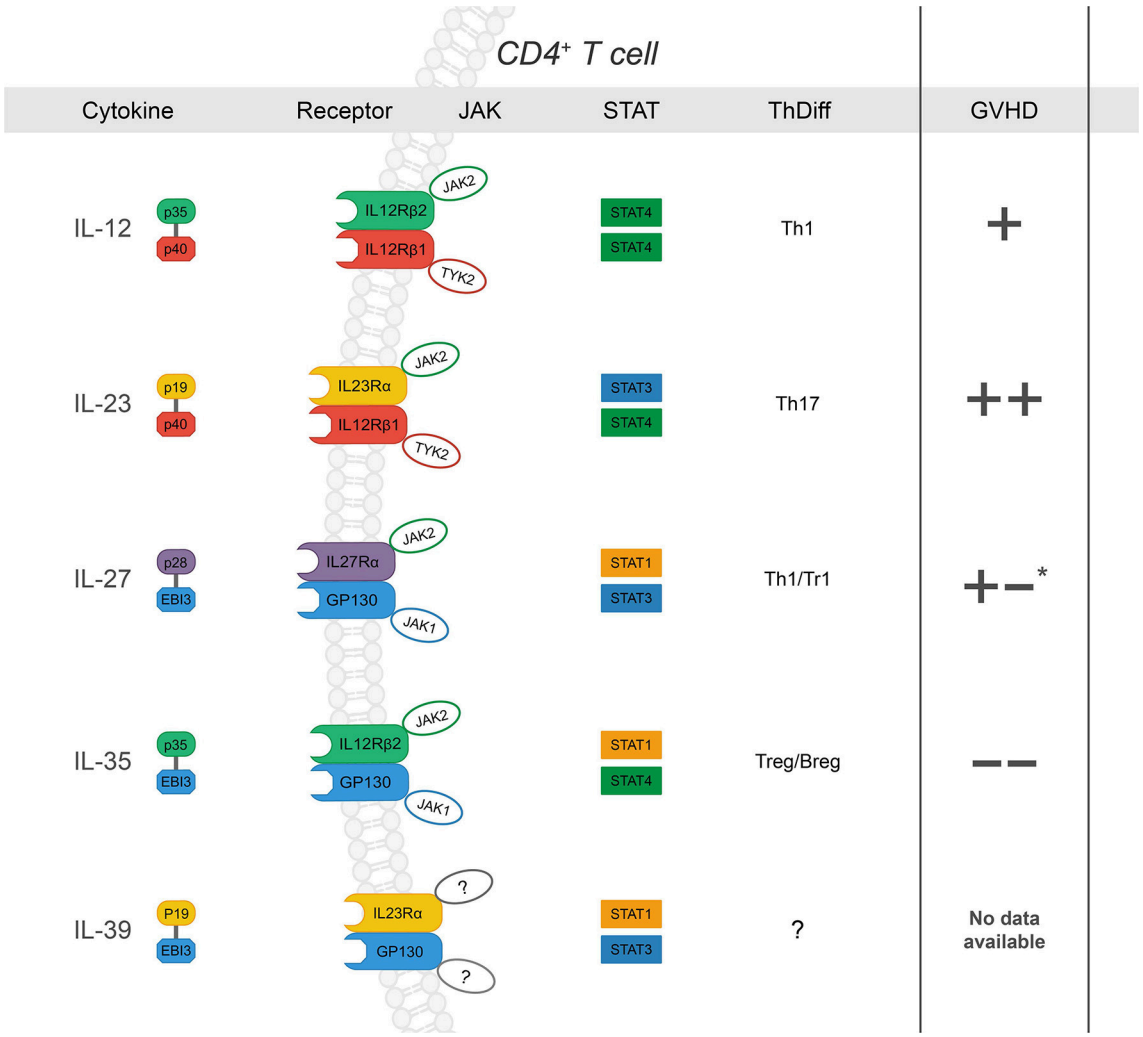


FIGURE 1 | The IL-12 family: cytokines, receptors, JAK-STAT signaling, influence on CD4$^+$ T cell differentiation and subsequent effect on GVHD severity after allo-HCT *("+" and "−" denote increase or decrease in GVHD severity, respectively. Listed from top to bottom)* IL-12 is a heterodimer composed of p35 and p40 (27). Upon ligation, IL-12 signals through IL-12Rβ1 and IL-12Rβ2, which form the receptor complex for IL-12 (IL-12R), subsequently leading to JAK2-STAT4 signal transduction and a positive feedback loop for Th1 differentiation (28–31). While its role in Th1 differentiation and IFNγ production has been shown to drive GVHD, there are conflicting reports concerning the specific requirement of IL-12 (32, 33); p35 can also associate with EBI3 to form IL-35, and p40 is the shared subunit with IL-23 (+). IL-23 is a heterodimer composed of p19 and p40 (34). IL-23Rα associates with JAK2 to induce primarily STAT3 phosphorylation but also STAT4 to a lesser degree (14, 35–37). IL-23 signaling results in stabilization cues for Th17 cells and has also been implicated exacerbation of intestinal GVHD (++) (38–42). IL-27, composed of p28 and EBI3, ligation to IL-27Rα/gp130 promotes IL-10 production by Tr1 cells via STAT1 at early time points post- BMT and plays a role in hindering GVHD- induced inflammation (43). However, IL-27 also inhibits Treg generation and may promote Th1 differentiation and function (+/-) (44, 45). IL-35 is composed of p35 and EBI3. IL-35 can signal through any combination of IL-12Rβ2 and gp130 was recently described as a potent immunoregulatory cytokine secreted by both T and B regulatory cells (46–48). IL-35 has been reported to suppress GVHD development. IL-39 is the most recent addition to the family and is composed of p19 and EBI3, which signal through STAT3 and STAT1. There are no reports of its function in the context of allo-HCT. *reported to promote GVHD through increased Th1 and decreased Treg differentiation; via dampen GVHD severity via Tr1 during induction phase.

subunits include p40 (IL-12/IL-23) and Epstein-Barr virus-induced gene 3 (EBI3) (IL-27/IL-35/IL-39) (50). Further, each cytokine signals through a distinct heterodimeric receptor that is associated with its cognate subunits: IL-12R (IL-12Rβ2/IL-12Rβ1), IL-23R (IL-23Rα/IL-12Rβ1), IL-27R (IL-27Rα/gp130), IL-39R (IL-23Rα/gp130), and IL-35R (IL-12Rβ2/gp130) (46, 50). The functionality of each respective cytokine and receptor combination ranges from proinflammatory to immune suppressive in a host of pathological and physiological conditions. Yet, similar subunits and receptors involved in proinflammatory functions can also form suppressive complexes, as in the case of IL-12Rβ2, involved in IL-12 and IL-35 signaling. Therefore, deciphering the contributions of each cytokine/receptor subunit combination is critical to understanding the immune response as a whole; the context of allo-HCT is no exception.

IL-12/IL-12R

Overview of IL-12/IL-12R Signaling

IL-12 consists of p35 and p40, and acts primarily on NK cells and T cells (23, 27). IL-12Rβ1 binds to IL-12Rβ2 to form the receptor for IL-12 (IL-12R) (27, 51). Upon ligation, IL-12Rβ1 binds to Tyrosine kinase 2 (Tyk2) while IL-12Rβ2 binds to Janus Kinase 2 (JAK2). Tyk2 and JAK2 then phosphorylate tyrosine residues primarily on signal transducer and activator of transcription 4 (STAT4). Ultimately, the STAT4 complex trans locates to the nucleus and binds to the IFNγ promoter; Jun oncogene (c-Jun) is also recruited to the IFNγ promoter via STAT4 (28, 29, 52), potentiating IFNγ transcription, and Th1 differentiation. In a STAT4 -dependent manner, IL-12 also promotes expression of Interferon regulatory factor 1 (IRF1) and 4 (IRF4), transcription factors required for Th1 differentiation (53, 54). Notwithstanding the contribution of IL-12 to Th1 differentiation, IL-12/IL-12R also promotes T-cell proliferation and adhesion during activation. It has been reported that IL-12 contributes to expression of Interleukin 2 receptor α (IL-2Rα) by recruiting STAT4 and c-Jun to the promoter of IL-2R, thereby enhancing T cell proliferation (55, 56). IL-12 -induced STAT4 activation also culminates in P-selectin ligand formation, which augments T cell adhesion during differentiation (57–60). Furthermore, activation of IL-12/IL-12R signaling induces both positive and negative feedback queues which can either strengthen or reduce IL-12 signaling, respectively. For instance, STAT4 activation fosters transcription of IL-12Rβ2 and Interleukin 18 receptor 1 (IL-18R1), which cooperate to amplify IL-12 signaling and Th1 cell differentiation. While IL-12R signaling can promote proliferation via STAT5-JAK2 interactions, evidence exists that STAT5A can suppress IL-12 -induced Th1 cell differentiation through the induction of Suppressor of cytokine signaling 3 (SOCS3) (35). However, this report demonstrated that SOCS3 activity inhibits IL-12 signaling by binding to the STAT4 docking site of the IL-12Rβ2 subunit (61, 62). Hence, IL-12 is predominately associated with Th1 differentiation, yet may simultaneously hinder this effect through mobilization of STAT5A depending on the context of disease or environment.

IL-12 in T Cell Responses and GVHD

IL-12 promotes the differentiation of primed $CD4^+$ T cells into Th1 cells, which express *Tbet*, produce IFNγ, and play a critical role in driving GVHD (15, 51). On the other hand, IL-12 negatively regulates T helper type 2 (Th2) transcription factors and associated cytokine production (63). As such, IL-12Rβ2 expression is absent on Th2 cells but upregulated in Th1; an increase in Th2 differentiation is associated with reduced acute GVHD yet can exacerbate chronic GVHD (64, 65). In addition, CD40-CD40L interactions between T cells and APCs can fuel IL-12 production by APCs, which amplify innate immune cell responses through IFNγ production (66). With regards to the IL-12 cytokine itself, the pool of available data is somewhat contradictory in the context of aGVHD. IL-12 has been reported to drive GVHD due to its stimulatory effect on Th1 cells (67, 68). IL-12 serum levels in aGVHD patients are increased compared to healthy controls, yet no correlation between higher grade GVHD (II-IV) and IL-12 has been observed (69) (**Table 1**). Conversely, exogenous IL-12 administration was suggested to be protective in GVHD via an IFNγ-dependent mechanism (72). Previous studies observed that a single injection of IL-12 at the time of allo-HCT stifles GVHD in myeloablative-conditioned recipients (32, 33, 72). The protective or pathogenic role of IL-12 seemingly relies on the dose and timing IL-12 injection, and irradiation type for the recipient conditioning regimen in murine BMT models (73). In NK cells, IL-12 induces cytotoxic events through STAT4 and subsequent activation of the Perforin 1 (perforin) gene promoter (74). A recent report a describes IL-12/IL-18 activated donor NK cells mitigate GVHD but enhance GVT activity (75).

TABLE 1 | Expression levels of IL-12 family cytokines in aGVHD patients.

aGVHD grade	0–1	2–4	References
IL-12	↑	↑	(69)
IL-23	↑	↑↑	(40)
IL-27	↓	↓	(70)
IL-35	↓	↓↓	(71)

Representative table of IL-12, IL-23, IL-27, and IL-35 levels detected in the serum of patients with aGVHD. Upward arrows indicate increases compared to healthy donors, while downward arrows indicate decreases.

Apart from advocating Th1 responses, IL-12 plays a critical role in T follicular helper cell (Tfh) differentiation and function through STAT4 and Tbet (76, 77). Consistent with the crucial role of Tfh cells in cGVHD pathogenesis, administration of anti-p40 mAb in recipient mice significantly reduced Tfh generation and scleroderma manifestations of cGVHD after allo-HCT[57]. Thus, targeting one or more of the IL-12 cytokine/receptor subunits represents a promising therapeutic strategy to reduce cGVHD.

IL-23/IL-23R

Overview of IL-23/IL-23R Signaling

IL-23 consists of p19 and p40. The IL-23R is a heterodimer comprised of IL-12Rβ1 and IL-23Rα. IL-23R associates with JAK2 and, in a ligand-dependent manner, with STAT3. IL-23- induced activation of STAT3 leads to direct binding of phosphorylated STAT3 to IL-17A and IL-17F promoters. STAT3 up-regulates the expression of RORγt, the master transcription factor of Th17, which is critical for the expression of two members of the Interleukin-17 family, IL-17A and IL-17F (78–80). SOCS3 inhibits JAK2 activity, hence decreasing IL-17A and IL-17F expression (78).

IL-23 signaling regulates Th17 cells. IL-23 plays an important role in expanding and maintaining the Th17 cell population, a T cell subset involved in homeostatic antimicrobial immune responses as well as in the propagation of many autoimmune diseases (81). IL-23 is an indispensable factor for promoting pathogenicity of Th17 cells, yet is not required for initial differentiation (82–85). IL-23 has been shown to control Th17 responses through regulating T cell metabolism. TCR stimulation induces GLUT-1 surface expression and subsequent lactate production, promoting glucose uptake (86, 87). T cells under Th17 polarizing conditions undergo a HIF1-α- dependent metabolic switch to glycolysis, and data indicates that IL-23 might contribute to this effect via PKM2 and HIF1-α (88). Notably,

allogeneic T cells were shown to depend on glycolysis for effector function during GVHD development, yet a connection to IL-23 and glycolysis has not been demonstrated. HIF1-α induction has also been associated with IL-23 production in dendritic cells; this link between HIF1-α and PKM2 has been previously established in cancer cells (89–91). Therefore, PKM2 may possess more than one function in addition to its role in glycolysis manifested by transcriptional activation as a protein kinase (92). Taken together, IL-23 signaling through PKM2/STAT3 may directly contribute to the metabolism of Th17 cells and, in concert with IL-6, could represent an essential factor for lineage commitment (93). Glucocorticoid-induced protein kinase 1 (SGK1) is critical for IL-23R expression through deactivating murine Foxo1, which directly represses IL-23R expression (94). SGK1 is essential for the induction of pathogenic Th17 cells and implicates environmental factors, such as a high-salt diet, as triggers to Th17 development and subsequent tissue inflammation (95). Lastly, while Blimp-1 IL-23-dependent Blimp-1 enhances Th17 pathogenic factors such as GM-CSF and IFNγ, and co-localizes with RORγt and STAT-3 at *Il23r, Il17a*, and *Csf2* enhancer sites (96).

IL-23 Signaling in Autoimmune Diseases

Studies show IL-23 signaling contributes to the pathogenesis of various autoimmune diseases. In mice, it was demonstrated that bacteria-driven innate colitis is associated with an increased production of IL-17A and IFNγ in the colon. Stimulation of intestinal leukocytes with IL-23 induced the production of IL-17 and IFNγ exclusively by innate lymphoid cells expressing IL-23R, which were demonstrated to accumulate in the inflamed colon. These results identified a previously unrecognized IL-23-responsive innate lymphoid population that mediates intestinal immune pathology and may therefore represent a target in inflammatory bowel disease (97–99). Intestinal IL-23-responsive innate cells are also a feature of T cell-dependent models of colitis, which resembles many of the features seen in intestinal GVHD with respect to T cell infiltration resulting in inflammation and gut injury. The transcription factor RORγt controls IL-23R expression, as it was shown that Rag/Rorc-null mice failed to develop innate colitis which is dependent on IL-23 (98). In addition, expression of IL-23 and IL-23R was increased in the tissues of patients with psoriasis (100). Injection of a neutralizing monoclonal antibody to IL-23p19 in a xenotransplant mouse model showed IL-23-dependent inhibition of psoriasis (100).

IL-23 Signaling in GVHD

Our group has demonstrated both Th1 and Th17 subsets are required to induce GVHD (15). Pharmacological inhibition of IL-23p19 results in reduced GVHD, and recent evidence suggests that IL-23R drives GVHD pathogenesis (38–41). These studies show that a $CD4^+CD11c^+IL\text{-}23R^+$ T cell population induces colonic inflammation during GVHD, indicating a key role for IL-23R expression on donor T cells in mediating damage to the gut after allo-HCT. Consistently, the gene expression levels of *IL-23* and *IL-23R* were upregulated in murine colons after allo-HCT (38). These studies demonstrate that the colon is specifically protected via IL-23p19 signaling blockade, and that GVL activity is maintained. In a patient cohort, Liu et al. observed IL-23 mRNA expressions in patients with aGVHD were significantly higher than those in healthy donors, and IL-23 and IL-23R expression were positively correlated with IL-17 expression (101). These studies additionally showed that IL-23 serum levels were elevated during the onset of aGVHD, yet decreased during disease remission (**Table 1**). In aGVHD, two out of three independent studies in patients found that a single nucleotide polymorphism (rs11209026) in IL-23R of donor origin reduced incidence of GVHD; the third study did not observe any effect (19, 102). Hence, blocking either p19 or p40 reduces aGVHD and IL-23R deficiency in donor T cells results in abrogated GVHD. These results indicate IL-23 also plays a key role in GVHD pathogenesis. Albeit, a recent paper demonstrated genetic inactivation of IL-23R, or the transcription factor RORγt, within donor T cells similarly ablated Th17 cell formation *in vivo* but preserved the T cells' ability to induce intestinal GVHD in an indistinguishable manner compared to wild-type controls (103).

Developing New Strategies to Target IL-23R

The crystal structure of IL-23Rα was recently reported (104). Hence, development of pharmaceutical compounds capable of specifically binding/inhibiting the IL-23R has been stagnant since its discovery in 2002. It appears that IL-23 binds IL-23R with an affinity of 44 nM, while binding IL-12Rβ1 with an affinity of 2 μM; nonetheless, the affinity of the IL-23:IL-23R complex for IL-12Rβ1 has been described as 25 nM, despite no apparent interaction of IL-23R with IL-12Rβ1, implying that there is a cooperative effect which is likely to be due to a conformational change of IL-23 upon binding IL-23R, which is indeed observed crystallographically (104–108). In a recent publication, hydrogen–deuterium exchange mass spectrometry (HDX-MS) was used to demonstrate IL-23 binding to the N-terminal immunoglobulin domain of IL-23R in both the solid state as well as under more physiologically relevant conditions. This data allowed specific identification of a binding epitope using a macrocyclic small molecule against IL-23R for the first time (106). However, IL-23R antagonism is not a new concept, as a peptide antagonist was shown to reduce inflammation in different models of autoimmune disease (109). The aforementioned data presents exciting new possibilities for future studies, yet efficacy of such prototype molecules requires vigorous testing in preclinical models.

Interplay Between IL-12 and IL-23

Recent findings have emphasized the need to develop therapeutics methods that enable targeting of IL-12 and IL-23 signaling. Interestingly, not only do the cytokines IL-12 and IL-23 share the same cytokine subunit, p40, but also the cognate receptor, IL-12Rβ1. Thus, these shared motifs provided the rationale for blocking Th1 and Th17 responses simultaneously through targeting p40/IL-12Rβ1. However, p40 itself has a diverse set of functions. For example, p40 has a chemo attractant role for macrophages mediated by IL-12Rβ1 alone, which is dependent on the intracellular domain of IL-12Rβ1 to signal; these reports were published with regard to IL-12Rβ1 signal transduction in response to a p40 homodimer (110, 111).

With respect to alloreactive T cells, this p40 homodimer was demonstrated to have antagonistic activity for $CD4^+$ IFNγ production, yet could amplify IFNγ production by $CD8^+$ T cells (112). Hence, targeting p40 in the context of GVHD may result in enhanced Th1 responses and potentially hinder CD8 mediated GVT responses (113). IL-12Rβ1 promiscuity among the IL-12 family has both assisted in the development of pharmaceuticals to target both pathways (as in the case of p40), yet also illuminated their complexity. Th1 and Th17 differentiation and stability converge at IL-12 and IL-23 signaling, respectively, as both signaling motifs share p40 at the cytokine level and IL-12Rβ1 for downstream signal transmission. Supported by studies done in mice and men, IL-12 is documented to induce IFNγ production by Th17 cells with respect to cytokines in the milieu, *in vivo* and *in vitro,* respectively (114, 115). This Th1/Th17 subset was shown in Crohns disease (114).

p40 and GVHD

Targeting p40, a shared subunit of IL-12 and IL-23 cytokines, consistently mitigates GVHD in clinical and preclinical studies. Our group and others have demonstrated that neutralization of the p40, using genetically deficient mice and pharmacological inhibition, alleviated acute and chronic GVHD in murine models through reducing Th1 and Th17 differentiation (116, 117). Recent data from Pidala et al. demonstrates *in vivo* IL-12/IL-23p40 neutralization with ustekinumab blocks the Th1/Th17 response and improves overall survival in patients after allo-HCT (118). Notably, in other models of autoimmunity, in which Th17 is the major mediator of diseases, much of the originally allocated inflammatory actions of IL-12 have since been shown to be influenced by IL-23, as many studies prior to IL-23 identification were conducted via targeting p40 (84, 119). Therefore, future studies should focus on the biological differences of IL-12 and IL-23 in order to determine why IL-12 can exacerbate GVHD in some contexts yet suppress it in others, yet pharmacologically targeting p40 can be efficacious in reducing GVHD severity in experimental and clinical settings.

IL-12Rβ1 Deficiency in Human Diseases

IL-12Rβ1 was identified in 1994 by Chua et al. as a member of the hemopoietin receptor superfamily, an amino acid type I transmembrane protein that resembled the IL-6 signal transducer, gp130 (120). It was not until 1996 that IL-12Rβ2 was identified, which subsequently led to the identification of a high affinity IL-12 receptor complex when IL-12Rβ1 and IL-12Rβ2 were coexpressed (27). Notably, the existing data with respect to IL-12Rβ1 in murine models of GVHD is sparse, although there is an abundance regarding its role in conferring immunity to mycobacteria and other infections (121–123) in human. However, given that deficiency of IL-12Rβ1 is relevant in patients, there are a plethora of case studies documenting related T cell responses (124). The IL12Rβ1 promoter, when deficient of the −265 to −104 region, suggested the existence of an important regulatory element. Furthermore, the −111A/T substitution appeared to cause decreased gene transcriptional activity, such that cells from−111A/A individuals were observed to have increased IL12Rβ1 mRNA levels compared with those from−111T allele carriers. Thus, in individuals with the−111T/T genotype, reduced IL12Rβ1 expression may lead to augmented Th2 cytokine production in the skin, and subsequently contribute to the development of atopic dermatitis and other associated allergic diseases (125).

Of particular interest is the role of IL-12/IL-12Rβ1 pathway in the induction of highly suppressive antigen-specific Th1-like Tregs from naïve Tregs (126). It was recently described that, in two patients with IL-12Rβ1 deficiency, features of systemic autoimmunity, and photosensitivity were observed (127). These features are similar to transgenic mice deficient for IL-12Rβ2, which develop an autoimmune syndrome consisting of anti-DNA positivity, immunocomplex glomerulonephritis, and multiorgan lymphoid infiltrates with features of vasculitis. However, IL-12Rβ1 deficient patients displayed substantially less circulating memory Tfh and memory B cells than healthy controls (77). In humans, TGFβ cooperates with IL-12 and IL-23 for expression of Tfh molecules: CXCR5, ICOS, IL-21, and the transcriptional regulator Bcl6 (128). Hence, data taken from studies in IL-12Rβ1 deficient patients suggests a regulatory role for IL-12, perhaps derived from Treg function, which may explain the contradictory results observed in murine models. Albeit, the role of IL-12 in Tfh/B cell axis seems at baseline consistent among experimental and clinical studies. While the aforementioned discrepancies are preliminary in comparison to the mass of studies documenting proinflammatory roles of IL-12, there is still much to learn in terms of IL-12 function; especially with respect to differences vs. IL-23.

IL-27/IL-27R

IL-27 is comprised of IL-27p28 and EBI3, binds to IL-27Rα/gp130, and is the only member of the family that is not secreted as a functional dimer (129). In fact, IL-27p28 is also known as IL-30. As such, the biological mechanisms associated with the role of IL-27 vs. IL-30 in the immune response are ambiguous, displaying both proinflammatory and suppressive functions that seem to be dependent on the disease model. IL-30 was previously reported to antagonize IL-27-mediated proinflammatory responses (130). Further, IL-30 inhibited activity by IL-6, IL-11, and IL-27 in the absence of EBI3 through gp130 binding (131). These findings support a role for IL-30 in hindering proinflammatory effects by such cytokines as IL-6. Yet, a recent report demonstrates that pharmacological blockade of IL-27p28 alleviates GVHD in mice, and resulted in augmented Treg responses (45). Consistently, our group found that IL-27Rα expression promotes T cell pathogenicity during GVHD induction, and was attributable to augmented Th1 effector function (44). However, a report by Zhang et al. elegantly demonstrated the function and prevalence of Tr1 early after allo-BMT; noting a significant role in IL-10 production which could ameliorate GVHD and which was dependent on IL-27 (43). Tr1 cells differentiate in the presence of IL-27 and are the central cell type implicated in IL-27- related suppressive activity, producing IFNγ, and IL-10 simultaneously. Of note, experiments performed in the aforementioned report depleted Tregs in donor grafts before transplantation, and therefore may be the reason they saw no difference compared to similar models

used in studies by Belle et al and those by our group. These studies can be connected in such a way that IL-27 promotes Tr1 cells early after BMT and can decrease GVHD independent of Tregs; yet in later stages IL-27 inhibits iTregs and Th2 cells and promotes Th1 differentiation; this ambiguous pattern is very similar to that seen in models of autoimmunity. Clinically, a study by Odile et al. demonstrated that membrane IL-27Rα existed in a soluble form (sIL-27Rα), functioning as a natural antagonist of IL-27, in healthy human serum, as well as in the serum of patients with Crohn's disease, suggesting that sIL-27Rα may play an immunoregulatory role in normal as well as pathological conditions (132). In extended studies by Liu et al. sIL-27Rα was identified as a potential biomarker for the development of aGVHD (70). However, IL-27 levels have only been shown to positively correlate with sIL-27R, which Liu et al. suggested was protective in GVHD. Higher levels of serum sIL-27Rα correlated with lower grade aGVHD, however, did not show any correlation for the prediction of cGVHD (70). In future investigations, studies should focus on how IL-27 blockade modulates established GVHD, which is characterized by Th1–mediated inflammation in the skin, gut, and liver. Additionally, it remains to be determined whether Treg cells deprived of Tbet induction by IL-27 will possess the transcriptional machinery sufficient to infiltrate into active GVHD sites. It will also be critical to determine the effects of IL-27 blockade on the GVT effect.

IL-35/IL-35R

IL-35 consists of p35 and EBI3, and functions as a regulatory cytokine released by $CD4^{+}Foxp3^{+}$ T regulatory cells (Tregs), as well as regulatory B cells (Bregs), to suppress inflammation, and subsequently reduce the severity of autoimmune diseases (50, 133). Interestingly, IL-35 signaling in Tregs was transduced via receptor combinations of IL-12Rβ2/gp130, IL-12Rβ2/IL-12Rβ2, or gp130/gp130, none of which could be clearly identified as the high affinity receptor (71). Currently, the suppressive effect of IL-35 in mouse models of aGVHD have been established by Zhang et al. (134) and Liu et al. (47). Importantly, IL-35 levels in the serum of aGVHD patients was significantly decreased in higher grade GVHD (II-IV) compared to lower grade (0-I) (47) (**Table 1**). Liu et al. (47) Collectively, these studies demonstrate that IL-35 is associated with higher frequencies of Tregs, reduced Th1 differentiation, reduced GVHD when combined with rapamycin, and evidence indicating maintenance of GVL activity (47, 134). However, IL-35 within the tumor microenvironment may oppose T cell responses required for GVT response by inducing effector exhaustion (135). Given the potential regulatory effects mediated by IL-35, we speculate the cytokine may be relevant in controlling cGVHD. Further, a recent publication by Yin et al. demonstrated that IL-35 administration skewed T cell differentiation from Th17 to Treg in islet cell transplantation models (136). IL-35 is clearly immunoregulatory and potentially useful in GVHD prevention, but a better understanding of its impact on T cell anti-tumor responses is needed prior to clinical translation.

IL-39/IL-39R

IL-39 has been proposed to consist of p19 and EBI3, and is the most recent addition to the family (137). Wang et al. published the first report describing the function of an additional heterodimer that involves p19 complexed with a subunit other than p40. IL-39 is secreted by activated B cells and was demonstrated to be significantly elevated in lupus models compared to other IL-12 members using MRL/lpr mice (137). The receptor for IL-39 was determined to be formed by dimerization of IL-23R and gp130 and signal through STAT1 and 3. While associated with neutrophil differentiation and expansion, the proinflammatory effects of IL-39 have yet to be fully defined. In a different report by Ramnath et al. IL-39 was shown to be secreted by keratinocytes and contribute to wound healing (138). While the function of IL-39 may be context dependent, these disparate reports indicate that IL-39 may also act on a broad range of cell types. Hence, further clarification regarding the general role of IL-39 in immunity is required in order to determine its effect in GVHD.

INTERPLAY BETWEEN IL-6 AND IL-12 SUPER FAMILIES

While promiscuity among IL-12 cytokine and/or receptor family is a common theme, the degree of association with glycoprotein 130 (gp130), better known for its role in IL-6 signaling, has become an intriguing area of research. Gp130 forms the link between "IL-6R/IL-12R" families, which collectively include Leukemia Inhibitory Factor Receptor (LIF-R), IL-12Rβ1, IL-12Rβ2, Granulocyte Colony-Stimulating Factor Receptor (GCSF-R), and Oncostatin-M Receptor (OSM-R); and serves as a shared signal-transducing subunit for IL-6, IL-11, Leukemia Inhibitory Factor (LIF), Oncostatin-M (OSM), Cilliary Neurotrophic Factor (CNTF), Cardiotrophin-1 (CT-1), Cardiotrophin-like cytokine (CLC), and IL-27(139, 140). Importantly, gp130 is well-documented for its capacity to transduce signals, especially for IL-6, a staple cytokine involved in inflammation. The complex of IL-6 and IL-6R binds to the ubiquitously expressed receptor subunit gp130, which forms a homodimer and thereby initiates intracellular signaling via the JAK/STAT and the MAPK pathways. IL-6R expressing cells can cleave the receptor protein to generate a soluble IL-6R (sIL-6R), which can still bind IL-6 and can associate with gp130 and induce signaling even on cells, which do not express IL-6R. This paradigm has been called IL-6 trans-signaling whereas signaling via the membrane bound IL-6R is referred to as classic signaling (139–141).

TRANSLATIONAL POTENTIAL FOR TARGETING THE IL-12/IL-12R FAMILY

Targeting IL-12 and IL-23 Cytokines

Regarding clinically translatable approaches targeting the IL-12 family, ustekinumab targets the p40 shared subunit between IL-23 and IL-12. Ustekinumab added to tacrolimus and rapamycin was shown to be safe and effective for GVHD prophylaxis after related or unrelated allo-HCT. In a randomized, blinded,

placebo-controlled study, ustekinumab significantly improved overall survival, and CRFS (Conditional Random Fields Score), a novel composite endpoint including moderate/severe cGVHD and relapse-free survival (118). Guselkumab and tildrakizumab, two monoclonal antibodies against p19, approved for treatment of plaque psoriasis (142, 143). However, these specific neutralizing antibodies against p19 have yet to be evaluated in GVHD patients. While inhibiting JAK2 signal transduction by IL-12 and IL-23 is a promising strategy, the question pertaining to how the shared or disparate receptors contribute to signal transduction and the consequential effect on T cell differentiation in allo-HCT remains unclear. The advancement of targeted pharmacological compounds specific for IL-12 or IL-23 signaling will be required to adequately dissect these scientific questions appropriately across species.

Targeting IL-12 Family and Infection

The use of any immunosuppressive agent carries the theoretical risk of impairing host defense responses to pathogens and/or decreased tumor surveillance. Relative risks of targeting IL-12 and/or IL-23 are well-documented with respect to potential risk of infections. When challenged with *Mycobacterium*, *Salmonella* or *Candida*, mice lacking IL-12p35, IL-12p19, and IL-12/23p40 have phenotypes that generally mirror what has been observed in humans. As mentioned earlier, studies of IL-12/23p40 and IL-12Rβ1 deficiencies indicate that human IL-12 and IL-23 are redundant in host defense to many pathogens. Importantly, allo-HCT recipients treated with ustekinumab did not experience any increase in opportunistic infections or reactivation of CMV, EBV, or HHV6 compared to the placebo arm (118).

DOWNSTREAM SIGNALING BY THE IL-12/IL-12R FAMILIES AND RELEVANT TRANSLATIONAL POTENTIAL

Jak2: The Center of IL-12/IL-12R Family Signaling

Both IL-12R and IL-23R have been demonstrated to signal via JAK2; JAK2 deficient donor T cells or JAK2 inhibition with pacritinib were demonstrated to significantly alleviate GVHD in murine models via spared Treg differentiation and reductions in Th1 and Th17 differentiation in in mouse and human T cells (144). This is consistent with reports describing a common reliance on JAK2 by both IL-12 and IL-23. A key difference in downstream signaling is that IL-12 phosphorylates primarily STAT4, while IL-23 mainly induces STAT3 phosphorylation. Betts et al. reported that at 20 days' post allo-HCT, pSTAT3 was significantly increased in $CD4^+$ T cells among patients who would later develop aGVHD; a signaling pathway known to directly drive the transcription of Th17 lineage-specific genes (14).

JAK2 signal transduction is implicated in human autoimmune syndromes and GVHD. IL-6, IL-12, and IL-23 mediate inflammation and activate T cells via JAK2 (14, 145–148). Blocking the IL-6 receptor with the monoclonal antibody tocilizumab has demonstrated efficacy in a phase II GVHD prevention trial (149). Tocilizumab, however, does not fully impair pathogenic Th1/Th17 responses (150), which may be attributed to the IL-12 and IL-23 receptor signaling-induced JAK2 activation to promote Th1 and Th17 differentiation, respectively. Neutralization of these p40 cytokines prevents GVHD in murine models, and may have activity in treating patients with steroid refractory GVHD (151).

JAK2 Inhibition in GVHD

JAK2 inhibition is an alternative approach to suppress IL-6 and p40 receptor signal transduction and induce durable tolerance to alloantigens. JAK2 inhibitors are clinically efficacious in myelofibrosis, a hematological disease often driven by constitutive JAK2 activation (152). The existing evidence regarding JAK2 as a therapeutic target for acute GVHD is primarily supported by observations using ruxolitinib, an equimolar inhibitor of JAK1 and JAK2 (153–156). Ruxolitinib has been previously demonstrated as efficacious in treating steroid-refractory GVHD, and is clearly immunosuppressive. In part, JAK1 mediates the biologic effects of common gamma chain cytokines, including IL-2 and IL-15. Ruxolitinib suppresses host-reactive T cells in mice and humans. Although not observed in murine transplant studies, ruxolitinib treatment reduces the quantity of Tregs as well as the beneficial effects of NK cells in myelofibrosis patients (157–159). Therefore, a JAK2 inhibitor has the potential to prevent GVHD without conceding JAK1-mediated functions provided by donor lymphocytes. Further research determining the differential effects of JAK1 and JAK2 is required to resolve these conundrums.

Given the recent discovery of IL-39 (p19/EBI3) and its cognate receptor (IL-23Ra/gp130), the question pertaining to the individual requirement for each particular cytokine/receptor complex becomes much more complex. IL-39R was shown to signal via STAT1/ STAT3 pathways, which overlaps with IL-27 and IL-23 signaling, respectively. The manner by which IL-39R and IL-23R on T cells may differentially or similarly impact the T cell response in allo-HCT requires further investigation.

CONCLUSION

The interplay between IL-6/IL-12 family members pertaining to T cell differentiation requires further investigation in the field of allo-HCT. Specific neutralizing antibodies against receptor subunits, such as IL-23Rα, are in development but have yet to be evaluated in preclinical models. While inhibiting JAK2 signal transduction by IL-12 and IL-23 is a promising strategy, the question pertaining to how the shared or disparate receptors contribute to signal transduction and the consequential effect on T cell differentiation in allo-HCT remains unclear. The advancement of targeted pharmacological compounds specific for IL-12 or IL-23 signaling will be required to adequately dissect these scientific questions appropriately across species.

AUTHOR CONTRIBUTIONS

DB wrote the manuscript. YW, X-ZY, and BB edited and revised the manuscript.

FUNDING

All extramural sources of funding received for the research are reported.

REFERENCES

1. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood.* (2008) 112:4371–83. doi: 10.1182/blood-2008-03-077974
2. Thomas ED, Lochte HL, Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med.* (1957) 257:491–6. doi: 10.1056/NEJM195709122571102
3. Cutler C, Logan B, Nakamura R, Johnston L, Choi S, Porter D, et al. Tacrolimus/sirolimus vs tacrolimus/methotrexate as GVHD prophylaxis after matched, related donor allogeneic HCT. *Blood.* (2014) 257:491–6. doi: 10.1182/blood-2014-04-567164
4. Ganguly S, Ross DB, Panoskaltsis-Mortari A, Kanakry CG, Blazar BR, Levy RB, et al. Donor CD4+Foxp3+regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood.* (2014) 124:2131–41. doi: 10.1182/blood-2013-10-525873
5. Scott BL, Pasquini MC, Logan BR, Wu J, Devine SM, Porter DL, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol.* (2017) 35:JCO.2016.70.709. doi: 10.1200/JCO.2016.70.7091
6. Pasquini, MC WZ, Pasquini M, Wang Z. Current use and outcome of hematopoietic stem cell transplantation. (2013) 23:1417–21. doi: 10.1016/j.bbmt.2017.05.035
7. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood.* (2006) 108:390–9. doi: 10.1182/blood-2006-01-0329
8. McDonald-Hyman C, Turka LA, Blazar BR. Advances and challenges in immunotherapy for solid organ and hematopoietic stem cell transplantation. *Sci Transl Med.* (2015) 7:280rv2. doi: 10.1126/scitranslmed.aaa6853
9. Dudakov JA, Hanash AM, van den Brink MRM. (2015). Interleukin-22: Immunobiology and Pathology. *Annu Rev Immunol.* doi: 10.1146/annurev-immunol-032414-112123
10. McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. *Nat Immunol.* (2009) 10:314–24.doi: 10.1038/ni.1698
11. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol.* (2011) 23:159–63. doi: 10.1093/intimm/dxr001
12. Longo DL, Zeiser R, Blazar BR. Acute graft-versus-host disease-biologic process, prevention, and therapy. *N Engl J Med.* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
13. Toubai T, Mathewson ND, Magenau J, Reddy P. Danger signals and graft-versus-host disease: current understanding and future perspectives. *Front Immunol.* (2016) 7:539. doi: 10.3389/fimmu.2016.00539
14. Betts BC, Sagatys EM, Veerapathran A, Lloyd MC, Beato F, Lawrence HR, et al. CD4 $^{+}$ T cell STAT3 phosphorylation precedes acute GVHD, and subsequent Th17 tissue invasion correlates with GVHD severity and therapeutic response. *J Leukoc Biol.* (2015) 97:807–819. doi: 10.1189/jlb.5A1114-532RR
15. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORγt in mice. *Blood.* (2011) 118:5011–20. doi: 10.1182/blood-2011-03-340315
16. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol.* (2007) 25:139–70. doi: 10.1146/annurev.immunol.25.022106.141606
17. Chen YB, McDonough S, Chen H, Kennedy J, Illiano C, Attar EC, et al. Expression of α4β7 integrin on memory CD8 + T cells at the presentation of acute intestinal GVHD. *Bone Marrow Transplant.* (2013) 48:598–603. doi: 10.1038/bmt.2012.191
18. Koyama M, Cheong M, Markey KA, Gartlan KH, Kuns RD, Locke KR, et al. Donor colonic CD103+ dendritic cells determine the severity of acute graft-versus-host disease. *J Exp Med.* (2015) 212:1303–21. doi: 10.1084/jem.20150329
19. Nguyen Y, Al-Lehibi A, Gorbe E, Li E, Haagenson M, Wang T, et al. Insufficient evidence for association of NOD2/CARD15 or other inflammatory bowel disease-associated markers on GVHD incidence or other adverse outcomes in T-replete, unrelated donor transplantation. *Blood.* (2010) 115:3625–3631. doi: 10.1182/blood-2009-09-243840
20. Lee SJ, Vogelsang G, Flowers MED. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* (2003) 9:215–33. doi: 10.1053/bbmt.2003.50026
21. Wu T, Young JS, Johnston H, Ni X, Deng R, Racine J, et al. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. *J Immunol.* (2013b) 191:488–99. doi: 10.4049/jimmunol.1300657
22. MacDonald KPA, Betts BC, Couriel D. Reprint of: emerging therapeutics for the control of chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* (2018) 24:S7–S14. doi: 10.1016/j.bbmt.2017.12.788
23. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med.* (2003) 198:1951–7. doi: 10.1084/jem.20030896
24. Deng R, Hurtz C, Song Q, Yue C, Xiao G, Yu H, et al. Extrafollicular CD4+T-B interactions are sufficient for inducing autoimmune-like chronic graft-versus-host disease. *Nat Commun.* (2017) 8:978. doi: 10.1038/s41467-017-00880-2
25. Jin H, Ni X, Deng R, Song Q, Young J, Cassady K, et al. Antibodies from donor B cells perpetuate cutaneous chronic graft-versus-host disease in mice. *Blood.* (2016) 127:2249–60. doi: 10.1182/blood-2015-09-668145
26. Du J, Paz K, Flynn R, Vulic A, Robinson TM, Lineburg KE, et al. Pirfenidone ameliorates murine chronic GVHD through inhibition of macrophage infiltration and TGF-β production. *Blood.* (2017) 129:2570–80. doi: 10.1182/blood-2017-01-758854
27. Presky DH, Yang H, Minetti LJ, Chua AO, Nabavi N, Wu CY, et al. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci USA.* (1996) 93:14002–7. doi: 10.1073/PNAS.93.24.14002
28. Jacobson NG, Szabo SJ, Weber-Nordt RM, Zhong Z, Schreiber RD, Darnell JE, et al. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J Exp Med.* (1995) 181:1755–62.
29. Kusaba H, Ghosh P, Derin R, Buchholz M, Sasaki C, Madara K, et al. Interleukin-12-induced interferon-gamma production by human peripheral blood T cells is regulated by mammalian target of rapamycin (mTOR). *J Biol Chem.* (2005) 280:1037–43 doi: 10.1074/jbc.M405204200
30. Bacon CM, Petricoin EF, Ortaldo JR, Rees RC, Larner AC, Johnston JA, et al. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. *Proc Natl Acad Sci USA.* (1995) 92:7307–11. doi: 10.1073/pnas.92.16.7307
31. Yao BB, Niu P, Surowy CS, Faltynek CR. Direct interaction of STAT4 with the IL-12 receptor. *Arch Biochem Biophys.* (1999) 368:147–55. doi: 10.1006/abbi.1999.1302
32. Dey BR, Yang YG, Szot GL, Pearson DA, Sykes M. Interleukin-12 inhibits graft-versus-host disease through an Fas-mediated mechanism associated with alterations in donor T-cell activation and expansion. *Blood.* (1998) 91:3315–22.
33. Sykes M, Szot GL, Nguyen PL, Pearson DA. *Interleukin-12 inhibits murine graft-versus-host disease. Blood.* (1995) 86:2429–38.

34. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* (2000) 13:715–25. doi: 10.1016/S1074-7613(00)00070-4
35. Ahn HJ, Tomura M, Yu WG, Iwasaki M, Park WR, Hamaoka T, et al. Requirement for distinct Janus kinases and STAT proteins in T cell proliferation versus IFN-gamma production following IL-12 stimulation. *J Immunol.* (1998) 161:5893–900.
36. Floss DM, Klocker T, Schroder J, Lamertz L, Mrotzek S, Strobl B, et al. Defining the functional binding sites of interleukin 12 receptor 1 and interleukin 23 receptor to Janus kinases. *Mol Biol Cell.* (2016) 27:2301–16. doi: 10.1091/mbc.E14-12-1645
37. Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S, et al. Jak2 is essential for signaling through a variety of cytokine receptors. *Cell.* (1998) 93:385–95. doi: 10.1016/S0092-8674(00)81167-8
38. Das R, Chen X, Komorowski R, Hessner MJ, Drobyski WR. Interleukin-23 secretion by donor antigen-presenting cells is critical for organ-specific pathology in graft-versus-host disease. *Blood.* (2009) 113:2352–62. doi: 10.1182/blood-2008-08-175448
39. Das R, Komorowski R, Hessner MJ, Subramanian H, Huettner CS, Cua D, et al. 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.* (2010) 115:5249–58. doi: 10.1182/blood-2009-11-255422
40. Thompson JS, Chu Y, Glass JF, Brown SA. Absence of IL-23p19 in donor allogeneic cells reduces mortality from acute GVHD. *Bone Marrow Transplant.* (2010) 45:712–22. doi: 10.1038/bmt.2009.215
41. Zhou V, Agle K, Chen X, Beres A, Komorowski R, Belle L, et al. A colitogenic memory CD4+T cell population mediates gastrointestinal graft-versus-host disease. *J Clin Invest.* (2016) 126:3541–55. doi: 10.1172/JCI80874
42. Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12R 1 and a novel cytokine receptor subunit, IL-23R. *J Immunol.* (2002) 168:5699–708. doi: 10.4049/jimmunol.168.11.5699
43. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A, et al. *Eomesodermin promotes the development of type 1 regulatory T (T R 1) cells. Sci Immunol.* (2017) 2:eaah7152. doi: 10.1126/sciimmunol.aah7152
44. Bastian D, Liu Y, Wu Y, Schutt S, Nguyen HD, Sofi MH, et al. IL-27 Receptor signaling on T cells augments GVHD severity through enhancing Th1 responses. *J Immunol Res Ther.* (2018) 2:151–157.
45. Belle L, Agle K, Zhou V, Yin-Yuan C, Komorowski R, Eastwood D, et al. Blockade of interleukin-27 signaling reduces GVHD in mice by augmenting Treg reconstitution and stabilizing Foxp3 expression. *Blood.* (2016) 128:2068–82. doi: 10.1182/blood-2016-02-698241
46. Vignali DAA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. *Nat Immunol.* (2012) 13:722–8. doi: 10.1038/ni.2366
47. Liu Y, Wu Y, Wang Y, Cai Y, Hu B, Bao G, et al. IL-35 mitigates murine acute graft-versus-host disease with retention of graft-versus-leukemia effects. *Leukemia.* (2015) 29:939–46. doi: 10.1038/leu.2014.310
48. Collison LW, Delgoffe GM, Guy CS, Vignali KM, Chaturvedi V, Fairweather D, et al. The composition and signaling of the IL-35 receptor are unconventional. *Nat Immunol.* (2012) 13:290–9. doi: 10.1038/ni.2227
49. Thompson A, Orr SJ. Emerging IL-12 family cytokines in the fight against fungal infections. *Cytokine.* (2018) 111:398–407. doi: 10.1016/j.cyto.2018.05.019
50. Garbers C, Hermanns HM, Schaper F, Müller-Newen G, Grötzinger J, Rose-John S, et al. Plasticity and cross-talk of Interleukin 6-type cytokines. *Cytokine Growth Factor Rev.* (2012) 23:85–97. doi: 10.1016/j.cytogfr.2012.04.001
51. Wu C-Y, Wang X, Gadina M, O'Shea JJ, Presky DH, Magram J. IL-12 Receptor 2 (IL-12R 2)-Deficient Mice Are Defective in IL-12-Mediated Signaling Despite the Presence of High Affinity IL-12 Binding Sites. *J Immunol.* (2000) 165:6221–8. doi: 10.4049/jimmunol.165.11.6221
52. Thierfelder WE, Van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature.* (1996) 382:171–4. doi: 10.1038/382171a0
53. Galon J, Sudarshan C, Ito S, Finbloom D, O'Shea JJ. IL-12 induces IFN regulating factor-1 (IRF-1) gene expression in human NK and T cells. *J Immunol.* (1999) 162:7256–62.
54. Lehtonen A, Lund R, Lahesmaa R, Julkunen I, Sareneva T, Matikainen S. IFN-α and IL-12 activate IFN regulatory factor 1 (IRF-1), IRF-4, and IRF-8 gene expression in human NK and T cells. *Cytokine.* (2003) 24:81–90. doi: 10.1016/j.cyto.2003.07.001
55. Foukas LC, Panayotou G, Shepherd PR. Direct interaction of major histocompatibility complex class II-derived peptides with class Ia phosphoinositide 3-kinase results in dose-dependent stimulatory effects. *J Biol Chem.* (2004) 279:7505–11. doi: 10.1074/jbc.M303999200
56. Yanagida T, Kato T, Igarashi O, Inoue T, Nariuchi H. Second signal activity of IL-12 on the proliferation and IL-2R expression of T helper cell-1 clone. *J Immunol.* (1994) 152:4919–28. doi: 10.4049/jimmunol.168.12.6506
57. Lim YC, Henault L, Wagers AJ, Kansas GS, Luscinskas FW, Lichtman AH. Expression of functional selectin ligands on Th cells is differentially regulated by IL-12 and IL-4. *J Immunol.* (1999) 162:3193–201.
58. Lim Y-C, Xie H, Come CE, Alexander SI, Grusby MJ, Lichtman AH, et al. IL-12, STAT4-dependent up-regulation of CD4+ T cell core 2−1,6-n-acetylglucosaminyltransferase, an enzyme essential for biosynthesis of P-selectin ligands. *J Immunol.* (2001) 167:4476–84. doi: 10.4049/jimmunol.167.8.4476
59. Underhill GH, Zisoulis DG, Kolli KP, Ellies LG, Marth JD, Kansas GS. A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. *Blood.* (2005) 106:3867–73. doi: 10.1182/blood-2005-03-0984
60. White SJ, Underhill GH, Kaplan MH, Kansas GS. Cutting edge: differential requirements for Stat4 in expression of glycosyltransferases responsible for selectin ligand formation in Th1 cells. *J Immunol.* (2001) 167:628–31. doi: 10.4049/jimmunol.167.2.628
61. Takatori H, Nakajima H, Kagami S, Hirose K, Suto A, Suzuki K, et al. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of suppressor of cytokine signaling 3 expression. *J Immunol.* (2005) 174:4105–12. doi: 10.4049/jimmunol.174.7.4105
62. Yamamoto K, Yamaguchi M, Miyasaka N, Miura O. SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor β2 subunit. *Biochem Biophys Res Commun.* (2003) 310:1188–93. doi: 10.1016/j.bbrc.2003.09.140
63. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, et al. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood.* (2009) 114:3101–12. doi: 10.1182/blood-2009-05-219402
64. Imanguli MM, Swaim WD, League SC, Gress RE, Pavletic SZ, Hakim FT. Increased T-bet + cytotoxic effectors and type i interferon-mediated processes in chronic graft-versus-host disease of the oral mucosa. *Blood.* (2009) 113:3620–30. doi: 10.1182/blood-2008-07-168351
65. Korholz D, Kunst D, Hempel L, Sohngen D, Heyll A, Bonig H, et al. Decreased interleukin 10 and increased interferon-gamma production in patients with chronic graft-versus-host disease after allogeneic bone marrow transplantation. *Bone Marrow Transpl.* (1997) 19:691–5. doi: 10.1038/sj.bmt.1700718
66. Rose-John S, Scheller J, Schaper F. "Family reunion" - A structured view on the composition of the receptor complexes of interleukin-6-type and interleukin-12-type cytokines. *Cytokine Growth Factor Rev.* (2015) 26:471–4. doi: 10.1016/j.cytogfr.2015.07.011
67. Williamson E, Garside P, Bradley J, a, Mowat, a M. IL-12 is a central mediator of acute graft-versus-host disease in mice. *J Immunol.* (1996) 157:689–99. doi: 10.4049/jimmunol.169.3.1535
68. Williamson E, Garside P, Bradley JA, More IA, Mowat AM. 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. *J Immunol.* (1997) 159:1208–15.
69. Reddy V, Winer AG, Eksioglu E, Meier-Kriesche HU, Schold JD, Wingard JR. Interleukin 12 is associated with reduced relapse without increased incidence of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2005) 11:1014–21. doi: 10.1016/j.bbmt.2005.08.032

70. Liu S, Han J, Gong H, Li Y, Bao X, Qi J, et al. Soluble interleukin-27 receptor alpha is a valuable prognostic biomarker for acute graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. *Sci Rep*. (2018) 8:10328. doi: 10.1038/s41598-018-28614-4
71. Wang R.-X, Yu C.-R, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev, Y. V, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med.* (2014) 20:633–41. doi: 10.1038/nm.3554
72. Yang YG, Dey B, Sergio JJ, Sykes M. Interleukin-12 prevents severe acute graft-versus-host disease (GVHD) and GVHD-associated immune dysfunction in a fully major histocompatibility complex haplotype-mismatched murine bone marrow transplantation model. *Transplantation.* (1997) 64:1343–52. doi: 10.1097/00007890-199711150-00018
73. Sykes M, Pearson DA, Taylor PA, Szot GL, Goldman SJ, Blazar BR. Dose and timing of interleukin (IL)-12 and timing and type of total-body irradiation: Effects on graft-vs.-host disease inhibition and toxicity of exogenous IL-12 in murine bone marrow transplant recipients. *Biol Blood Marrow Transplant.* (1999) 5:277–84. doi: 10.1016/S1083-8791(99)70002-9
74. Yamamoto K, Shibata F, Miyasaka N, Miura O. (2002). The human perforin gene is a direct target of STAT4 activated by IL-12 in NK cells. *Biochem. Biophys. Res. Commun.* doi: 10.1016/S0006-291X(02)02378-1
75. Song Y, Hu B, Liu Y, Jin Z, Zhang Y, Lin D, et al. IL-12/IL-18-preactivated donor NK cells enhance GVL effects and mitigate GvHD after allogeneic hematopoietic stem cell transplantation. *Eur J. Immunol.* (2018) 48:670–82. doi: 10.1002/eji.201747177
76. Linterman MA, Vinuesa CG. Signals that influence T follicular helper cell differentiation and function. *Semin Immunopathol.* (2010) 32:183–96. doi: 10.1007/s00281-009-0194-z
77. Schmitt N, Bustamante J, Bourdery L, Bentebibel SE, Boisson-Dupuis S, Hamlin F, et al. IL-12 receptor β1 deficiency alters *in vivo* T follicular helper cell response in humans. *Blood*. (2013) 121:3375–85. doi: 10.1182/blood-2012-08-448902
78. Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu B-M, Tato C, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci USA*. (2006) 103:8137–42. doi: 10.1073/pnas.0600666103
79. Laurence A, O'Shea JJ. TH-17 differentiation: of mice and men. *Nat Immunol.* (2007) 8:903–5. doi: 10.1038/ni0907-903
80. Yang XO, Panopoulos AD, Nurieva R, Seon HC, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem.* (2007) 282:9358–63. doi: 10.1074/jbc.C600321200
81. McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. *Immunity.* (2008) 28:445–53. doi: 10.1016/j.immuni.2008.03.001
82. Codarri L, Gyülvészii G, Tosevski V, Hesske L, Fontana A, Magnenat L, et al. RORγ3t drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol.* (2011) 12:560–7. doi: 10.1038/ni.2027
83. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of TH17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol.* (2011) 12:568–75. doi: 10.1038/ni.2031
84. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* (2005) 201:233–40. doi: 10.1084/jem.20041257
85. Meyer zu Horste G, Wu C, Wang C, Cong L, Pawlak M, Lee Y, et al. RBPJ controls development of pathogenic Th17 cells by regulating IL-23 receptor expression. *Cell Rep.* (2016) 16:392–404. doi: 10.1016/j.celrep.2016.05.088
86. Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, et al. The CD28 signaling pathway regulates glucose metabolism. *Immunity.* (2002)16:769–77. doi: 10.1016/S1074-7613(02)00323-0
87. Jacobs SR, Herman CE, MacIver NJ, Wofford JA, Wieman HL, Hammen JJ, et al. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol.* (2008) 180:4476–86. doi: 10.4049/jimmunol.180.7.4476
88. Hanahan D, Weinberg RA, Palazon A, Aragones J, Morales-Kastresana A, de Landazuri MO, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Immunol.* (2011) 208:1367–76. doi: 10.1084/jem.201 10278
89. Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell.* (2011) 145:732–44. doi: 10.1016/j.cell.2011.03.054
90. Peter K, Rehli M, Singer K, Renner-Sattler K, Kreutz M. Lactic acid delays the inflammatory response of human monocytes. *Biochem Biophys Res Commun.* (2015) 457:412–8. doi: 10.1016/j.bbrc.2015.01.005
91. Semenza GL. Regulation of metabolism by hypoxia-inducible factor 1. *Cold Spring Harb. Symp Quant Biol.* (2011) 76:347–53. doi: 10.1101/sqb.2011.76.010678
92. Gao X, Wang H, Yang JJ, Liu X, Liu ZR. Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol Cell*. (2012) 45:598–609. doi: 10.1016/j.molcel.2012.01.001
93. Lochmatter C, Fischer R, Charles PD, Yu Z, Powrie F, Kessler BM. Integrative phosphoproteomics links IL-23R signaling with metabolic adaptation in lymphocytes. *Sci Rep.* (2016) 6:24491. doi: 10.1038/srep24491
94. Kleinewietfeld M, Manzel A, Titze J, Kvakan H, Yosef N, Linker RA, et al. Sodium chloride drives autoimmune disease by the induction of pathogenic TH 17 cells. *Nature.* (2013) 496:518–22. doi: 10.1038/nature11868
95. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH 17 cells by inducible salt-sensing kinase SGK1. *Nature.* (2013) 496:513–7. doi: 10.1038/nature11984
96. Jain R, Chen Y, Kanno Y, Joyce-Shaikh B, Vahedi G, Hirahara K, et al. Interleukin-23-induced transcription factor blimp-1 promotes pathogenicity of T helper 17 cells. *Immunity.* (2016) 44:131–142. doi: 10.1016/j.immuni.2015.11.009
97. Ahern PP, Schiering C, Buonocore S, McGeachy MJ, Cua DJ, Maloy KJ, et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity.* (2010) 33:279–88. doi: 10.1016/j.immuni.2010.08.010
98. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature.* (2010) 464:1371–5. doi: 10.1038/nature08949
99. Hue S, Ahern P, Buonocore S, Kullberg MC, Cua DJ, McKenzie BS, et al. Interleukin-23 drives innate and T cell–mediated intestinal inflammation. *J Exp Med.* (2006) 203:2473–83. doi: 10.1084/jem.20061099
100. Tonel G, Conrad C, Laggner U, Di Meglio P, Grys K, McClanahan TK, et al. Cutting edge: a critical functional role for IL-23 in psoriasis. *J Immunol.* (2010) 185:5688–91. doi: 10.4049/jimmunol.1001538
101. Liu Y, Cai Y, Dai L, Chen G, Ma X, Wang Y, et al. The expression of Th17-associated cytokines in human acute graft-versus-host disease. *Biol Blood Marrow Transplant.* (2013) 19:1421–9. doi: 10.1016/j.bbmt.2013.06.013
102. Elmaagacli AH, Koldehoff M, Landt O, Beelen DW. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell transplantation. *Bone Marrow Transplant.* (2008) 41:821–6. doi: 10.1038/sj.bmt.1705980
103. Buchele V, Abendroth B, Büttner-Herold M, Vogler T, Rothamer J, Ghimire S, et al. Targeting inflammatory T helper cells via retinoic acid-related orphan receptor gamma t is ineffective to prevent allo-response-driven colitis. *Front Immunol.* (2018) 9:1138. doi: 10.3389/fimmu.2018.01138
104. Bloch Y, Bouchareychas L, Merceron R, Składanowska K, Van den Bossche L, Detry S, et al. Structural activation of pro-inflammatory human cytokine IL-23 by cognate IL-23 receptor enables recruitment of the shared receptor IL-12Rβ1. *Immunity*. (2018) 48:45–58.e6. doi: 10.1016/j.immuni.2017.12.008
105. Desmet J, Verstraete K, Bloch Y, Lorent E, Wen Y, Devreese B, et al. Structural basis of IL-23 antagonism by an Alphabody protein scaffold. *Nat Commun.* (2014) 5:5237. doi: 10.1038/ncomms6237
106. Sayago C, Gonzalez Valcarcel IC, Qian Y, Lee J, Alsina-Fernandez J, Fite NC, et al. Deciphering binding interactions of IL-23R with HDX-MS: mapping protein and macrocyclic dodecapeptide ligands. *ACS Med Chem Lett.* (2018) 9:912–6. doi: 10.1021/acsmedchemlett.8b00255
107. Schröder J, Moll JM, Baran P, Grötzinger J, Scheller J, Floss DM. Non-canonical interleukin 23 receptor complex assembly: P40 protein recruits interleukin 12 receptor β1 via site II and induces P19/interleukin 23 receptor interaction via site III. *J Biol Chem.* (2015) 290:359–70. doi: 10.1074/jbc.M114.617597
108. Yoon C, Johnston SC, Tang J, Stahl M, Tobin JF, Somers WS. Charged residues dominate a unique interlocking topography in the heterodimeric cytokine interleukin-12. *EMBO J.* (2000) 19:3530–41. doi: 10.1093/emboj/19.14.3530

109. Quiniou C, Dominguez-Punaro M, Cloutier F, Erfani A, Ennaciri J, Sivanesan D, et al. Specific targeting of the IL-23 receptor, using a novel small peptide noncompetitive antagonist, decreases the inflammatory response. *AJP Regul Integr Comp Physiol.* (2014) 307:R1216–30. doi: 10.1152/ajpregu.00540.2013
110. Heinzel FP, Hujer AM, Ahmed FN, Rerko RM. *In vivo* production and function of IL-12 p40 homodimers. *J Immunol.* (1997) 158:4381–8.
111. Russell TD, Yan Q, Fan G, Khalifah AP, Bishop DK, Brody SL, et al. IL-12 p40 homodimer-dependent macrophage chemotaxis and respiratory viral inflammation are mediated through IL-12 receptor beta 1. *J Immunol.* (2003) 171:6866–74.
112. Piccotti JR, Chan SY, Li K, Eichwald EJ, Bishop DK. Differential effects of IL-12 receptor blockade with IL-12 p40 homodimer on the induction of CD4+ and CD8+ IFN-gamma-producing cells. *J Immunol.* (1997) 158:643–8.
113. Gillessen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, et al. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur J Immunol.* (1995) 25:200–6. doi: 10.1002/eji.1830250133
114. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) 204:1849–61. doi: 10.1084/jem.20070663
115. Lexberg MH, Taubner A, Förster A, Albrecht I, Richter A, Kamradt T, et al. Th memory for interleukin-17 expression is stable *in vivo*. *Eur J Immunol.* (2008) 38:2654–64. doi: 10.1002/eji.200838541
116. Okamoto S, Fujiwara H, Nishimori H, Matsuoka K, Fujii N, Kondo E, et al. Anti–IL-12/23 p40 antibody attenuates experimental chronic graft-versus-host disease via suppression of IFN-γ/IL-17–producing cells. *J Immunol.* (2015) *194:1357–63.* doi: 10.4049/jimmunol.1400973
117. Wu Y, Bastian D, Schutt S, Nguyen H, Fu J, Heinrichs J, et al. Essential role of interleukin-12/23p40 in the development of graft-versus-host disease in mice. *Biol. Blood Marrow Transplant.* (2015) 21:1195–204. doi: 10.1016/j.bbmt.2015.03.016
118. Pidala J, Beato F, Kim J, Betts B, Jim H, Sagatys E, et al. *In vivo* IL-12/IL-23p40 neutralization blocks Th1/Th17 response after allogeneic hematopoietic cell transplantation. *Haematologica.* (2018) 103:531–9. doi: 10.3324/haematol.2017.171199
119. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature.* (2003) 421:744–8. doi: 10.1038/nature01355
120. Chua A, Presky DH, Cubler U. *Cloning and characterization of a mouse IL-12 receptor-beta component*. *J Immunol.* (1995) 55:4286–94.
121. Altare F, Durandy A, Lammas D, Emile JF, Lamhamedi S, Le Deist F, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science.* (1998) 280:1432–5. doi: 10.1126/science.280.5368.1432
122. De Jong R, Altare F, Haagen IA, Elferink DG, De Boer T, Van Breda Vriesman PJC, et al. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science.* (1998) 280:1435–8. doi: 10.1126/science.280.5368.1435
123. Losana G, Rigamonti L, Borghi I, Assenzio B, Ariotti S, Jouanguy E, et al. Requirement for both IL-12 and IFN-γ signaling pathways in optimal IFN-γ production by human T cells. *Eur J Immunol.* (2002) 32:693–700. doi: 10.1002/1521-4141(200203)32:3<693::AID-IMMU693>3.0.CO;2-Q
124. Fieschi C, Casanova JL. The role of interleukin-12 in human infectious diseases: Only a faint signature. *Eur J Immunol.* (2003) 33:1461–4. doi: 10.1002/eji.200324038
125. Takahashi N, Akahoshi M, Matsuda A, Ebe K, Inomata N, Obara K, et al. Association of the IL12RB1 promoter polymorphisms with increased risk of atopic dermatitis and other allergic phenotypes. *Hum Mol Genet.* (2005) 14:3149–59. doi: 10.1093/hmg/ddi347
126. Koch MA, Thomas KR, Perdue NR, Smigiel KS, Srivastava S, Campbell DJ. T-bet+Treg Cells Undergo Abortive Th1 Cell Differentiation due to Impaired Expression of IL-12 Receptor β2. *Immunity.* (2012) 37:501–10. doi: 10.1016/j.immuni.2012.05.031
127. Ling G, Ling E, Broides A, Poran Feldman H, Levy J, Garty BZ, et al. IL-12 receptor 1β deficiency with features of autoimmunity and photosensitivity. *Autoimmunity.* (2016) 49:143–6. doi: 10.3109/08916934.2015.1134513
128. Schmitt N, Liu Y, Bentebibel SE, Munagala I, Bourdery L, Venuprasad K, et al. The cytokine TGF-β 2 co-opts signaling via STAT3-STAT4 to promote the differentiation of human TFHcells. *Nat Immunol.* (2014) 15:856–65. doi: 10.1038/ni.2947
129. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+T cells. *Immunity.* (2002) 16:779–90. doi: 10.1016/S1074-7613(02)00324-2
130. Shimozato O, Sato A, Kawamura K, Chiyo M, Ma G, Li Q, et al. The secreted form of p28 subunit of interleukin (IL)-27 inhibits biological functions of IL-27 and suppresses anti-allogeneic immune responses. *Immunology.* (2009) 128(1 Suppl.):e816–25. doi: 10.1111/j.1365-2567.2009.03088.x
131. Stumhofer JS, Tait ED, Iii WJQ, Hosken N, Spudy B, Goenka R, et al. A role for IL-27p28 as an antagonist of gp130-mediated signaling. *Nat Immunol.* (2010) 11:1119–26. doi: 10.1038/ni.1957
132. Dietrich C, Candon S, Ruemmele FM, Devergne O. A soluble form of IL-27R is a natural IL-27 antagonist. *J Immunol.* (2014) 192:5382–9. doi: 10.4049/jimmunol.1303435
133. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature.* (2014) 507:366–70. doi: 10.1038/nature12979
134. Zhang X.-H, Zhou Y, Zhou S, Feng F, Wang Q, Han W, et al. IL-35 and rapamycin reduce acute graft-versus-host disease associated with decreased platelet aggregation in a mouse model. *Blood.* (2014) 124:3814.
135. Turnis ME, Sawant DV, Szymczak-Workman AL, Andrews LP, Delgoffe GM, Yano H, et al. Interleukin-35 limits anti-tumor immunity. *Immunity.* (2016) 44:316–29. doi: 10.1016/j.immuni.2016.01.013
136. Zongyi Y, Funian Z, Hao L, Xin W, Ying C, Jialin Z, et al. Interleukin-35 mitigates the function of murine transplanted islet cells via regulation of Treg/Th17 ratio. *PLoS ONE.* (2017) 12:e0189617. doi: 10.1371/journal.pone.0189617
137. Wang X, Wei Y, Xiao H, Liu X, Zhang Y, Han G, et al. A novel IL-23p19/Ebi3 (IL-39) cytokine mediates inflammation in Lupus-like mice. *Eur J Immunol.* (2016) 46:1343–50. doi: 10.1002/eji.201546095
138. Ramnath D, Tunny K, Hohenhaus DM, Pitts CM, Bergot AS, Hogarth PM, et al. TLR3 drives IRF6-dependent IL-23p19 expression and p19/EBI3 heterodimer formation in keratinocytes. *Immunol Cell Biol.* (2015) 93:771–9. doi: 10.1038/icb.2015.77
139. Silver JS, Hunter CA. gp130 at the nexus of inflammation, autoimmunity, and cancer. *J Leukoc Biol.* (2010) 88:1145–56. doi: 10.1189/jlb.0410217
140. Wolf J, Rose-John S, Garbers C. Interleukin-6 and its receptors: a highly regulated and dynamic system. *Cytokine.* (2014) 70:11–20. doi: 10.1016/j.cyto.2014.05.024
141. Wang X, Lupardus P, LaPorte SL, Garcia KC. Structural biology of shared cytokine receptors. *Annu Rev Immunol.* (2009) 27:29–60. doi: 10.1146/annurev.immunol.24.021605.090616
142. Blauvelt A, Reich K, Mehlis S, Vanaclocha F, Sofen H, Abramovits W, et al. Secukinumab demonstrates greater sustained improvements in daily activities and personal relationships than ustekinumab in patients with moderate-to-severe plaque psoriasis: 52-week results from the CLEAR study. *J Eur Acad Dermatol Venereol.* (2017) 31:1693–9. doi: 10.1111/jdv.14391
143. Reich K, Papp KA, Blauvelt A, Tyring SK, Sinclair R, Thaçi D, et al. Tildrakizumab versus placebo or etanercept for chronic plaque psoriasis (reSURFACE 1 and reSURFACE 2): results from two randomised controlled, phase 3 trials. *Lancet.* (2017) 390:276–88. doi: 10.1016/S0140-6736(17)31279-5
144. Betts BC, Bastian D, Iamsawat S, Nguyen H, Heinrichs JL, Wu Y, et al. Targeting JAK2 reduces GVHD and xenograft rejection through regulation of T cell differentiation. *Proc Natl Acad Sci USA.* (2018) 115:1582–7. doi: 10.1073/pnas.1712452115
145. Betts BC, Abdel-Wahab O, Curran SA, St Angelo ET, Koppikar P, Heller G, et al. Janus kinase-2 inhibition induces durable tolerance to alloantigen by human dendritic cell-stimulated T cells yet preserves immunity to recall antigen. *Blood.* (2011a) 118:5330–9. doi: 10.1182/blood-2011-06-363408
146. Spoerl S, Mathew NR, Bscheider M, Schmitt-Graeff A, Chen S, Mueller T, et al. Activity of therapeutic JAK 1/2 blockade in graft-versus-host disease. *Blood.* (2014) 123:3832–42. doi: 10.1182/blood-2013-12-543736

147. Steward-Tharp SM, Laurence A, Kanno Y, Kotlyar A, Villarino AV, Sciume G, et al. A mouse model of HIES reveals pro- And anti-inflammatory functions of STAT3. *Blood.* (2014) 123:2978–87. doi: 10.1182/blood-2013-09-523167
148. Teng MWL, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, et al. IL-12 and IL-23 cytokines: From discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med.* (2015) 21:719–29. doi: 10.1038/nm.3895
149. Kennedy GA, Varelias A, Vuckovic S, Le Texier L, Gartlan KH, Zhang P, et al. Addition of interleukin-6 inhibition with tocilizumab to standard graft-versus-host disease prophylaxis after allogeneic stem-cell transplantation: a phase 1/2 trial. *Lancet Oncol.* (2014) 15:1451–9. doi: 10.1016/S1470-2045(14)71017-4
150. Betts BC, St Angelo ET, Kennedy M, Young JW. Anti-IL6-receptor-alpha (tocilizumab) does not inhibit human monocyte-derived dendritic cell maturation or alloreactive T-cell responses. *Blood.* (2011b) 118:5340–3. doi: 10.1182/blood-2011-06-363390
151. Pidala J, Perez L, Beato F, Anasetti C. Ustekinumab demonstrates activity in glucocorticoid-refractory acute GVHD. *Bone Marrow Transplant.* (2011) 47:747. doi: 10.1038/bmt.2011.172
152. Harrison C, Kiladjian, J.-J, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V, et al. JAK inhibition with Ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med.* (2012) 366:787–98. doi: 10.1056/NEJMoa1110556
153. Carniti C, Gimondi S, Vendramin A, Recordati C, Confalonieri D, Bermema A, et al. Pharmacologic inhibition of JAK1/JAK2 signaling reduces Experimental murine acute GVHD while preserving GVT effects. *Clin Cancer Res.* (2015) 21:3740–9. doi: 10.1158/1078-0432.CCR-14-2758
154. Choi J, Ziga ED, Ritchey J, Collins L, Prior JL, Cooper ML, et al. IFNγR signaling mediates alloreactive T-cell trafficking and GVHD. *Blood.* (2012) 120:4093–103. doi: 10.1182/blood-2012-01-403196
155. Choi J, Cooper ML, Alahmari B, Ritchey J, Collins L, Holt M, et al. Pharmacologic blockade of JAK1/JAK2 reduces GvHD and preserves the graft-versus-leukemia effect. *PLoS ONE.* (2014) 9:e109799. doi: 10.1371/journal.pone.0109799
156. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in corticosteroid-refractory graft-versus-host disease after allogeneic stem cell transplantation: a multicenter survey. *Leukemia.* (2015) 29:2062–8. doi: 10.1038/leu.2015.212
157. Curran SA, Shyer JA, St. Angelo ET, Talbot LR, Sharma S, Chung DJ, et al. Human Dendritic cells mitigate NK-cell dysfunction mediated by nonselective JAK1/2 blockade. *Cancer Immunol Res.* (2017) 5:52–60. doi: 10.4102/sajhrm.v12i1.650
158. Parampalli Yajnanarayana S, Stübig T, Cornez I, Alchalby H, Schönberg K, Rudolph J, et al. JAK1/2 inhibition impairs T cell function *in vitro* and in patients with myeloproliferative neoplasms. *Br J Haematol.* (2015) 169:824–33. doi: 10.1111/bjh.13373
159. Schönberg K, Rudolph J, Vonnahme M, Yajnanarayana SP, Cornez I, Hejazi M, et al. JAK inhibition impairs NK cell function in myeloproliferative neoplasms. *Cancer Res.* (2015) 75:2187–99. doi: 10.1158/0008-5472.CAN-14-3198

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fimmu.2019.00624

Donor Allospecific CD44^{high} Central Memory T Cells Have Decreased Ability to Mediate Graft-vs.-Host Disease

Wei Huang[1], *Wenjian Mo*[1,2], *Jieling Jiang*[1,3], *Nelson J. Chao*[1,4] *and Benny J. Chen*[1,4*]

[1] *Division of Hematologic Malignancies and Cellular Therapy, Duke University Medical Center, Durham, NC, United States,* [2] *Department of Hematology, School of Medicine, Guangzhou First People's Hospital, South China University of Technology, Guangzhou, China,* [3] *Department of Hematology, Shanghai General Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, China,* [4] *Duke Cancer Institute, Duke University Medical Center, Durham, NC, United States*

Data from both animal models and humans have demonstrated that effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) from unprimed donors have decreased ability to induce graft-vs-host disease (GVHD). Allospecific T_{EM} from primed donors do not mediate GVHD. However, the potential of alloreactive T_{CM} to induce GVHD is not clear. In this study, we sought to answer this question using a novel GVHD model induced by T cell receptor (TCR) transgenic OT-II T cells. Separated from OT-II mice immunized with OVA protein 8 weeks earlier, the allospecific CD44^{high} T_{CM} were able to mediate skin graft rejection after transfer to naive mice, yet had dramatically decreased ability to induce GVHD. We also found that these allospecific CD44^{high} T_{CM} persisted in GVHD target organs for more than 30 days post-transplantation, while the expansion of these cells was dramatically decreased during GVHD, suggesting an anergic or exhausted state. These observations provide insights into how allospecific CD4$^+$ T_{CM} respond to alloantigen during GVHD and underscore the fundamental difference of alloresponses mediated by allospecific T_{CM} in graft rejection and GVHD settings.

Keywords: alloreactive memory T cells, T_{CM}, GVHD, skin graft rejection, OT-II, OVA

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina, United States

Reviewed by:
Xiao Chen,
Medical College of Wisconsin, United States
Tomomi Toubai,
Yamagata University, Japan
Defu Zeng,
City of Hope, United States

****Correspondence:***
Benny J. Chen
chen0032@mc.duke.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *02 October 2018*
Accepted: *08 March 2019*
Published: *02 April 2019*

Citation:
Huang W, Mo W, Jiang J, Chao NJ and Chen BJ (2019) Donor Allospecific CD44^{high} Central Memory T Cells Have Decreased Ability to Mediate Graft-vs.-Host Disease. Front. Immunol. 10:624. doi: 10.3389/fimmu.2019.00624

INTRODUCTION

Graft-vs.-host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation caused by alloreactive donor T cells (1). After bone marrow transplantation, the alloreactive donor T cells recognize the alloantigens presented by MHC in the recipients, and initiate the pathogenesis of GVHD. The contribution of different subsets of donor T cells to GVHD is different (2). T cells can be further separated into naive and memory T cells according to the expression of the cell-trafficking molecule CD62L and T cells activation molecule CD44. It has been proven that naive T cells, with the phenotype CD62L$^+$ CD44$^-$, have the strongest ability to induce vigorous GVHD in MHC-mismatch murine models. On the contrary, the memory T cells, including effector memory T cells (T_{EM}, CD62L$^-$ CD44$^+$) and central memory T cells (T_{CM}, CD62L$^+$ CD44$^+$) from either untreated or allo-antigen primed donors, do not cause GVHD or cause only minor GVHD after transplantation (3–6). Specifically, we have previously identified a population of T_{CM} that express high level of CD44 do not induce GVHD (5).

It has previously been reported that common virus specific memory T cells including EBV-specific and CMV-specific memory T cells do not GVHD in humans (7–11). However, since alloreactive memory T cells can be generated either by cross-reaction or allospecific memory reaction, it is important to further understand the biology and pathogenesis of the true allospecific memory T cells in GVHD. In the previous research, we used an antigen-specific murine model to study allospecific T_{EM} in GVHD (12). By transferring the naive TEa cells into Rag-$1^{-/-}$ mice following by *in vivo* priming with splenocytes from CB6F1 ($H2^b$/I-E^+ strain), T_{EM} cells from the primed animals maintained the memory function to mediate skin graft rejection, but did not mediate GVHD when transplanted into lethally irradiated CB6F1 hosts. However, allospecific T_{CM} population could not be generated in this model. To study the potential of alloreactive TCM to induce GVHD, we utilized a novel GVHD model induced by T cell receptor (TCR) transgenic OT-II T cells. Using this model, we were able to generate antigen-specific T_{CM} by immunizing donor mice directly and further demonstrated that these cells mediated secondary skin graft rejection while did not induce GVHD.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) mice and C57BL/6-Tg(CAG-OVA)916Jen/J (OVA) mice (13) were purchased from The Jackson Laboratory as breeders, and were bred and maintained at Duke University in a specific pathogen-free facility during the study. To enable cell tracing, OT-II mice were further crossed with GFP^+ mice and $Luciferase^+$ mice (a generous gift from Dr. Andreas Beilhack and Dr. Robert Negrin, Stanford University) to generate OT-II^+ $Luciferase^+$ GFP^+ triple positive mice. For all the strains, both female and male mice were used in this study. The donor mice were primed at 6–8 weeks old. The recipient mice were between 7 and 16 weeks old at the time of transplantation. All animal care and experimental procedures were approved by National Institute of Health and Duke University Institutional Animal Care and Use Committee.

Generation of Allospecific T Cells

To generate allospecific OT-II memory T cells *in vivo*, OT-II mice between the age of 6–8 weeks were immunized with OVA protein (Sigma-Aldrich, MO, USA) emulsified in complete Freund's adjuvant (Sigma-Aldrich, MO, USA) i.p. at 100 ug/mouse (14). Mice were then hosted in a pathogen-free facility for 8 weeks before use.

T-Cell Depletion From Bone Marrow

OVA mice between age 7–16 weeks were used as T-cell depleted (TCD) bone marrow donors. T cells were depleted from bone marrow using anti-CD90.2 antibody and complement as previously published. In brief, bone marrow cells were flushed out from the long bones of donor mice and strained through a 70 μm cell strainer (Becton Dickinson labware, NJ, USA). Cells were then resuspended in cytotoxicity medium, incubated with anti-CD90.2 monoclonal antibody (clone 30H12; BD Pharmingen, CA USA) at 4°C for 1 h. The cells were washed once and then resuspended in cytotoxicity medium containing 1:10 Low-Tox-M Rabbit Complement (Cedarlane, Canada). The cells were then incubated at 37°C for 60 min and washed twice before use.

T Cell Separation

OT-II mice primed for 8 weeks were used as T-cell donors. Purified T cells were separated from splenocytes using mouse Pan T Cell Isolation Kit II (Miltenyi, Germany). The purified T cells were then stained with APC–conjugated anti-CD62L (clone MEL-14), PE-conjugated anti-CD4 (clone CT-CD4), PerCy5.5-conjugated anti-CD44 (clone IM7) from BD PharmMingen (CA, USA), and sorted into different T-cell subsets according to **Figure 1** Panel using MoFlo Astrios Cell Sorter (Backman Coulter, IN, USA). Sorted cells were washed and counted before use. The purity after sorting was 92–96% for T_N, 90∼95% for T_{EM} with 2–3% T_{CM} contamination, and 86∼92% for T_{CM} with 2∼9% T_{EM} contamination and 1–2% T_N contamination.

Mixed Lymphocyte Reaction (MLR)

The proliferation assay was performed as described previously (5). Graded numbers of purified OT-II T cells as indicated were plated in 96-wells, flat-bottomed culture plates with 5×10^5 irradiated (20Gy) OVA splenocytes in a final volume of 200 μl. After incubation at 37°C in 5% CO_2 for a specified period as indicated, cultures were pulsed with ^{3}H-thymidine (1μCi [0.037MBq]/well). Cells were harvested after another 16 h of incubation, and counted in a MicroBeta Trilux liquid scintillation counter (EG&G Wallac, Turku, Finland). Triplicate cultures were set up for each cell population tested.

GVHD Model

OVA mice were lethally irradiated (10.5 Gy) using Cs irradiator and injected with 1×10^7 TCD BM and different numbers of purified OT-II cells through tail vein. Survival and clinical scores of GVHD including body weight change, fur ruffling, skin changes, hunching posture, diarrhea, and activity were monitored daily. Moribund mice were sacrificed according to protocol approved by the Duke University Institutional Animal Care and Use Committee.

Skin Transplantation

The skin transplantation protocol was modified as previously published (12). In brief, tail skin from OVA mice was removed from sacrificed donors, cut into ∼0.5×0.5 cm^2 pieces, and kept on swab damped with cold PBS. The C57BL/6 recipient mice were anesthetized with isoflurane (Halocarbon, GA, USA) with the right lumbar region shaved and sanitized with iodine solution followed by alcohol. A graft bed was prepared by removing an area of skin down to the level of the intrinsic muscle using fine scissors. The graft was fitted to the prepared bed, sutured with 5-0 surgical suture, and wrapped with an adhesive plastic bandage. The bandage was removed 4 days

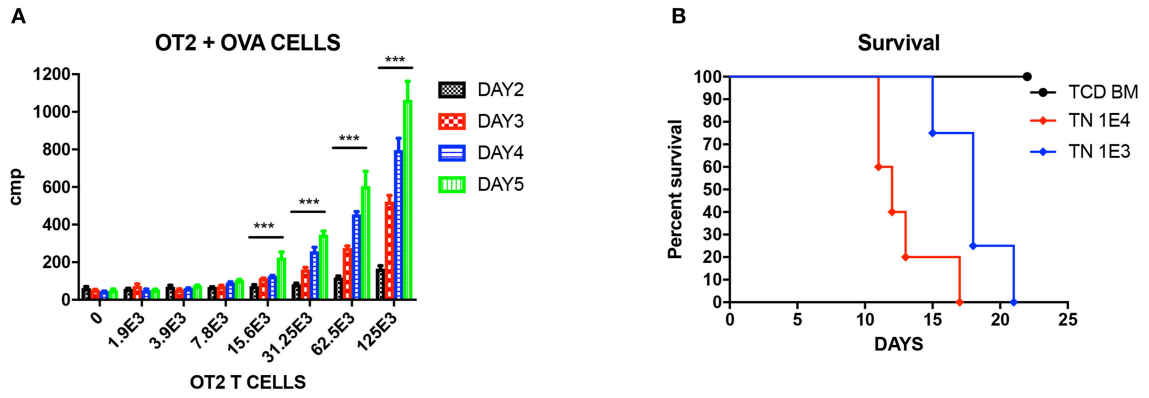


FIGURE 1 | Unprimed OT-II T cells reacting to OVA cells. **(A)** Mixed lymphocyte reaction (MLR) of unprimed OT-II T cells cultured with different doses of OVA splenocytes, cultured for different days. Three wells each condition. Experiment repeated twice. ***$P < 0.001$ for four titrations. Analyzed using multiple *t* test. **(B)** Titration of unprimed sorted T_N from OT-II mice and injected into OVA mice to induce GVHD. $P < 0.01$ for both doses compared to TCD BM. $N = 5$ each group. Experiment repeated twice.

after surgery. Skin graft survival was assessed everyday by visual and caliper measuring. Rejection was defined as the first day when the entire epidermal surface area of the graft was <10% of original.

Bioluminescent Imaging

Mice were monitored for T-cell tracking once per week after bone marrow transplantation. For *in vivo* imaging, mice were anesthetized with isoflurane and injected intraperitoneally with 50 mg/kg D-Luciferin (PerkinElmer, CT, USA) 10 min before imaging with a Xenogen IVIS 100 imaging system (Xenogen Corporation, Alameda, CA, USA) at maximum signal intensity using 5 min exposure time. Regions of Interest (ROIs) were drawn using Living Image 2.5 software (Caliper, MA, USA).

Flow Cytomery Analysis

Single cell suspension of splenocytes were prepared as described before (5, 12). In brief, organs were removed from the sacrificed mice, and gently crunched using the gridded end of a syringe on a 70 μm cell strainer. Cells were then strained, treated with red blood cell lysis, washed, and stained with antibodies for flow cytometry per manufacturer's protocol. The antibodies used were as follow: PE anti-mouse Vα2 TCR (B20.1), PE/Cy7 anti-mouse CD62L (MEL-14), APC anti-mouse CD4 (RM4-5), PerCP-Cy5.5^{TM} CD44 (IM7) (all from BD Biosciences, CA, USA). Flow cytometry was performed using a BD FACSCanto (BD Biosciences). Data were analyzed with BD FACSDiva^{TM} Software (BD Biosciences).

Statistical Analysis

Statistical analysis was performed using Prism GraphPad (GraphPad Software, CA, USA) and Excel (Microsoft, WA, USA). For survival studies, log-rank Mantel-Cox test was used. For MLR, body weight changes, GVHD score, and bioluminescent measurement, Student's *t* test, multiple *t* test, and multi-way ANOVA test were used. Level of significance was set at $P < 0.05$. Bar graphs represent mean ± SEM.

RESULTS

Unprimed OT-II T Cells React to OVA Cells

We first tested the reactivity of OT-II T cells again OVA cells in vitro. Unprimed OT-II T_N were sorted from OT-II mice as responding cells, and cocultured with 5×10^5 lethally irradiated OVA splenocytes as stimulators at graded ratio for different time period from 2 to 5 days. By analyzing the ^{3}H-thymidine uptakes, it is shown that from T cells: OVA splenocytes 1:10 on, OT-II unprimed T_N can be efficiently stimulated and proliferated (**Figure 1A**. $P < 0.01$). When we titrated these unprimed T_N into lethally irradiated OVA mice, we proved that unprimed T_N could cause lethal GVHD at a low dose of 1,000 cells, and the GVHD effect was dose dependent (**Figure 1B**).

Generation of Functional OVA Antigen-Specific OT-II Memory T Cells

In order to study the role of antigen-specific central memory T cells in GVHD, we first generated a T-cell mediated antigen-specific GVHD model using the OT-II/OVA system as showed in **Figure 2A**. We first immunized the OT-II donor mice by injecting emulsified OVA protein intraperitoneally and housed the mice for 8 weeks to generate OVA-specific memory OT-II cells. OT-II T cells as identified as $CD4^+$ $V\alpha2^+$ cells were sorted into naive (T_N, $CD62L^+$ $CD44^{low}$), effector memory (T_{EM}, $CD62L^-$), and central memory (T_{CM}, $CD62^+CD44^{high}$) T cell subsets. Flow cytometry also confirmed that all the sorted antigen specific cells are GFP^+ (**Figure 2B**).

To verify the immune function of these immunized OT-II cells, we introduced the secondary skin graft rejection model. C57BL/6 mice were transferred with 1×10^3 OT-II T_N, T_{CM}, or T_{EM}. On the subsequent day, a 0.5 × 0.5 cm^2 piece of tail skin peeled from OVA mice were transplanted

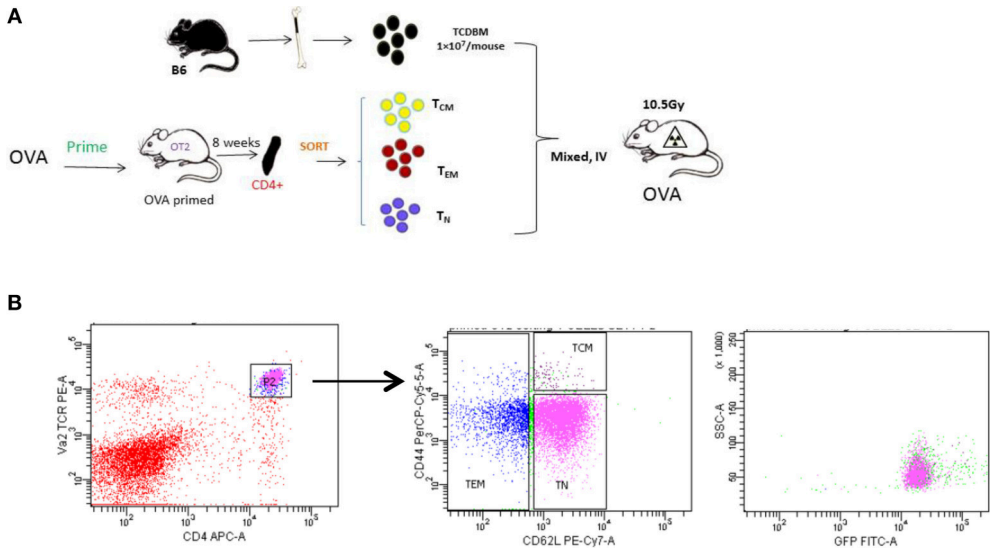


FIGURE 2 | Generating allospecifc T_{CM} cells. **(A)** A schematic for antigen-specific T-cell generation. OT-II transgenic mice were primed with OVA peptide 8 weeks before transplantation. Primed OT-II transgenic T cells were sorted into three subsets (T_N, T_{CM}, T_{EM}) and transplanted into lethally irradiated OVA mice at the dose of 1×10^3 cells/mouse, along with 1×10^7 T-cell-depleted BM cells. **(B)** Gating of antigen-specific T cells. Flow cytometry analysis was performed in OT-II mice at least 8 weeks after priming. All OT-II T cells were GFP positive.

to the right lumbar region of the recipients (**Figure 3**). By measuring the area of the live graft daily, we demonstrated that the graft survival times in mice that received T_{EM} or T_{CM} injections were significantly shorter ($P = 0.0002$) or trended to be shorter ($P = 0.078$) compared to that in T_N recipients These data are consistent with the previous publications (2, 4, 12) and indicate that the different OT-II subsets sorted from OVA-immunized OT-II mice were functionally anti-OVA memory T cells.

Antigen-Specific T_{EM} and T_{CM} Do Not Cause GVHD

After confirming the anti-OVA function of the memory OT-II T cells, we tested these cells in the OT-II anti-OVA antigen specific GVHD model by injecting 1×10^3 sorted T_N, T_{CM}, or T_{EM} subsets of OT-II T cells from OVA-primed OT-II mice together with 1×10^7 TCD-BM from OVA mice into lethally irradiated OVA recipients. The survival, body weight changes, and GVHD clinical score were monitored daily. Unlike what was observed in skin rejection model, the mice that received T_N cells had the earliest death related to GVHD, with all the mice died within 56 days, while mice that received T_{EM} had 100% survival over 100 days, and mice that received T_{CM} had 70% survival till 100 days (**Figure 4A**). As to body weight recovery and GVHD clinical score, T_N recipients had the worst performance compared to mice that received memory T cells. Mice receiving either T_{EM} or T_{CM} had similar recovery status compared to TCD BM mice, which were the negative controls (**Figures 4B,C**). Using higher T cell dose at 1×10^4 for all cell types led to similar conclusion (**Supplemental Figure 1**). These result indicate that, although antigen-specific T_{CM} cells result in some mortality in acute phase,

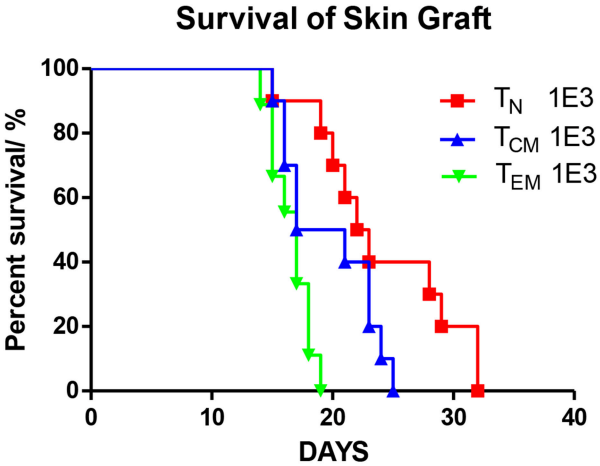


FIGURE 3 | Allospecific T_{CM} mediate secondary skin graft rejection. OT-II T cells were sorted into three subsets (T_N, T_{CM}, T_{EM}) after 8 weeks priming. 1×10^3 T cells of each subset were transplanted into C57BL/6 female mice which were transplanted with OVA tail skin graft on the subsequent day. Graft survival was observed daily. $P = 0.0002$, T_{EM} recipients vs. T_N recipients. $P = 0.078$, T_{CM} vs. T_N. $n = 10$ for each group. Data were pooled from two independent experiments.

neither T_{EM} nor T_{CM} cause significantly clinical GVHD in the survivors in long-term follow-up.

To verify this, we further collected the target organs of GVHD including spleen, liver, small and large intestines, when sacrificing the mice because of morbidity or at Day 28, and accessed for histopathological changes (**Figure 4D**). In the organs from TCD BM mice, the histological structure of the organs was clear with cells well aligned. However, in the organs from

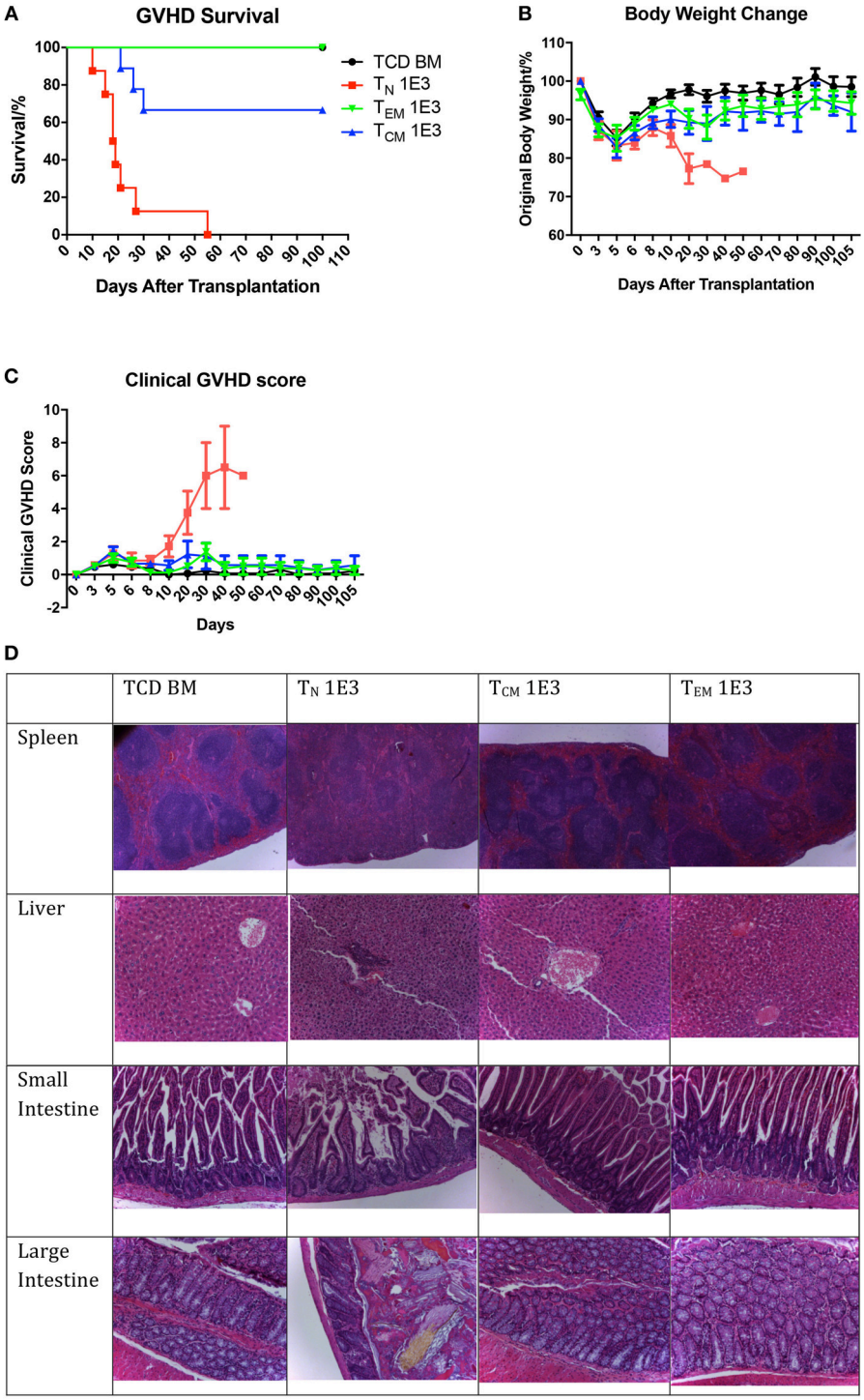


FIGURE 4 | Allospecific T_{CM} have decreased ability to induce GVHD. Primed OT-II T cells were sorted into three subsets and transplanted into lethally irradiated OVA mice at the dose of 1×10^3 along with 1×10^7 TCD BM. Mice survival, body weight, and GVHD scores (body weight, posture, activity, fur, skin integrity, diarrhea) were monitored daily. $n = 9$ for each group. Data pooled from two independent experiments. **(A)** T_{EM} and T_{CM} recipients have better survival comparing to T_N recipients. $P < 0.0001$, T_N vs. TCD BM. $P = 0.065$, T_{CM} vs. TCD BM. Estimate hazard ratio between T_N and T_{CM} is 7.4821. **(B)** T_{EM} and T_{CM} recipients have better body weight recovery comparing to T_N recipients. $P < 0.0001$, T_N vs. TCD BM. $P = 0.043$, T_{CM} vs. TCD BM. $P = 0.1136$, T_{EM} vs. TCD BM. **(C)** T_{EM} and T_{CM} recipients have lower GVHD score comparing to T_N recipients. $P < 0.001$, T_N vs. TCD BM. $P = 0.0937$, T_{CM} vs. TCD BM. $P = 0.5324$, T_{EM} vs. TCD BM. $P < 0.001$, T_N vs. T_{CM} and T_{EM}. **(D)** Histology on GVHD target organs. In T_N recipients, GVHD pathological changes can be found in spleen as fibrosis and hypocellularity, in liver as portal triad lymphocyte infiltration with bile duct injury and cholangitis, in intestines as crype/gland destruction with epithelial cell apoptosis and lymphocyte infiltration. TCD BM, T_{CM}, and T_{EM} recipients have relatively normal organ morphology.

T_N mice, significant GVHD histological structure changes were seen, including the blurred edges between the white pulps and red pulps in the spleen, portal vein thrombosis and lymphocyte infiltration in the liver, disruptions of the villi and crypts with lost of epithelial cells and goblet cells in the small and large intestines. These pathological changes in the organs were not presented in the organs from mice received T_{EM} or T_{CM} cells. The histological results further confirmed that although OT-II antigen-specific memory T cells had the memory function to reject OVA-expressed skin grafts faster compared to T_N cells, neither T_{EM} nor T_{CM} caused histopathological GVHD changes in the GVHD target organs.

Antigen-Specific T_{EM} and T_{CM} Proliferated Less but Persisted in GVHD Hosts

To understand why antigen-specific memory T cells did not cause GVHD, we used bioluminescent imaging (BLI) and flow cytometry to track the antigen-specific T cell expansion *in vivo* after BMT. To generate GFP- and luciferase-expressing OT-II T cells, OT-II mice were crossed with Luciferase-reporter mice, and further crossed with GFP positive mice. Cells were sorted as described in **Figure 1** and transplanted into irradiated OVA mice at two different cell doses to enable cell tracing. BLI revealed that, at both cell doses, T_N recipients had a much higher number of photon counts compared to either T_{EM} or T_{CM} recipients, indicating the robust expansion of T_N after BMT. T_{CM} recipients also had a higher T-cell signal on Day 21 compared to T_{EM} recipients, but soon declined to a comparable low level after 28 days (**Figure 5A**). The GFP^+ cell number in the peripheral blood detected by flow cytometry also showed the same trend. Similar but different to BLI, in the peripheral blood, GFP^+ OT-II T_{CM} cells had the peak around Day 14 and started to decline afterwards (**Figure 5B**). Although the mice receiving the lower dose of T cells did not have detectable significant expansion peak due to limited cell numbers and technical sensitivity, the same trend detected in both methods using different cell doses indicated the robust expansion of T_N, the transient expansion of T_{CM}, and the limited expansion of T_{EM} in an antigen specific GVHD model. Since the detected number of T_{EM} cells were very limited in both methods, we further confirmed the existence of the GFP cells in the spleens of T_{EM} and T_{CM} recipients 30 days after BMT using flow cytometry (**Figure 5C**). Although the limited cell number in the recipients prevented us to further analyze the cell surface markers for mechanistic studies, the existence of GFP^+ antigen-specific memory T cells inside the target organs without causing GVHD suggested the exhausted status of these cells in GVHD model.

DISCUSSION

In our study, we successfully utilized the OT-II / OVA system to generate allo-specific T_{EM} and T_{CM} by directly immunizing donor mice with alloantigens. These T_{EM} and T_{CM} were phenotypically the same as those isolated from polyclonal mice (5). We further confirmed that T_{EM} and T_{CM} separated from primed OT-II mice were functionally memory T cells because they rejected second-set skin grafts faster than T_N did. By transplanting these different subsets of primed T cells into OVA mice, we proved that OT-II T_N cells mediated the vigorous GVHD, while T_{EM} did not cause GVHD in OVA mice. Although primed OT-II T_{CM} resulted in some death within the first 3 weeks, the survival rate was still significantly higher than T_N group, while the body weight recovery, GVHD score, and histological changes in the target organs were all similar to T_{EM} recipients, indicating that T_{CM} do not cause or cause very minor GVHD. These results are consistent with the previously published data demonstrating that alloreactive T_{EM} and T_{CM} would not cause GVHD (3–6, 12, 15). Our finding further verified this conclusion under the antigen-specific condition with no interference of antigen cross-presentation.

Our study has the important clinical significance in T-cell therapy in BMT patients. Antigen-specific T cells against host antigen are believed to be the major players in inducing GVHD. In our study, we demonstrated that not only antigen-specific T_{EM} but also T_{CM} against host antigen do not cause GVHD. Currently, naive T cells depletion and anti-virus memory T cells transfusion are under clinical trial for BMT patients to preserve T-cell anti-infection function while preventing GVHD (11, 16–20). Similar studies are also under investigation using tumor specific T cells (21–24). Our study further supports the safety and feasibility of naive T cell depletion and using virus- and tumor-specific memory T cells to prevent infections and tumor relapses for BMT patients without causing GVHD.

One major difference between human and mouse memory T cells is that human memory T cells may contain true alloantigen specific T cells while those from normal mice do not. In humans, alloantigen specific memory T cells are generated when naïve T cells are exposed to alloantigens during transfusion or pregnancy (25). Even though multiple groups have demonstrated in several different animal models that memory T cells do not induce GVHD, (3–6, 15) one major concern when translating these findings into clinic is that human memory T cells may behave differently because they contain true alloantigen specific T cells. The findings from the current study at least partially address this concern because we demonstrate that even true alloantigen specific T cells have decreased ability to induce GVHD.

We also further investigate the primed OT-II T cells proliferation and retention in the organ after transplantation. By using bioluminescent imaging, we proved that compared to T_N cells that underwent vigorous proliferation in the first 3 weeks, OT-II T_{EM} and T_{CM} had very limited proliferation in the spleens after transplantation. This finding was supported by Dr. Brede's research, and further advanced his findings (26). Compared to T_{EM} cells, T_{CM} had a more potent proliferation in the peripheral blood between Day 10 to Day 21. This may explain some of the GVHD related death in the first 3 weeks. When we further traced these cells, we found that even 30 days after the BMT, in the survivors' spleens we could still identify the retention of transplanted T_{CM} and T_{EM} cells. This result confirmed that the antigen specific memory T cells persist in the hosts after BMT but failed to induce GVHD, suggesting a potential status of T-cell exhaustion. Due to the limited number of T cells that we could recover from the recipient mice, we

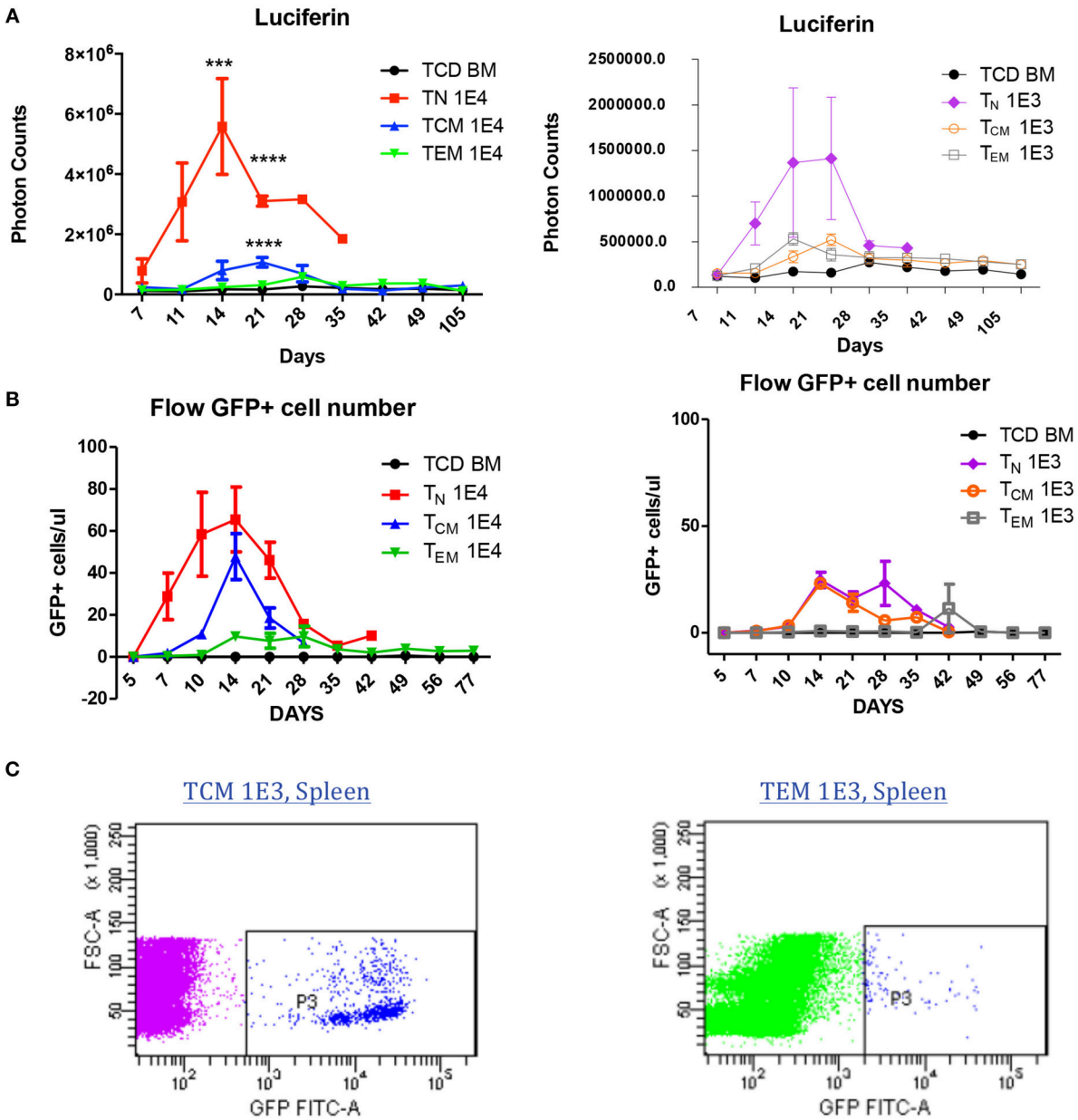


FIGURE 5 | Allospecific T_{CM} are exhausted in GVHD model. Luciferase and GFP labeled OT-II T cells were primed and transplanted into OVA mice at two doses (1×10^4, 1×10^3). $n = 7$ each group. Experiments repeated twice. **(A)** Significant T-cell proliferation was observed in T_N recipients, but not in T_{CM} and T_{EM} groups using luciferin tracing. Luciferin tracing were performed on day 7, 11, 14 after transplantation and once every week. **(B)** GFP^+ labeled OT-II T cells in peripheral blood were significantly increased in T_N and T_{CM} groups but not in T_{EM} group. Peripheral blood was collected from transplanted OVA mice ($n = 4$) on day 5, 7, 10, 14 after transplantation and once every week. GFP^+ cells were counted using flow cytometry. The different pattern of T-cell proliferation between **(A)** and **(B)** may be caused by T-cell distribution in the body. **(C)** T cells were detectable in GVHD survivors' organs. $^{***}P < 0.001$; $^{****}P < 0.0001$, compared with TCD BM.

were unable to completely understand the mechanism by which alloreactive T_{CM} could reject skin graft but could not induce GVHD, and why the existing alloreactive T_{CM} remained for more than 30 days but did not induce GVHD. Hypothetically, we speculate that the fate of antigen-specific memory T cells would be different in the environment that encounters a small amount of removable antigens vs. the environment that is surrounded by a large amount of non-removable antigens. According to the previous research studying T-cell immunology in viral infections, (27) memory T cells were the dominant T-cell population in peripheral blood in acute viral infection when virus titer was low and the virus could be eliminated. On the contrary, in chronic viral infection when virus load was high and the virus sustained, naive T cells were the dominant T cells, and memory T cells had a limited clonal expansion within the first week compared to acute infection. These memory T cells were exhausted and

underwent clonal depletion within 7–21 days (13, 28–31). The way that we challenged the alloreactive T-cell transplanted mice with skin graft was very similar to acute viral infection, while the BMT especially GVHD situation was very similar to chronic viral infection. This explains why the antigen-specific T_{CM} could reject skin graft, but could not induce GVHD later. We speculate that the long-term existing T_{CM} would get exhausted to a specific non-removable alloantigen in an GVHD setting.

In the previous studies, different TCR Tg T-cell of a single specificity models were used to study to alloreactive GVHD (3). These models include the CD8-mediated major MHC-mismatched 2C Tg model which is L^d-specific, the CD4-mediated MHC-mismatched 3BBM74 model which is I-A^{bm12}-specific and D10 model which is I-A^b-specific, and the CD4-mediated miHAg-mismatched TEa model, and the CD4-mediated TS1 TCR Tg model which recognize the S1 epitope of HA on the HA104 Tg mice (3, 12, 32–36). In most of these studies, T cells were immunized and activated *in vivo* or *in vitro*, and transferred and expanded in RAG1$^{-/-}$ mice. In these models, T-cell homeostasis is unpreventable, while the separation of T_{CM} is hard to achieve due to continually CD44 expression. In Juchem et al. study, the use of *in vitro* immunized TS1 cells injecting into HA mice, which was a single-antigen TCR Tg model, reached similar conclusion about T_{EM} with what we have seen in OT-II T-cell OVA host model (3). However, in the TS1-HA model, the T_{CM} could not be well distinguished from the T_N cells due to the continually CD44 expression, and mice receiving T_{CM} had shown signs of GVHD. Thinking that the high potent of T_N to cause GVHD, the different phenomenon of T_{CM} in TS1-HA model and in our model may be caused by the very small number of T_N contamination. In our OT-II-OVA model, due to a clearer separation of T_{CM} subsets, we were able to focus on the CD44^{high} expression population, and proved that antigen-specific T_{CM} did not cause GVHD.

Based on the previous findings from Strober's group demonstrating that memory CD4$^+$ T cells do not directly mediate GVT effect by themselves (2), we do not expect OT-II T_{CM} are able to mediate direct GVT effect because they are CD4$^+$ T cells. However, based on our finding that antigen-specific T_{CM} was able to reject OVA-expressing skin graft (**Figure 3**) and the ability of primed CD4 cells to facilitate tumor killing (37), we believe it is reasonable to speculate that these antigen specific T_{CM} maintain at least indirect GVT activity.

The OT-II into OVA murine model that we provided is novel to study the antigen-specific memory T cells in murine GVHD model. There are two major advantages that contributed to the study. First, in OT-II mice, the memory T cells can be directly generated and expanded *in vivo*, and further sorted into well differentiated subsets including T_N, T_{EM}, and T_{CM}. This enables the study of single peptide antigen-specific subsets of T cells generated in a physical condition *in vivo*. Second, OVA is a commonly used labeled antigen on various cancer cell lines. OT-II / OVA GVHD model facilitates the study of the anti-tumor effect of different antigen-specific T-cell subsets in the GVHD model.

There are also some limitations and unanswered questions that need to be aware of. First, the antigen-specific model is very sensitive to T-cell number. Only 1,000 antigen-specific T_N cells can cause lethal GVHD, and 1,000 T_{CM} cells can partially cause GVHD. The limitation of small number of cells becomes the obstacle for further cell tracing and cellular and molecular mechanism study. Secondly, the model is still preliminary. Further information about the exhaustion markers and functional assays of the cells injected would be more helpful to define the status and biological characteristics of the antigen specific memory T cells in GVHD.

In conclusion, we have established a feasible antigen-specific TCR Tg GVHD model by immunizing OT-II mice *in vivo* to generate memory T cells, and transplanting these T cells into OVA mice to induce GVHD. We have demonstrated that antigen-specific T_{EM} and T_{CM} model do not cause GVHD due to a decreased proliferation potency after BMT, but can exist in the hosts' organs for long possibly due to exhaustion.

AUTHOR CONTRIBUTIONS

BJC and WH designed the experiments. WH, WM, JJ performed the experiments. WH did the statistic analysis. WH, BJC, and NJC wrote the manuscript.

FUNDING

This study was supported by National Institutes of Health grant P01-CA048841 (BJC and NJC).

ACKNOWLEDGMENTS

We thank Duke University's Division of Laboratory Animal Resources (DLAR) for caring of the experimental mice. The cell sorting was performed by Duke Cancer Institute Flow Cytometry Shared Resource.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00624/full#supplementary-material

Supplemental Figure 1 | Alloreactive T_{CM} cells at higher dose have decreased ability to induce GVHD. Primed OT-II T cells were sorted into three subsets and transplanted into lethally irradiated OVA mice at the dose of 1×10^4 along with 1×10^7 TCD BM. Mice survival, body weight, and GVHD scores (body weight, posture, activity, fur, skin integrity, diarrhea) were monitored daily. **(A)** T_{EM} and T_{CM} recipients had better survival comparing to T_N recipients. $P < 0.0001$, T_N vs. TCD BM. $P < 0.0001$, T_{CM} vs. TCD BM. **(B)** T_{EM} and T_{CM} recipients had better body weight recovery comparing to T_N recipients. $P < 0.0001$, T_N vs. T_{CM} and T_{EM} on Day 8 and Day 10. **(C)** T_{EM} and T_{CM} recipients had lower GVHD score comparing to T_N recipients. $P < 0.001$, T_N vs. T_{CM} and T_{EM} on Day 8 and Day 10. $n = 14$–16 for each group. Data pooled from three independent experiments.

REFERENCES

1. Perkey E, Maillard I. New insights into graft-versus-host disease and graft rejection. *Annu Rev Pathol*. (2018) 13:219–45. doi: 10.1146/annurev-pathol-020117-043720
2. Dutt S, Tseng D, Ermann J, George TI, Liu YP, Davis CR, et al. Naive and memory T cells induce different types of graft-versus-host disease. *J Immunol*. (2007) 179:6547–54. doi: 10.4049/jimmunol.179.10.6547
3. Juchem KW, Anderson BE, Zhang C, McNiff JM, Demetris AJ, Farber DL, et al. A repertoire-independent and cell-intrinsic defect in murine GVHD induction by effector memory T cells. *Blood*. (2011) 118:6209–19. doi: 10.1182/blood-2011-01-330035
4. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest*. (2003) 112:101–8. doi: 10.1172/JCI17601
5. Chen BJ, Deoliveira D, Cui X, Le NT, Son J, Whitesides JF, et al. Inability of memory T cells to induce graft-versus-host disease is a result of an abortive alloresponse. *Blood*. (2007) 109:3115–23. doi: 10.1182/blood-2006-04-016410
6. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, et al. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood*. (2008) 111:2476–84. doi: 10.1182/blood-2007-08-109678
7. Muller AM, Shashidhar S, Küpper NJ, Kohrt HEK, Florek M, Negrin RS, et al. Co-transplantation of pure blood stem cells with antigen-specific but not bulk T cells augments functional immunity. *Proc Natl Acad Sci USA*. (2012) 109:5820–5. doi: 10.1073/pnas.1120237109
8. Chan WK, Suwannasaen D, Throm RE, Li Y, Eldridge PW, Houston J, et al. Chimeric antigen receptor-redirected CD45RA-negative T cells have potent antileukemia and pathogen memory response without graft-versus-host activity. *Leukemia*. (2015) 29:387–95. doi: 10.1038/leu.2014.174
9. Yarkoni S, Stein J, Yaniv I, Askenasy N. Antigen-specific priming is dispensable in depletion of apoptosis-sensitive T cells for GvHD prophylaxis. *Front Immunol*. (2014) 5:215. doi: 10.3389/fimmu.2014.00215
10. Heinrichs JL. *Antigen Specific Induced T Regulatory Cellular Therapy for Graft-Versus-Host Disease Following Allogeneic Bone Marrow Transplantation*. Graduation Theses and Dissertations (2016), University of South Florida. Available online at: https://scholarcommons.usf.edu/etd/6093
11. Melenhorst JJ, Leen AM, Bollard CM, Quigley MF, Price DA, Rooney CM, et al. Allogeneic virus-specific T cells with HLA alloreactivity do not produce GVHD in human subjects. *Blood*. (2010) 116:4700–2. doi: 10.1182/blood-2010-06-289991
12. Zhang P, Wu J, Deoliveira D, Chao NJ, Chen BJ. Allospecific CD4(+) effector memory T cells do not induce graft-versus-host disease in mice. *Biol Blood Marrow Transplant*. (2012) 18:1488–99. doi: 10.1016/j.bbmt.2012.07.009
13. Kuchroo VK, Anderson AC, Petrovas C. Coinhibitory receptors and CD8 T cell exhaustion in chronic infections. *Curr Opin HIV AIDS*. (2014) 9:439–45. doi: 10.1097/COH.0000000000000088
14. Ke Y, Li Y, Kapp JA. Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses. *Eur J Immunol*. (1995) 25:549–53. doi: 10.1002/eji.1830250237
15. Huang W, Chao NJ. Memory T cells: a helpful guard for allogeneic hematopoietic stem cell transplantation without causing graft-versus-host disease. *Hematol Oncol Stem Cell Ther*. (2017) 10:211–9. doi: 10.1016/j.hemonc.2017.05.006
16. Bollard CM, Heslop HE. T cells for viral infections after allogeneic hematopoietic stem cell transplant. *Blood*. (2016) 127:3331–40. doi: 10.1182/blood-2016-01-628982
17. Fuji S, Kapp M, Einsele H. Alloreactivity of virus-specific T cells: possible implication of graft-versus-host disease and graft-versus-leukemia effects. *Front Immunol*. (2013) 4:330. doi: 10.3389/fimmu.2013.00330
18. Gerdemann U, Vera JF, Rooney CM, Leen AM. Generation of multivirus-specific T cells to prevent/treat viral infections after allogeneic hematopoietic stem cell transplant. *J Vis Exp*. (2011) 51:2736. doi: 10.3791/2736
19. Houghtelin A, Bollard CM. Virus-specific T cells for the immunocompromised patient. *Front Immunol*. (2017) 8:1272. doi: 10.3389/fimmu.2017.01272
20. Vadakekolathu J, Rutella S. T-cell manipulation strategies to prevent graft-versus-host disease in haploidentical stem cell transplantation. *Biomedicines*. (2017) 5:E33. doi: 10.3390/biomedicines5020033
21. Ghosh A, Holland AM, van den Brink MR. Genetically engineered donor T cells to optimize graft-versus-tumor effects across MHC barriers. *Immunol Rev*. (2014) 257:226–36. doi: 10.1111/imr.12142
22. Melenhorst JJ, Castillo P, Hanley PJ, Keller MD, Krance RA, Margolin J, et al. Graft versus leukemia response without graft-versus-host disease elicited by adoptively transferred multivirus-specific T-cells. *Mol Ther*. (2015) 23:179–83. doi: 10.1038/mt.2014.192
23. Pidala J, Anasetti C. Can antigen-specific regulatory T cells protect against graft versus host disease and spare anti-malignancy alloresponse? *Haematologica*. (2010) 95:660–5. doi: 10.3324/haematol.2009.015818
24. Warren EH, Deeg HJ. Dissecting graft-versus-leukemia from graft-versus-host-disease using novel strategies. *Tissue Antigens*. (2013) 81:183–93. doi: 10.1111/tan.12090
25. Bingaman AW, Farber DL. Memory T cells in transplantation: generation, function, and potential role in rejection. *Am J Transplant*. (2004) 4:846–52. doi: 10.1111/j.1600-6143.2004.00453.x
26. Brede C. *Peripheral Alloantigen Expression Directs the Organ Specific T Cell Infiltration After Hematopoietic Cell Transplantation*. Doctoral Thesis. Universität Würzburg, Graduate Schools (2013).
27. West EE, Youngblood B, Tan WG, Jin HT, Araki K, Alexe G, et al. Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load. *Immunity*. (2011) 35:285–98. doi: 10.1016/j.immuni.2011.05.017
28. Hashimoto M, Kamphorst AO, Im SJ, Kissick HT, Pillai RN, Ramalingam SS, et al. CD8 T cell exhaustion in chronic infection and cancer: opportunities for interventions. *Annu Rev Med*. (2018) 69:301–18. doi: 10.1146/annurev-med-012017-043208
29. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA*. (2010) 107:14733–8. doi: 10.1073/pnas.1009731107
30. Kahan SM, Wherry EJ, Zajac AJ. T cell exhaustion during persistent viral infections. *Virology*. (2015) 479–80:180–93. doi: 10.1016/j.virol.2014.12.033
31. McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. *Annu Rev Immunol*. (2019). doi: 10.1146/annurev-immunol-041015-055318. [Epub ahead of print].
32. Dey B, Yang YG, Preffer F, Shimizu A, Swenson K, Dombkowski D, et al. The fate of donor T-cell receptor transgenic T cells with known host antigen specificity in a graft-versus-host disease model. *Transplantation*. (1999) 68:141–9.
33. Haspot F, Fehr T, Gibbons C, Zhao G, Hogan T, Honjo T, et al. Peripheral deletional tolerance of alloreactive CD8 but not CD4 T cells is dependent on the PD-1/PD-L1 pathway. *Blood*. (2008) 112:2149–55. doi: 10.1182/blood-2007-12-127449
34. O'Shaughnessy MJ, Chen ZM, Gramaglia I, Taylor PA, Panoskaltsis-Mortari A, Vogtenhuber C, et al. Elevation of intracellular cyclic AMP in alloreactive CD4(+) T Cells induces alloantigen-specific tolerance that can prevent GVHD lethality *in vivo*. *Biol Blood Marrow Transplant*. (2007) 13:530–42. doi: 10.1016/j.bbmt.2007.01.071
35. Yu XZ, Albert MH, Anasetti C. Alloantigen affinity and CD4 help determine severity of graft-versus-host disease mediated by CD8 donor T cells. *J Immunol*. (2006) 176:3383–90. doi: 10.4049/jimmunol.176.6.3383
36. Yu XZ, Bidwell S, Martin PJ, Anasetti C. Visualization, fate, and pathogenicity of antigen-specific CD8+ T cells in the graft-versus-host reaction. *J Immunol*. (1999) 163:4780–7.
37. Haabeth OA, Tveita AA, Fauskanger M, Schjesvold F, Lorvik KB, Hofgaard PO, et al. How do CD4(+) T cells detect and eliminate tumor cells that either lack or express MHC class II molecules? *Front Immunol*. (2014) 5:174. doi: 10.3389/fimmu.2014.00174

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 05 March 2019
doi: 10.3389/fimmu.2019.00295

Metabolic Targets for Improvement of Allogeneic Hematopoietic Stem Cell Transplantation and Graft-vs.-Host Disease

Natalia M. Tijaro-Ovalle[1,2], *Theodoros Karantanos*[1,2], *Hong-Tao Wang*[1,2] *and Vassiliki A. Boussiotis*[1,2]*

[1] *Division of Hematology-Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States,* [2] *Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States*

Utilization of the adaptive immune system against malignancies, both by immune-based therapies to activate T cells *in vivo* to attack cancer and by T-cell therapies to transfer effector cytolytic T lymphocytes (CTL) to the cancer patient, represent major novel therapeutic advancements in oncologic therapy. Allogeneic hematopoietic stem cell (HSC) transplantation (HSCT) is a form of cell-based therapy, which replaces the HSC in the patient's bone marrow but also serves as a T-cell therapy due to the Graft-vs.-leukemia (GVL) effect mediated by donor T cells transferred with the graft. Allogeneic HSCT provides one potentially curative option to patients with relapsed or refractory leukemia but Graft-vs.-Host-Disease (GVHD) is the main cause of non-relapse mortality and limits the therapeutic benefit of allogeneic HSCT. Metabolism is a common cellular feature and has a key role in the differentiation and function of T cells during the immune response. Naïve T cells and memory T cells that mediate GVHD and GVL, respectively, utilize distinct metabolic programs to obtain their immunological and functional specification. Thus, metabolic targets that mediate immunosuppression might differentially affect the functional program of GVHD-mediating or GVL-mediating T cells. Components of the innate immune system that are indispensable for the activation of alloreactive T cells are also subjected to metabolism-dependent regulation. Metabolic alterations have also been implicated in the resistance to chemotherapy and survival of malignant cells such as leukemia and lymphoma, which are targeted by GVL-mediating T cells. Development of novel approaches to inhibit the activation of GVHD-specific naïve T cell but maintain the function of GVL-specific memory T cells will have a major impact on the therapeutic benefit of HSCT. Here, we will highlight the importance of metabolism on the function of GVHD-inducing and GVL-inducing alloreactive T cells as well as on antigen presenting cells (APC), which are required for presentation of host antigens. We will also analyze the metabolic alterations involved in the leukemogenesis which could differentiate leukemia initiating cells from normal HSC, providing potential therapeutic opportunities. Finally, we will discuss the immuno-metabolic effects of key drugs that might be repurposed for metabolic management of GVHD without compromising GVL.

Keywords: T cells, antigen presenting cells (APCs), GVHD, metabolism, GVL

OPEN ACCESS

Edited by:
Brian Christopher Betts, University of Minnesota Twin Cities, United States

Reviewed by:
Robert James Salmond, University of Leeds, United Kingdom
Mark A. Schroeder, Washington University in St. Louis, United States
Craig Alan Byersdorfer, University of Pittsburgh, United States

***Correspondence:**
Vassiliki A. Boussiotis
vboussio@bidmc.harvard.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *14 November 2018*
Accepted: *05 February 2019*
Published: *05 March 2019*

Citation:
Tijaro-Ovalle NM, Karantanos T, Wang H-T and Boussiotis VA (2019) Metabolic Targets for Improvement of Allogeneic Hematopoietic Stem Cell Transplantation and Graft-vs.-Host Disease. Front. Immunol. 10:295. doi: 10.3389/fimmu.2019.00295

INTRODUCTION

Quiescent immune cells use glucose, amino acids, and lipids to meet their bioenergetic demands. ATP, the key energy-transporting molecule, is generated in every cell during the breakdown of such nutrients by glycolysis and OXPHOS. Depending on the functional demands, cell metabolism can be shifted toward anabolic reactions leading to production of molecules involved in biosynthesis necessary for cell growth, or toward catabolic reactions leading to breakdown of macromolecules and the generation of byproducts, which are subsequently used for energy generation or for construction of anabolic pathways. A balance of these anabolic and catabolic processes is mandatory for maintenance of metabolism homeostasis (1).

Glucose is the most abundant extracellular nutrient and, although ATP production during glucose catabolism by glycolysis is significantly lower compared to the ATP generated by OXPHOS reactions, it is faster and more efficient in increasing cellular ATP than mitochondrial metabolism. Glycolysis also supports cell growth because glycolytic intermediates provide a bridge to multiple biosynthetic pathways, including PPP that has an important role in building blocks necessary for nucleotide biosynthesis, rapid generation of metabolic intermediates, and cell growth (2, 3). Additionally, glycolysis fuels production of NADPH, which is mandatory not only for the support of anabolic pathways but also plays a crucial role in decreasing the oxidative stress in rapidly proliferating cells and maintaining the redox state of the cell (4). Pyruvate derived from glucose in glycolysis can be converted into lactate in the cytoplasm or into acetyl-CoA in the mitochondria to subsequently enter the TCA cycle (also known as Krebs cycle). In addition to producing intermediates that feed multiple biosynthetic pathways, the oxidative reactions of the TCA cycle generate NADH and $FADH_2$ which are required for the donation of electrons to the electron-transport chain for OXPHOS.

Rapidly proliferating malignant cells preferentially use glucose to sustain their rapid growth in the hazardous TME (5). The preference of cells to ferment glucose to lactic acid, even in the presence of oxygen that could support OXPHOS, is known as the Warburg effect (6). Although originally observed in cancer cells, it is now known that the Warburg effect is used by most cell types, including immune cells, to generate energy during times of rapid growth, because using glucose for energy generation through glycolysis, spares other nutrients for usage in anabolic reactions.

Metabolic aberration provides a key signature that differentiates malignant hematopoietic cells from normally differentiating hematopoietic progenitors that give rise to committed progenitors and mature myeloid cells (7). As in other cancer types, the Warburg effect dominates the metabolic preference of leukemia cells (7, 8), whereas during normal HSC differentiation glycolysis declines and mitochondrial metabolism and FAO gradually increases (9) (**Figure 1**). It has been hypothesized that leukemia cells that are resistant to treatment and responsible for relapses, have features of "LSC" that have the ability to reproduce the disease in animal models (10). These LSC, also known as leukemia initiating cells, appear to have unique metabolic features that differentiate them not only from normal HSCs but also from other leukemia cells. These findings underline the significance of metabolism in leukemia initiation and relapse.

AlloHSCT provides the only curative therapeutic approach for aggressive leukemias and lymphomas that are resistant to chemotherapy and immunotherapy. One of the key benefits of this therapy comes directly from the T cell-mediated offense to cancer, a process known as GVL effect (11). Nonetheless, T lymphocytes can also drive GVHD, the principal cause of non-relapse mortality among alloHSCT recipients. GVHD results from the attack of healthy recipient tissue by donor T cells that recognize host's alloantigens. Detailed, extensive studies have identified that T cells involved in GVHD are substantially different from the ones that mediate GVL (12–14). Specifically, naïve αβ TCR-positive T cells appear to be the main mediators of GVHD upon activation by host antigens (15). Conversely, T_{MEM} subsets have been found to sustain GVL function (12–14), suggesting that the immunologic and metabolic mechanisms implicated in these two effects after allotransplantation are distinct. Moreover, Treg also play a role in GVHD and GVL as they have the ability to suppress GVHD without compromising GVL (16). As a consequence, Tregs have been considered a

Abbreviations: ACC1, acetyl-CoA carboxylase 1; acetyl-CoA, acetyl coenzyme A; AEB071, Sotrastaurin; aGVHD, Acute graft-vs.-host-disease; ALL, Acute lymphoblastic leukemia; AML, Acute myelogenous leukemia; alloHSCT, Allogeneic hematopoietic stem-cell transplantation; AKT, Protein Kinase B; AMPK, AMP-activated protein kinase; APC, Antigen-presenting cell; ATP, Adenosine triphosphate; BEZ235, Dactolisib; BCL-2, B-cell lymphoma 2; Bz-423, Benzodiazepine (Bz)-423; C75, *trans*-4-Carboxy-5-octyl-3-methylene-butyrolactone; cGVHD, Chronic graft-vs.-host-disease; CML, Chronic myelogenous leukemia; CoA, Coenzyme A; CPT1a, carnitine palmitoyl transferase; DC, Dendritic cell; ECAR, extracellular acidification rate; F1F0-ATPase, F1 portion and a transmembrane FO portion of ATP synthase; $FADH_2$, Flavin adenine dinucleotide; FAO, Fatty acid oxidation; FAS, Fatty acid synthesis; FOXP3, Forkhead-Box P3; Glut1, Glucose transporter 1; Glut 3, Glucose transporter 3; GVL, Graft-vs.-leukemia; GVHD, Graft-vs.-host-disease; HIF-1α, Hypoxia-inducible factor 1α; HK2, Hexokinase 2; HSC, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; ICOS, Inducible T-cell costimulator; IFNγ, Interferon γ; IL-1/2/4/6/7/10/13/15, Interleukin 1/2/4/6/7/10/13/15; iTreg, inducible regulatory T cell; KO, knock out; LC-FA, Long-chain fatty acids; LPS, Lipopolisacharide; LSC, leukemia stem cells; LDH-A, Lactate dehydrogenase-A; M1, Classically activated macrophage; M2, Alternatively activated macrophage; MHC, Major Histocompatibility Complex; MHC-II, Major Histocompatibility Complex type 2; MM, Multiple myeloma; MRD, Minimal residual disease; MP, Myeloid progenitors; Mct4, Monocarboxylate transporter 4; mTOR, Mechanistic/mammalian target of rapamycin; mTORC1, Mechanistic/mammalian target of rapamycin complex 1; mTORC2, Mechanistic/mammalian target of rapamycin complex 2; Myc, Myc proto-oncogene; NADH, Nicotinamide adenine dinucleotide; NADPH, Nicotinamide adenine dinucleotide phosphate; NFAT, Nuclear factor of activated T cells; NOS, Nitric oxide synthase; OXPHOS, Oxidative phosphorylation; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PKM2, Pyruvate kinase M2; PPP, Pentose phosphate pathway; ROS, Reactive oxygen species; SREBPs, Sterol regulatory element-binding proteins; SC-FA, Short-chain fatty acids; SLC7A5, Solute Carrier Family 7 member 5; TCA cycle, Tricarboxylic acid cycle; TCR, T cell receptor; T_{EFF}, T effector cells ; Th, T helper cells; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; Tfh, follicular T helper; TLR, Toll-Like Receptor; TKI, Tyrosine kinase inhibitors; TME, Tumor microenvironment; T_{MEM}, T memory; TNF α, Tumor necrosis factor α; Treg, Regulatory T cells.

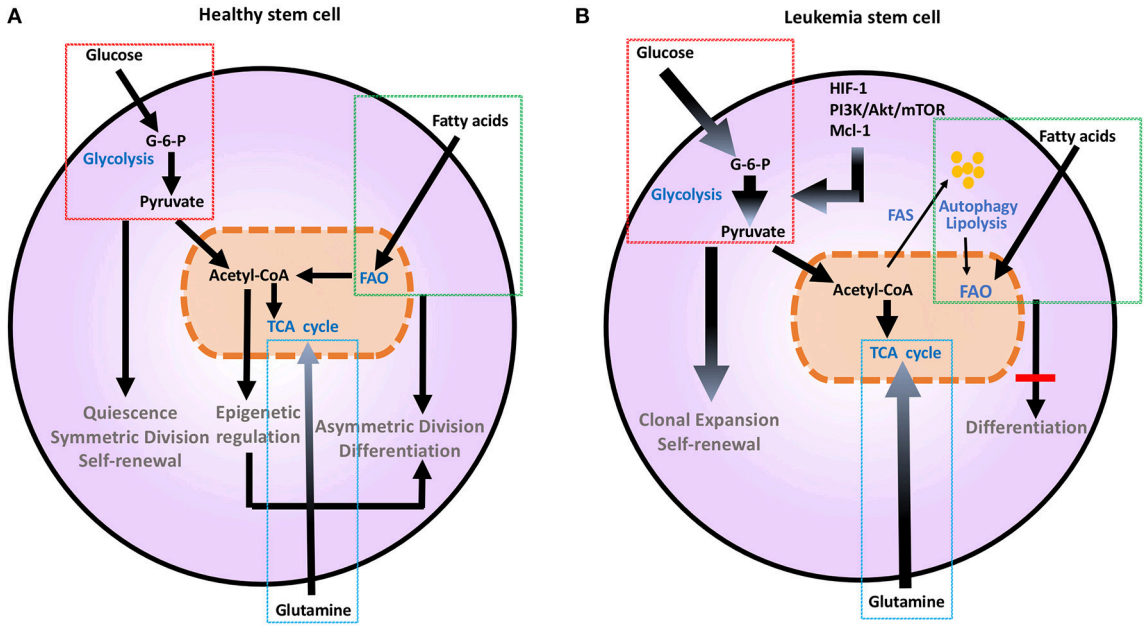


FIGURE 1 | Metabolic changes in healthy **(A)** and leukemia stem cells **(B)**. Metabolic changes drive stem cell differentiation in healthy subsets and clonal expansion in leukemia stem cells. Glucose uptake and glycolysis supports pluripotency and self-renewal of HSC, and is associated to the persistent ability of HSC to engage glycolysis, converting glucose to G-6-P and pyruvate, while sustaining a low ATP state. During differentiation, HSCs engage mitochondrial metabolic programs, including TCA cycle and FAO. This shift toward more efficient ATP generation is important for maturation and long-term survival. Mutations and aberrant protein expression related to malignant conversion of HSCs, including upregulation of HIF-1, PI3K/Akt/mTOR, and Mcl-1, induce metabolic changes. Leukemia stem cells support their uncontrolled clonal expansion by significantly increasing glucose uptake and metabolism (thick arrows). Although LSCs are highly dependent on glycolysis under steady state conditions, they have a high degree of metabolic plasticity and adaptation potential and can utilize autophagy and catabolic pathways such as OXPHOS and FAO in the setting of energy stress to support their survival and proliferation. Glutamine addiction is also an important feature common to malignant hematopoietic cells (thick arrow). Together these changes support extensive self-renewal without differentiation of LSC. Akt, Protein Kinase B; ATP, Adenosine triphosphate; G-6-P, Glucose-6-phosphate; FAO, Fatty acid oxidation; HIF-1, hypoxia induced factor 1; HSCs, hematopoietic stem cells; Mcl-1, myeloid cell leukemia 1; mTOR, Mechanistic/mammalian target of rapamycin; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; TCA, Tricarboxylic acid cycle.

therapeutic target for the control of GVHD either as a cell-based immunotherapy (17, 18) or as an *in vivo* therapeutic target by using approaches that induce Treg differentiation and expansion (19, 20).

GVHD is the leading cause of non-relapse mortality after HSCT because its prevention and treatment remain challenging. Global immunosuppression is the mainstay of therapy for GVHD but responses are only partial in most cases. Moreover, complications of chronic immunosuppression are detrimental (21, 22). As an alternative, the administration of T cell depleted donor grafts has been tested, but the high relapse and infection rates seen in patients who receive these graft variants mostly guide against the use of this strategy (23). This renders the discovery of new strategies that can ameliorate GVHD, while preserving the benefits from GVL effect, a real necessity.

Metabolism is an attractive tentative target for therapeutic intervention both in cancer immunotherapy and GVHD. T cell subsets are poised to distinct metabolic pathways that can determine their function and differentiation (24, 25). Upon activation, naïve T cells rely on glycolytic metabolism to rapidly meet the bioenergetic needs required for their proliferation, TCR rearrangement, production of growth factors, and differentiation to T_{EFF}. On the contrary, the function of Treg and T_{MEM} cells depends on enhanced FAO (26, 27). Because distinct T cell subsets mediate GVHD vs. GVL, the dominant metabolic properties of these distinct subsets might serve as new therapeutic targets that can be exploited for prevention or suppression of GVHD without compromising GVL.

Although in the context of GVHD and GVL, emphasis has been placed on T cells, the innate immune cells of the host, particularly macrophages and dendritic cells, have an indispensable role in the activation of alloreactive T cells (28–31). Differentiation, proliferation and function of innate immune cells are also subjected to metabolism-dependent regulation (3). After allogeneic HSCT, these components of the immune system function in the context of the engrafted and rapidly expanding allogeneic HSC, residual leukemia cells potentially remaining at the state of MRD and rapidly dividing cells in host non-hematopoietic tissues that are the targets of GVHD, such as the gut (32, 33).

Based on the above, it is apparent that targeting metabolism for therapy of GVHD will require thorough understanding of the unique metabolic properties and programs of the multiple cellular components involved in GVHD and GVL. In the following sections we will briefly highlight the metabolic features of malignant hematopoietic cells and we will discuss the metabolic features that guide the function of T cells and APCs during processes involved in GVHD and GVL. We will

also provide rationale for potential therapeutic interventions by targeting metabolic pathways that guide the differentiation and function of these immune cells in the context of alloHSCT.

METABOLISM IN NORMAL AND MALIGNANT HEMATOPOIETIC CELLS

Metabolic changes drive division and differentiation of HSC and MP (9). HSCs are predominantly quiescent, in G_0 phase, but divide approximately every 145 days, as a consequence of a cell-cycle-linked maturation process (34, 35). Their dormancy is important to sustain adult HSC pluripotency and to prevent HSC exhaustion (36). In order to maintain this state, HSCs utilize aerobic glycolysis and suppress oxidative phosphorylation, thereby maintaining an environment of low ROS (37). HSCs respond rapidly to stimuli to either maintain themselves via self-renewal by sustaining glycolytic metabolism and symmetric division or give rise to committed progenitors, by shifting their metabolism toward mitochondrial metabolism and activation of TCA cycle or FAO and asymmetric division (9) (**Figure 1A**). This is supported by the observation that depleting the mitochondrial oxidative phosphatase *PTPMT1* blocks the entry into the cell cycle and differentiation of HSC (38). Maturation from a pluripotent state to a committed progenitor state also requires precise epigenetic modifications (39). Defects in DNA methyltransferases Dnmt3a and Dnmt3b that regulate such epigenetic effects are associated with impaired stem cell differentiation, leading to leukemia-inducing events (40).

Similarly to other malignant cell types, *anabolic metabolism* is the signature of malignant hematopoietic cells including AML, MM, and ALL (7, 41–43). This is mediated by upregulation of glucose transporters and increase of glucose uptake and glycolysis. Such changes are induced by molecular aberrations and inappropriate activation of signaling pathways such as PI3K/Akt/mTOR, enhanced pro-survival mechanisms, and hypoxia (5). Normal and malignant hematopoietic cells also highly depend on the use of glutamine. This is related to the expression of *myc*, which is proportional to HSC multipotency, cell-maintenance, and self-renewal (44). Upregulation of c-*myc* in high-grade lymphomas increases glutaminolysis and leads to glutamine dependence and addiction of malignant cells to support their biosynthesis pathways. Anaplerosis via glutamine usage in the TCA cycle may be a c-*myc*-mediated mechanism critical for survival and growth of malignant hematopoietic cells (45). An additional important anabolic pathway in malignant hematopoietic cells is fatty acid synthesis. Non-Hodgkin B-cell lymphoma cells are particularly sensitive to C-75, a fatty acid synthase inhibitor, supporting the premise that rapidly proliferative lymphoma cells are not only dependent on aerobic glycolysis but on other anabolic pathways for their growth and proliferation (46).

LSCs, which are responsible for survival and persistence of leukemia, are more dependent on aerobic glycolysis (**Figure 1B**) and display higher expression of the glycolysis enzymes PKM2 and LDH-A compared to normal HSCs. In turn, combined inhibition of PKM2 and LDH-A leads to eradication of LSCs (7). LSCs also rely on *catabolic pathways* for the production of energy and can utilize fatty acids for FAO in order to escape the detrimental effects of chemotherapy and maintain their survival under conditions of stress (47). Deletion of AMPK, an important sensor of energetic stress that maintains metabolic homeostasis by activating catabolic metabolism and autophagy, synergizes with metabolic stress caused by nutrient restriction in LSCs and profoundly suppresses leukemogenesis (48). In CML, autophagy acts as a possible mechanism of survival and resistance of leukemia to TKI treatment (49). Under these conditions, inhibition of mitochondrial OXPHOS can eradicate TKI-resistant CML LSCs (50). Thus, although LSCs are highly dependent on glycolysis under steady state conditions, they have a high degree of metabolic plasticity and adaptation potential and can utilize catabolic pathways in the setting of energy stress to support their survival and proliferation. The clinical relevance of the increased metabolic plasticity that is pivotal in LSCs is supported by the observation that BCL-2 blockage, which reduces OXPHOS, selectively eliminates this quiescent leukemia subset (51).

Our knowledge regarding the metabolic features of leukemia cells in relapsed or resistant disease in patients who undergo allogeneic HSCT is limited because relevant studies are currently missing. As mentioned above, relapsed or resistant leukemia cells display features of LSC, which are highly depend on glycolysis but also have the metabolic plasticity to adopt other metabolic programs for energy generation, including mitochondrial metabolism, FAO and autophagy. Thus, although therapeutic approaches to target glycolytic metabolism to inhibit activation of GVHD-mediating T_{EFF} cells are expected to suppress or eradicate MRD, it is possible that plasticity and metabolic adaptation will allow LSC to survive by shifting their metabolic preferences. Focused studies are required to address this issue.

IMMUNO-METABOLIC REPROGRAMING AND HEMATOPOIETIC STEM CELL TRANSPLANTATION

Role of Metabolism in T Cell Differentiation and Relevance to Alloreactive T Cell Function

Resting T cells rely on mitochondrial respiratory capacity and OXPHOS for their metabolism and bioenergetic demands. Upon activation, they demand higher energetic supply, met mostly by the engagement of glycolytic pathway and mitochondrial OXPHOS (52). Similar to cancer cells, activated T cells predominantly depend on glycolysis for energy production and generation of biosynthetic intermediates while sparing other nutrients for anabolic reactions. Glycolysis has a key role in the differentiation of T effector cells. Conversely, glucose deprivation impairs the ability of $CD8^+$ T cells to express IFN-γ gene, a signature of their differentiation into the effector state (53). Extracellular glucose that T cells uptake during the effector phase, supports fatty acid *de novo* synthesis and these newly synthesized lipids form the fuel used after the transition and differentiation

of T_{EFF} to T_{MEM} cells (27). Environmental cues that promote T_{MEM} cell differentiation, such as IL-15, promote mitochondrial biogenesis and the expression of Cpt1a, which allows entry of long chain fatty acids to the mitochondria and functions as the rate limiting enzyme for FAO. These immune-metabolic properties are associated with longevity and survival in high-stress environments (54). In contrast, pathologically activated lymphocytes, such as those in autoimmune diseases, activate mitochondrial metabolism but utilize glucose for OXPHOS (55).

Metabolic pathways are also linked to the functional differentiation and polarization of T cell subsets. Th1, Th2, Th17 and Tfh preferentially undergo glycolysis by increasing the expression of Glut1 and by activating the PI3K/Akt/mTOR pathway (26). mTOR plays a role as a cell nutrient sensor and is a crucial regulator of T cell metabolism (56) by activating anabolic reactions including glycolysis, but also fatty acid metabolism, by targeting SREBPs (57). Through these coordinated effects, mTORC1 leads to Th1 and Th17 differentiation along with regulation of T cell priming and generation of iTregs, while mTORC2 drives differentiation to Th2 (58). Although Th17 cells are known to depend on glycolysis (59), inhibiting ACC1, a key mediator for *de novo* fatty acid synthesis, impairs Th17 development in both human and mouse models, favoring the formation of anti-inflammatory $Foxp3^+$ Tregs (60). The significance of these complex effects mediated by mTOR on pathways that regulate glycolysis and fatty acid metabolism are also supported by the implications induced on T cell differentiation and function by AMPK signaling (61) which negatively regulates mTOR-mediated glycolytic metabolism (62). AMPK promotes FAO by multiple mechanisms, including the direct regulation of key lipid metabolizing enzymes, the negative regulation of the mTOR and the intracellular transport of fatty acids (63–65). These coordinated processes, leading to lipid synthesis and utilization, provide two key properties of T_{MEM} cells, namely longevity and immune quiescence (66).

It is therefore apparent that mTOR actively influences the differentiation of all T cell subsets that are involved in GVHD, including Th1, Th2, Th17 and Tfh cells. Th1, Th2 and Th17 have essential roles in the induction of aGVHD, while Tfh cells are pathogenic in cGVHD (67). Intriguingly, Tregs and T_{MEM} cells, which appear to be protective from GVHD, also depend on mTOR for their differentiation and function (68, 69). Due to their overall inhibitory effect on T effector cell function, mTOR antagonists such as sirolimus are routinely used for the prophylaxis or treatment of GVHD in alloHSCT recipients (70). The addition of RGI-2001, a synthetic CD1 ligand that expands Tregs *in vivo*, to sirolimus results in a greater decrease in GVHD rates, as compared to the ones achieved by either compound alone (71).

Although these results indicate that mTOR antagonists support the activation and differentiation of Treg *in vivo*, the mechanistic role of mTOR in Treg biology remains controversial. The absence of mTORC1 signaling during T cell differentiation has been associated with lack of Th1/Th2 polarization and enhanced conversion to Treg phenotype (58). Surprisingly, conditional targeting of mTORC1 in Treg cells by deletion of the mTORC1 partner, Raptor, resulted in impaired fatty acid and cholesterol synthesis, leading to defective Treg generation and function (72). Conversely, the absence of mTORC2 signaling by deletion of the mTORC2 partner, Rictor, potentiated the generation of short-lived effector and memory precursor $CD8^+$ T cells (73). The combined administration of the mTOR inhibitor, Rapamycin, and IL-2 not only preserved but also promoted Treg expansion and increased the donor $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, resulting in decreased aGVHD-related mortality (74). This implies that Treg differentiation and function is positively regulated by mTOR inhibition and mTOR-independent IL-2-mediated signaling. Conversely, cyclosporin, which inhibits IL-2 production by targeting NFAT signaling, compromised Treg proliferation *in vivo* (68). In light of their specific effects on Treg differentiation and expansion, the mechanisms of these immunosuppressive agents in the prevention and treatment of GVHD should be revisited and their Treg-dependent immunoregulatory effects should be considered when these compounds are used for the prevention or treatment of GVHD.

Metabolism of Alloactivated GVHD-Mediating T Cells

After HSCT, naïve donor T cells are directed to the recipient secondary lymphoid tissues, where they become activated by recipient's alloantigens (32, 33).When this happens, an increase in glycolysis and OXPHOS is induced (75–77). Overall, glycolysis escalates, becoming the principal source of energy for GVHD-causing T cells that under these conditions convert to T effector cells (75). For this mechanism to be efficient, carbohydrate catabolism mediators are also highly upregulated. These metabolic changes alter the functional profile of GVHD-mediating T cells, which are no longer naïve, but undergo differentiation to T effectors, simultaneously with metabolic reprogramming and proliferation in response to alloantigen-mediated stimulation (75) (**Figure 2**). In comparison to mice that received syngeneic BMT, mice undergoing allogeneic transplant displayed higher ECAR, accumulation of glycolytic intermediates, increased levels of LDH-A, Mct4, Glut1 and Glut3 mRNA, along with higher glucose-6-phosphate levels, all of which imply higher glycolytic activity (75). Consistent with the key role of glycolysis in regulating alloreactive T_{EFF} function, similarly to T_{EFF} of different specificity, overexpression of Glut1 results in superior T cell survival (78).

Glutamine metabolism is also a central component of T cell metabolic reprogramming during activation. T helper cell responses are supported by the upregulation of the glutamine/leucine transporter Slc7a5, and *Slc7a5* null cells are unable to complete metabolic reprogramming and fail to undergo differentiation and clonal expansion (79). The role of glutamine in T_{EFF} differentiation is supported by the observations that glutamine availability in the culture media increases IL-2 receptor expression, lymphocyte proliferation and cytokine production (79). Thus, glutamine is another critical source of energy and macromolecule production in activated T cells and might be involved in the development of alloreactive T cell responses and GVHD during alloHSCT (**Figure 2**).

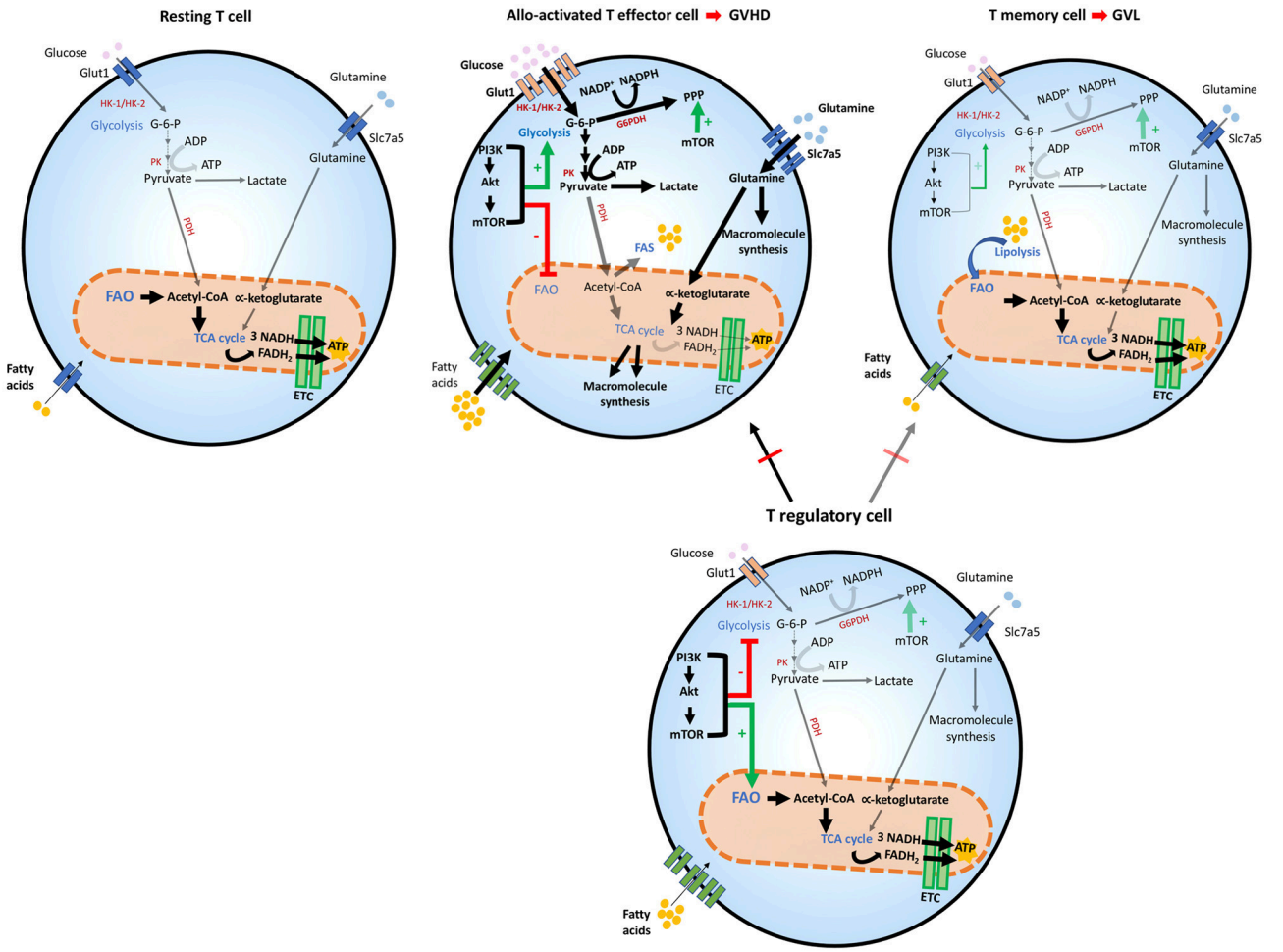


FIGURE 2 | Metabolic reprogramming of T cells after allogeneic stem cell transplantation. T cells adapt to distinct stressors in order to meet their bioenergetic demand. After HSCT, the engagement of distinct metabolic pathways is correlated with T cell role and function. Glycolysis is the preferred pathway for GVHD-mediating alloactivated T naïve cells after exposure to host antigens, which differentiate them to their effector phenotype and increase the expression of Glut1 receptor. Glycolytic reactions also provide a bridge for macromolecule synthesis, redox balance and cell growth, by producing intermediate metabolites that favor PPP. The generation of pyruvate from glucose yields lactate in the cytoplasm or acetyl-CoA, which enters the TCA cycle in the mitochondria. In alloactivated GVHD-mediating T cells, expression of glucose, amino acid, and fatty acid transporters increase leading to enhanced entry of these compounds (thick arrows). The PI3K/Akt/mTOR pathway favors glycolysis and suppresses FAO. mTOR also potentiates PPP in these cells. GVHD-mediating T cells also use amino-acids, especially glutamine, which enters the cell by the increased expression of Slc7a5 transporters. Glutaminolysis yields α-ketoglutarate that enters the TCA cycle in the mitochondria although mitochondrial metabolism does not seem to be the dominant metabolic profile of alloreactive T cells. In T effector memory cells, the main T subset involved in mediating GVL function, FAO is the dominant energy source and mitochondrial metabolism is significantly enhanced, leading to OXPHOS reactions in the ETC, where higher amounts of ATP are produced, thereby providing sustained energy for cell survival. T regulatory cells are protective against GVHD, by inhibiting predominantly alloactivated GVHD-mediating T cells but inhibit GVL-mediating T memory cells to a lesser extent thereby preserving GVL. Treg cells share metabolic features with GVL cells, as they use FAO as their preferred energy source. mTOR regulation appears to have a distinct effect on Treg cells, as it is necessary for optimizing FAO that leads to adequate Treg differentiation and function. ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; Akt, Protein Kinase B; ETC, Electron transport chain; FAD, Flavin adenine dinucleotide (oxidized state); FADH2, Flavin adenine dinucleotide (reduced state); G-6-P, Glucose-6-phosphate; G6PDH, Glucose-6-phosphate dehydrogenase; Glut1, Glucose transporter1; GVHD, graft-vs.-host diseases; GVL, graft-vs.-leukemia; HK-1/2, Hexokinase-1/2; HSCT, hematopoietic stem cell transplant; mTOR, Mechanistic/mammalian target of rapamycin; NAD, Nicotinamide adenine dinucleotide (oxidized state); NADH, Nicotinamide adenine dinucleotide (reduced state); NADP+, Nicotinamide adenine dinucleotide phosphate (oxidized state); NADPH, Nicotinamide adenine dinucleotide phosphate (reduced state); OXPHOS, Oxidative phosphorylation; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PPP, Pentose phosphate pathway; PK, Pyruvate kinase; Slc7a5, Solute carrier family 7 member 5; TCA cycle, Tricarboxylic acid cycle.

Although mitochondrial metabolism has a role in the function of alloactivated T cells (76, 77, 80), it was also observed that regulators of fatty acid uptake and FAO are significantly reduced after autologous or allogeneic HSCT, compared to resting T cells. This correlated with metabolic reprogramming of alloreactive T cells to favor glycolytic metabolism and glutaminolysis as the key pathways for energy generation (75). FAO seems to increase in GVHD T_{EFF} cells only after the fifth cell division, around 3 days post BMT (76), suggesting that these metabolic pathways might have distinct roles during the life of alloreactive T_{EFF} cells

in vivo. Nevertheless, most experimental evidence indicates that enhanced FAO is critical for T_{MEM} (27) and Treg cell activity (26, 72) and for this reason it would be protective against GVHD (12, 15, 16, 19). Thus, the precise role of FAO in alloreactive T cell function and the details of its regulation in GVHD remain to be determined.

Studies have indicated that administration of metformin, which activates AMPK thereby promoting FAO, might attenuate GVHD by supporting the differentiation of Treg and altering the balance between Th17 and Treg cells (81). This observation potentially provides an opportunity to repurpose metformin for the prevention or treatment of GVHD. However, two important issues should be taken into consideration: First, AMPK does not exclusively affect the function of Treg because T_{EFF} cells from AMPK KO mice display impaired differentiation and metabolic fitness, and impaired glutamine-dependent mitochondrial metabolism that allows T_{EFF} metabolic plasticity and survival under low-glucose conditions (61); Second, in addition to activating AMPK, metformin can inhibit complex I of the electron transport chain (ETC) (82) which may impact the metabolism and function of all T cell subsets independently of AMPK. Thus, glycolysis, FAO and AMPK remain attractive metabolic targets to explore for therapeutic immunomodulation of alloreactive T cells by individual or combinatorial approaches.

Microbiota in T Cell Metabolism and GVHD

The investigation of the role of microbiota in host immunity in health and disease is a highly active topic with major biological relevance. Commensal bacteria are closely related to the host's nutritional status and the function of the immune system. Our understanding about their role in disease pathogenesis is rapidly expanding. It is now well-known that the development, differentiation and polarization of T lymphocytes is affected by gut microbes (83, 84). Commensal microbe-derived SC-FA, butyrate and propionate, can promote the differentiation of Treg cells (85). In mice, *Clostridia* strains can induce $CD4^+Foxp3^+$ Treg differentiation by producing SC-FA (86). Tregs induced by these microbiota can also induce IL-10 and ICOS, affect the intestinal immune function, and prevent colitis and allergic diarrhea (86). Conversely, segmented filamentous bacteria in mice and *Bifidobacterium adolescentis* in humans, promote Th17 differentiation, enhance Th17 cell survival (87, 88) and exacerbate autoimmune arthritis (88). The latter reinforces the importance of understanding the harmful impact of symbiont-driven T helper cells in the context of inflammatory conditions.

Not unexpectedly, SC-FA can influence the development of GVHD (89). SC-FA regulate both T_{EFF} and Treg cells accumulation by increasing histone H3 acetylation in the locus of *Foxp3* and activating the mTOR pathway (90, 91). Consequently, Butyrate restoration in intestinal epithelial cells, implemented to overcome the reduction caused by the inflammatory cascade seen in alloHSCT, promotes histone acetylation and correlates with lower GVHD clinical scores (92). These findings indicate that microbial-derived metabolic products have a potential use in GVHD, probably due their impact on T cell subset differentiation and survival.

Metabolism of Leukemia-Activated GVL Effector Cells

In mice, GVL effect is driven by $CD4^+$ effector T_{MEM} that require cognate interaction with MHC-II and leukemia antigens (12). Although mouse models do not fully recapitulate human T_{MEM} life-long repertoire and CD4/CD8 ratio, it should be noted that the infusion of $CD4^+$ T_{MEM} to recipients of T cell-depleted human allografts effectively enhanced GVL and immune reconstitution without increasing GVHD (93). This observation provided an important insight on the potential use of sorted T cell populations to promote GVL, instead of administering unfractionated donor lymphocyte infusions, which are associated with GVHD (94). The therapeutic efficacy of this approach was explored by implementing selective depletion of T naïve cells from allografts given to high-risk leukemia patients. This modification of the allografts resulted in comparable rates of aGVHD but significantly improved responsiveness of aGVHD to steroid treatment. In addition, these patients had decreased cGVHD rates and improved immune reconstitution characterized by rapid T cell recovery and transfer of protective anti-viral immunity (93). Thus, selective utilization of donor T_{MEM} cells might be the most preferred approach to preserve immunity while decreasing GVHD-mediated morbidity.

The reason for the differential action of T naïve and T_{MEM} cells after allogeneic HSCT, has been hypothesized to rely on their differential responses. Unlike T naïve cells, T effector memory alloreactive cells cannot expand or sustain high magnitude responses and while they are less likely to induce cGVHD, they are sufficient to mediate GVL function (12, 15). Moreover, cytokine production by memory T cells is also suboptimal, compared to naïve T cells that rapidly increase aGVHD-associated cytokines TNF-α, IL-1, IL-6, and IFN-γ or the cGVHD-associated cytokine IL-17 (95). Because T naive cells that convert to effectors and T memory cells engage different metabolic pathways to meet their energetic demands, the distinct nature of GVL-specific and GVHD-specific alloreactive T cell populations might provide an excellent opportunity to introduce selective metabolism-targeting therapies, to optimize GVL and prevent the development of GVHD. Moreover, the differentiation of Treg cells that have the ability to suppress GVHD but not GVL (16, 17) are also supported by metabolic pathways similar to those engaged by T_{MEM} cells such as oxidative metabolism and FAO (26, 54). This metabolic program of T_{MEM} cells is supported by utilization of LC-FA for FAO (54). Although the mitochondrial transporter of LC-FAs, Cpt1a, is involved in this mechanism (54), subsequent studies discovered that pharmacologic inhibition or genetic ablation of *Cpt1a* did not affect the generation of T_{MEM} (96), suggesting that T cells may metabolize short-chain fatty acid, in the absence of Cpt1a activity. Further, in *Cpt1a* KO T cells, the use the Cpt1a inhibitor, etomoxir, used in concentrations significantly higher than those required to inhibit Cpt1a, suppressed the generation of Tregs *in vitro*, suggesting an a Cpt1a-independent action (96). Thus, differentiation of T_{MEM} and Treg depends on FAO that is regulated by CPT1a-dependent and independent

mechanisms. FAO might be a tentative therapeutic target to induce T_{MEM} and Treg differentiation in order to prevent GVHD and preserve GVL.

Metabolism of Antigen Presenting Cells and Relevance to Allogeneic-HSCT

The role of APCs, both from host and donor, in the setting of GVHD and GVL is a growing research focus during the past few years, given the recent understanding of their key role in both processes in alloHSCT (33, 97, 98). Recipient APCs are also important mediators of graft rejection, due to their potential to activate graft-infiltrating T cells (99). Today, it is well-known that the activation of the innate arm of the immune system is essential for the unfolding of GVHD, as APCs mediate T-cell priming and imprinting to GVHD target organs after transplantation (30, 98). Professional APCs, comprised DCs, B cells and macrophages, are capable of processing and presenting antigens to T cells through MHC proteins, promoting the formation of the immunological synapse that allows development of adaptive immune responses (98). During HSCT, host bone marrow APCs are mostly ablated by the conditioning regimen (100). Under these circumstances, skin macrophages and, to a lesser extent, dendritic cells engage in most of the antigen-presenting activities, mainly due to their resistance to myeloablative regimens (101, 102). Host and donor APCs have different roles in the development of GVHD. Host APCs seem to be involved in the induction of aGVHD, while donor macrophages contribute to cGVHD by cross-priming alloactivated $CD8^+$ T cells (98, 103, 104). Replacing host APCs with donor APCs reverses T cell activation, as it decreases the interaction between GVHD-related host APCs and donor $CD8^+$ T cells (28). Additionally, depletion of host liver and spleen-resident APCs results in decreased recruitment of allogeneic $CD8^+$ T cells, thereby suppressing hepatic aGVHD but not skin involvement (29). Paradoxically, host APCs also take part in GVL function, whereas donor APCs only have a limited role in this process (105).

APCs activate different metabolic pathways, depending on the engagement of specific accessory surface receptors, cytokine stimulation and other microenvironmental cues. DCs increase their glycolytic activity upon their TLR activation as a means to produce enough pyruvate that can activate TCA cycle reactions and OXPHOS (3, 106). IFN-γ-mediated signals can direct macrophages into the classic M1 proinflammatory phenotype, in which glucose uptake via GLUT1 mediated influx predominates. LPS expressed on the outer membrane of gram-negative bacteria, after interacting with TLR4 on M1 macrophages, induces glycolysis, leading to lactate accumulation and production of TCA cycle metabolites, particularly succinate, which induces the IL1-β production and inflammation (107). Conversely, IL-10, IL-4, and IL-13, induce the alternative anti-inflammatory M2 phenotype, which relies mostly on mitochondrial respiration and instead of inducing tissue inflammation, promotes resolution of inflammation, tissue remodeling, and repair (108–110).

These extensive studies indicate that the metabolic profile of M1 macrophages has similarities to that exhibited by activated effector-like T cells (such as those inducing GVHD), while the metabolic phenotype of M2 macrophages parallels that of memory-like T cells (such as those inducing GVL). Thus, concomitant metabolic reprogramming of APCs and T cells will have an important role in the net outcome of GVHD and GVL and these outcomes might vary dependent on the metabolic polarization of one or both these immune populations. For instance, the preferential engagement of APCs and T cells in glycolytic metabolism will allow immune cells to sustain inflammatory GVHD-mediating functions by promoting the generation of GVHD-inducing M1 macrophages and effector-like T cells. Conversely, the metabolic shift of these cell populations toward oxidative phosphorylation might selectively promote the differentiation of GVL-inducing memory-like T cells while supporting M2 differentiation and resolution of inflammation thereby preventing or suppressing GVHD. Indeed, inhibition of FAO by etomoxir suppressed M2 polarization of macrophages (111) and T_{MEM} cell differentiation in a Cpt1a dependent and independent manner (96). It should be noted that immune cell polarization *in vivo* is not an all or nothing event but rather a continuum that leads to an immune signature depending on the dominating metabolic balance, thereby providing an opportunity for therapeutic intervention through implementation of subtle metabolic changes that will influence both APCs and T cells. Future studies are needed to investigate whether targeting glycolytic metabolism will have a similar simultaneous effect to suppress both M1 macrophage polarization and generation of alloreactive T effector cells that mediate GVHD.

THERAPEUTIC RELEVANCE OF METABOLISM IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

The central goal of post-transplant therapeutic immunomodulation is the prevention or treatment of GVHD, without diminishing GVL activity. As outlined in the previous sections, the distinct metabolic pathways in these processes point to potential new therapeutic targets. Although FAO and glutamine metabolism might have a role in the activation of GVHD-inducing alloreactive T cells, elegant work has provided evidence that glycolysis is the dominant metabolic pathway of alloactivated donor T cells and inhibition of glycolysis by targeting HK-2 or the rate-limiting PFKFB3 prevents alloreactivity *in vivo* and attenuates GVHD in HSCT recipient mice (75). Blocking PFKFB3 in cancer cells also downregulates glucose influx, thereby interfering with tumor growth and disease progression (112). Thus, targeting PFKB3 might control GVHD while suppressing metabolic activity and growth of residual leukemia cells. Other means of reducing glycolytic activity also translate into amelioration of GVHD severity. The role of glucose metabolism in GVHD is also supported by the observation that Glut1 transporter-deficient murine $CD4^+$ effector T cells are unable to expand and induce GVHD *in vivo*, while, in this context, Treg population increases, showing independence from Glut1 (113). Notably, inhibiting glycolysis

by using 2-deoxyglucose, not only diminished the expansion of T_{EFF} cells but enhanced the differentiation of $CD8^+$ T_{MEM} cells (114). Similarly, IL-15-driven overexpression of Cpt1a induced T_{MEM} cell production and supported their survival (54). Such modifications in the abundance of T cell subsets by targeting glycolysis might selectively prevent GVHD while preserving GVL.

Targeting PI3K/AKT/mTOR pathway has been explored in the context of alloHSCT, because this pathway is central to the activation, expansion and differentiation of $T_{EFF,}$ T_{MEM}, and Treg cells. Inhibition of PI3K/AKT/mTOR with BEZ235, a dual PI3K/mTOR inhibitor, resulted in decreased T cell activation and diminished GVHD grade (115). Importantly, using rapamycin, an mTORC1 inhibitor, enforced FAO and increased T_{MEM} cell differentiation (116). Because rapamycin also promotes the differentiation of Treg (69), such approach might selectively suppress GVHD and promote GVL by inducing T_{MEM} and Treg. Thus, rapamycin might be repurposed and used not simply as an immunosuppressant but also as an immunomodulator to alter metabolism-driven differentiation of T cell subsets in recipients of alloHSCT.

Utilization of mitochondrial F1F0-ATPase inhibitor, Bz-423, promotes apoptosis of alloactivated cells, thereby reducing GVHD rates and improving survival without impairing immune reconstitution (77). The Cpt1a inhibitor, etomoxir, was also reported to decrease GVHD severity in mice after day 30 post-transplant, without impairing immune reconstitution (76). As mentioned above, etomoxir, used in concentrations significantly higher than those required to inhibit Cpt1a, suppressed the generation of Tregs *in vitro*, suggesting a Cpt1a-independent action (96). Such Cpt1a-independent effect of etomoxir was also observed in bone marrow derived macrophages from Cpt1/2 KO mice, in which etomoxir retained the ability to disrupt IL-4-mediated M2 macrophage polarization possibly by causing depletion of intracellular coA (111). Thus, the combined effects of such metabolism-targeting compounds might have implications in the components of the innate and adoptive immune system resulting in clinical effects on GVHD, GVL and immune reconstitution that are driven by the altered function of more than one immune population or by previously unidentified selective effects on a certain immune cell population. This is also supported by the observation that AEB071, an inhibitor of protein kinase C-θ that preferentially halts Treg differentiation and activation, preserves graft survival and GVL but prevents IFN-γ production and GVHD by enhancing the function of Treg (117).

Together, the results of targeting studies in various mouse models (54, 75, 77, 113, 115–117) are of direct clinical relevance and indicate that therapeutic targeting of selective components of signaling and metabolic pathways might have distinct outcomes on T cell differentiation and distinct clinical implications in the prevention and treatment of GVHD and GVL. Because possibly distinct metabolic mechanisms dominate during different phases of alloreactive T cell lifespan, it will be critical to determine the metabolic signatures of alloreactive GVHD- and GVL-specific T cells during various times after alloHSCT. Such knowledge will allow the design of proper therapeutic combinatorial therapies to selectively induce the desired metabolism-driven immune cell differentiation.

A major challenge when targeting metabolism for therapy of GVHD, will be to preserve the metabolic properties of pathogen-specific T_{EFF} cells, which are mandatory for their function under conditions of stress and response to pathogens. Future work is required to identify and dissect the potential metabolic differences of pathogen-specific vs. host antigen-specific T cells that induce GVHD. Identifying pathways that dominate in each of these populations during their lifespan will allow the development of experimental approaches and clinical trials to implement and evaluate metabolic interventions in these distinct T_{EFF} cell populations in parallel to studies dissecting the effects of such approaches on GVHD vs. GVL.

CONCLUDING REMARKS

Metabolism is a rapidly growing subject in immunology and malignant hematology. LSC that survive under intensive chemotherapy are responsible for MRD and relapse. These LSC use both anabolic and catabolic pathways, depending on the environmental cues. Our knowledge regarding the metabolic features of leukemia cells in relapsed or resistant disease in patients who undergo allogeneic HSCT is limited because relevant studies are currently missing. Based on current data, relapsed or resistant leukemia cells display features of LSC, which are highly dependent on glycolysis, but also have the metabolic plasticity to adopt other metabolic programs for energy generation, including mitochondrial metabolism, FAO and autophagy. Thus, although therapeutic approaches to target glycolytic metabolism employed to suppress GVHD-mediating T_{EFF} cells are expected to suppress or eradicate leukemia cells, it is possible that the high degree of plasticity and metabolic adaptation of LSC may provide them the means to survive by shifting their metabolic preferences. Identifying LSC dominant pathways upfront and modulating them by metabolism-targeting interventions, together with chemotherapy, will be highly beneficial because will eradicate LSC, thereby minimizing the risk for relapse. It is particularly intriguing and hopeful to attempt achieving this objective, together with selective metabolism-driven differentiation of immune cell subsets, with the goal to minimize GVHD and enhance GVL after allogeneic HSCT.

AUTHOR CONTRIBUTIONS

NT-O: wrote the main body of the manuscript and prepared figures; TK: wrote several sections of the manuscript and provided relevant citations; H-TW: wrote sections of the manuscript and provided relevant citations; VB: supervised NT-O, TK, and H-TW and was responsible for the overall preparation of the manuscript. All authors read and approved the content of the manuscript.

FUNDING

This work was supported by NIH/NCI grants: RO1CA183605-01, R01 CA229784-01, and RO1CA212605.

REFERENCES

1. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: the metabolic requirements of cell proliferation. *Science.* (2009) 324:1029–33. doi: 10.1126/science.1160809
2. MacIver NJ, Michalek RD, Rathmell JC. Metabolic regulation of T lymphocytes. *Annu Rev Immunol.* (2013) 31:259–83. doi: 10.1146/annurev-immunol-032712-095956
3. O'Neill LAJ, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med.* (2016) 213:15–23. doi: 10.1084/jem.20151570
4. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab.* (2008) 7:11–20. doi: 10.1016/j.cmet.2007.10.002
5. Herbel C, Patsoukis N, Bardhan K, Seth P, Weaver JD, Boussiotis VA. Clinical significance of T cell metabolic reprogramming in cancer. *Clin Transl Med.* (2016) 5:29. doi: 10.1186/s40169-016-0110-9
6. Warburg O. On the origin of cancer cells. *Science.* (1956) 123:309–14.
7. Wang Y-H, Israelsen WJ, Lee D, Yu VWC, Jeanson NT, Clish CB, et al. Cell-state-specific metabolic dependency in hematopoiesis and leukemogenesis. *Cell.* (2014) 158:1309–23. doi: 10.1016/j.cell.2014.07.048
8. Suganuma K, Miwa H, Imai N, Shikami M, Gotou M, Goto M, et al. Energy metabolism of leukemia cells: glycolysis versus oxidative phosphorylation. *Leuk Lymphoma.* (2010) 51:2112–9. doi: 10.3109/10428194.2010.512966
9. Ito K, Ito K. Hematopoietic stem cell fate through metabolic control. *Exp Hematol.* (2018) 64:1–11. doi: 10.1016/j.exphem.2018.05.005
10. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature.* (1994) 367:645–8. doi: 10.1038/367645a0
11. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood.* (1990) 75:555–62.
12. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, et al. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood.* (2008) 111:2476–84. doi: 10.1182/blood-2007-08-109678
13. Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L- memory T cells without graft-versus-host disease. *Blood.* (2004) 103:1534–41. doi: 10.1182/blood-2003-08-2987
14. Dutt S, Baker J, Kohrt HE, Kambham N, Sanyal M, Negrin RS, et al. CD8+CD44hi but not CD4+CD44hi memory T cells mediate potent graft antilymphoma activity without GVHD. *Blood.* (2011) 117:3230–9. doi: 10.1182/blood-2010-10-312751
15. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T cells do not induce graft-versus-host disease. *J Clin Invest.* (2003) 112:101–8. doi: 10.1172/JCI17601
16. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med.* (2003) 9:1144–50. doi: 10.1038/nm915
17. Taylor PA, Lees CJ, Blazar BR. The infusion of *ex vivo* activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood.* (2002) 99:3493–9. doi: 10.1182/blood.V99.10.3493
18. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of *ex vivo* expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood.* (2011) 117:1061–70. doi: 10.1182/blood-2010-07-293795
19. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *New Engl J Med.* (2011) 365:2055–66. doi: 10.1056/NEJMoa1108188
20. Matsuoka K, Koreth J, Kim HT, Bascug G, McDonough S, Kawano Y, et al. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med.* (2013) 5:179ra43. doi: 10.1126/scitranslmed.3005265
21. Inamoto Y, Flowers MED, Lee SJ, Carpenter PA, Warren EH, Deeg HJ, et al. Influence of immunosuppressive treatment on risk of recurrent malignancy after allogeneic hematopoietic cell transplantation. *Blood.* (2011) 118:456–63. doi: 10.1182/blood-2011-01-330217
22. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol.* (2012) 12:443–58. doi: 10.1038/nri3212
23. Saad A, Lamb LS. *Ex vivo* T-cell depletion in allogeneic hematopoietic stem cell transplant: past, present and future. *Bone Marrow Transplant.* (2017) 52:1241–8. doi: 10.1038/bmt.2017.22
24. Almeida L, Lochner M, Berod L, Sparwasser T. Metabolic pathways in T cell activation and lineage differentiation. *Semin Immunol.* (2016) 28:514–24. doi: 10.1016/j.smim.2016.10.009
25. Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. *J Exp Med.* (2015) 212:1345–60. doi: 10.1084/jem.20151159
26. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol.* (2011) 186:3299–303. doi: 10.4049/jimmunol.1003613
27. O'Sullivan D, van der Windt GJW, Ching-Cheng Huang S, Curtis JD, Chang C-H, Buck MD, et al. Memory CD8+ T cells use cell intrinsic lipolysis to support the metabolic programming necessary for development. *Immunity.* (2014) 41:75–88. doi: 10.1016/j.immuni.2014.06.005
28. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science.* (1999) 285:412–5.
29. Zhang Y, Shlomchik WD, Joe G, Louboutin J-P, Zhu J, Rivera A, et al. APCs in the liver and spleen recruit activated allogeneic CD8+ T cells to elicit hepatic graft-versus-host disease. *J Immunol.* (2002) 169:7111–8. doi: 10.4049/jimmunol.169.12.7111
30. Zhang Y, Louboutin J-P, Zhu J, Rivera AJ, Emerson SG. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell–mediated acute graft-versus-host disease. *J Clin Invest.* (2002) 109:1335–44. doi: 10.1172/JCI14989
31. Oberbarnscheidt MH, Zeng Q, Li Q, Dai H, Williams AL, Shlomchik WD, et al. Non-self recognition by monocytes initiates allograft rejection. *J Clin Invest.* (2014) 124:3579–89. doi: 10.1172/JCI74370
32. Beilhack A, Schulz S, Baker J, Beilhack GF, Wieland CB, Herman EI, et al. *In vivo* analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood.* (2005) 106:1113–22. doi: 10.1182/blood-2005-02-0509
33. MacDonald KP, Shlomchik WD, Reddy P. Biology of graft-versus-host responses: recent Insights. *Biol Blood Marrow Transplant.* (2013) 19:S10–4. doi: 10.1016/j.bbmt.2012.11.005
34. Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell.* (2008) 135:1118–29. doi: 10.1016/j.cell.2008.10.048
35. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell.* (2008) 132:631–44. doi: 10.1016/j.cell.2008.01.025
36. Orford KW, Scadden DT. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet.* (2008) 9:115–28. doi: 10.1038/nrg2269
37. Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, et al. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab.* (2015) 21:392–402. doi: 10.1016/j.cmet.2015.02.002
38. Yu W-M, Liu X, Shen J, Jovanovic O, Pohl EE, Gerson SL, et al. Metabolic regulation by the mitochondrial phosphatase PTPMT1 is required for hematopoietic stem cell differentiation. *Cell Stem Cell.* (2013) 12:62–74. doi: 10.1016/j.stem.2012.11.022
39. Bernitz JM, Kim HS, MacArthur B, Sieburg H, Moore K. Hematopoietic stem cells count and remember self-renewal divisions. *Cell.* (2016) 167:1296–309.e10. doi: 10.1016/j.cell.2016.10.022
40. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet.* (2011) 44:23–31. doi: 10.1038/ng.1009
41. McBrayer SK, Cheng JC, Singhal S, Krett NL, Rosen ST, Shanmugam M. Multiple myeloma exhibits novel dependence on GLUT4, GLUT8,

and GLUT11: implications for glucose transporter-directed therapy. *Blood.* (2012) 119:4686–97. doi: 10.1182/blood-2011-09-377846
42. Frolova O, Samudio I, Benito JM, Jacamo R, Kornblau SM, Markovic A, et al. Regulation of HIF-1α signaling and chemoresistance in acute lymphocytic leukemia under hypoxic conditions of the bone marrow microenvironment. *Cancer Biol Ther.* (2012) 13:858–70. doi: 10.4161/cbt.20838
43. Chen W-L, Wang J-H, Zhao A-H, Xu X, Wang Y-H, Chen T-L, et al. A distinct glucose metabolism signature of acute myeloid leukemia with prognostic value. *Blood.* (2014) 124:1645–54. doi: 10.1182/blood-2014-02-554204
44. Laurenti E, Varnum-Finney B, Wilson A, Ferrero I, Blanco-Bose WE, Ehninger A, et al. Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity. *Cell Stem Cell.* (2008) 3:611–24. doi: 10.1016/j.stem.2008.09.005
45. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci.* (2010) 35:427–33. doi: 10.1016/j.tibs.2010.05.003
46. Bhatt AP, Jacobs SR, Freemerman AJ, Makowski L, Rathmell JC, Dittmer DP, et al. Dysregulation of fatty acid synthesis and glycolysis in non-Hodgkin lymphoma. *Proc Natl Acad Sci USA.* (2012) 109:11818–23. doi: 10.1073/pnas.1205995109
47. Ye H, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, et al. Leukemic stem cells evade chemotherapy by metabolic adaptation to an adipose tissue niche. *Cell Stem Cell.* (2016) 19:23–37. doi: 10.1016/j.stem.2016.06.001
48. Saito Y, Chapple RH, Lin A, Kitano A, Nakada D. AMPK protects leukemia-initiating cells in myeloid leukemias from metabolic stress in the bone marrow. *Cell Stem Cell.* (2015) 17:585–96. doi: 10.1016/j.stem.2015.08.019
49. Ianniciello A, Dumas P-Y, Drullion C, Guitart A, Villacreces A, Peytour Y, et al. Chronic myeloid leukemia progenitor cells require autophagy when leaving hypoxia-induced quiescence. *Oncotarget.* (2017) 8:96984–92. doi: 10.18632/oncotarget.18904
50. Kuntz EM, Baquero P, Michie AM, Dunn K, Tardito S, Holyoake TL, et al. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nat Med.* (2017) 23:1234–40. doi: 10.1038/nm.4399
51. Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell.* (2013) 12:329–41. doi: 10.1016/j.stem.2012.12.013
52. Chang C-H, Curtis JD, Maggi LB, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell.* (2013) 153:1239–51. doi: 10.1016/j.cell.2013.05.016
53. Cham CM, Gajewski TF. Glucose availability regulates IFN- production and p70S6 kinase activation in CD8+ effector T cells. *J Immunol.* (2005) 174:4670–7. doi: 10.4049/jimmunol.174.8.4670
54. van der Windt GJW, Everts B, Chang C-H, Curtis JD, Freitas TC, Amiel E, et al. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity.* (2012) 36:68–78. doi: 10.1016/j.immuni.2011.12.007
55. Wahl DR, Petersen B, Warner R, Richardson BC, Glick GD, Opipari AW. Characterization of the metabolic phenotype of chronically activated lymphocytes. *Lupus.* (2010) 19:1492–501. doi: 10.1177/0961203310373109
56. Powell JD, Delgoffe GM. The mammalian target of rapamycin (mTOR) provides a critical link between T cell differentiation, function and metabolism. *Immunity.* (2010) 33:301–11. doi: 10.1016/j.immuni.2010.09.002
57. Düvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell.* (2010) 39:171–83. doi: 10.1016/j.molcel.2010.06.022
58. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity.* (2009) 30:832–44. doi: 10.1016/j.immuni.2009.04.014
59. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med.* (2011) 208:1367–76. doi: 10.1084/jem.20110278
60. Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrolfs K, et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nat Med.* (2014) 20:1327–33. doi: 10.1038/nm.3704
61. Blagih J, Coulombe F, Vincent EE, Dupuy F, Galicia-Vázquez G, Yurchenko E, et al. The energy sensor AMPK regulates T cell metabolic adaptation and effector responses *in vivo*. *Immunity.* (2015) 42:41–54. doi: 10.1016/j.immuni.2014.12.030
62. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell.* (2008) 30:214–26. doi: 10.1016/j.molcel.2008.03.003
63. Jäger S, Handschin C, St.-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proc Natl Acad Sci USA.* (2007) 104:12017–22. doi: 10.1073/pnas.0705070104
64. Rolf J, Zarrouk M, Finlay DK, Foretz M, Viollet B, Cantrell DA. AMPKα1: a glucose sensor that controls CD8 T-cell memory. *Eur J Immunol.* (2013) 43:889–96. doi: 10.1002/eji.201243008
65. MacIver NJ, Blagih J, Saucillo DC, Tonelli L, Griss T, Rathmell JC, et al. The liver kinase B1 is a central regulator of T cell development, activation, and metabolism. *J Immunol.* (2011) 187:4187–98. doi: 10.4049/jimmunol.1100367
66. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang L-S, et al. Enhancing CD8 T cell memory by modulating fatty acid metabolism. *Nature.* (2009) 460:103–7. doi: 10.1038/nature08097
67. Forcade E, Kim HT, Cutler C, Wang K, Alho AC, Nikiforow S, et al. Circulating T follicular helper cells with increased function during chronic graft-versus-host disease. *Blood.* (2016) 127:2489–97. doi: 10.1182/blood-2015-12-688895
68. Zeiser R, Leveson-Gower DB, Zambricki EA, Kambham N, Beilhack A, Loh J, et al. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood.* (2008) 111:453–62. doi: 10.1182/blood-2007-06-094482
69. Battaglia M, Stabilini A, Roncarolo M-G. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood.* (2005) 105:4743–8. doi: 10.1182/blood-2004-10-3932
70. Lutz M, Mielke S. New perspectives on the use of mTOR inhibitors in allogeneic haematopoietic stem cell transplantation and graft-versus-host disease. *Br J Clin Pharmacol.* (2016) 82:1171–9. doi: 10.1111/bcp.13022
71. Chen Y-B, Efebera YA, Johnston L, Ball ED, Avigan D, Lekakis LJ, et al. Increased Foxp3+Helios+ Regulatory T cells and decreased acute graft-versus-host disease after allogeneic bone marrow transplantation in patients receiving sirolimus and RGI-2001, an activator of invariant natural killer T cells. *Biol Blood Marrow Transplant.* (2017) 23:625–34. doi: 10.1016/j.bbmt.2017.01.069
72. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature.* (2013) 499:485–90. doi: 10.1038/nature12297
73. Zhang L, Tschumi BO, Lopez-Mejia IC, Oberle SG, Meyer M, Samson G, et al. Mammalian Target of rapamycin complex 2 controls CD8 T cell memory differentiation in a foxo1-dependent manner. *Cell Rep.* (2016) 14:1206–17. doi: 10.1016/j.celrep.2015.12.095
74. Shin H-J, Baker J, Leveson-Gower DB, Smith AT, Sega EI, Negrin RS. 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.* (2011) 118:2342–50. doi: 10.1182/blood-2010-10-313684
75. Nguyen HD, Chatterjee S, Haarberg KMK, Wu Y, Bastian D, Heinrichs J, et al. Metabolic reprogramming of alloantigen-activated T cells after hematopoietic cell transplantation. *J Clin Invest.* (2016) 126:1337–52. doi: 10.1172/JCI82587
76. Byersdorfer CA, Tkachev V, Opipari AW, Goodell S, Swanson J, Sandquist S, et al. Effector T cells require fatty acid metabolism during murine graft-versus-host disease. *Blood.* (2013) 122:3230–7. doi: 10.1182/blood-2013-04-495515
77. Gatza E, Wahl DR, Opipari AW, Sundberg TB, Reddy P, Liu C, et al. Manipulating the bioenergetics of alloreactive T cells causes their selective

apoptosis and arrests graft-versus-host disease. *Sci Transl Med.* (2011) 3:67ra8. doi: 10.1126/scitranslmed.3001975
78. Jacobs SR, Herman CE, MacIver NJ, Wofford JA, Wieman HL, Hammen JJ, et al. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol.* (2008) 180:4476–86. doi: 10.4049/jimmunol.180.7.4476
79. Sinclair LV, Rolf J, Emslie E, Shi Y-B, Taylor PM, Cantrell DA. Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. *Nat Immunol.* (2013) 14:500–8. doi: 10.1038/ni.2556
80. Glick GD, Rossignol R, Lyssiotis CA, Wahl D, Lesch C, Sanchez B, et al. Anaplerotic metabolism of alloreactive T cells provides a metabolic approach to treat graft-versus-host disease. *J Pharmacol Exp Ther.* (2014) 351:298–307. doi: 10.1124/jpet.114.218099
81. Park M-J, Lee S-Y, Moon S-J, Son H-J, Lee S-H, Kim E-K, et al. Metformin attenuates graft-versus-host disease via restricting mammalian target of rapamycin/signal transducer and activator of transcription 3 and promoting adenosine monophosphate-activated protein kinase-autophagy for the balance between T helper 17 and Tregs. *Transl Res.* (2016) 173:115–30. doi: 10.1016/j.trsl.2016.03.006
82. El-Mir MY, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
83. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell.* (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
84. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science.* (2012) 336:1268–73. doi: 10.1126/science.1223490
85. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature.* (2013) 504:446–50. doi: 10.1038/nature12721
86. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature.* (2013) 500:232–6. doi: 10.1038/nature12331
87. Atarashi K, Tanoue T, Ando M, Kamada N, Nagano Y, Narushima S, et al. Th17 cell induction by adhesion of microbes to intestinal epithelial cells. *Cell.* (2015) 163:367–80. doi: 10.1016/j.cell.2015.08.058
88. Tan TG, Sefik E, Geva-Zatorsky N, Kua L, Naskar D, Teng F, et al. Identifying species of symbiont bacteria from the human gut that, alone, can induce intestinal Th17 cells in mice. *Proc Natl Acad Sci USA.* (2016) 113:E8141–50. doi: 10.1073/pnas.1617460113
89. Teshima T, Reddy P, Zeiser R. Acute graft-versus-host disease: novel biological insights. *Biol Blood Marrow Transplant.* (2016) 22:11–6. doi: 10.1016/j.bbmt.2015.10.001
90. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. *Clin Transl Immunol.* (2016) 5:e73. doi: 10.1038/cti.2016.17
91. *Short-Chain Fatty Acids Induce Both Effector and Regulatory T Cells by Suppression of Histone Deacetylases and Regulation of the mTOR-S6K Pathway*. Available online at: https://www.ncbi.nlm.nih.gov/pubmed/24917457 (Accessed October 24, 2018).
92. Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat Immunol.* (2016) 17:505–13. doi: 10.1038/ni.3400
93. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest.* (2015) 125:2677–89. doi: 10.1172/JCI81229
94. Or R, Hadar E, Bitan M, Resnick IB, Aker M, Ackerstein A, et al. Safety and efficacy of donor lymphocyte infusions following mismatched stem cell transplantation. *Biol Blood Marrow Transplant.* (2006) 12:1295–301. doi: 10.1016/j.bbmt.2006.07.014
95. Henden AS, Hill GR. Cytokines in graft-versus-host disease. *J Immunol.* (2015) 194:4604–12. doi: 10.4049/jimmunol.1500117
96. Raud B, Roy DG, Divakaruni AS, Tarasenko TN, Franke R, Ma EH, et al. Etomoxir actions on regulatory and memory T cells are independent of Cpt1a-mediated fatty acid oxidation. *Cell Metab.* (2018) 28:504–15.e7. doi: 10.1016/j.cmet.2018.06.002
97. Kwan W-H, Hashimoto D, Paz-Artal E, Ostrow K, Greter M, Raedler H, et al. Antigen-presenting cell-derived complement modulates graft-versus-host disease. *J Clin Invest.* (2012) 122:2234–38. doi: 10.1172/JCI61019
98. Chakraverty R, Sykes M. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. *Blood.* (2007) 110:9–17. doi: 10.1182/blood-2006-12-022038
99. Zhuang Q, Liu Q, Divito SJ, Zeng Q, Yatim KM, Hughes AD, et al. Graft-infiltrating host dendritic cells play a key role in organ transplant rejection. *Nat Commun.* (2016) 7:12623. doi: 10.1038/ncomms12623
100. Ogonek J, Kralj Juric M, Ghimire S, Varanasi PR, Holler E, Greinix H, et al. Immune Reconstitution after allogeneic hematopoietic stem cell transplantation. *Front Immunol.* (2016) 7:507. doi: 10.3389/fimmu.2016.00507
101. Auffermann-Gretzinger S, Eger L, Bornhäuser M, Schäkel K, Oelschlaegel U, Schaich M, et al. Fast appearance of donor dendritic cells in human skin: dynamics of skin and blood dendritic cells after allogeneic hematopoietic cell transplantation. *Transplantation.* (2006) 81:866–73. doi: 10.1097/01.tp.0000203318.16224.57
102. Durakovic N, Bezak KB, Skarica M, Radojcic V, Fuchs EJ, Murphy GF, et al. Host-derived langerhans cells persist after MHC-matched allografting independent of donor T cells and critically influence the alloresponses mediated by donor lymphocyte infusions. *J Immunol.* (2006) 177:4414–25. doi: 10.4049/jimmunol.177.7.4414
103. Matte CC, Liu J, Cormier J, Anderson BE, Athanasiadis I, Jain D, et al. Donor APCs are required for maximal GVHD but not for GVL. *Nat Med.* (2004) 10:987–92. doi: 10.1038/nm1089
104. Alexander KA, Flynn R, Lineburg KE, Kuns RD, Teal BE, Olver SD, et al. CSF-1–dependant donor-derived macrophages mediate chronic graft-versus-host disease. *J Clin Invest.* (2014) 124:4266–80. doi: 10.1172/JCI75935
105. Reddy P, Maeda Y, Liu C, Krijanovski OI, Korngold R, Ferrara JLM. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nat Med.* (2005) 11:1244–9. doi: 10.1038/nm1309
106. Everts B, Pearce EJ. Metabolic control of dendritic cell activation and function: recent advances and clinical implications. *Front Immunol.* (2014) 5:203. doi: 10.3389/fimmu.2014.00203
107. Everts B, Amiel E, Huang SC-C, Smith AM, Chang C-H, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nat Immunol.* (2014) 15:323–32. doi: 10.1038/ni.2833
108. Galván-Peña S, O'Neill LAJ. Metabolic reprograming in macrophage polarization. *Front Immunol.* (2014) 5:420. doi: 10.3389/fimmu.2014.00420
109. Odegaard JI, Chawla A. Alternative macrophage activation and metabolism. *Annu Rev Pathol.* (2011) 6:275–97. doi: 10.1146/annurev-pathol-011110-130138
110. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med.* (1992) 176:287–92.
111. Divakaruni AS, Hsieh WY, Minarrieta L, Duong TN, Kim KKO, Desousa BR, et al. Etomoxir inhibits macrophage polarization by disrupting CoA homeostasis. *Cell Metab.* (2018) 28:490–503.e7. doi: 10.1016/j.cmet.2018.06.001
112. Lu L, Chen Y, Zhu Y. The molecular basis of targeting PFKFB3 as a therapeutic strategy against cancer. *Oncotarget.* (2017) 8:62793–802. doi: 10.18632/oncotarget.19513
113. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, et al. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. *Cell Metab.* (2014) 20:61–72. doi: 10.1016/j.cmet.2014.05.004
114. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *J Clin Invest.* (2013) 123:4479–88. doi: 10.1172/JCI69589

115. Herrero-Sánchez MC, Rodríguez-Serrano C, Almeida J, San Segundo L, Inogés S, Santos-Briz Á, et al. Targeting of PI3K/AKT/mTOR pathway to inhibit T cell activation and prevent graft-versus-host disease development. *J Hematol Oncol.* (2016) 9:113. doi: 10.1186/s13045-016-0343-5
116. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature.* (2009) 460:108–12. doi: 10.1038/nature08155
117. Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M, et al. Protein kinase C-θ mediates negative feedback on regulatory T cell function. *Science.* (2010) 328:372–6. doi: 10.1126/science.1186068

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 22 February 2019
doi: 10.3389/fimmu.2019.00163

Inflammatory Cytokine Networks in Gastrointestinal Tract Graft vs. Host Disease

Clint Piper[1] *and William R. Drobyski*[1,2]*

[1] Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, WI, United States, [2] Department of Medicine, Bone Marrow Transplant Program, Medical College of Wisconsin, Milwaukee, WI, United States

Graft vs. host disease (GVHD) is the major non-relapse complication associated with allogeneic hematopoietic stem cell transplantation (HSCT). Damage to the gastrointestinal (GI) tract from acute GVHD is a particularly serious event that can result in significant morbidity and mortality. Proinflammatory cytokines play a critical role in the pathophysiology of intestinal GVHD, in part by activating donor T cell populations which subsequently induce tissue damage. In this review, we summarize pre-clinical data derived from experimental murine models that have examined the role of inflammatory cytokine pathways that play critical roles in the pathophysiology of GVHD of the GI tract. Specific areas of focus are on STAT 3-dependent cytokines (e.g., IL-6, IL-23, and IL-21), and members of the IL-1 cytokine family, both of which have been shown to induce pathological damage within the GI tract during this disease. We also review established and ongoing efforts to translate these pre-clinical findings into the clinic in an effort to reduce morbidity and mortality due to this complication.

Keywords: graft vs. host disease, inflammatory cytokines, allogeneic hematopoietic stem cell transplantation, gastrointestinal tract, mouse models

OPEN ACCESS

Edited by:
Brian Christopher Betts, University of Minnesota Twin Cities, United States

Reviewed by:
Rizwan Romee, Dana–Farber Cancer Institute, United States
Yi Zhang, Temple University, United States

***Correspondence:**
William R. Drobyski
wdrobysk@mcw.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *01 October 2018*
Accepted: *18 January 2019*
Published: *22 February 2019*

Citation:
Piper C and Drobyski WR (2019) Inflammatory Cytokine Networks in Gastrointestinal Tract Graft vs. Host Disease. Front. Immunol. 10:163. doi: 10.3389/fimmu.2019.00163

GRAFT vs. HOST DISEASE

Graft-vs.-host disease (GVHD) is the major complication that occurs after allogeneic hematopoietic stem cell transplantation (HSCT) and is the leading cause of transplant-related mortality (1, 2). Mature T cells, which are present in the donor stem cell graft, are instrumental in the development of GVHD in HSCT recipients (1, 2). These pathogenic T cells are activated and clonally expand in response to recognition of a cognate recipient-derived peptide on an antigen presenting cell (APC), mounting an adaptive immune response against healthy recipient tissues. Current evidence in the literature suggests that two phases of antigen presentation occur (3). GVHD is initiated by recipient hematopoietic and non-hematopoietic APCs by a process termed direct alloantigen presentation (4, 5). Following the elimination of recipient-derived APCs post-transplantation, donor-derived APCs sustain GVHD by presenting recipient-derived peptides through the indirect pathway (6–9). Studies have revealed that the most important donor-derived APCs in this process are classical dendritic cells (7, 9).

GVHD has been divided into two phases, termed acute and chronic, which are distinguishable based on the timing of onset as well as unique clinical and pathological manifestations

(10–12). During the acute phase, which is responsible for significant mortality (13), GVHD targets a restricted set of organs including the skin, gastrointestinal (GI) tract, lung, and liver (1, 2, 4). Compelling data in experimental models have shown that the GI tract plays a primary role in the propagation of this disease (14, 15). Damage to the gastrointestinal mucosa from the conditioning regimen results in the release of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) (16, 17), which activate cells of the innate immune system through the ligation of pattern recognition receptors (PRRs) (2). This ultimately leads to the generation of clonally-expanded alloreactive T cells which mediate further damage, creating an inflammatory cascade (18). From a clinical perspective, involvement of the GI tract is a major cause of morbidity and can result in significant complications including protracted diarrhea, requirement for parenteral nutrition, and infectious complications due to translocation of bacteria across a damaged mucosal barrier (19). Given the pivotal role that the GI tract plays in acute GVHD biology, strategies designed to reduce inflammation in this target organ have the potential to significantly decrease morbidity and mortality associated with this disease.

STAT3 SIGNALING IN GVHD OF THE GI TRACT

During the formation of an immune response, the transduction of signals from the T cell receptor, costimulatory ligands, and cytokines into the nucleus is required for the differentiation of naïve T cells into effector lineages. During GVHD, these effector alloreactive cells are then able to secrete inflammatory cytokines and acquire cytotoxic capability, leading to tissue damage. Signal transducer and activator of transcription (STAT) proteins are responsible for much of these gene expression changes as a result of signaling through cytokine receptors. Upon ligation of a cytokine to its receptor, Janus kinases (JAKs) bind to the cytoplasmic domain of the receptor and become active, phosphorylating the appropriate STAT proteins, which then dimerize and translocate to the nucleus where they execute their function in driving transcriptional changes (20). Of these STAT proteins, STAT3 is particularly important in T cell pathogenicity during GVHD (21). In fact, treatment with a small molecule that inhibits STAT3 phosphorylation (22) or transplantation with STAT3-deficient T cells (21) significantly reduced GVHD-related mortality and pathological damage within the colon, providing support for the premise that STAT3-depednent cytokines play a prominent role in the induction of inflammation within this tissue site. Abrogation of STAT3 signaling was associated with a reduction in donor effector T cells with a corresponding increase in the number of regulatory T cells (Tregs). These results suggested that the STAT3 signaling pathway plays a critical role in balancing the effector and regulatory arms of the immune system within the context of GVHD. This basic premise has been confirmed *in vitro* using human cells where small molecule inhibition of STAT3 signaling suppressed alloreactive T cell proliferation while enhancing expansion of induced Tregs (iTregs) (23). In addition, $CD4^+$ T cell STAT3 activation has been associated with an increase in T_H17 cells and corresponding pathological damage within the GI tract in patients (23).

In contrast to the proinflammatory nature of STAT3 signaling in alloreactive T cells, expression of STAT3 in recipient myeloid cells was found to exacerbate GVHD (24). Notably, this analysis was limited to LysM-expressing cells, which are predominantly of the macrophage/monocyte lineage. While this study did not explore a mechanism for why STAT3 signaling in recipient myeloid cells elicits a paradoxical anti-inflammatory effect, the authors did note an increase in the number of donor $CD4^+$ and $CD8^+$ T cells in the spleen and an elevation in serum IFN-γ and IL-17 in LysM-Cre $STAT3^{fl/-}$ recipients compared to WT recipients, suggesting that this subset of recipient myeloid cells might indirectly regulate donor T cell responses. Interestingly, deficiency in donor myeloid cells had no impact on overall GVHD severity (24). Thus, the proinflammatory effects of STAT3 signaling appear to be mediated through T cells and not myeloid cell populations. The potential clinical significance of these observations derives from the fact that a number of the inflammatory cytokines that have been implicated in the pathophysiology of GVHD, specifically within the gastrointestinal tract, use STAT3 as part of their signaling pathway, and therefore are amenable to blockade with appropriate and specific antibodies. The STAT-dependent cytokines which have been most critically examined with respect to GVHD within the GI tract are IL-6, IL-23, and IL-21.

INTERLEUKIN 6

IL-6 is a proinflammatory cytokine that is crucial in initiating a T_H17 immune response. In the presence of IL-6 and TGF-β, naive T cells are able to differentiate into cells of the T_H17 lineage, whereas in the absence of this cytokine, these same cells are directed to become Tregs (25, 26). Specifically, TGF-β-induced Foxp3 is able to inhibit the transcriptional activation of RORγt which prevents the differentiation of T_H17 cells from naïve $CD4^+$ T cells (27). Thus, IL-6 appears to have a pivotal role in facilitating inflammatory responses by the immune system. In experimental murine studies, IL-6 and soluble IL-6R levels have both been shown to be increased in the gastrointestinal tract during GVHD (28). Moreover, blockade of IL-6 signaling by the administration of an antibody that binds to the IL-6 receptor significantly reduces GVHD-associated mortality and, specifically, pathologic damage within the colon (28–30). In one study (28), this was attributed to a significant increase in the absolute number of Tregs that was due to augmentation of both thymic-dependent and thymic-independent Treg production. Notably, when GVHD protection was dependent solely upon the ability to generate iTregs, blockade of IL-6 signaling resulted in a reduction in GVHD severity only within the colon (30). These results support the premise that IL-6 has an important role in mediating GVH responses within this tissue site, and that inhibition of this signaling pathway serves to recalibrate the effector and regulatory arms of the immune system in the GI tract. It should be noted that augmented Treg reconstitution

has not been observed in all studies (29), although this may be due, in part, to a more abbreviated anti-IL-6R antibody administration schedule that did not provide sufficient IL-6 blockade to positively affect Treg regeneration. The requirement for more protracted anti-IL-6R antibody administration to observe robust Treg reconstitution is supported by findings in a murine sclerodermatous chronic GVHD model (31).

The potential efficacy of IL-6 blockade for the treatment and prevention of GVHD has also been examined in humans. This is due to the availability of tocilizumab which is a humanized anti-IL-6R antibody that has been FDA-approved for the treatment of patients with rheumatoid and juvenile arthritis (32, 33). Off label use of tocilizumab has therefore been possible in HSCT patients. Initial studies using tocilizumab have been in patients with steroid refractory (SR) GVHD. A total of three studies comprising 31 patients have reported results on the use of tocilizumab for the therapy of SR acute GVHD (34–36). In nearly all patients (i.e. 30/31), treatment was instituted for disease involving the lower GI tract. In two of the three studies, response rates (PR and CR) were quite similar (67 and 69%, respectively). In a third trial, however, responses were observed in only 44% of patients and were short-lived. One potential explanation for the discrepancy in these results is that the majority of patients in this latter trial had concurrent liver GVHD, and tocilizumab has not been shown in any study to have any efficacy for the treatment of disease in the liver. The reasons for this are not entirely clear, although the fact that one of the primary side effects of tocilizumab is transaminitis suggests that this agent may induce some degree of liver inflammation which could be deleterious in the setting of concurrent liver GVHD.

Inhibition of IL-6 has also been examined for the prevention of acute GVHD in allogeneic HSCT patients. The first report was by Kennedy et al. (37) who treated 48 patients (median age 48) with a single dose of tocilizumab on the day prior to transplantation in addition to standard immune suppression consisting of tacrolimus and methotrexate. The primary end point of the study was grade 2–4 acute GVHD at day 100. Conditioning was with either total body irradiation and cyclophosphamide (myeloablative) or fludarabine and melphalan (reduced intensity) and patients were transplanted with stem cell grafts from either HLA-matched sibling or matched unrelated donors. The incidence of grades II-IV and III-IV acute GVHD at day 100 was 12 and 3%, respectively, which was lower than historical controls, although this was not a randomized trial nor were the patients demographically matched to a historical or contemporaneous cohort. Of note, GVHD in the GI tract occurred in only 8% of patients and it was not specified as to whether this involved the lower or upper GI tract. Therefore, it is possible that the incidence of lower tract GI GVHD was even lower which is noteworthy given studies that have shown that upper GI tract GVHD is generally responsive to modest doses of steroids and does not impact overall survival (38, 39). Immune reconstitution was also preserved in these patients which suggested that blockade of IL-6 signaling did not deleteriously impact overall immunity (37). Flow cytometric and gene expression analysis of both monocytes and $CD4^+$ T cells of patients treated with Tocilizmab revealed that there was a reduction in STAT3 phosphoyrylation and an attenuation in expression of STAT3-driven genes (37), demonstrating that IL-6 is a prominent inducer of this signaling cascade in human patients during GVHD.

A more recent study (40) also administered tocilizumab in addition to tacrolimus and methotrexate for the prevention of GVHD in an older aged population (median age 66) who underwent reduced intensity or myeloablative stem cell transplantation. All patients received busulfan-based conditioning which distinguished this trial from the previous publication. The tocilizumab administration schedule, however, was identical to that employed in the study of Kennedy et al. The cumulative incidences of grades II-IV and III-IV acute graft vs. host disease were 14 and 3% at day 100 which was similar to that observed in the prior trial. Importantly, we observed that there were no cases of GVHD of the lower gastrointestinal tract within the first 100 days. To provide additional context to these results, the authors obtained a control population from the database of the Center for International Blood and Marrow Transplant Research consisting of patients who were demographically matched for age, performance status, conditioning regimen, disease, and donor type, but who had received only tacrolimus and methotrexate for GVHD prophylaxis. This analysis revealed a lower cumulative incidence of grades II-IV acute graft vs. host disease (17 vs. 45%) and a significant increase in grades II-IV acute graft vs. host disease-free survival at 6 months (69 vs. 42%) in patients who were treated with tocilizumab, tacrolimus, and methotrexate. Collectively, these studies provided evidence that inhibition of IL-6 signaling had efficacy for the prevention of GVHD in the GI tract in allogeneic HSCT patients.

INTERLEUKIN 23

IL-23 is a member of the IL-12 family, signals through STAT3, and is secreted by DCs, as well as other APCs such as macrophages and monocytes (41). This cytokine shares a p40 subunit with IL-12, but also contains an IL-23-specific p19 component. The p19/p40 complex binds to a heterodimer of IL-12Rβ1, which is shared with the IL-12 receptor and a unique IL-23 receptor subunit that together is present on memory/activated T cells, DCs and macrophages (42). Early studies demonstrated that IL-23 plays a critical role in disorders such as experimental allergic encephalomyelitis (EAE) (43), collagen-induced arthritis (44), and inflammatory bowel disease (45) implicating this cytokine as a pivotal mediator in the pathogenesis of inflammatory disorders and autoimmunity. Pre-clinical murine BMT studies have demonstrated that IL-23 has a selective role in the promotion of inflammation within the colon during acute GVHD. In addition, this cytokine also functions as a critical mediator linking mucosal injury and LPS translocation that occurs as a consequence of the conditioning regimen to subsequent proinflammatory cytokine production and GVHD-associated pathological damage (46, 47). In these murine models, transplantation of IL-23–deficient marrow grafts or the administration of a p19-specific antibody resulted in

a significant amelioration in the severity of acute GVHD. This was shown to be due to the preferential reduction in colonic GVHD-induced pathology, accompanied by a decrease in proinflammatory cytokine production within this target organ. Donor, as opposed to host, APC production of IL-23 was demonstrated to be crucial for inducing GVHD-associated inflammation in the colon. These findings established that IL-23 has a novel organ-specific role in GVHD biology within the context of a broader systemic inflammatory disorder. Further mechanistic studies revealed that the proinflammatory effects of IL-23 were mediated at least in part by IFN-γ, not IL-17, and that an intact upstream LPS/TLR4 signaling pathway was required for IL-23-mediated colonic inflammation. Moreover, blockade of this pathway did not abrogate the graft vs. leukemia effect when tested in both acute and chronic models of leukemia (46).

More recent studies have shown that blockade of the IL-23 receptor using either genetic or antibody-based approaches similarly protect mice from lethal GVHD and pathological damage in the colon (47). In the course of these studies, a unique colitogenic $CD4^+$ T cell population was identified that constitutively expresses the β2 integrin CD11c, has a biased central memory phenotype, possesses innate-like properties by gene expression analysis, and has augmented expression of the gut-homing molecules, α4β7 and CCR9. Adoptive transfer of these cells resulted in increased overall mortality, proinflammatory cytokine production, and pathology specifically in the colon. The pathogenicity of these cells was critically dependent upon co-expression of the IL-23 receptor. The fact that these $CD4^+$ T cells possess an innate-like transcriptional signature suggests that they are positioned at the interface of the innate and adaptive immune systems where they are able to mediate early inflammatory events during GI tract GVHD.

There are currently two p19-specific antibodies (i.e., guselkumab and tildrakinumab) that have received FDA approval for the therapy of psoriasis (48, 49). However, specific blockade of the IL-23 signaling pathway has not been examined for the prevention or treatment of GVHD in humans. Ustekunimab which binds to the p40 subunit and thereby inhibits both IL-12 and IL-23 has been administered to patients for the prevention of GVHD in a randomized, placebo-controlled study (50). The results of this trial showed that patients treated with Ustekunimab had no difference in the incidence of grades 2–4 acute or chronic GVHD, and there was no specific protective effect on the severity of GVHD within the GI tract. Interestingly, despite the lack of effect on GVHD, there was a significant reduction in transplant-related mortality which translated to an improvement in survival. However, since the trial was not powered to assess these clinical outcomes and therefore were not the primary endpoints of the trial, the significance of these findings is not entirely clear.

INTERLEUKIN 21

IL-21 is produced by $CD4^+$ T cells, $CD8^+$ T cells, and NKT cells, while the receptor for IL-21 is expressed on T cells, B cells, NK cells, dendritic cells macrophages, and epithelial cells (51). The role of IL-21 in the biology of GVHD has been examined in a number of murine transplantation models (52–55). A common finding in all of these studies has been that blockade of IL-21 signaling by either antibody-based strategies or genetic approaches is able to significantly reduce the severity of GVHD. In some instances, this was shown to be due to an increase in the reconstitution of Tregs accompanied by a commensurate reduction in the expansion of donor effector T cells, suggesting that blockade of IL-21 signaling recalibrates the effector and regulatory arms of the immune system, similar to IL-6 blockade. Notably, several studies have confirmed that blockade of IL-21 signaling results in decreased pathological damage specifically within the GI tract (52–55). More recently, tissue from the GI tract of patients with active GVHD also revealed increased IL-21 expression in mononuclear cells in the colon when comparted to samples obtained from patients with no GVHD (56), suggesting that IL-21 may play a role in gastrointestinal GVHD in humans as well. As of yet, however, there have been no trials examining whether blockade of IL-21 signaling is able to reduce the severity of GVHD in humans.

INTERLEUKIN 1 FAMILY MEMBERS

Interleukin-1 (IL-1) was one of the first cytokines described and was named for the soluble product of macrophages during inflammation (57). Since then, IL-1 has been discovered to be not a single gene product but a family of cytokines (58), collectively referred to as the "IL-1 superfamily" (59, 60). Most of the genes in the IL-1 family are clustered together on the same chromosome, likely attributable to a gene duplication event that is evident in their similarity in sequence, structure, and function (61). IL-1 family cytokines direct a host of events in the immune system ranging from acute inflammatory processes initiated by the cells of the innate immune system (57), to T cell differentiation (62), to the regulation of inflammation (63, 64). In this section, we will focus on the "classical" IL-1 cytokines (IL-1α and IL-1β) as well as the IL-1 receptor antagonist (IL-1RA), which binds the IL-1 receptor and blocks binding of other ligands but elicits no signaling itself (64). Furthermore, we will discuss new insights into the role of IL-33, another IL-1 family member, and its cognate receptor ST2 in mediating inflammation in the GI tract during GVHD.

INTERLEUKIN 1

A role for the IL-1 family in the biology of GVHD was first postulated in 1991 when increased levels of IL-1α mRNA was detected in the skin of GVHD recipients in an MHC-matched, minor antigen mismatched murine transplant model (65). In addition, the administration of a recombinant human IL-1Ra was found to significantly increase survival in transplant recipients (66), supporting a role for this cytokine in the pathophysiology of this disease. Subsequent studies yielded more conflicting results, raising the issue as to whether this observed variability might, to some extent, be model-specific (66, 67). These preclinical

studies, however, did lay the foundation for human trials testing the efficacy of IL-1 blockade which were conducted in patients with steroid-refractory GVHD. In a phase I/II trial, which involved a 7 day continuous infusion of recombinant IL-1Ra, 10 out of 16 patients had an overall reduction in GVHD grade, including 8 out of 11 patients with GI-tract involvement who showed improvement (68). In another phase I/II trial using a recombinant IL-1 decoy receptor, eight of 14 patients had an overall reduction in GVHD grade, with 2 of 6 patients with GI tract involvement showing organ-specific improvement (69). The largest clinical trial examining anti-IL-1-directed therapy was conducted nearly a decade after the initial observation in murine models (70). This was a double-blind, placebo-controlled trial involving 186 patients in which the study arm consisted of treatment with an IL-1 receptor antagonist for GVHD prophylaxis. The primary endpoints of this study were event-free survival, overall survival, and incidence of GVHD. Unfortunately, patients that received IL-1Ra treatment had no improvement in any of these outcome measures. Notably, recombinant IL-1Ra was administered in the peritransplant period, with IL-1Ra levels in the serum returning to baseline by day 14 (70), before most patients develop acute GVHD. Whether this administration schedule may have been responsible for the lack of any perceived effect is not clear and the ability of IL-1 blockade to prevent GVHD within the GI tract in humans remains unproven.

INFLAMMASOME SIGNALING

With the discovery of the inflammasome (71) there has been renewed interest in the role of IL-1 cytokines in gastrointestinal GVHD. This is due to the fact that the GI tract is a source of innate immune activating pathogen- and damage-associated molecular patterns (15), some of which can cause activation and assembly of the inflammasome. In the case of one particular NLRP3, the ligation of TLR4 by lipopolysaccharide (LPS) causes the up regulation of inflammasome substrates and components such as pro-IL-1β and NLRP3 (72), as well as the deubiquitination and stabilization of NLRP3 (73). As a complementary step, the ligation of the purinergic receptor P2X7 by ATP provides the second step in inflammasome activation, ultimately leading to the assembly of NLRP3 with ASC, cleavage of pro-caspase-1, and caspase-1-mediated conversion of pro-IL-1β to its secreted and biologically active form (73).

Jankovic and colleagues provided the first evidence that the NLRP3 inflammasome is important in the pathophysiology of gastrointestinal GVHD (74). This report showed that in a MHC-mismatched murine model, pretransplant but not post-transplant treatment of mice with an IL-1 receptor antagonist or an IL-1β blocking antibody prevented GVHD. Furthermore, they showed that the NLRP3 inflammasome was required for production of IL-1 in the GI tract and corresponding lethality post-transplant. Biopsies from patients with GVHD had a higher proportion of cleaved (active) caspase-1 staining cells by immunohistochemistry compared to biopsies from transplant patients without GVHD. PBMCs isolated from patients with GVHD produced more IL-1β than those from BMT patients without GVHD and healthy controls. These results provide strong evidence that the NLRP3 inflammasome/IL-1β pathway is involved in the pathogenesis of gastrointestinal GVHD and that further investigation into these pathophysiological mechanisms is warranted to determine whether this will have potential therapeutic implications. In this regard, it is noteworthy that transplantation of microRNA(mir)-155-deficient dendritic cells has also been shown to cause less GVHD in the GI tract due to defective inflammasome activation (75). This observation could potentially be exploited clinically using antagomir administration to inhibit mir-155.

INTERLEUKIN 33/ST2 AXIS

One of the more recently identified pathways in GVHD biology is the IL-33/ST2 axis. IL-33 is a member of the IL-1 superfamily that has been identified as an alarmin and is released during cell injury and necrosis to initiate the immune response (76). IL-33 is produced primarily by a variety of non-hematopoietic cells which include endothelial cells, fibroblasts, and epithelial cells in the intestines and bronchi. Within the GI tract, in particular, IL-33 is expressed by α-SMA$^+$ subepithelial myelofibroblasts which have also been termed pericryptal fibroblasts (77). Release of IL-33 leads to binding to its membrane receptor, ST2, which is expressed on a large number of immune cells (i.e., T_H2 cells, regulatory T cells, type 2 innate lymphoid cells, macrophages, and granulocyte populations) (78). Notably, a soluble form of the receptor, sST2, lacks transmembrane and cytoplasmic domains and serves as a decoy receptor to IL-33, hampering the ability of this cytokine to elicit and effect in target cells (76).

In preclinical studies, IL-33 and sST2 have both been shown to be increased in the blood of mice during GVHD (79, 80). IL-33 is specifically increased in the GI tract where it is produced by non-hematopoietic cells in both murine models and patients with stage IV acute GVHD (79). The mechanism by which the IL-33/ST2 axis manipulates GVHD is complex. Transplant recipients that are deficient for IL-33 are protected from GVHD and administration of IL-33 in the early post transplantation period (days 3–7 post-transplant) was shown to exacerbate this disease (79), suggesting a proinflammatory role. Post-transplant IL-33 administration was associated with reduced survival, higher serum TNF-α, and an increased number of infiltrating intestinal donor T cells (79). Of note, post-transplantation blockade of IL-33 with an sST2-Fc receptor fusion protein also attenuated GVHD. In contrast, peri-transplant IL-33 administration (days −10 to +4) attenuated disease (81). This reduction in GVHD severity was associated with an increase in recipient mST2$^+$ regulatory T cells, a cell population that is able to survive after total body irradiation. A reduction in GVHD was also observed in mice that received peritranspant sST2 blockade, which potentiates the effects of IL-33 due to an inhibition of its decoy receptor (80). Notably, sST2 blockade did not affect expression of mST2 on specific lymphocyte populations, such as T_H2 cells and regulatory T cells (80). These data indicate a

paradoxical role for IL-33/ST2 signaling in GVHD, whereby early treatment of IL-33 downregulates inflammation in the GI tract, whereas administration of IL-33 is proinflammatory once disease has been established. Thus, the pro or anti-inflammatory effects of IL-33 in GVHD appear to be highly schedule dependent. While the reasons for this are still not completely clear, this disparity could be due to differences in cell populations which populate the gastrointestinal tract at various stages post-transplantation. For example, recipient Tregs of which there is an $ST2^+$ subset which can be expanded with peritransplant IL-33 treatment are present early post-transplantation and can suppress GVHD (81). In fact, the depletion of Tregs during peritransplant IL-33 administration results in the loss of protection against GVHD (81). Conversely, IL-33 signaling appears to act primarily on $ST2^+$ conventional T cells later post-transplantation resulting in an exacerbation of the disease (79). The temporal effects of IL-33 in these models may also be, to some extent, dose-dependent, as mice which received IL-33 peritransplant and were protected from GVHD received more than twice the dose over a much longer treatment window (81) than those that received posttransplant IL-33 (79).

In allogeneic HSCT patients, ST2 has emerged as a powerful biomarker that is predictive for GVHD severity in patients. Specifically, biomarker panels obtained early post transplantation which incorporated ST2 have been shown in several studies to be predictive for increased non-relapse-related mortality (82–84). In addition, in one of these studies (83), severe GI tract GVHD was also significantly greater in those patients who were in the cohort with highest levels of ST2. ST2 has also emerged as a critical component of a biomarker panel that has been shown to be predictive for response or lack thereof in steroid refractory acute GVHD (85). This disease carries a particularly high mortality which is typically attributable to disease involving the GI tract which is often the proximate cause of death. To date, there have been no clinical studies which have attempted to interrupt signaling through the IL-33/ST2 pathway in order to reduce inflammation. However, recent efforts to develop small molecule inhibitors that interfere with this pathway has shown promise in murine studies where there has been a reduction in sST2 plasma levels, reduced GVHD, and improved survival (86). Thus, these data provide hope that targeting of this pathway may soon be clinically feasible.

OTHER CYTOKINES

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF was originally characterized and designated as a hematopoietic growth factor which could promote myelopoiesis in the bone marrow. However, more recent studies have demonstrated that GM-CSF is largely redundant in the development of the hematopoietic system as mice deficient in either the cytokine or its receptor have only limited defects in steady-state myelopoiesis (87, 88). Rather, GM-CSF has been implicated as a key signaling molecule which is able to activate the innate immune system in autoimmune and proinflammatory syndromes such as experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis (89). In EAE, for example, GM-CSF is required for the induction of autoimmunity (90, 91) and serves as a conduit between $CD4^+$ T cells and $CCR2^+$ macrophages (92). In the latter cell population, GM-CSF institutes a proinflammatory transcriptional program which facilitates pathological damage within the central nervous system (92). Recently, a role for GM-CSF in GVHD has been posited by Ulrich et al. who described a population of BATF-dependent $IL\text{-}7R^{hi}$ T cells that produce GM-CSF (93). In this report, which primarily focused on inflammation in the gastrointestinal tract, $GM\text{-}CSF^{-/-}$ T cells induced less GVHD in the colon as evidenced by reduced colonoscopy and clinical scores, as well as increased overall survival. This finding was replicated in a MHC-mismatched and haploidentical transplant models by Tughes et al. (94). Interestingly, GVHD was attenuated when GM-CSF receptor deficient bone marrow donors were used, suggesting that the proinflammatory effects of GM-CSF are at least in part mediated through donor myeloid cells. However, a mechanism or specific target myeloid populations for GM-CSF signaling were not definitively established. Thus, further studies are needed to elucidate the full role of this proinflammatory cytokine. Given that mavrilimumab (95), an anti-GM-CSF receptor alpha monoclonal antibody, and MOR103 (96), a humanized anti-GM-CSF antibody, are currently in clinical trials for the treatment of rheumatoid arthritis and multiple sclerosis, respectively, GM-CSF could represent a new target for the prevention of GVHD in the GI tract.

Interferon-Gamma (IFN-γ)

Donor T cells with a T_H1 cytokine phenotype have an important role in the pathophysiology of GVHD (1, 2). The signature cytokine of these cells, IFN-γ, has also been demonstrated to induce pathological damage in the GI tract during GVHD. However, inhibition of IFN-γ signaling has divergent effects on GVHD target organs in pre-clinical murine models. Specifically, these studies have shown that mice transplanted with IFN-γ deficient grafts have reduced pathology in the GI tract (14, 97), but rapidly develop an idiopathic pneumonia syndrome (IPS)-like disease early post-transplantation resulting in increased mortality (97–99). The protective effect of IFN-γ is mediated through host non-hematopoietic cells, likely the lung parenchyma itself (97, 99), which inhibit the production of IL-6 by signaling through the IFN-γR (99). In contrast, IFN-γR signaling in T cells appears to be proinflammatory. A reduction in GVHD severity was observed when recipients were transplanted with IFN-γR-deficient T cells (97, 100). Additionally, reduced pathological damage occurred in the GI tract of these recipients without a commensurate increase in tissue damage in the lung (100). This was attributable to reduced expression of the chemokine receptor, CXCR3, which altered trafficking into this tissue site. To date, likely due to the divergent effects observed with inhibition of IFN-γ signaling in these pre-clinical models, there have been no clinical studies targeting this pathway to ameliorate GVHD in the GI tract.

CONCLUSIONS

The GI tract is the target organ which induces the most profound morbidity in patients who develop GVHD after allogeneic HSCT, and is responsible for much of the mortality associated with this disease. Inflammatory cytokines have been shown to play a pivotal role in this process and serve to amplify the pathogenic effects of alloreactive donor T cells. Ongoing research, primarily in murine models, has identified a number cytokines (IL-6, IL-21, IL-23, IL-1, IL-33, GM-CSF) and cytokine pathways (e.g., STAT3 signaling dependent, inflammasome-mediated) that are operative in the pathophysiology of GVHD of the GI tract. Notably, many of these cytokines or specific pathways can be targeted with existing, clinically available antibodies or small molecules designed to inhibit their activity in human transplant recipients. Thus, there is now optimism that the further evolution of this work will lead to the rational development of new strategies designed to reduce the severity of this complication in man and ultimately result in improved overall survival.

AUTHOR CONTRIBUTIONS

CP and WD wrote and edited the manuscript.

FUNDING

This research was supported by grants from the National Institutes of Health (HL064605, HL126166, and HL139008) and by awards from the Midwest Athletes Against Childhood Cancer Fund.

REFERENCES

1. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol*. (2007) 7:340–52. doi: 10.1038/nri2000
2. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. (2012) 12:443–58. doi: 10.1038/nri3212
3. Chakraverty R, Sykes M. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. *Blood* (2007) 110:9–17. doi: 10.1182/blood-2006-12-022038
4. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen presenting cells. *Science* (1999) 285:412–5. doi: 10.1126/science.285.5426.412
5. Koyama M, Kuns RD, Olver SD, Raffelt NC, Wilson YA, Don AL, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat Med*. (2012) 18:135–42. doi: 10.1038/nm.2597
6. Matte CC, Liu J, Cormier J, Anderson BE, Athanasiadis I, Jain D, et al. Donor APCs are required for maximal GVHD but not for GVL. *Nat Med*. (2004) 10:987–92. doi: 10.1038/nm1089
7. Koyama M, Cheong M, Markey KA, Gartlan KH, Kuns RD, Locke KR, et al. Donor colonic CD103+ dendritic cells determine the severity of acute graft-versus-host disease. *J Exp Med*. (2015) 212:1303–21. doi: 10.1084/jem.20150329
8. Toubai T, Tawara I, Sun Y, Liu C, Nieves E, Evers R, et al. Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radiosensitive host hematopoietic-derived antigen-presenting cells. *Blood* (2012) 119:3844–53. doi: 10.1182/blood-2011-10-384057
9. Markey KA, Banovic T, Kuns RD, Olver SD, Don AL, Raffelt NC, et al. Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. *Blood* (2009) 113:5644–9. doi: 10.1182/blood-2008-12-191833
10. Zeiser R, Socie G, Blazar BR. Pathogenesis of acute graft-versus-host disease: from intestinal microbiota alterations to donor T cell activation. *Br J Haematol*. (2016) 175:191–207. doi: 10.1111/bjh.14295
11. Vriesendorp HM, Heidt PJ. History of graft-versus-host disease. *Exp Hematol*. (2016) 44:674–88. doi: 10.1016/j.exphem.2016.05.011
12. Socie G, Ritz J. Current issues in chronic graft-versus-host disease. *Blood* (2014) 124:374–84. doi: 10.1182/blood-2014-01-514752
13. Sorror ML, Martin PJ, Storb RF, Bhatia S, Maziarz RT, Pulsipher MA, et al. Pretransplant comorbidities predict severity of acute graft-versus-host disease and subsequent mortality. *Blood* (2014) 124:287–95. doi: 10.1182/blood-2014-01-550566
14. Das R, Chen X, Komorowski R, Hessner MJ, Drobyski WR. Interleukin-23 secretion by donor antigen-presenting cells is critical for organ-specific pathology in graft-versus-host disease. *Blood* (2009) 113:2352–62. doi: 10.1182/blood-2008-08-175448
15. Hill GR, Ferrara JLM. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood* (2000) 95:2754–9.
16. Wilhelm K, Ganesan J, Müller T, Dürr C, Grimm M, Beilhack A, et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat Med*. (2010) 16:1434–8. doi: 10.1038/nm.2242
17. Apostolova P, Zeiser R. The role of purine metabolites as DAMPs in acute graft versus host disease. *Front Immunol*. (2016) 7:439e. doi: 10.3389/fimmu.2016.00439
18. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft versus host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* (1997) 90:3204–13.
19. Naymagon S, Naymagon L, Wong SY, Ko HM, Renteria A, Levine J, et al. Acute graft-versus-host disease of the gut: considerations for the gastroenterologist. *Nat Rev Gastroenterol Hepatol*. (2017) 14:711–26. doi: 10.1038/nrgastro.2017.126
20. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. *Immunity* (2008) 28:477–87. doi: 10.1016/j.immuni.2008.03.002
21. Lu SX, Alpdogan O, Lin J, Balderas R, Campos-Gonzalez R, Wang X, et al. Stat3 and Erk1/2 phosphorylation are critical for T cell alloactivation and graft versus host disease. *Blood* (2008) 112:5254–8. doi: 10.1182/blood-2008-03-147322
22. Betts BC, Veerapathran A, Pidala J, Yu XZ, Anasetti C. Stat5 polarization promotes iTregs and suppresses human T cell alloresponses while preserving CTL activity. *J Leuk Biol*. (2013) 95:205–13. doi: 10.1189/jlb.0313154
23. Betts BC, Sagatys EM, Veerapathran A, Lloyd MC, Beato F, Lawrence HR, et al. CD4+ T cell Stat3 phosphorylation precedes acute GVHD and subsequent TH17 tissue invasion correlates with GVHD severity and therapeutic response. *J Leuk Biol*. (2015) 97:807–19. doi: 10.1189/jlb.5A1114-532RR
24. Nieves EC, Toubai T, Peltier DC, Oravecz-Wilson K, Liu C, Tamaki H, et al. Stat3 expression in host myeloid cells controls graft versus host disease severity. *Biol Blood Marrow Transplant* (2017) 23:1622–30. doi: 10.1016/j.bbmt.2017.06.018
25. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* (2006) 441:235–8. doi: 10.1038/nature04753
26. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* (2006) 441:231–4. doi: 10.1038/nature04754

27. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, et al. TGF-beta-induced Foxp3 inhibits TH17 cell differentiation by antagonizing ROR gammat function. *Nature* (2008) 453:236–40. doi: 10.1038/nature06878
28. Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, et al. Blockade of interleukin 6 signaling augments regulatory T cell reconstitution and attenuates the severity of graft versus host disease. *Blood* (2009) 114:891–900. doi: 10.1182/blood-2009-01-197178
29. Tawara I, Koyama M, Liu C, Toubai T, Thomas D, Evers R, et al. Interleukin 6 modulates graft versus host responses after experimental allogeneic bone marrow transplantation. *Clin Can Res*. (2010) 17:77–88. doi: 10.1158/1078-0432.CCR-10-1198
30. Beres A, Komorowski R, Mihara M, Drobyski WR. Instability of Foxp3 expression limits the ability of induced regulatory T cells to mitigate graft versus host disease. *Clin Can Res*. (2011) 17:3969–83. doi: 10.1158/1078-0432.CCR-10-3347
31. Le Huu D, Matsushita T, Jin G, Hamaguchi Y, Hasegawa M, Takehara K, et al. IL-6 blockade attenuates the development of murine sclerodermatous chronic graft versus host disease. *J Inv Dermatol*. (2012) 132:2752–61. doi: 10.1038/jid.2012.226
32. Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, et al. Treatment of rheumatoid arthritis with humanized anti-interleukin 6 receptor antibody. *Arthritis Rheum*. (2004) 50:1761–9. doi: 10.1002/art.20303
33. Yokota S, Imagawa T, Mori M, Miyamae T, Aihara Y, Takei S, et al. Efficacy and safety of tocilizumab in patients with systemic-onset juvenile idiopathic arthritis: a randomized, double-blind, placebo-controlled, withdrawal phase III trial. *Lancet* (2008) 371:998–1006. doi: 10.1016/S0140-6736(08)60454-7
34. Drobyski WR, Pasquini M, Kovatovic K, Palmer J, Douglas Rizzo J, Saad A, et al. Tocilizumab for the treatment of steroid refractory graft versus host disease. *Biol Blood Marrow Transplant* (2011) 17:1862–8. doi: 10.1016/j.bbmt.2011.07.001
35. Roddy JV, Haverkos BM, McBride A, Leininger KM, Jaglowski S, Penza S, et al. Tocilizumab for steroid refractory acute graft versus host disease. *Leuk Lymphoma* (2016) 57:81–5. doi: 10.3109/10428194.2015.1045896
36. Ganetsky A, Frey N, Hexner E, Loren A, Gill S, Luger S, et al. Tocilizumab for the treatment of severe steroid refractory acute graft versus host disease of the lower gastrointestinal tract. *Bone Marrow Transplant* (2018). doi: 10.1038/s41409-018-0236-z. [Epub ahead of print].
37. Kennedy GA, Varelias A, Vuckovic S, Le Texier L, Gartlan KH, Zhang P., et al. Addition of interleukin 6 inhibition with tocilizumab to standard graft versus host disease prophylaxis after allogeneic stem cell transplantation: a phase 1/2 trial. *Lancet Oncol*. (2014) 15:1451–9. doi: 10.1016/S1470-2045(14)71017-4
38. Mielcarek M, Storer BE, Boeckh M, Carpenter PA, McDonald GB, Deeg HJ, et al. Initial therapy of acute graft versus host disease with low dose prednisone does not compromise patient outcomes. *Blood* (2009) 113:2888–94. doi: 10.1182/blood-2008-07-168401
39. Castilla C, Pérez-Simón JA, Sanchez-Guijo FM, Díez-Campelo M, Ocio E, Pérez-Persona E, et al. Oral beclomethasone dipropionate for the treatment of gastrointestinal acute graft versus host disease (GVHD). *Biol Blood Marrow Transplant* (2006) 12:936–41. doi: 10.1016/j.bbmt.2006.05.010
40. Drobyski WR, Szabo A, Zhu F, Keever-Taylor CA, Hebert KM, Dunn R, et al. Tocilizumab, tacrolimus and methotrexate for the prevention of acute graft versus host disease: Low incidence of lower gastrointestinal tract disease. *Haematologica* (2018) 103:717–27. doi: 10.3324/haematol.2017.183434
41. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* (2000) 13:715–25. doi: 10.1016/S1074-7613(00)00070-4
42. Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R. *J Immunol*. (2002) 168:5699–708. doi: 10.4049/jimmunol.168.11.5699
43. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin 23 rather than interleukin 12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* (2003) 421:744–8. doi: 10.1038/nature01355
44. Murphy, CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med*. (2003) 198:1951–7. doi: 10.1084/jem.20030896
45. Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest*. (2006) 116:1310–6. doi: 10.1172/JCI21404
46. Das R, Komorowski R, Hessner MJ, Subramanian H, Huettner CS, Cua D, et al. 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* (2010) 115:5249–58. doi: 10.1182/blood-2009-11-255422
47. Zhou V, Agle K, Chen X, Beres A, Komorowski R, Belle L, et al. Identification of a colitogenic memory CD4+ T cell population that mediates gastrointestinal GVHD. *J Clin Invest*. (2016) 126:3541–55. doi: 10.1172/JCI80874
48. Blauvelt A, Papp KA, Griffiths CE, Randazzo B, Wasfi Y, Shen YK., et al. Efficacy and safety of guselkumab, an anti-interleukin 23 monoclonal antibody, compared with adalimumab for the continuous treatment of patients with moderate to severe psoriasis: results from the phase III, double-blinded, placebo and active comparator-controlled VOYAGE 1 trial. *J Am Acad Dermatol*. (2017) 76:405–17. doi: 10.1016/j.jaad.2016.11.041
49. Reich K, Papp KA, Blauvelt A, Tyring SK, Sinclair R, Thaci D, et al. Tildrakizumab versus placebo or etanercept for chronic plaque psoriasis (reSURFACE 1 and reSURFACE2): results from two randomized controlled, phase 3 trials. *Lancet* (2017) 390:276–88. doi: 10.1016/S0140-6736(17)31279-5
50. Pidala J, Beato F, Kim J, Betts B, Jim H, Sagatys E, et al. *In vivo* IL-12/IL-23p40 neutralization blocks TH1/TH17 response after allogeneic hematopoietic cell transplantation. *Haematologica* (2018) 103:531–9. doi: 10.3324/haematol.2017.171199
51. Sploski R, Leonard WJ. The yin and yang of interleukin 21 in allergy, autoimmunity and cancer. *Curr Opin Immunol*. (2008) 20:295–301. doi: 10.1016/j.coi.2008.02.004
52. Bucher C, Koch L, Vogtenhuber C, Goren E, Munger M, Panoskaltsis-Mortari A, et al. IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. *Blood* (2009) 114:5375–84. doi: 10.1182/blood-2009-05-221135
53. Oh I, Ozaki K, Meguro A, Hatanaka K, Kadowaki M, Matsu H, et al. Altered effector CD4+ T cell function in IL-21R—/— CD4+ T cell-mediated graft versus host disease. *J Immunol*. (2010) 185:1920–6. doi: 10.4049/jimmunol.0902217
54. Hanash AM, Kappel LW, Yim NL, Nejat RA, Goldberg GL, Smith OM, et al. Abrogation of donor T cell IL-21 signaling leads to tissue-specific modulation of immunity and separation of GVHD from GVL. *Blood* (2011) 118:446–55. doi: 10.1182/blood-2010-07-294785
55. Meguro A, Ozaki K, Oh I, Hatanaka K, Matsu H, Tatara R, et al. IL-21 is critical for GVHD in a mouse model. *Bone Marrow Transplant*. (2010) 45:723–9. doi: 10.1038/bmt.2009.223
56. Hippen KL, Bucher C, Schirm DK, Bearl AM, Brender T, Mink KA, et al. Blocking IL-21 signaling ameliorates xenogeneic GVHD induced by human lymphocytes. *Blood* (2012) 119:619–28. doi: 10.1182/blood-2011-07-368027
57. Dinarello CA. IL-1: Discoveries, controversies and future directions. *Eur J Immunol*. (2010) 40:599–606. doi: 10.1002/eji.201040319
58. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, et al. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* (1985) 315:641–7. doi: 10.1038/315641a0
59. Dinarello CA. Overview of the interleukin-1 family of ligands and receptors. *Semin Immunol*. (2013) 25:389–93. doi: 10.1016/j.smim.2013.10.001
60. Dinarello CA. Immunological and Inflammatory Functions of the Interleukin-1 Family. *Ann Rev Immunol*. (2009) 27:519–50. doi: 10.1146/annurev.immunol.021908.132612
61. Rivers-Auty J, Daniels MJD, Colliver I, Robertson DL, Brough D. Redefining the ancestral origins of the interleukin-1 superfamily. *Nat Commun*. (2018) 9:1156. doi: 10.1038/s41467-018-03362-1
62. Santarlasci V, Cosmi L, Maggi L, Liotta F, Annunziato F. IL-1 and T helper immune responses. *Front Immunol*. (2013) 4:182. doi: 10.3389/fimmu.2013.00182

63. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *J Exp Med*. (2000) 191:313–20. doi: 10.1084/jem.191.2.313
64. Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, et al. Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* (1990) 343:336–40. doi: 10.1038/343336a0
65. McCarthy PL, Abhyankar S, Neben S, Newman G, Sieff C, Thompson RC, et al. Inhibition of interleukin-1 by an interleukin-1 receptor antagonist prevents graft-versus-host disease. *Blood* (1991) 78:1915–8.
66. Abhyankar S, Gilliland DG, Ferrara JL. Interleukin-1 is a critical effector molecule during cytokine dysregulation in graft versus host disease to minor histocompatibility antigens. *Transplantation* (1993) 56:1518–23. doi: 10.1097/00007890-199312000-00045
67. Vallera DA, Taylor PA, Vannice JL, Panoskaltsis-Mortari A, Blazar BR. Interleukin-1 or tumor necrosis factor-alpha antagonists do not inhibit graft-versus-host disease induced across the major histocompatibility barrier in mice. *Transplantation* (1995) 60:1371–4.
68. Antin J, Weinstein H, Guinan E, McCarthy P, Bierer B, Gilliland D, et al. Recombinant human interleukin-1 receptor antagonist in the treatment of steroid-resistant graft-versus-host disease. *Blood* (1994) 84:1342–8.
69. McCarthy PL, Williams L, Harris-Bacile M, Yen J, Przepiorka D, Ippoliti C, et al. A clinical phase I/II study of recombinant human interleukin-1 receptor in glucocorticoid-resistant graft-versus-host disease. *Transplantation* (1996) 62:626–31. doi: 10.1097/00007890-199609150-00015
70. Antin JH, Weisdorf D, Neuberg D, Nicklow R, Clouthier S, Lee SJ, et al. Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* (2002) 100:3479–82. doi: 10.1182/blood-2002-03-0985
71. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of pro-IL-beta. *Mol Cell*. (2002) 10:417–26. doi: 10.1016/S1097-2765(02)00599-3
72. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol*. (2009) 183:787–91. doi: 10.4049/jimmunol.0901363
73. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. *Nature* (2012) 481:278–86. doi: 10.1038/nature10759
74. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber FC, Guarda G, et al. The Nlrp3 inflammasome regulates acute graft-versus-host disease. *J Exp Med*. (2013) 210:1899–910. doi: 10.1084/jem.20130084
75. Chen S, Smith BA, Iype J, Prestipino A, Pfeifer D, Grundmann S, et al. MicroRNA-155-deficient dendritic cells cause less severe GVHD through reduced migration and defective inflammasome activation. *Blood* (2015) 126:103–12. doi: 10.1182/blood-2014-12-617258
76. Cayrol C, Girard J-P. Interleukin 33 (IL-33): a nuclear cytokine from the IL-1 family. *Immunol Rev*. (2018) 281:154–68. doi: 10.1111/imr.12619
77. Hodzic Z, Schill EM, Bolock AM, Good M. IL-33 and the intestine: the good, the bad, and the inflammatory. *Cytokine* (2017) 100:1–10. doi: 10.1016/j.cyto.2017.06.017
78. Griesenauer B, Paczesny S. The ST2/IL-33 axis in immune cells during inflammatory diseases. *Front Immunol*. (2017) 8:1–17. doi: 10.3389/fimmu.2017.00475
79. Reichenbach DK, Schwarze V, Matta BM, Tkachev V, Lieberknecht E, Liu Q, et al. The IL-33/ST2 axis augments effector T cell responses during acute GVHD. *Blood* (2015) 125:3183–92. doi: 10.1182/blood-2014-10-606830
80. Zhang J, Ramadan AM, Griesenauer B, Li W, Turner MJ, Liu C, et al. ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft versus host disease. *Sci Trans Med*. (2015) 7:308ra. doi: 10.1126/scitranslmed.aab0166
81. Matta BM, Reichenbach DK, Zhang X, Mathews L, Koehn BH, Dwyer GK, et al. Peri-alloHCT IL-33 administration expands recipient T regulatory cells that protect mice against acute GVHD. *Blood* (2016) 128:427–39. doi: 10.1182/blood-2015-12-684142
82. Levine JE, Braun TM, Harris AC, Holler E, Taylor A, Miller H, et al. A prognostic score for acute graft versus host disease based on biomarkers: a multicenter study. *Lancet Haematol*. (2015) 2:e21–9. doi: 10.1016/S2352-3026(14)00035-0
83. Hartwell MJ, Özbek U, Holler E, Renteria AS, Major-Monfried H, Reddy P, et al. An early biomarker algorithm predicts lethal graft versus host disease and survival. *JCI Insight*. (2017) 2:e89798. doi: 10.1172/jci.insight.89798
84. Abu Zaid M, Wu J, Wu C, Logan BR, Yu J, Cutler C, et al. Plasma biomarkers of risk for death in a multicenter phase 3 trial with uniform transplant characteristics post-allogeneic HCT. *Blood* (2017) 129:162–70. doi: 10.1182/blood-2016-08-735324
85. Major-Monfried H, Renteria AS, Pawarode A, Reddy P, Ayuk F, Holler E, et al. MAGIC biomarkers predict long term outcomes for steroid-resistant acute GVHD. *Blood* (2018) 131:2846–55. doi: 10.1182/blood-2018-01-822957
86. Ramadan AM, Daguindau E, Rech JC, Chinnaswamy K, Zhang J, Hura GL, et al. From proteomics to discovery of first in class ST2 inhibitors active *in vivo*. *JCI Insight* (2018) 3:pii:99208. doi: 10.1172/jci.insight.99208
87. Stanley E, Lieschke GJ, Grail D, Metcalf D, Hodgson G, Gall JA, et al. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci USA*. (1994) 91:5592–6. doi: 10.1073/pnas.91.12.5592
88. Becher B, Tugues S, Greter M. GM-CSF: from growth factor to central mediator of tissue inflammation. *Immunity* (2016) 45:963–73. doi: 10.1016/j.immuni.2016.10.026
89. Cornish AL, Campbell IK, McKenzie BS, Chatfield S, Wicks IP. G-CSF and GM-CSF as therapeutic targets in rheumatoid arthritis. *Nat Rev Rheumatol*. (2009) 5:554–9 doi: 10.1038/nrrheum.2009.178
90. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of TH17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol*. (2011) 12:568–75. doi: 10.1038/ni.2031
91. Ponomarev ED, Shriver LP, Maresz K, Pedras-Vasconcelos J, Verthelyi D, Dittel BN. GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. *J Immunol*. (2007) 178:39–48. doi: 10.4049/jimmunol.178.1.39
92. Croxford AL, Lanzinger M, Hartmann FJ, Schreiner B, Mair F, Pelczar P, et al. The cytokine GM-CSF drives the inflammatory signature of CCR2+ monocytes and licenses autoimmunity. *Immunity* (2015) 43:502–14. doi: 10.1016/j.immuni.2015.08.010
93. Ullrich E, Abendroth B, Rothamer J, Huber C, Büttner-Herold M, Buchele V, et al. BATF-dependent IL-7RhiGM-CSF+ T cells control intestinal graft-versus-host disease. *J Clin Invest*. (2018) 128:916–30. doi: 10.1172/JCI89242
94. Tugues S, Amorim A, Spath S, Martin-Blondel G, Schreiner B, De Feo D, et al. Graft-versus-host disease, but not graft-versus-leukemia immunity, is mediated by GM-CSF-licensed myeloid cells. *Sci Trans Med*. (2018) 10:469. doi: 10.1126/scitranslmed.aat8410
95. Burmester GR, McInnes IB, Kremer J, Miranda P, Korkosz M, Vencovsky J, et al. A randomized phase IIb study of mavrilimumab, a novel GM-CSF receptor alpha monoclonal antibody, in the treatment of rheumatoid arthritis. *Ann Rheum Dis*. (2017) 76:1020–30. doi: 10.1136/annrheumdis-2016-210624
96. Constantinescu CS, Asher A, Fryze W, Kozubski W, Wagner F, Aram J, et al. Randomized phase 1b trial of MOR103, a human antibody to GM-CSF, in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm*. (2015) 2:e117. doi: 10.1212/NXI.0000000000000117
97. Burman AC, Banovic T, Kuns RD, Clouston AD, Stanley AC, Morris ES, et al. IFN gamma differentially controls the development of idiopathic pneumonia syndrome and GVHD of the gastrointestinal tract. *Blood* (2007) 110:1064–72. doi: 10.1182/blood-2006-12-063982
98. Mauermann N, Burian J, von Garnier C, Dirnhofer S, Germano D, Schuett C, et al. Interferon-γ regulates idiopathic pneumonia syndrome, a Th17+

CD4+ T-cell–mediated graft-versus-host disease. *Am J Respir Crit Care Med.* (2008) 178:379–88. doi: 10.1164/rccm.200711-1648OC

99. Varelias A, Gartlan KH, Kreijveld E, Olver SD, Lor M, Kuns RD, et al. Lung parenchyma-derived IL-6 promotes IL-17A-dependent acute lung injury after allogeneic stem cell transplantation. *Blood* (2015) 125:2435–44. doi: 10.1182/blood-2014-07-590232
100. Choi J, Ziga ED, Ritchey J, Collins L, Prior JL, Cooper ML, et al. IFNγR signaling mediates alloreactive T cell trafficking and GVHD. *Blood* (2012) 120:4093–103. doi: 10.1182/blood-2012-01-403196

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 13 February 2019
doi: 10.3389/fimmu.2019.00186

Posttransplant Hemophagocytic Lymphohistiocytosis Driven by Myeloid Cytokines and Vicious Cycles of T-Cell and Macrophage Activation in Humanized Mice

Satoshi Yoshihara[1], *Yuying Li*[1,2], *Jinxing Xia*[1], *Nichole Danzl*[1], *Megan Sykes*[1] *and Yong-Guang Yang*[1,2]*

[1] *Columbia Center for Translational Immunology, Department of Medicine, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, United States,* [2] *Institute of Translational Medicine, First Hospital of Jilin University, Changchun, China*

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina, United States

Reviewed by:
Benny Chen,
Duke University, United States
Grégory Ehx,
Université de Montréal, Canada
Ronald Sluyter,
University of Wollongong, Australia

***Correspondence:**
Yong-Guang Yang
yy2324@columbia.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *02 October 2018*
Accepted: *22 January 2019*
Published: *13 February 2019*

Citation:
Yoshihara S, Li Y, Xia J, Danzl N, Sykes M and Yang Y-G (2019) Posttransplant Hemophagocytic Lymphohistiocytosis Driven by Myeloid Cytokines and Vicious Cycles of T-Cell and Macrophage Activation in Humanized Mice. Front. Immunol. 10:186. doi: 10.3389/fimmu.2019.00186

Hemophagocytic lymphohistiocytosis (HLH) has recently been increasingly reported as an important complication after stem cell transplantation, in line with the increase in the number of HLA-mismatched transplantation. Although previous clinical studies have shown an elevation of inflammatory cytokines in patients with HLH after hematopoietic stem cell transplantation, as well as those after viral infection or autoimmune disease, the disease pathogenesis remains poorly understood. Here we explored this issue in humanized mice with functional human lymphohematopoietic systems, which were constructed by transplantation of human $CD34^+$ cells alone, or along with human fetal thymus into NOD/SCID/$\gamma c^{-/-}$ (NSG) or NSG mice carrying human SCF/GM-CSF/IL-3 transgenes (SGM3). In comparison with humanized NSG (huNSG) mice, huSGM3 mice had higher human myeloid reconstitution and aggressive expansion of human $CD4^+$ memory T cells, particularly in the absence of human thymus. Although all huNSG mice appeared healthy throughout the observation period of over 20 weeks, huSGM3 mice developed fatal disease characterized by severe human T cell and macrophage infiltrations to systemic organs. HuSGM3 mice also showed severe anemia and thrombocytopenia with hypoplastic bone marrow, but increased reticulocyte counts in blood. In addition, huSGM3 mice showed a significant elevation in human inflammatory cytokines including IL-6, IL-18, IFN-α, and TNF-γ, faithfully reproducing HLH in clinical situations. Our study suggests that posttransplant HLH is triggered by alloresponses (or xenoresponses in our model), driven by myeloid cytokines, and exacerbated by vicious cycles of T-cell and macrophage activation.

Keywords: allogeneic SCT, hypercytokinemia, inflammation, immune activation, hemophagocytic lymphohistiocytosis (HLH), graft-vs.-host disease, posttransplant complication

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH), also known as hemophagocytic syndrome (HPS) or macrophage activation syndrome (MAS), is a disease triggered by hypercytokinemia (1). Although the most frequent trigger for HLH is viral infection, other immune activated conditions including autoimmune diseases and malignant lymphoma also serve as a trigger. Allogeneic transplantation may also induce inflammatory cytokine production as a consequence of tissue damages following preconditioning regimen and allogeneic immune responses. Indeed, HLH after allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been increasingly reported (2–4). As well known, graft-vs.-host disease (GVHD) is also triggered by cytokine storm (5). While mechanisms of GVHD have been extensively examined, the pathogenesis of HLH remains poorly understood.

To explore this issue, we utilized humanized (hu) mice with functional human hematopoietic and lymphoid systems. Previous studies have shown that hu-mouse models serve as a highly useful tool to study human hematopoiesis, immune function, and various diseases (6). However, human myeloid reconstitution remains relatively poor in these hu-mice, presumably due to the lack of or insufficient cross-reactivity between mouse and human cytokines (7). To solve this problem, various strategies have been examined to express human myeloid cytokines in humanized mice (8–10). Besides the expression of human cytokines in the immunodeficient mice, there are considerable variations in the protocols used for the generation of hu-mice with a human immune system. The variations arise from human stem cell source (fetal, cord blood, or adult), age of mice (neonatal or adult), and the use of human thymus graft (11). We have previously shown that NSG mice receiving fetal thymic grafts and partially-matched allogeneic adult $CD34^+$ cells show robust T-cell reconstitution, providing a new model for individualized analysis of human immune responses (12).

In this study we generated humanized mice by transplantation of human $CD34^+$ cells either alone or combined with fetal thymic tissue into adult NSG or transgenic NSG mice expressing human stem cell factor (SCF), GM-CSF and IL-3 (referred to as SGM3 mice). We show that humanized SGM3 (huSGM3), but not huNSG, mice developed lethal HLH, regardless of whether or not human thymus was transplanted. Moreover, huSGM3 mice showed aberrant expansion of human T cells developing in the native mouse thymus, presumably reflecting poor negative selection of human thymocytes in the mouse thymus.

METHODS

Animals and Human Tissues

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NOD/SCID/$\gamma c^{-/-}$ or NSG) and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG-SGM3 or SGM3) mice were purchased from The Jackson Laboratory and were housed in a specific pathogen-free microisolator environment. Human fetal liver and thymus tissues of gestational age of 17–21 weeks were obtained from Advanced Bioscience Resource. Human $CD34^+$ fetal liver cells (FLCs), isolated by a magnetic-activated cell sorter separation system using anti-CD34 microbeads (Miltenyi Biotec), and thymus tissues were cryopreserved in liquid nitrogen until use. Protocols involving the use of human tissues and animals were approved by the Institutional Review Board and the Institutional Animal Care and Use Committee of Columbia University (New York, NY), and all of the experiments were performed in accordance with the protocols.

Humanized Mouse Preparation

Female NSG or SGM3 mice at the age of 10–11 weeks were conditioned with sublethal (1.2 Gy) total body irradiation using a RS2000 X-ray irradiator (Rad Source Technologies) and received human $CD34^+$ FLCs (7×10^4/mouse, intravenously) alone, or along with a human fetal thymic tissue fragment measuring approximately 1 mm^3 (under the recipient kidney capsule) from the same fetal donor, as previously described (13–15). Humanized mice were monitored daily, body weight was checked weekly, and peripheral blood was collected from the retro-orbital sinus every 2–3 weeks starting 4 weeks after transplantation. RBC lysis using BD Pharm Lyse (BD Biosciences) was performed for leukocyte chimerism analysis, mononuclear cell purification by density gradient centrifugation ($400 \times g$ for 30 min at room temperature) with Histopaque 1077 (Sigma-Aldrich) was performed for human lymphocyte analysis, and whole blood was used for RBC chimerism analysis. Humanized mice were sacrificed when they became moribund and complete necropsy was performed.

Isolation of Leukocytes From Organs in the Sacrificed Humanized Mice

Liver, spleen, lungs, and lymph nodes were minced and digested by Liberase TM (Roche) for 15 min at 37°C. Digested liver and lung cells were purified for mononuclear cells by density gradient centrifugation ($400 \times g$ for 30 min at room temperature) with Histopaque 1077 (Sigma-Aldrich). Digested spleen cells received RBC lysis by ACK lysing buffer (Lonza). Human thymus graft and mouse thymus were strained with a 40 μm nylon cell strainer (Falcon) to obtain a single cell suspension. The bone marrow (BM) cells, which were obtained from tibia and femur, received RBC lysis. Number of the cells were counted using a hemocytometer.

Flow Cytometry

Flow cytometry was performed with LSR II (BD Biosciences) using various combinations of the following mAbs: anti-human CD45 (2D1), CD19 (HIB 19), CD3 (UCHT1), CD4 (RPA-T4), CD8 (SK1), CD33 (WM53), CCR7 (G043H7), CD45RA (HI100), CD31 (WM59), CD127 (A019D5), CD25 (M-A251), CD235a (HI264); anti-mouse CD45 (30-F11), and TER119 (TER-119); and isotype control mAbs (purchased from BD Biosciences PharMingen or Biolegend). Intracellular FoxP3 staining was performed with FoxP3 Staining Kit (Biolegend) according to the manufacturer's instructions.

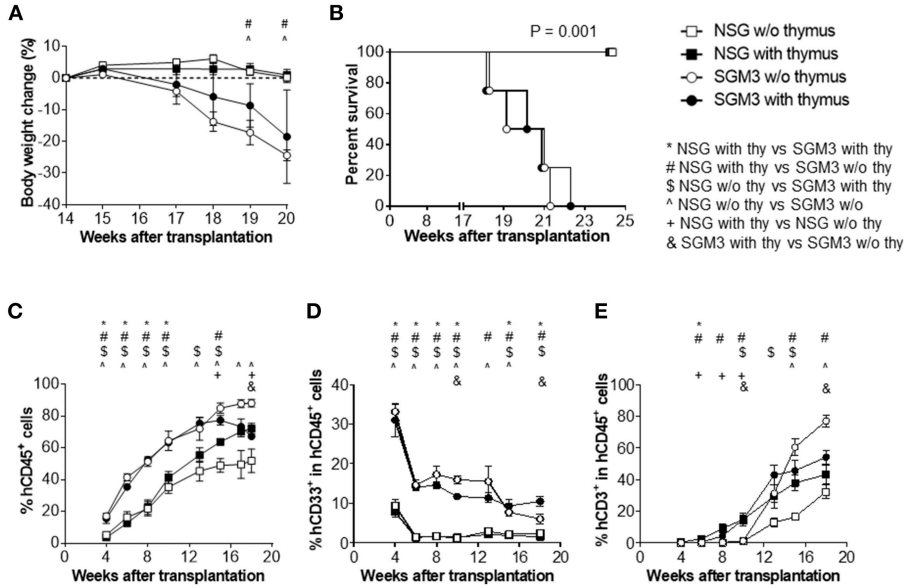


FIGURE 1 | Body weight, survival and human blood cell reconstitution in huNSG and huSGM3 mice with or without human thymus. NSG or SGM3 mice were transplanted with human CD34$^+$ cells alone or along with human fetal thymus tissue ($n = 4$ per group). **(A)** Body weight changes in the indicated groups of humanized mice between 14 and 20 weeks after transplantation. Body weight at 14 weeks was used as baseline value. **(B)** Survival of humanized mice after transplantation. **(C)** Levels (%) of human CD45$^+$ cell chimerism in WBCs at the indicated time points after transplantation. **(D–E)** Kinetics of the frequencies of human CD33$^+$ myeloid **(D)** and CD3$^+$ T cells **(E)** within human CD45$^+$ cells. For **(A,C–E)**, repeated measures analysis of variance was used to determine main effects ($P < 0.05$) between groups. All of the panels had main effects, and *post-hoc* Bonferroni was used to compare groups at each time point. For $p < 0.05$ for *post-hoc* test are indicated as *, #, $, or & for group comparisons indicated in the legend. Error bars represent SEMs.

Cytologic and Histologic Analysis and Immunohistochemical Staining

Leukocytes isolated from organs underwent cytospin and Wright-Giemsa staining by conventional methods. Tissue samples underwent H&E staining and Prussian blue staining by conventional methods. Immunohistochemical staining was performed using rabbit anti-human CD3 antibody (SP7, Thermo Scientific) and mouse anti-human CD68 antibody (KP1, DAKO) as primary antibodies and appropriate secondary antibodies were used for detection.

Quantification of WBC, Hemoglobin, Platelets, and Reticulocytes

Quantification of WBC, hemoglobin, platelets, and reticulocytes was performed using VetHemaChemRX (Oxford Science).

Quantification of Cytokines in Plasma

Quantification of cytokines in cryopreserved plasma was performed by Luminex multiplex assay using ProcartaPlex® Multiplex Immunoassay Panels according to the manufacturer's instructions (eBioscience).

Statistical Analyses

Statistical analysis was conducted using the Student's *t*-test, one-way ANOVA with Bonferroni's *post-hoc* multiple comparison test, two-way ANOVA, or log-rank test. A *P*-value of less than or equal to 0.05 was considered significant in all analyses herein.

RESULTS

HuSGM, but Not HuNSG, Mice Develop Fetal Disease

Hu-mice were generated by intravenous injection of human CD34$^+$ cells alone or along with implantation of human fetal thymus under the kidney capsule in sublethally irradiated NSG or SGM3 mice. All huSGM3 mice, regardless of with or without human thymus, became lethargic and started losing body weight from 17 weeks after transplantation (**Figure 1A**), and became moribund 18–22 weeks after transplantation (**Figure 1B**). With exception of one mouse with mild diarrhea, none showed signs of GVHD, such as ruffled fur or loss of skin integrity. In contrast to huSGM3 mice, huNSG mice, regardless of whether or not human thymus was transplanted, appeared healthy throughout the observation period (22 weeks), which is consistent with our previous studies (14).

Higher Human Leukocyte Chimerism With Better Myeloid Reconstitution in HuSGM3 Mice

Peripheral blood was collected every 2–3 weeks starting from 4 weeks after transplantation and analyzed for human cell chimerism by flow cytometry. HuSGM3 mice (both with and without human thymus) showed higher human leukocyte (CD45$^+$ cell) chimerism levels than huNSG mice (**Figure 1C**). Although the chimerism levels were similar

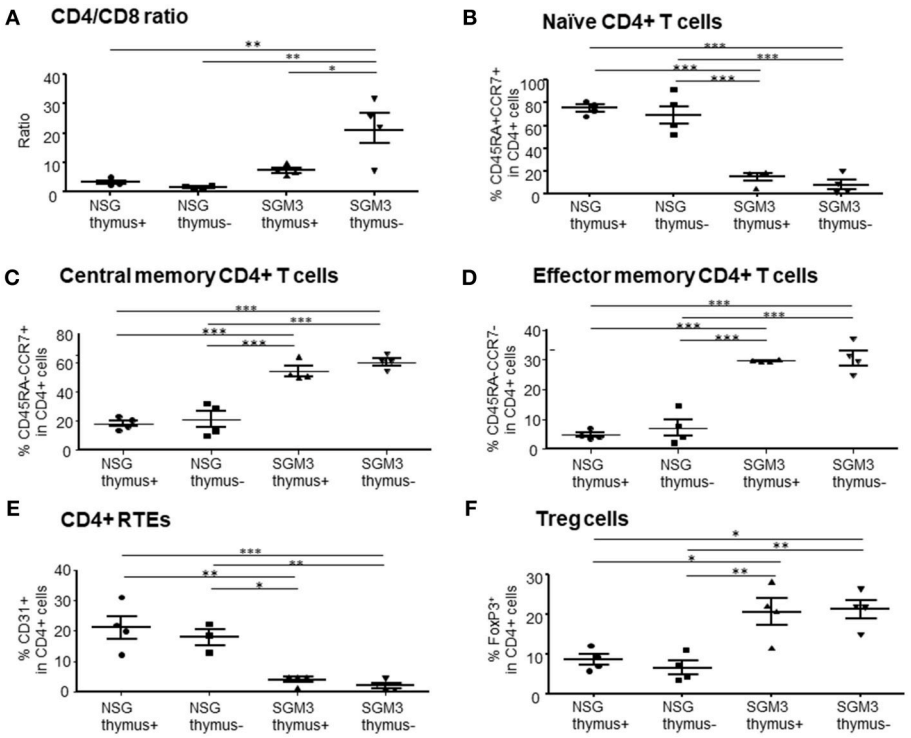


FIGURE 2 | Characterization of human T cells in huNSG and huSGM3 mice with or without human thymus. Human T cells in peripheral blood at 15 weeks after transplantation were analyzed for the frequencies of T cell subsets ($n = 4$ per group). **(A)** Ratios of human $CD4^+$ to $CD8^+$ T cells within $CD3^+$ population. **(B)** Percentages of human $CD45RA^+CCR7^+$ naïve CD4 T cells. **(C)** Percentages of human $CD45RA^-CCR7^+$ central memory CD4 T cells. **(D)** Percentages of human $CD45RA^-CCR7^-$ effector memory CD4 T cells. **(E)** Frequencies of $CD31^+CD45RA^+$ recent thymic emigrant CD4 T cells. **(F)** Frequencies of human $FoxP3^+CD25^{hi}$ Treg cells. Group differences were determined by one-way ANOVA with Bonferroni's *post-hoc* multiple comparison test; each symbol represents an individual mouse. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars indicate SEMs.

between huSGM3 mice with and without human thymus until 13 weeks, those in the group without human thymus started to decline from 15 weeks after transplantation. Among huNSG mice, mice with human thymus had higher human leukocyte chimerism levels than those without throughout the observation period. Furthermore, huSGM3 mice had higher $CD33^+$ myeloid cell frequencies than huNSG mice (**Figure 1D**). High myeloid cell frequencies at the early time (by 4 weeks) and subsequent decreases suggest that myeloid cells recovered first, followed by B cells and T cells as in the case of the patients undergoing hematopoietic stem cell transplantation. Human RBCs were almost undetectable in both huNSG and huSGM3 mice (data not shown), consisting with previous studies (14).

Aggressive Peripheral T Cell Expansion in huSGM3 Mice, Particularly in the Absence of Human Thymus

It is known that human T cell reconstitution does not occur after $CD34^+$ cells transplantation in the absence of human thymus graft when adult NOD/SCID mice are used to generate humanized mice (11). In fact, in both huNSG and huSGM3 mice, the no thymus group had scarce T cell reconstitution up to 10 weeks after transplantation (**Figure 1E**). However, T cell frequencies in these mice started to increase from 13 weeks, and the increase was strikingly steeper in huSGM3 than in huNSG mice. T cell frequencies in huSGM3 mice without human thymus even exceeded those in huSGM3 with human thymus by 15 weeks after transplantation. Interestingly, in groups with human thymus, the huSGM3 mice had only a slightly higher T cell frequency (not statistically significant) than the huNSG mice.

We also performed detailed phenotypic analysis of T cells in peripheral blood at 15 weeks when the huSGM3 mice appeared to start losing weight. First, the $CD4^+$ to $CD8^+$ T cell ratio in huSGM3 mice was significantly higher than that in huNSG mice, in particular the huSGM3 mice without human thymus, in which more than 90% of $CD3^+$ T cells were $CD4^+$ T cells (**Figure 2A**). In huNSG mice (in both with and without human thymus groups), the majority of human $CD4^+$ T cells expressed a naïve ($CD45RA^+CCR7^+$) phenotype (**Figure 2B**). In contrast, the majority of $CD4^+$ T cells in huSGM3 mice expressed a central memory ($CD45RA^-CCR7^+$) or effector memory ($CD45RA^-CCR7^-$) phenotype (**Figures 2C,D**). Similar results were obtained from other experiments, in which augmented $CD4^+$ T cell activation and expansion were detected in huSGM3 mice that were constructed by transplantation of bone marrow

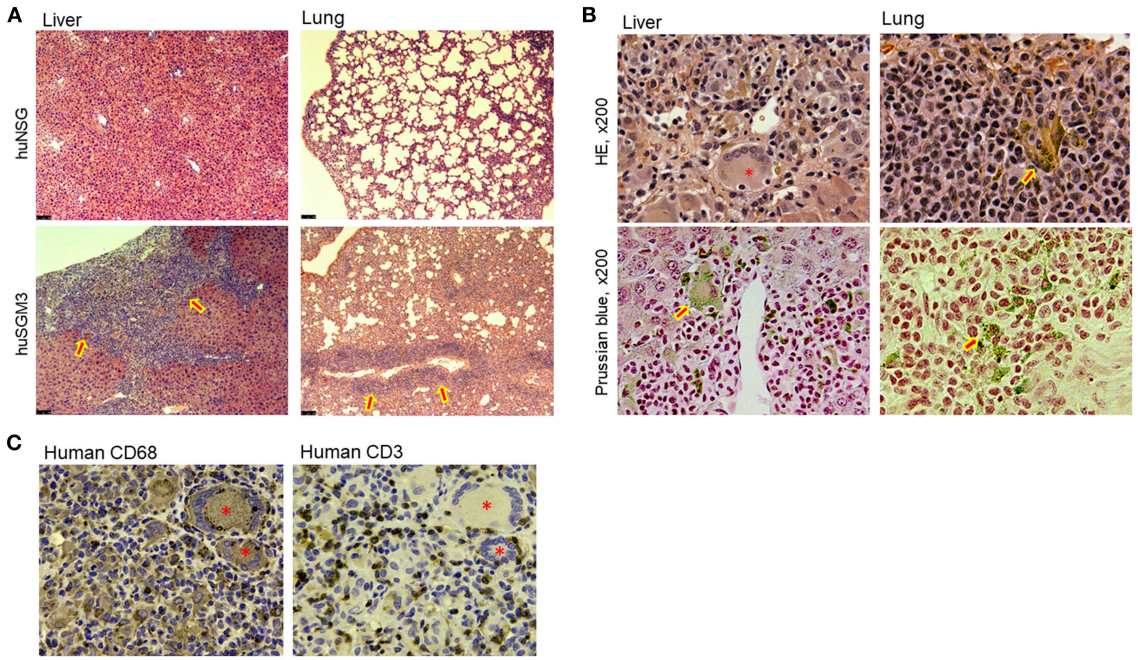


FIGURE 3 | Tissue infiltration of T cells and hemophagocytic macrophages in huSGM3 mice. Liver and lung tissue samples were prepared from huNSG and huSGM3 mice 18–22 weeks after transplantation, and examined histologically ($n = 4$ per group). **(A)** Representative H&E staining of liver and lung tissue sections from huNSG and huSGM3 mice (without human thymus). Original magnification: x50. Arrow signs in huSGM3 mice organs show severe cellular infiltrations. **(B)** Representative staining images infiltration of hemosiderin-containing hemophagocytic histiocytes (macrophages) in liver and lungs from huSGM3 mice (without human thymus). Hemosiderin-containing hemophagocytic histiocytes, indicated by arrow signs, are stained with brown by H&E and blue by Prussian blue. *denotes a representative giant cell (i.e., fused macrophages) containing ingested erythrocytes. Original magnification: x200. **(C)** Representative immunohistochemical staining of huSGM3 mouse (without human thymus) liver sections (two consecutive sections) with anti-human CD3 and anti-human CD68 antibodies. *denotes a $CD68^+$ giant cell.

$CD34^+$ cells and HLA-partially-matched fetal thymus compared to similarly constructed huNSG mice (**Figure S1**). CD31 has been described as a marker to identify the $CD4^+$ recent thymic emigrants among the naive $CD4^+$ T cell population (16). HuSGM3 mice also had significantly fewer $CD31^+$ $CD4^+$ T cells than huNSG mice (**Figure 2E**), likely due to abnormal human $CD4^+$ T cell expansion and reduced human thymopoiesis (see below) in these mice. The results indicate an aggressive, human cytokine-driven expansion of human $CD4^+$ T cells in huSGM3 mice, particularly in the absence of human thymus. The frequency of regulatory T cells (Tregs) defined as $FoxP3^+$ cells within $CD4^+$ T cells were higher in huSGM3 than in huNSG mice (**Figure 2F**), in concordance with a previous report (9).

Severe Tissue Infiltration by Human T Cells and Hemophagocytic Macrophages in HuSGM3 Mice

To determine the cause of mortality in huSGM3 mice, comprehensive histopathological analysis was performed on various organs including liver, lungs, spleen, kidney, small intestine, colon, and skin. There were no significant findings in skin, small intestine, or colon (data not shown). However, liver from both of huSGM3 mice with or without thymus had strikingly severe cellular infiltration, which destructed normal structure (**Figure 3A**). Infiltrated cells consisted of lymphocytes and macrophages, and many of the latter are hemosiderin-containing hemophagocytic macrophages that were visualized brown by H&E staining and blue by Prussian blue staining (**Figure 3B**). In addition, giant cells, which are fused macrophages upon activation having multiple nucleus, were observed. Immunohistochemical staining with anti-human CD3 antibody and anti-human CD68 antibody confirmed that liver-infiltrating cells were predominantly human T cells and human macrophages, and distributed in a pattern with activated hemophagocytic macrophages being surrounded by T cells (**Figure 3C**). This pattern is different from that of GVHD in which T cells infiltrate predominantly into portal areas. Lungs also showed severe cellular infiltration that resulted in scarce alveolar space (**Figure 3A**), and the presence of large numbers of hemophagocytic macrophages that were stained positive with Prussian blue (**Figure 3B**). These observations indicate that the huSGM3 mice developed a fatal disease similar to HLH in human.

We also performed flow cytometric analysis on single cell suspensions prepared from these tissues. In accordance with the histopathological analysis, absolute numbers of human $CD45^+$ total leukocytes isolated from the liver of huSGM3 mice were significantly higher than those in huNSG mice (**Figure 4A**). Moreover, absolute numbers of human $CD33^+$ myeloid cells isolated from the liver of huSGM3 mice were also significantly higher than those in huNSG mice, and were similar between

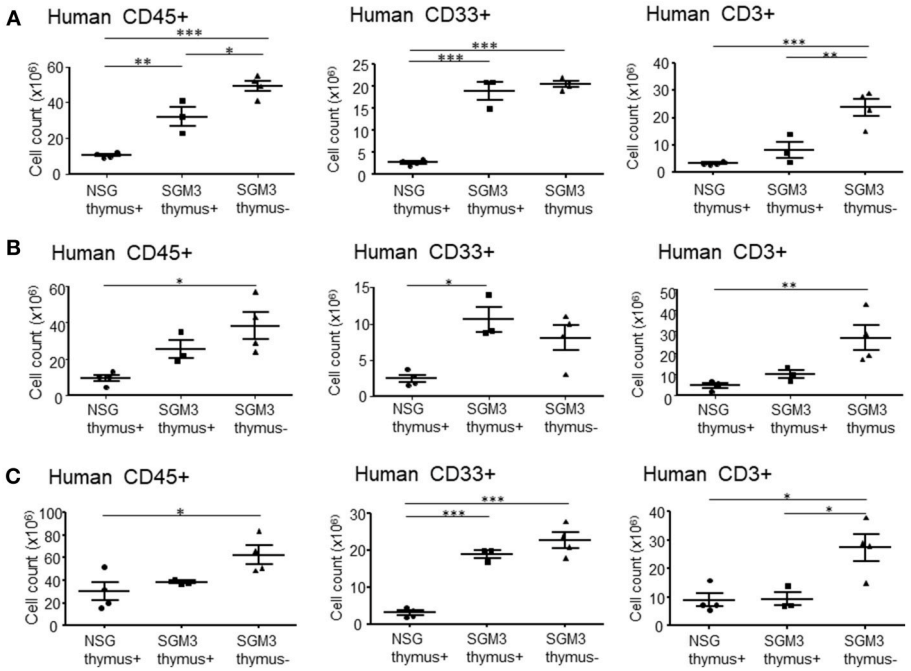


FIGURE 4 | Absolute numbers of human T cells and myeloid cells isolated from huNSG and huSGM3 mice. HuSGM3 mice with or without human thymus graft (sacrificed when moribund between 18 and 22 weeks) and huNSG mice with human thymus graft (sacrificed at 21–22 weeks after transplantation) were analyzed for human cell infiltrations in various tissues ($n = 4$ per group). Absolute cell numbers of human $CD45^+$ cells, T cells, and myeloid cells in liver **(A)**, lungs **(B)** and spleen **(C)** are calculated by multiplying the total cell count after mononuclear cell-purification using density gradient centrifugation with the percentage of each cell population determined by flow cytometry. One-way ANOVA with Bonferroni's *post-hoc* multiple comparison test; each symbol represents an individual mouse. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars indicate SEMs.

with and without human thymus groups. Interestingly, huSGM3 mice without human thymus, but not those with human thymus showed significantly increased human T cell infiltration in the liver compared to huNSG mice. Lungs (**Figure 4B**) and spleen (**Figure 4C**) showed a similar pattern as the liver in absolute numbers of human $CD45^+$ total leukocytes, $CD33^+$ myeloid cells, and $CD3^+$ T cells. These results indicate that human cytokines in the huSGM3 mice induced human myeloid and T cell infiltrations into the liver, lung and spleen, and suggested that the human thymus graft may suppress the infiltration of human T cells, but not myeloid cells, in these mice.

Cytopenia in HuSGM3 Mice

Clinical symptoms of HLH in human include pancytopenia due to hypoplastic marrow caused by the myelosuppressive effects of inflammatory cytokines and hemophagocytosis (17). Despite a significantly increased white cell counts, moribund huSGM3 mice showed severe anemia and thrombocytopenia (**Figure 5A**). Interestingly, absolute reticulocyte counts in huSGM3 mice were significantly higher than that in huNSG mice (**Figure 5A**). Although hematopoietic stem cell exhaustion has been reported previously in the huSGM3 mice, (9, 18) the high reticulocyte counts in these mice suggest that destruction by activated macrophages, not poor production, is the major mechanism for anemia. In support of this possibility, histopathological analysis revealed hypoplastic marrow, with the presence of hemophagocytic macrophages and eosinophils in the huSGM3 mice (**Figure 5B**). Bone marrow hypoplasia was also confirmed by flow cytometry analysis showing a markedly reduced cellularity of human $CD45^+$ and $CD33^+$ myeloid cells in the bone marrow from huSGM3 mice (**Figure 5C**).

Human Thymopoiesis in the Human Thymic Graft and Native Mouse Thymus in HuNSG and HuSGM3 Mice

We also examined the function of human and mouse thymus in huNSG and huSGM3 mice. Briefly, we first identified human $CD45^+$ cells, and then analyzed the expression of CD4 and CD8. Since some of the non-thymocytes might be contaminated in the $CD4^-CD8^-$ double negative (DN) population, we calculated the frequency of $CD4^+CD8^+$ double positive (DP) cells using DP cells plus CD4 and CD8 single positive (SP) cells as a denominator (**Figure 6**). In huNSG mice, human thymus graft had a higher DP cell frequency than mouse thymus. In contrast, in huSGM3, both human and mouse thymus had scarce DP cells, suggesting that thymopoiesis was severely damaged in these mice.

Hypercytokinemia in HuSGM3 Mice

Plasma were prepared from huNSG and huSGM3 mice at 18 weeks after transplantation, and human cytokine levels were measured using the multiplex immunoassay system (**Figure 7**).

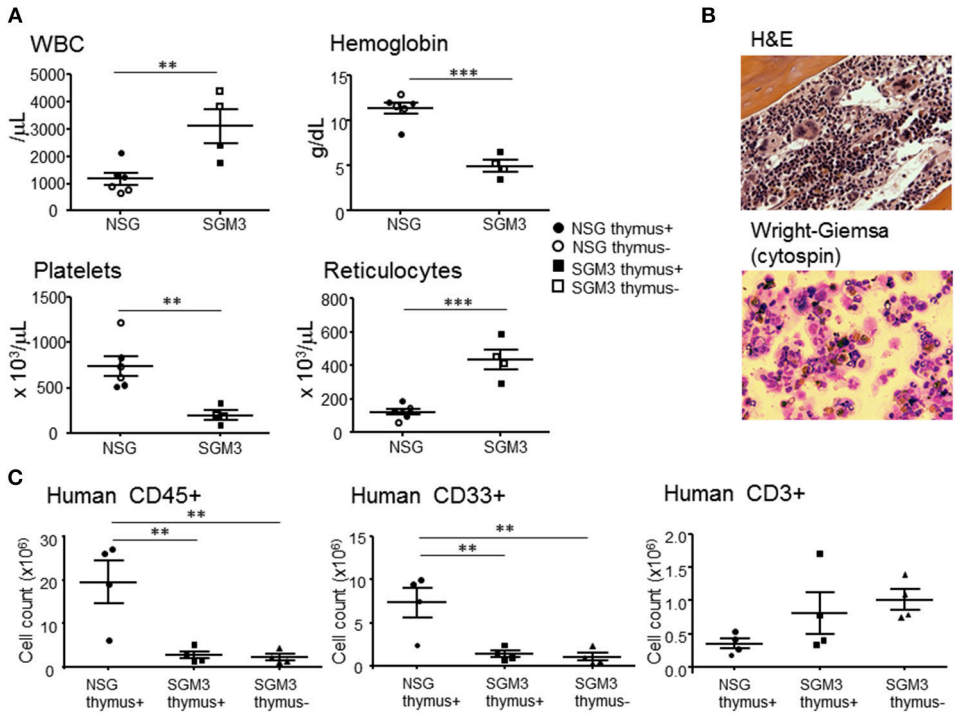


FIGURE 5 | Cytopenia and bone marrow hypoplasia in huSGM3 mice. HuSGM3 mice with (closed square) or without (open square) human thymus were sacrificed when moribund between 18 and 22 weeks. HuNSG mice with (closed circle) or without (open circle) human thymus were sacrificed at 21–22 weeks after transplantation as controls. Since there was no apparent difference between the "thymus+" and "thymus-" groups, the data were pooled (but samples from different groups are distinguishable by their respective symbols). **(A)** White blood cell count (WBC), hemoglobin concentration, platelet count, and reticulocyte count measured by hematometry. Unpaired *t*-test; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars indicate SEMs. **(B)** Histology of BM analyzed by H&E staining and cytology of BM analyzed by Wright-Giemsa staining. **(C)** Absolute cell numbers of human CD45$^+$ cells, CD33$^+$ myeloid cells and CD3$^+$ T cells in BM, which were calculated by multiplying the total cell count after mononuclear cell-purification using density gradient centrifugation with the percentage of each cell population determined by flow cytometry. One-way ANOVA with Bonferroni's *post-hoc* multiple comparison test; each symbol represents an individual mouse. $^{**}P < 0.01$.

The levels of human IL-4, IL-6, IL-10, IL-13, IL-18, IFN-γ, and TNF-α were significantly elevated in huSGM3 mice compared to huNSG mice. Plasma levels of IL-1β, IL-2, IL-5, and IL-12 were below sensitivity in both groups (data now shown). Interestingly, GM-CSF levels in huSGM3 mice were significantly higher than non-humanized SGM3 mice, reflecting the production of GM-CSF by activated human macrophages and/or T cells in the huSGM3 mice.

DISCUSSION

In the current study, we successfully reproduced a posttransplant HLH by utilizing huSGM3 mice and showed that posttransplant HLH is triggered by alloresponses (or xenoresponses in our model), driven by myeloid cytokines, and exacerbated by vicious cycles of T-cell and macrophage activation. HLH was seen in huSGM3 mice with or without human thymic grafts, indicating that mouse thymus-derived T cells were largely responsible for the disease development. Given that the huSGM3 mice with human thymus have a normal human immune T cell pool consisting of more naïve T cells and immune regulatory T cells (e.g., Tregs), these mice are considered more clinically relevant. However, because the disease is much more severe in huSGM3 mice without human thymus, these mice could also be helpful in testing the efficacy of HLH therapies. This study not only offers a humanized HLH mouse model, but also raises an alert on the use of human cytokine transgenic mice in the construction of hu-mice with functional human lymphohematopoietic systems in an attempt to improve myeloid reconstitution.

HLH is a life-threatening disease of severe hyper inflammation caused by uncontrolled proliferation of activated macrophages and lymphocytes that secrete high amounts of inflammatory cytokines, including IL-1, IL-6, IL-18, and TNF-α from macrophages and IFN-γ from T-cells. IL-10, an anti-inflammatory cytokine, is also elevated in HLH patients, (19) possibly as a response to hyper-inflammation (20). The cardinal symptoms include prolonged fever, hepatosplenomegaly, cytopenia, and hemophagocytosis by activated, morphologically benign macrophages. Recent studies have shown that hypercytokinemia is the driving cause of immunopathology of HLH, and most of above mentioned symptoms are attributable to hypercytokinemia. For example, fever is induced by IL-1 and IL-6, splenomegaly is the direct result of infiltration by lymphocytes and macrophages, and cytopenia can be explained by high concentrations of TNF-α and IFN-γ (21–23) as well as direct hemophagocytosis.

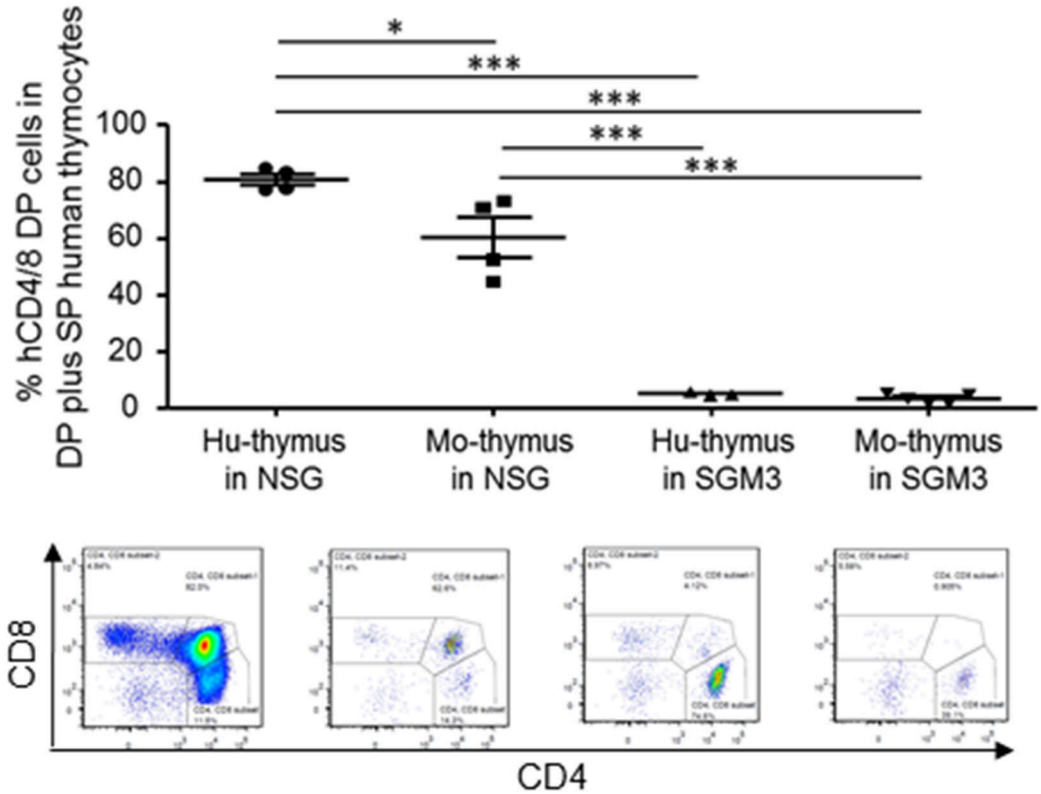


FIGURE 6 | Thymopoiesis in the human and mouse thymus in NSG and SGM3 humanized mice. Frequencies (%) of CD4 and CD8 double positive cells (double positive cells / (double positive cells + single positive cells) × 100%) in the human and mouse thymus from huNSG and huSGM3 mice (with human thymic grafts) were shown with representative flow cytometric profiles. One-way ANOVA with Bonferroni's *post-hoc* multiple comparison test; each symbol represents an individual mouse. $^{*}P < 0.05$; $^{***}P < 0.001$. Error bars indicate SEMs. DP, double positive, SP, single positive cells.

While HLH after allo-SCT has been recognized to be rare complication, recent studies have suggested that the incidence of posttransplant HLH is higher than previously thought, particularly in HLA-mismatched transplantation settings. Takagi reported that 11.8% of patients who received HLA-mismatched cord blood transplantation following reduced-intensity conditioning regimen developed HLH (3) Interestingly, they subsequently reported that the intensification of immune suppressant after SCT reduced the incidence of HLH (24). Jaiswal reported that 12.2% of patients who received HLA-haploidentical peripheral blood stem cell transplantation with posttransplant cyclophosphamide developed HLH (2). These findings support the theory that alloresponse is the trigger for the posttransplant HLH. Notably, huSGM3 mice in the current study closely resembled posttransplant HLH in the clinic. These mice manifested hepatosplenomegaly (**Figure S2**) with T cell and macrophage infiltrations and cytopenia, and had a significant elevation in multiple inflammatory cytokines including IL-6, IL-18, IFN-γ, and TNF-α. Compared to mouse models of primary HLH, which are generated by deletion or mutation of the murine orthologs of the genes involved in human primary HLH, mouse models of secondary HLH are scarce (25). Moreover, the majority of the limited number of secondary HLH models, including a model of EBV-associated HLH in hu-mice (26), are infection associated HLH/HPS models.

Although the current study did not specify which cytokine played a major role in the pathogenesis of HLH, GM-CSF is assumed to be the primary pathogenic cytokine, as the huSGM3 mice shared many of the findings observed in a mouse HLH model driven by GM-CSF overexpression, such as short survival, splenomegaly, lymphadenopathy, thymic atrophy, and multiple abnormalities in blood cell populations including progressing anemia (27). Nonetheless, such a humanized mouse model of HLH allows for examining the pathogenesis of secondary HLH caused by human immune systems, including the cross-talk between human T cells and macrophages via cytokines, and therefore, is more clinically relevant than previous mouse models and ideal for evaluating therapeutic options. Immunohistological analysis demonstrated severe tissue infiltration by activated human T cells and phagocytic macrophages, indicating that these human immune cells are the primary pathogenic effectors in HLH development in the huSGM3 mice. Moreover, since both human IL-6 and TNF-α, the major inflammatory cytokines driving the development of wasting syndrome or cachexia in HLH, are cross-reactive with mouse cells, (28) it is likely that the body weight loss and wasting

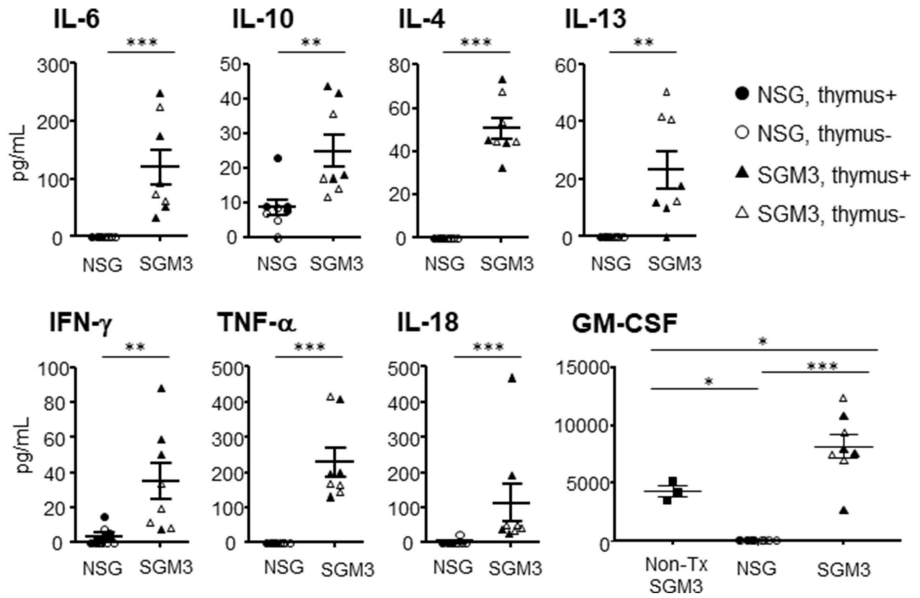


FIGURE 7 | Plasma levels of human cytokines in huNSG and huSGM3 mice. Human cytokines levels in plasma at 18 weeks after transplantation were measured using multiplex immunoassay system. HuNSG and huSGM3 mice are shown as circle and square symbols, respectively, and each symbol represents an individual mouse (closed and open symbols represent mice with and without human thymus, respectively). Since there was no apparent difference between the "thymus+" and "thymus-" groups, the data were pooled (but samples from different groups are distinguishable by their respective symbols). Unpaired *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars indicate SEMs.

syndrome are largely attributed to these human inflammatory cytokines. It has been reported that TNFα is capable of suppressing hematopoiesis by several mechanisms including direct cytotoxicity and induction of apoptosis (21–23). Therefore, elevated human TNFα production may also be one of the mechanisms causing severe hypocellularity in the BM in huSGM3 mice.

Another striking finding in our model was the delayed, but aggressive T cell reconstitution in huSGM3 mice without human thymus graft. We found that T cell frequencies in peripheral blood from both huNSG and huSGM3 mice without human thymus graft remained extremely low or almost absent until 10 weeks, but increased remarkably steeply thereafter in huSGM3 mice, and exceeded huSGM3 mice with human thymus by 15 weeks. Absolute T cell numbers from the organs proved that T cell expansion in the mice without human thymus graft was more aggressive than in the mice with human thymus.

These results suggest a possible aberrant human thymopoiesis in the mouse native thymus, leading to generation of functionally abnormal human T cells. Nevertheless, huSGM3 mice without human thymus did not show characteristic GVHD findings when they became moribund except for one mouse that had moderate diarrhea. These mice showed severe T cell infiltration of the liver; however, infiltration was not restricted to portal area as shown in GVHD, indicating it is more likely caused by uncontrolled proliferation due to hypercytokinemia. While it is plausible to assume that the huSGM3 mice developed lethal HLH prior to GVHD, another possible explanation could be the balance of myeloid and lymphoid cytokines determines the disease manifestations i.e., HLH or GVHD.

In conclusion, we successfully produced a disease model of posttransplant HLH. Our study suggests that posttransplant HLH is triggered by alloresponse (or xenoresponse in our model), driven by myeloid cytokines, and exacerbated by vicious cycles of T-cell and macrophage activation.

AUTHOR CONTRIBUTIONS

SY designed and performed experiments, analyzed data, and wrote the paper. YL, JX, and ND designed and performed experiments, and analyzed data. MS designed experiments and analyzed data. Y-GY conceived the research project, designed experiments, analyzed data, and wrote the paper. All authors edited and approved the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. Remi J. Creusot for critical review of the manuscript. This work was supported by grants from MOST of China (2015CB964400), NSFC (81273334), and NIH (P01AI045897). Flow Cytometric analysis and cell sorting were performed in the CCTI Flow Cytometry Core funded in part through an NIH Shared Instrumentation Grant (1S10RR027050).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00186/full#supplementary-material

REFERENCES

1. Janka GE, Lehmberg K. Hemophagocytic syndromes - An update. *Blood Rev.* (2014) 28:135–42. doi: 10.1016/j.blre.2014.03.002
2. Jaiswal SR, Chakrabarti A, Chatterjee S, Bhargava S, Ray K, Chakrabarti S. Hemophagocytic syndrome following haploidentical peripheral blood stem cell transplantation with post-transplant cyclophosphamide. *Int J Hematol.* (2016) 103:234–42. doi: 10.1007/s12185-015-1905-y
3. Takagi S, Masuoka K, Uchida N, Ishiwata K, Araoka H, Tsuji M, et al. High incidence of haemophagocytic syndrome following umbilical cord blood transplantation for adults. *Br J Haematol.* (2009) 147:543–53. doi: 10.1111/j.1365-2141.2009.07863.x
4. Abdelkefi A, Jamil WB, Torjman L, Ladeb S, Ksouri H, Lakhal A, et al. Hemophagocytic syndrome after hematopoietic stem cell transplantation: a prospective observational study. *Int J Hematol.* (2009) 89:368–73. doi: 10.1007/s12185-009-0261-1
5. Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. *Int J Hematol.* (2003) 78:181–7. doi: 10.1007/BF02983793
6. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. *Nat Rev Immunol.* (2012) 12:786–98. doi: 10.1038/nri3311
7. Rongvaux A, Takizawa H, Strowig T, Willinger T, Eynon EE, Flavell RA, et al. Human hemato-lymphoid system mice: current use and future potential for medicine. *Annu Rev Immunol.* (2013) 31:635–74. doi: 10.1146/annurev-immunol-032712-095921
8. Chen Q, Khoury M, Chen J. Expression of human cytokines dramatically improves reconstitution of specific human-blood lineage cells in humanized mice. *Proc Natl Acad Sci USA.* (2009) 106:21783–8. doi: 10.1073/pnas.0912274106
9. Billerbeck E, Barry WT, Mu K, Dorner M, Rice CM, Ploss A. Development of human $CD4^+FoxP3^+$ regulatory T cells in human stem cell factor-, granulocyte-macrophage colony-stimulating factor-, and interleukin-3-expressing NOD-SCID IL2Rgamma(null) humanized mice. *Blood* (2011) 117:3076–86. doi: 10.1182/blood-2010-08-301507
10. Rongvaux A, Willinger T, Martinek J, Strowig T, Gearty SV, Teichmann LL, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol.* (2014) 32:364–72. doi: 10.1038/nbt.2858
11. Onoe T, Kalscheuer H, Chittenden M, Zhao G, Yang YG, Sykes M. Homeostatic expansion and phenotypic conversion of human T cells depend on peripheral interactions with APCs. *J Immunol.* (2010) 184:6756–65. doi: 10.4049/jimmunol.0901711
12. Kalscheuer H, Danzl N, Onoe T, Faust T, Winchester R, Goland R, et al. A model for personalized *in vivo* analysis of human immune responsiveness. *Sci Trans Med.* (2012) 4:125ra30. doi: 10.1126/scitranslmed.3003481
13. Tonomura N, Habiro K, Shimizu A, Sykes M, Yang YG. Antigen-specific human T-cell responses and T cell-dependent production of human antibodies in a humanized mouse model. *Blood* (2008) 111:4293–6. doi: 10.1182/blood-2007-11-121319
14. Hu Z, Van Rooijen N, Yang YG. Macrophages prevent human red blood cell reconstitution in immunodeficient mice. *Blood* (2011) 118:5938–46. doi: 10.1182/blood-2010-11-321414
15. Hu Z, Yang YG. Full reconstitution of human platelets in humanized mice after macrophage depletion. *Blood* (2012) 120:1713–6. doi: 10.1182/blood-2012-01-407890
16. Kohler S, Thiel A. Life after the thymus: $CD31^+$ and $CD31^-$ human naive $CD4^+$ T-cell subsets. *Blood* (2009) 113:769–74. doi: 10.1182/blood-2008-02-139154
17. George MR. Hemophagocytic lymphohistiocytosis: review of etiologies and management. *J Blood Med.* (2014) 5:69–86. doi: 10.2147/JBM.S46255
18. Nicolini FE, Cashman JD, Hogge DE, Humphries RK, Eaves CJ. NOD/SCID mice engineered to express human IL-3, GM-CSF and Steel factor constitutively mobilize engrafted human progenitors and compromise human stem cell regeneration. *Leukemia* (2004) 18:341–7. doi: 10.1038/sj.leu.2403222
19. Xu XJ, Tang YM, Song H, Yang SL, Xu WQ, Zhao N, et al. Diagnostic accuracy of a specific cytokine pattern in hemophagocytic lymphohistiocytosis in children. *J Pediatr.* (2012) 160:984–90 e1. doi: 10.1016/j.jpeds.2011.11.046
20. Weaver LK, Behrens EM. Hyperinflammation, rather than hemophagocytosis, is the common link between macrophage activation syndrome and hemophagocytic lymphohistiocytosis. *Curr Opin Rheumatol.* (2014) 26:562–9. doi: 10.1097/BOR.0000000000000093
21. Broxmeyer HE, Williams DE, Lu L, Cooper S, Anderson SL, Beyer GS, et al. The suppressive influences of human tumor necrosis factors on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: synergism of tumor necrosis factor and interferon-gamma. *J Immunol.* (1986) 136:4487–95.
22. Maciejewski J, Selleri C, Anderson S, Young NS. Fas antigen expression on $CD34^+$ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression *in vitro*. *Blood* (1995) 85:3183–90.
23. Selleri C, Sato T, Anderson S, Young NS, Maciejewski JP. Interferon-gamma and tumor necrosis factor-alpha suppress both early and late stages of hematopoiesis and induce programmed cell death. *J Cell Physiol.* (1995) 165:538–46. doi: 10.1002/jcp.1041650312
24. Uchida N, Wake A, Nakano N, Ishiwata K, Takagi S, Tsuji M, et al. Mycophenolate and tacrolimus for graft-versus-host disease prophylaxis for elderly after cord blood transplantation: a matched pair comparison with tacrolimus alone. *Transplantation* (2011) 92:366–71. doi: 10.1097/TP.0b013e318223d7ac
25. Strippoli R, Caiello I, De Benedetti F. Reaching the threshold: a multilayer pathogenesis of macrophage activation syndrome. *J Rheumatol.* (2013) 40:761–7. doi: 10.3899/jrheum.121233
26. Sato K, Misawa N, Nie C, Satou Y, Iwakiri D, Matsuoka M, et al. A novel animal model of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in humanized mice. *Blood* (2011) 117:5663–73. doi: 10.1182/blood-2010-09-305979
27. van Nieuwenhuijze AE, Coghill E, Gray D, Prato S, Metcalf D, Alexander WS, et al. Transgenic expression of GM-CSF in T cells causes disseminated histiocytosis. *Am J Pathol.* (2014) 184:184–99. doi: 10.1016/j.ajpath.2013.09.014
28. Manz MG. Human-hemato-lymphoid-system mice: opportunities and challenges. *Immunity* (2007) 26:537–41. doi: 10.1016/j.immuni.2007.05.001

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 01 February 2019
doi: 10.3389/fimmu.2019.00093

Dendritic Cell Regulation of Graft-vs.-Host Disease: Immunostimulation and Tolerance

Hongshuang Yu[1], *Yuanyuan Tian*[1], *Ying Wang*[1], *Shin Mineishi*[2] *and Yi Zhang*[1,3]*

[1] Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia, PA, United States, [2] Department of Medicine, Pennsylvania State University, Hershey, PA, United States, [3] Department of Microbiology & Immunology, Temple University, Philadelphia, PA, United States

Graft-vs.-host disease (GVHD) remains a significant cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Significant progresses have been made in defining the dichotomous role of dendritic cells (DCs) in the development of GVHD. Host-derived DCs are important to elicit allogeneic T cell responses, whereas certain donor-types of DCs derived from newly engrafted hematopoietic stem/progenitor cells (HSPCs) can amply this graft-vs.-host reaction. In contrast, some DCs also play non-redundant roles in mediating immune tolerance. They induce apoptotic deletion of host-reactive donor T cells while promoting expansion and function of regulatory T cells (Treg). Unfortunately, this tolerogenic effect of DCs is impaired during GVHD. Severe GVHD in patients subject to allo-HSCT is associated with significantly decreased number of circulating peripheral blood DCs during engraftment. Existing studies reveal that GVHD causes delayed reconstitution of donor DCs from engrafted HSPCs, impairs the antigen presentation function of newly generated DCs and reduces the capacity of DCs to regulate Treg. The present review will discuss the importance of DCs in alloimmunity and the mechanism underlying DC reconstitution after allo-HSCT.

Keywords: graft-vs.-host, disease, dendritic cells, transcription factors, alloreactive T cells, immunostimulation, immune tolerance

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina, United States

Reviewed by:
Xuefang Cao,
University of Maryland, Baltimore, United States
Tomomi Toubai,
Yamagata University, Japan

***Correspondence:**
Yi Zhang
yi.zhang@temple.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *06 November 2018*
Accepted: *14 January 2019*
Published: *01 February 2019*

Citation:
Yu H, Tian Y, Wang Y, Mineishi S and Zhang Y (2019) Dendritic Cell Regulation of Graft-vs.-Host Disease: Immunostimulation and Tolerance. Front. Immunol. 10:93. doi: 10.3389/fimmu.2019.00093

INTRODUCTION

Allogenic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for many hematological malignancies, such as leukemia, lymphoma, and multiple myeloma (1, 2). This beneficial effect is largely derived from infused donor immune cells that can eliminate malignant cells, a process known as graft-vs.-leukemia (GVL) response (3–5). However, the success of the procedure is limited by the life-threatening complication graft-vs.-host disease (GVHD), in which the gastrointestinal (GI) tract, skin and liver are preferentially damaged (2, 6–9).

GVHD is mediated by infused donor T cells that recognize and react to histocompatibility differences between the host and donor (9–12). Host-derived antigen-presenting cells (APCs) can directly present antigens to prime allogenic donor T cells, whereas donor-derived APCs can present host antigens to donor T cells via indirect antigen presentation (10, 12, 13). Initial studies demonstrate that host APCs are critical for donor $CD8^+$ T cell-mediated GVHD. Subsequent studies indicate that either host or donor APCs are sufficient to induce $CD4^+$ T cell-dependent GVHD (9–12, 14–17). Importantly, unlike T cell responses to pathogens in which hematopoietic

APCs prime T cells, alloreactive T cell responses in the setting of allo-HSCT may be primed by both hematopoietic and non-hematopoietic APCs (9–12, 14–17).

DCs are the most potent professional APCs known to elicit primary T cell responses (18–20). Based on their surface phenotype, anatomical location and function, DCs at the steady state are broadly categorized into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Under inflammatory condition, both DC subsets undergo profound changes in their phenotype and functionality (21–25). For example, in response to inflammatory stimuli, DCs may be primed selectively to produce special types of cytokines (e.g., IL-12, IL-23) and Notch ligands (e.g., Delta-like 1 (DLL1) and DLL4). These DC-derived molecules are important to instruct antigen-activated T cells to differentiate into distinct lineages of effector T cells, such as T helper (TH)1, TH17 cells, and cytotoxic T cells (CTLs) (26–33).

Over the past two decades, both clinical and preclinical studies have demonstrated dichotomous roles of DCs in GVHD (9, 34, 35). While some DCs induce alloreactive T cell responses mediating host tissue injury, other DC subsets induce donor T cell tolerance against the host tissue. In this review, we will discuss these effects of DC-mediated immunogenicity and tolerogenicity during GVHD.

DC INDUCTION OF GVHD

DCs are specialized APCs that play non-redundant roles in regulating both immunity and tolerance (9, 18, 36–44). In the setting of allo-HSCT, host-derived DCs are important for donor T cell-mediated GVHD in the liver, colon and skin (9–12, 16, 17, 35, 45). *De novo* generated donor APCs, including DCs, are also required to induce maximal GVHD through a complex mechanism (9–11, 35).

Host DCs and Initiation of Alloreactive T Cell Responses

Shlomchik and colleagues demonstrate, for the first time, that host hematopoietic APCs are critical for induction of the disease, and donor APCs can mediate maximal GVHD (10, 12). Subsequent studies reveal that host DCs, which are activated during preparative conditioning for allo-HSCT, present host antigens to prime donor $CD4^+$ and $CD8^+$ T cells and promote their proliferation and differentiation into alloreactive effector cells (17, 46). Add-back of WT host-type cDCs or pDCs causes severe GVHD in mice lacking MHC class-I or MHC class-II, respectively (47), further strengthening the importance of host DCs in mediating GVHD (**Table 1**). However, these studies do not explain whether host DCs contribute to GVHD when all the other types of host APCs, including B cells, macrophages and non-hematopoietic APCs, are intact. For example, host B cells produced high levels of IL-10 to modulate alloreactive T cell responses *in vivo* (57), Recipient macrophages, which resist the conditioning regimen, persisted in patients for several weeks following allo-HSCT and limited the severity of GVHD (58). In contrast, non-hematopoietic APCs activated by irradiation induce potent allo-specific responses in peripheral tissues(14, 59).

The role of host DCs in the development of GVHD in the presence of functional macrophages and non-hematopoietic APCs has been studied by several groups. Merad et al. examined the role of host Langerhans cells (LCs), a distinct subset of DCs located in the skin (19), in cutaneous GVHD (40). Administration of donor T cells to bone marrow (BM)-chimeric mice with persistent host LCs, but not to mice whose LCs had been replaced, resulted in marked skin GVHD (40), suggesting that host LCs are important for mediating the disease in the skin. Intriguingly, other studies show that LCs were dispensable for the induction of skin GVHD (48). In one of those studies, donor T cells and BM cells were transferred into lethally irradiated transgenic recipient mice in which epidermal LCs expressed the Diphtheria toxin A (DTA) under the control of the human Langerin locus (48). Deficiency of LCs did not affect the development of either $CD8^+$ T cell- or $CD4^+$ T cell-mediated GVHD (48). How to reconcile these observations remains controversial.

Donor DCs Amplify GVH Reaction by Cross-Presenting Host-Type Antigen

In the setting of allo-HSCT, *de novo* generated donor APCs are also found to be important for GVHD (9–11, 35). Studies by Markey et al. suggested that donor cDCs isolated from the spleen were the most effective population in presenting alloantigens and stimulating naïve donor T cell responses early after allo-HSCT (49). Intriguingly, upon exposure to GVH inflammation, donor $CD103^+CD11b^-$ cDCs, which are independent of the transcription factor IRF4 for their development (60, 61), captured alloantigen in the colon and migrated into the mesenteric lymph node to amplify alloreactive T cell responses (13). This suggests that tissue resident DCs may play important roles in regulating GVH reactions, which is supported by our early studies. We found that selective depletion of both host- and donor-type APCs, including DCs, in visceral organs led to significantly reduced GVHD in the liver but not in the skin (11). These observations suggest that donor DCs possess great capacity to orchestrate the alloreactive T cell response both in the lymphoid organ and non-lymphoid tissues, eliciting different types of GVHD.

DC-Derived IL-12 and Notch Ligands Shape Alloreactive T Cell Responses

DCs produce multiple molecules capable of shaping allogeneic T cell responses (**Figure 1**). For example, IL-12 produced by DCs drives expansion and differentiation of antigen-activated T cells (13, 18, 27, 30, 62, 63). Donor BM cells lacking IL-12 p40 had significantly decreased capacity to promote effector differentiation and expansion in the mesenteric lymph nodes of mice receiving allogenic T cells. IL-12 derived from $CD103^+CD11b^-$ cDCs promoted IFN-γ production in host-reactive T cells (13). Notch signaling pathway is demonstrated as an important regulator of alloreactive T cell responses. Using a genetic approach, we reported that inhibition of pan-Notch

TABLE 1 | Effect of different DC subsets in GVHD.

DC subset	Effect on GVHD	Origin	Method	GVHD model	Outcome	Mechanism	Authors/ References
cDCs or pDCs	Induction	Host	Add-back of cDCs or pDCs	Balb/c → B6 AKR → C3H	GVHD↑	Prime allo-T cell response	Koyama et al. (47)
LCs	Induction	Host	Depletion of LCs	B6 → Balb/c	skin GVHD↓	Increase donor T cell infiltrating in the skin	Merad et al. (40)
LCs	No effect	Host	Depletion of LCs	B6 → Balb/c C3H → B6	GVHD maintained	-	Li et al. (48)
cDCs	Induction	Donor	Depletion of $CD11c^+$ cDCs	B6 → Balb/c C3H → B6 B6 → B6D2F1	GVHD↓	Inhibit donor T cell proliferation	Markey et al. (49)
$CD103^+$ $CD11b^-$DCs	Induction	Donor	Depletion of $CD103^+CD11b^-$ cDCs	B6 → Balb/c	GVHD↑	Induce expansion and differentiation of donor T cells within the mLNs	Koyama et al. (13)
$CD8\alpha^+$ DCs	Tolerance	Host	Depletion of $CD8^+$ cDCs	Balb/c → B6	GVHD↑	Reduce numbers of Tregs and TGF- β levels	Weber et al. (50)
$CD8\alpha^+$ DCs	Tolerance	Host	Pre-treatment of the recipient with Flt3L	B6 → B6D2F1	GVHD↓	Suppress donor T cell responses to host antigens	Teshima et al. (41)
$CD8\alpha^+$ DCs	Tolerance	Host	Pre-treatment of recipients with Flt3L	C3H → B6 B6 → B6D2F1	GVHD↓	Functionally delete of the alloreactive T-cell	Markey et al. (51)
$CD8\alpha^+$ cDCs	No effect	Host	Depletion of $CD8^+$ cDCs	C3H → B6	GVHD maintained	-	Toubai et al. (52)
$CCR9^+$ pDCs	Tolerance	Host	Transfer of $CCR9^+$ pDCs	Balb/c → B6	GVHD↓	Promote Treg expansion and function Suppress antigen-specific T responses	Hadeiba et al. (53)
SAHA treated moDCs	Tolerance	Host	Transfer of moDC treated with SAHA	Balb/c → B6	GVHD↓	Promote Treg expansion and function	Reddy et al. (54)
pre-pDCs	Tolerance	Donor	Depletion of pre-pDCs from BM grafts	B6 → Balb/c B6 → B6D2F1	GVHD↑	Inhibit T cell proliferation in a contact-dependent fashion	Banovic et al. (55)
pre-pDCs	Tolerance	Donor	Transfer of BM pre-pDCs	B6→ B10	GVHD↓	Increase Tregs and decrease alloreactive effector T cells	Lu et al. (56)

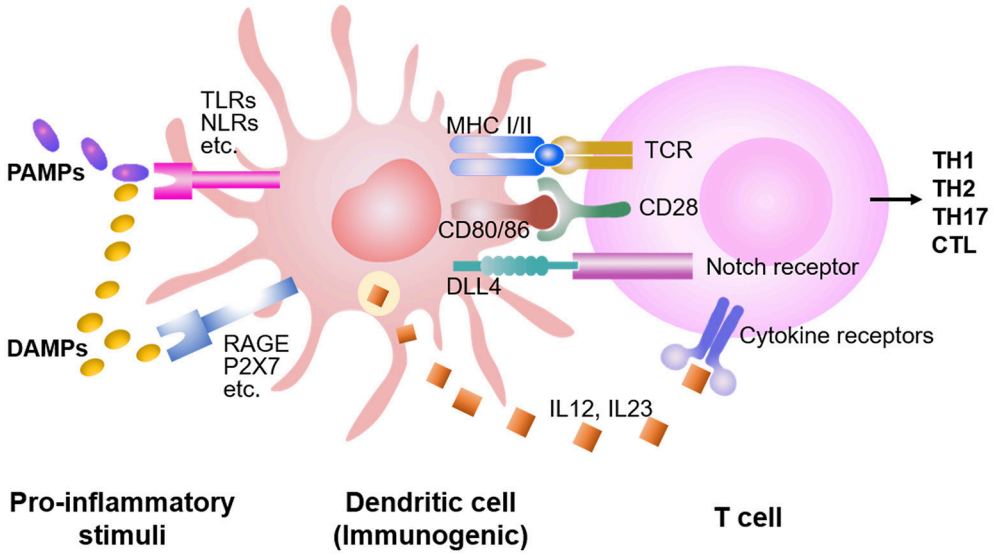


FIGURE 1 | DC stimulation of allogeneic T cell responses. Preparative conditioning regimens before the allo-HSCT induce host tissue injuries, leading to the release of DAMPs and PAMPs. Consequently, DCs are activated by DAMPs and PAMPs through multiple receptors, capable to present antigens and prime the T cells. While DAMPs activate DCs mainly through TLRs (i.e., TLR 1-13), PAMPs activate DCs through RAGE, P2X7, etc., in addition to the TLRs. Both costimulatory molecules (e.g., CD28) and cytokines (e.g., IL-12, IL-23) synergize with the TCR signaling to promote proliferation and expansion of antigen-activated T cells. DCs also produce higher levels of Notch ligands (e.g., DLL1 and DLL4) to trigger Notch signaling in the T cell, instructing differentiation into distinct lineages of effector cells.

receptor signaling in donor T cells significantly reduced severity and mortality of GVHD in mouse models (32). Notch-deprived T cells proliferated and expanded in response to alloantigen *in vivo*, but failed to produce inflammatory cytokines, including IFN-γ, IL-17, and TNF-α (31, 32). In a separate study, we further observed that host DCs expressing DLL4 (named DLL4$^+$ DCs), one of the ligands of Notch receptors, had greater ability to stimulate the generation of alloreactive effector T cells that produced IFN-γ and IL-17 compared to DLL4$^-$ DCs (64–66). Studies by Maillard et al. have shown that blockade of DLL4 could abrogate this effect and prevented GVHD while preserving anti-tumor activity (67, 68). Intriguingly, recent studies demonstrate that chemokine CCL19-expressing host cells, including both fibroblastic reticular cells and follicular DCs, were also the essential source of DLL4 for shaping alloreactive T cell response in mice subject to allo-HSCT (69). Collectively, DC-derived IL-12 and DLL4 are important for the generation of alloreactive effector T cells during GVHD.

Activation of DCs After the Conditioning Regimens for Allo-HSCT

DCs express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and nod-like receptors (NLRs) to respond to pathogen-associated molecular patterns (PAMPs) (70–72). In addition, DCs are also capable to detect certain intracellular molecules, called damage-associated molecular patterns (DAMPs), that are released from cells stressed, damaged and/or dying in the local tissue (73). When PAMPs or DAMPs are present, DCs are stimulated to migrate to lymphoid tissues and present both antigen and costimulatory molecules to T cells (73–75). Preparative conditioning regimens for allo-HSCT, including high-dose chemotherapy and/or total body irradiation, cause host tissue injuries. This leads to the release of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) as well as DAMPs and PAMPs (74, 76).

Both PAMPs and DAMPs activate DCs through stimulating TLRs (i.e., TLR1-13) (**Figure 1**) (1, 8, 76–78). TLR expression among DC subsets is heterogeneous: pDC mainly express TLR1, 7 and 9; CD8α$^+$ DCs preferentially produce high levels of TLR3; whereas other cDC subsets express certain TLR subtypes but TLR9 (73–75, 79–85). Data from our studies and others suggested that Notch ligands DLL1 and DLL4 played non-redundant roles in activating Notch signaling to drive alloreactive T cell responses (32, 64–66, 68). LPS (TLR4 agonist) rapidly induces Dll4 expression in human and murine DCs (65, 66, 81–83). Combined stimulation of human DCs with LPS with TLR7 agonist R848 further increases the expression of DLL4 (65, 83). TLR3 is critical for presentation of viral double-stranded RNA (83, 86). Reddy and colleagues found that TLR3 stimulation enhanced GVL response without exacerbating GVHD in mice (52). These observations explain, at least in part, how different pro-inflammatory stimuli induce distinct types of immune responses.

DC-MEDIATED DONOR T CELL TOLERANCE AGAINST HOST TISSUES

Self-tolerance can be induced and maintained in different compartments of the immune system. During thymopoietic

development, self-reactive T cells are clonally deleted in the thymus as a result of negative selection (8, 23, 36, 87). However, thymopoiesis is impaired during GVHD (88, 89), which is associated with generation of alloreactive T cells that mediate chronic-like GVHD in mice (90, 91). Considered as a supplemental mechanism to central tolerance, peripheral tolerance however, is important to prevent autoimmunity (8, 23, 36, 87). DCs are the crucial players mediating peripheral tolerance (27, 36, 37, 44, 87). Therefore, we will review the tolerogenic role of DCs in the context of allo-HSCT.

cDCs

Both host and donor DCs may contribute to the induction of donor T cell tolerance against host tissues in mice undergoing allo-HSCT. Early studies by Teshima et al. reported that Flt3 ligand (Flt3L) treatment of recipient mice induced expansion of CD8α$^+$ DCs that were poor stimulators of allogeneic T cells in cultures and had great ability to suppress donor T cell responses to host antigens *in vivo* (**Table 1**) (41). These Flt3L-treated recipient mice developed much less severe GVHD compared to untreated controls (41). However, whether these *in vivo* expanded CD8α$^+$ DCs have direct effects on reducing GVHD was not examined in this study (41). Subsequent studies show that deletion of host CD11c$^+$ cells in CD11c. DTR (diphtheria toxin receptor) transgenic recipient mice caused a strong increase in GVHD-related mortality (50). Since CD11c is also expressed on the surface of some macrophages (18, 19, 62), the possibility that DT treatment might delete CD11c$^+$ macrophages that mediate immune suppression cannot be ruled out. Other studies examined the impact of deleting CD8α$^+$ DCs on GVHD development in recipient mice lacking Batf3 (50), which is a transcription factor crucial for the generation of CD8α$^+$ DCs and migratory CD103$^+$ cDCs (92, 93). Recipient mice lacking Batf3 developed more severe GVHD compared to WT mice and marked increase of proliferative donor T cells (50). This finding is further supported independently by studies from Hill and colleagues (51), but not from Reddy's group (52). However, whether transfer of CD8α$^+$ DCs may directly suppress GVHD in mice has never been reported. Thus, the exact DC subset induced upon Flt3L treatment capable to reduce GVHD has never been clearly addressed.

pDCs

The important role of pDCs in modulating GVH response was initially shown in a mouse model of GVHD. Transfer of host-type CCR9$^+$ pDCs inhibited GVHD in mice receiving MHC- or miHA-mismatched donor T cells (53). CCR9$^+$ pDCs migrate to the GI tract through chemotaxis via their own chemokine receptor CCR9 and the ligand CCL25 in the environment. Upon stimulation with TLR9 agonist CpG ODNs, CCR9$^+$ pDCs rapidly downregulate CCR9 from the original immature state and decrease the capacity to attenuate GVHD *in vivo* (53). Furthermore, precursor pDCs (pre-pDCs) were found to modulate GVHD in mouse models (55, 56). *In vivo* depletion of pre-pDCs using the antibody specific to PDCA-1, which is expressed on the surface of pDC lineage, significantly increased the severity of GVHD compared to recipient mice with intact donor pre-pDCs (55). Mechanistic analysis reveals that CCR9$^+$ pDCs and pre-pDCs are capable to promote Treg expansion and function, as well as to suppress antigen-specific immune responses both *in vitro* and *in vivo* (55, 56). These observations identify the tolerogenic effect of pDCs on inducing donor T cells against host tissues.

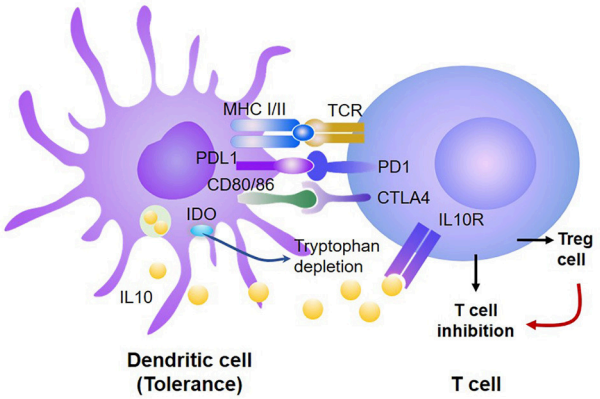


FIGURE 2 | DCs induce donor T cell tolerance via both T-cell intrinsic and extrinsic mechanisms. Tolerogenic DCs produce high levels of PD-L1 and CD80/CD86, which, respectively, bind PD-1 and CTLA4, leading to inhibition of antigen-activated T cells and generation of Treg. Treg further suppress the proliferation and survival of those antigen-activated T cells. In addition, tolerogenic DCs produce high levels of IDO, which can inhibit antigen-reactive T cells and promote Treg expansion and function.

Molecular Mechanisms by Which DCs Induce Donor T Cell Tolerance

Emerging evidence indicate that the mechanism responsible for DC-induced peripheral T cell tolerance can be broadly classified into two categories: intrinsic and extrinsic (18, 23, 62, 87, 94). T cell intrinsic signal acts directly on the responding T cells, such as inhibition or deletion of specific T cells, while T cell extrinsic signal acts through additional cells or factors, such as Treg or suppressive cells (**Figure 2**).

Whether to induce immune activation or tolerance was initially correlated to the maturation state of DCs (62). Immature DCs generated from murine BM induced T cell unresponsiveness *in vitro* and prolonged cardiac allograft survival (43, 95). The immune tolerance induced by immature DCs was associated with their low expression of CD40 (which is essential to activation of CD4$^+$ T cells) and the capacity to produce high levels of IL-10 (which inhibits T cell response). Probst et al. reported that resting DCs induced peripheral CD8$^+$ T cell tolerance through activating the inhibitory signals PD-1 and CTLA4 on the T cell via PD-L1 and CD80/86, respectively (96–104). Under physiological conditions, these inhibitory molecules keep autoreactive T cells in check without causing autoimmunity. Blocking either PD-1 or CTLA4 abrogated CD8$^+$ T cell tolerance induction and enhanced T cell priming while blocking both resulted in an synergistic effect on inducing CD8$^+$ T cell

tolerance (96). These observations suggest that DC-derived PD-L1 may promote T cell tolerance through triggering the T-cell intrinsic mechanism.

Treg and suppressive cells (e.g., myeloid-derived suppressive cells) play crucial roles in establishing and maintaining peripheral tolerance and are known to be important for reducing GVHD (105–108). Waller and colleagues have demonstrated that transfer of donor BM pre-pDCs attenuated GVHD in mice (56). They identified that donor pDCs activated donor T cells to produce IFN-γ, which then enhanced pDC synthesis of indoleamine 2,3-dioxygenase (IDO). Increased production of IDO by pDCs altered the balance between donor Treg and alloreactive effector T cells, thereby limiting the severity of GVHD (56). Other studies showed that GVH reaction also impaired the antigen presentation function of *de novo* generated donor cDCs, leading to dramatically decreased Treg expansion and function, leading to severe chronic GVHD (109). These observations suggest that reconstitution of tolerogenic DCs from engrafted donor hematopoietic cells may be crucial for preventing the occurrence of severe GVHD.

RECONSTITUTION OF DONOR DCS AFTER ALLO-HSCT

Clinical studies indicated that impaired reconstitution of donor DCs correlates with the occurrence of severe GVHD (110–115). Wingard and colleagues examined the number of donor DCs in the circulating peripheral blood from a group of 50 allo-HSCT patients. They found that low number of circulating DCs was not only associated with significantly increased risk of relapse and acute GVHD, but also predicative of patient death after allogeneic HSCT (110). Notably, clinical studies from 39 children with allo-HSCT indicated that while normal cDC numbers were observed by 300–400 days after transplantation, pDC numbers were always lower than those of age-matched control patients during the entire follow-up period of up to 7 years (112). In contrast, patients with high pDC recovery profile often had improved overall survival (114). Data from preclinical studies also showed a marked deficit in all lineages of DCs ($CD8^+$ DCs, $CD11b^+$ DCs, and pDCs) in GVHD compared with non-GVHD mice (55, 109, 116, 117). Thus, the recovery of DCs from engrafted HSPCs in allo-HSCT patients may predict the occurrence of severe GVHD and non-relapse mortality.

Donor DCs Mediate Protective Immunity

DCs are critical for eliciting T cell immune responses, protecting the host against viral infection (18, 19, 118). Viral infection remains a major challenge for the success of allo-HSCT. Clinical studies have shown that after allo-HSCT, patients with lower numbers of circulating peripheral blood DCs often have increased risk of infections (1, 110). Cytomegalovirus (CMV) is a major cause of post-transplant mortality in patients subject to allo-HSCT, with ∼25% of CMV-seropositive recipients developing CMV-related disease within 3 months after transplantation (119–121). It is well-established that induction of adaptive T cell immunity is critical to control CMV replication and resolve viral reactivation-mediated disease (100, 122, 123). cDCs are essential to the generation of effector T cells reactive to CMV, especially during a primary response. However, GVHD induces a profound DC defect that leads to a failure in the generation of CMV-specific $CD8^+$ T-cells and dramatically decreased antiviral immunity (116). Collectively, improving the reconstitution of DCs following allo-HSCT may represent an effective strategy to re-establish the protective immunity in the recipient. Since pDCs produce high levels of IL-12 and IFN-α upon activation (29, 124–126), improving pDC recovery after allo-HSCT may also provide efficient antiviral immunity.

GVHD Impairs the Generation of DC Progenitors

GVHD-associated inflammatory responses may influence the reconstitution of donor DCs via a complex mechanism. DCs developed from HSPCs through successive steps of lineage commitment and differentiation: HSCs → multiple potent progenitors (MPP) → common DC progenitors (CDP) → cDCs and pDCs (22, 42, 125, 127–132). Inflammatory cytokines, such as TNF-α and IFN-γ, directly inhibit the proliferation of HSPCs and their generation of DCs (117). In addition, Matsushima and colleagues have shown that GVHD induced damage to the BM niche, leading to dramatically decreased hematopoiesis, including the reduction of CDP (133). However, the specific cellular component(s) within the niche that are responsible for the generation of DC progenitors have yet to be determined.

Transcriptional Regulation of DC Development

The generation of DCs is regulated by a group of functionally distinctive transcription factors (TFs). Analysis of gene-targeted mice has identified many critical TFs in DC development. Some of these TFs, such as Pu.1 and Stat3, influence the generation of all DC subsets. HSPCs lacking Pu.1 showed defective DC differentiation potential (134, 135). Targeted deletion of Stat3 impaired the generation of both cDCs and pDCs *in vivo* (128, 136). Thus, both Pu.1 and Stat3 are pioneer TFs in the regulation of DC commitment and differentiation from MPP (42, 128, 130).

DC subset-specifying TFs are required for committed CDP to become functionally distinct DC lineages (42, 127, 128, 130). For example, Batf3 has a non-redundant role in $CD103^+$ cDC development and partial effect on inducing $CD8\alpha^+$ DCs in lymph organs (52, 92, 127). Irf8-deficient animals lack spleen-resident $CD8\alpha^+$ cDCs and nonlymphoid tissue $CD103^+$ cDCs (42, 127, 137). Other TFs, such as Irf4, Klf4, Notch2, and Relb, also play important roles in the regulation of other types of cDCs localized in different tissues (94, 138). Among them, Irf4 is required for cDCs to prime $CD4^+$ T cells and promote Th17 differentiation in both the lung and intestine (60, 139). In addition, several TFs, such as Tcf4, Irf8, and Spib, are known to regulate pDC differentiation (140–142). The absence of Tcf4 leads to the loss of pDCs in mice (142).

There are limited number of studies investigating how GVH reactions influence the expression and function of these TFs required for DC development. Notably, a recent

study revealed that inflammation cascades in GVH reaction favor the development of $CD103^+D11b^-$ DCs in the GI tract (42), which require the presence of functional Irf4 (93). These data indicate that distinct TFs in DCs and their progenitors may have different susceptibility to the regulatory effect of inflammatory environments. This may result in a skewed expression and activation of transcriptional programs, promoting the generation of specific subset(s) of DCs and feed-forward action on alloreactive T cell responses during the GVHD progression. Delineating the mechanisms underlying this dysregulated donor DC reconstitution during GVH reaction will be important for understanding the pathophysiology of GVHD and the development of effective treatments for the disease.

DC MODULATION OF ALLOIMMUNITY

Manipulation of DC precursors in the HSPC graft may facilitate the establishment of a balance between GVHD and GVL effects (2, 9, 16, 34, 35, 56). Preclinical studies have shown that transfer of donor pre-pDCs derived from donor mice treated with Flt3L induced markedly augmented GVL activity of donor T cells without aggravating GVHD (56). These donor pre-pDCs persisted long in that they expanded *in vivo* for 2 weeks after transplantation (56). These findings perfectly supported the clinical value of donor DCs in modulating alloimmunity to improve the efficacy and safety of allo-HSCT.

Use of Tolerogenic DCs to Reduce GVHD

The capacity of DCs to induce tolerance has led to numerous therapeutic studies using these cells in an effort to control harmful immune responses in models of allograft rejection, GVHD and autoimmune disorders (18, 27, 34, 36, 37, 62, 87, 143). While transfer of immature $CCR9^+$ pDCs reduced GVHD, transfer of mature donor pDCs did not as expected (144). Furthermore, transfer of highly purified immature pDCs derived from donors was technically challenging and typically required *in vivo* expansion step to generate the number enough for modulating GVH reaction *in vivo* (53, 56, 144).

With this technical bottle neck, many studies had to assess the therapeutic effect of *ex-vivo*-generated DCs. Tolerogenic DCs were tried to be generated through propagating human monocytes *in vitro* in the presence of various agents, such as IL-10, Vitamin D3, and immunosuppressive drugs (e.g., dexamethasone and rapamycin) (34, 36, 37, 87, 143). Yet, none of the approaches generated the best clinically applicable DCs with the expected tolerogenic capacity to modulate alloreactive T cells (34, 36, 37, 87, 143). Reddy and colleagues report that upon pre-treatment with the HDAC inhibitor SAHA, these moDCs produced high levels of IDO and promoted Treg expansion and function *in vivo*, leading to attenuated GVHD in mice (54). These findings indicate that targeting epigenetic regulators in DCs may prove to be an effective strategy to induce the generation of DCs with tolerogenic properties for reducing GVHD.

DC Induction of GVL Effects After Allo-HSCT

Emerging evidence indicated that DCs were required for optimal GVL response without aggravating GVHD. Reddy and colleagues report that as compared to allogeneic wild-type (WT) hosts, allogeneic Batf3-deficient recipient mice developed severe GVHD but with significantly reduced GVL response (52). This indicates the importance of $CD8\alpha^+$ DCs in GVL response. Indeed, co-transfer of WT host-type spleen DCs (which contain $CD8\alpha^+$ DCs) and T cells into allogeneic $B2m^{-/-}$ recipients, which are functionally deficient in antigen presentation, induced a significant $CD8^+$ T cell-mediated GVL response, leading to prolonged survival of recipients without tumor. In contrast, all of the $B2m^{-/-}$ mice receiving $Batf3^{-/-}$ spleen DCs, which lack $CD8\alpha^+$ DCs (92), died from tumor despite the presence of other DC subsets (52). This confirms the crucial role of $CD8\alpha^+$ DCs in eliciting anti-tumor immunity. However, these experiments did not examine the direct effect of $CD8\alpha^+$ DCs on T cell-mediated GVL response.

In human recipients of unrelated donor BM grafts, but not granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood grafts, a higher number of donor pDCs is associated with increased survival and reduced GVHD (145). Data from experimental studies indicate that transfer of pDC-enriched BM grafts preserved GVL effects without aggravating GVHD in mice (56, 146). pDCs produce high levels of IFN-α and IL-12 (147, 148), cytokines important to promote differentiation and expansion of antigen-specific effector cells (149). In these studies, Waller and colleagues have demonstrated that *in vivo* administration of Flt3L to donor mice induced 5-fold increase in pDC content without significant changes in the number of HSCs, T cells and B cells. Most importantly, transfer of pDC-enriched BM graft from Flt3L-treated donors decreased GVHD while retaining GVL effects in allogeneic recipient mice (146).

We have recently established a novel platform to produce $Dll4^+$ DCs from murine BM using Flt3L and TLR agonists (64). Upon allogeneic $Dll4^+$ DC stimulation, $CD4^+$ naïve T cells underwent effector differentiation and produced high levels of IFN-γ and IL-17 *in vitro,* depending on Dll4 activation of Notch signaling. Adoptive transfer of these $Dll4^+$ DC-induced T cells eliminated leukemic cells without causing severe GVHD, leading to significantly improved survival of leukemic mice undergoing allo-HSCT. This strategy may potentially improve the anti-leukemic response after HSCT and overcome some barriers to the GVL response such as high disease burden and pharmacologic immunosuppression (64). Since DC activation of naïve T cells allows them to be primed with antigens, $Dll4^+$ DCs loaded with leukemia-associated antigens may facilitate the selective expansion of leukemic cell-reactive T cells and specifically boost the anti-leukemia activity.

CONCLUSION

While traditional therapies have been targeting T cells, extensive research in murine HSCT models convincingly showed the ability of DCs to preserve GVL response without aggravating GVHD.

Targeting donor DCs *in vivo* or *ex vivo* may potentially subvert alloreactive T cell responses and reduce GVHD (53). Given the role of DCs in maintaining Treg after allo-HSCT (56, 109), co-transfer of tolerogenic DCs and Treg could be more effective on reducing GVH reactions *in vivo*. A randomized Phase I study has shown the safety of infusing the host tolerogenic DCs into diabetes patients (150). It will be interesting to test whether these *ex vivo* generated tolerogenic DCs given in the peri-transplant period may prevent GVHD while preserving GVL effects.

One major challenge is to produce large number of donor-type tolerogenic DCs that can persist sufficient time to execute their function following adoptive transfer to modulate alloimmunity. We propose that donor-type DCs have several advantages compared to host-type DCs. For example, donors are healthy, and their hematopoietic system is not compromised by accompanied disease state and treatment conditions. Furthermore, available data from both clinical and pre-clinical studies suggest that donor-derived pDCs have potent capability to modulate GVH reactions (55, 56, 145, 146). These data provide a proof of concept that *in vivo* administration of pDCs is promising for enhancing GVL response without causing severe GVHD.

Most recent studies have shown that the fate of pDCs is determined early at the stage of HSCs (127, 130, 136, 151–153). This suggests that induction of tolerogenic DCs should start from the HSPC stage. Better understanding how the fate of tolerogenic DCs are determined and regulated may have significant implication in the production of DCs for efficiently modulating alloimmunity.

AUTHOR CONTRIBUTIONS

HY, YT, YW, SM, and YZ collected all materials for reviewing and wrote the review.

ACKNOWLEDGMENTS

This work was supported by Department of Defense (YZ) and NIH (CA172106-01, YZ, HL127351-01A1, YZ).

REFERENCES

1. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. *N Engl J Med*. (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
2. Choi SW, Reddy P. Current and emerging strategies for the prevention of graft-versus-host disease. *Nat Rev Clin Oncol*. (2014) 11:536–47. doi: 10.1038/nrclinonc.2014.102
3. Dickinson AM, Norden J, Li S, Hromadnikova I, Schmid C, Schmetzer H, et al. Graft-versus-leukemia effect following hematopoietic stem cell transplantation for leukemia. *Front Immunol*. (2017) 8:496. doi: 10.3389/fimmu.2017.00496
4. Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. *Blood* (2004) 103:767–776. doi: 10.1182/blood-2003-02-0342
5. Wu CJ, Ritz J. Induction of tumor immunity following allogeneic stem cell transplantation. *Adv Immunol*. (2006) 90:133–73. doi: 10.1016/S0065-2776(06)90004-2
6. Ruutu T, Gratwohl A, de Witte T, Afanasyev B, Apperley J, Bacigalupo A, et al. Prophylaxis and treatment of GVHD: EBMT-ELN working group recommendations for a standardized practice. *Bone Marrow Transplant*. (2014) 49:168–73. doi: 10.1038/bmt.2013.107
7. Hamadani M, Hofmeister CC, Jansak B, Phillips G, Elder P, Blum W, et al. Addition of infliximab to standard acute graft-versus-host disease prophylaxis following allogeneic peripheral blood cell transplantation. *Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant*. (2008) 14:783–9. doi: 10.1016/j.bbmt.2008.04.006
8. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. (2012) 12:443–58. doi: 10.1038/nri3212
9. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol*. (2007) 7:340–52. doi: 10.1038/nri2000
10. Matte CC, Liu J, Cormier J, Anderson BE, Athanasiadis I, Jain D, et al. Donor APCs are required for maximal GVHD but not for GVL. *Nat Med*. (2004) 10:987–92. doi: 10.1038/nm1089
11. Zhang Y, Shlomchik WD, Joe G, Louboutin JP, Zhu J, Rivera A, et al. APCs in the liver and spleen recruit activated allogeneic CD8+ T cells to elicit hepatic graft-versus-host disease. *J Immunol*. (2002) 169:7111–8. doi: 10.4049/jimmunol.169.12.7111
12. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* (1999) 285:412–5. doi: 10.1126/science.285.5426.412
13. Koyama M, Cheong M, Markey KA, Gartlan KH, Kuns RD, Locke KR, et al. Donor colonic CD103+ dendritic cells determine the severity of acute graft-versus-host disease. *J Exp Med*. (2015) 212:1303–21. doi: 10.1084/jem.20150329
14. Koyama M, Kuns RD, Olver SD, Raffelt NC, Wilson YA, Don AL, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat Med*. (2011) 18:135–42. doi: 10.1038/nm.2597
15. Tawara I, Shlomchik WD, Jones A, Zou W, Nieves E, Liu C, et al. A crucial role for host APCs in the induction of donor CD4+CD25+ regulatory T cell-mediated suppression of experimental graft-versus-host disease. *J Immunol*. (2010) 185:3866–72. doi: 10.4049/jimmunol.1001625
16. Reddy P, Maeda Y, Liu C, Krijanovski OI, Korngold R, Ferrara JL. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nat Med*. (2005) 11:1244–9. doi: 10.1038/nm1309
17. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med*. (2002) 8:575–81. doi: 10.1038/nm0602-575
18. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature* (2007) 449:419–26. doi: 10.1038/nature06175
19. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* (1998) 392:245–52. doi: 10.1038/32588
20. Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. *Annu Rev Immunol*. (1990) 8:579–621. doi: 10.1146/annurev.iy.08.040190.003051
21. Naik SH. Demystifying the development of dendritic cell subtypes, a little. *Immunol Cell Biol*. (2008) 86:439–52. doi: 10.1038/icb.2008.28
22. Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol*. (2007) 7:19–30. doi: 10.1038/nri1996
23. Reizis B, Colonna M, Trinchieri G, Barrat F, Gilliet M. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat Rev Immunol*. (2011) 11:558–65. doi: 10.1038/nri3027
24. Zuniga EI, McGavern DB, Pruneda-Paz JL, Teng C, Oldstone MB. Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection. *Nat Immunol*. (2004) 5:1227–34. doi: 10.1038/ni1136
25. Schlitzer A, Loschko J, Mair K, Vogelmann R, Henkel L, Einwachter H, et al. Identification of CCR9- murine plasmacytoid DC precursors with

plasticity to differentiate into conventional DCs. *Blood* (2011) 117:6562–70. doi: 10.1182/blood-2010-12-326678
26. Strioga MM, Felzmann T, Powell DJ, Jr., Ostapenko V, Dobrovolskiene NT, Matuskova M, et al. Therapeutic dendritic cell-based cancer vaccines: the state of the art. *Crit Rev Immunol.* (2013) 33:489–547. doi: 10.1615/CritRevImmunol.2013008033
27. Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity* (2013) 39:38–48. doi: 10.1016/j.immuni.2013.07.004
28. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology* (2013) 140:22–30. doi: 10.1111/imm.12117
29. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol*. (2004) 5:1219–26. doi: 10.1038/ni1141
30. Benlahrech A, Duraisingham S, King D, Verhagen L, Rozis G, Amjadi P, et al. Human blood CD1c dendritic cells stimulate IL-12-independent IFN-gamma responses and have a strikingly low inflammatory profile. *J Leukocyte Biol.* (2015) 97:873–85. doi: 10.1189/jlb.1A0114-058RR
31. Sandy AR, Chung J, Toubai T, Shan GT, Tran IT, Friedman A, et al. T cell-specific notch inhibition blocks graft-versus-host disease by inducing a hyporesponsive program in alloreactive CD4+ and CD8+ T cells. *J Immunol.* (2013) 190:5818–28. doi: 10.4049/jimmunol.1203452
32. Zhang Y, Sandy AR, Wang J, Radojcic V, Shan GT, Tran IT, et al. Notch signaling is a critical regulator of allogeneic CD4+ T-cell responses mediating graft-versus-host disease. *Blood* (2011) 117:299–308. doi: 10.1182/blood-2010-03-271940
33. Amsen D, Antov A, Flavell RA. The different faces of Notch in T-helper-cell differentiation. *Nat Rev Immunol*. (2009) 9:116–24. doi: 10.1038/nri2488
34. Stenger EO, Turnquist HR, Mapara MY, Thomson AW. Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity. *Blood* (2012) 119:5088–103. doi: 10.1182/blood-2011-11-364091
35. MacDonald KP, Shlomchik WD, Reddy P. Biology of graft-versus-host responses: recent insights. *Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant.* (2013) 19:S10–14. doi: 10.1016/j.bbmt.2012.11.005
36. Audiger C, Rahman MJ, Yun TJ, Tarbell KV, Lesage S. The importance of dendritic cells in maintaining immune tolerance. *J Immunol.* (2017) 198:2223–31. doi: 10.4049/jimmunol.1601629
37. Devi KS, Anandasabapathy N. The origin of DCs and capacity for immunologic tolerance in central and peripheral tissues. *Semin Immunopathol.* (2017) 39:137–52. doi: 10.1007/s00281-016-0602-0
38. Fugier-Vivier IJ, Rezzoug F, Huang Y, Graul-Layman AJ, Schanie CL, Xu H, et al. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *J Exp Med*. (2005) 201:373–83. doi: 10.1084/jem.20041399
39. Cardenas PA, Huang Y, Ildstad ST. The role of pDC, recipient T(reg) and donor T(reg) in HSC engraftment: mechanisms of facilitation. *Chimerism* (2011) 2:65–70. doi: 10.4161/chim.17588
40. Merad M, Hoffmann P, Ranheim E, Slaymaker S, Manz MG, Lira SA, et al. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. *Nat Med.* (2004) 10:510–7. doi: 10.1038/nm1038
41. Teshima T, Reddy P, Lowler KP, KuKuruga MA, Liu C, Cooke KR, et al. Flt3 ligand therapy for recipients of allogeneic bone marrow transplants expands host CD8 alpha(+) dendritic cells and reduces experimental acute graft-versus-host disease. *Blood* (2002) 99:1825–32. doi: 10.1182/blood.V99.5.1825
42. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. (2013) 31:563–604. doi: 10.1146/annurev-immunol-020711-074950
43. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol*. (2007) 7:610–21. doi: 10.1038/nri2132
44. Chung CY, Ysebaert D, Berneman ZN, Cools N. Dendritic cells: cellular mediators for immunological tolerance. *Clin Dev Immunol*. (2013) 2013:972865. doi: 10.1155/2013/972865
45. Chakraverty R, Sykes M. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. *Blood* (2007) 110:9–17. doi: 10.1182/blood-2006-12-022038
46. Zhang Y, Louboutin JP, Zhu J, Rivera AJ, Emerson SG. Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. *J Clin Invest*. (2002) 109:1335–44. doi: 10.1172/JCI0214989
47. Koyama M, Hashimoto D, Aoyama K, Matsuoka K, Karube K, Niiro H, et al. Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells. *Blood* (2009) 113:2088–95. doi: 10.1182/blood-2008-07-168609
48. Li H, Kaplan DH, Matte-Martone C, Tan HS, Venkatesan S, Johnson K, et al. Langerhans cells are not required for graft-versus-host disease. *Blood* (2011) 117:697–707. doi: 10.1182/blood-2010-07-299073
49. Markey KA, Banovic T, Kuns RD, Olver SD, Don AL, Raffelt NC, et al. Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. *Blood* (2009) 113:5644–9. doi: 10.1182/blood-2008-12-191833
50. Weber M, Rudolph B, Stein P, Yogev N, Bosmann M, Schild H, et al. Host-derived CD8(+) dendritic cells protect against acute graft-versus-host disease after experimental allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant.* (2014) 20:1696–704. doi: 10.1016/j.bbmt.2014.08.005
51. Markey KA, Kuns RD, Browne DJ, Gartlan KH, Robb RJ, Martins JP, et al. Flt-3L expansion of recipient CD8alpha(+) dendritic cells deletes alloreactive donor T cells and represents an alternative to posttransplant cyclophosphamide for the prevention of GVHD. *Clin Cancer Res Offic J Am Assoc Cancer Res.* (2018) 24:1604–16. doi: 10.1158/1078-0432.CCR-17-2148
52. Toubai T, Sun Y, Luker G, Liu J, Luker KE, Tawara I, et al. Host-derived CD8+ dendritic cells are required for induction of optimal graft-versus-tumor responses after experimental allogeneic bone marrow transplantation. *Blood* (2013) 121:4231–41. doi: 10.1182/blood-2012-05-432872
53. Hadeiba H, Sato T, Habtezion A, Oderup C, Pan J, Butcher EC. CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. *Nat Immunol*. (2008) 9:1253–60. doi: 10.1038/ni.1658
54. Reddy P, Sun Y, Toubai T, Duran-Struuck R, Clouthier SG, Weisiger E, et al. Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. *J Clin Invest*. (2008) 118:2562–73. doi: 10.1172/JCI 34712
55. Banovic T, Markey KA, Kuns RD, Olver SD, Raffelt NC, Don AL, et al. Graft-versus-host disease prevents the maturation of plasmacytoid dendritic cells. *J Immunol.* (2009) 182:912–20. doi: 10.4049/jimmunol.182.2.912
56. Lu Y, Giver CR, Sharma A, Li JM, Darlak KA, Owens LM, et al. IFN-gamma and indoleamine 2,3-dioxygenase signaling between donor dendritic cells and T cells regulates graft versus host and graft versus leukemia activity. *Blood* (2012) 119:1075–85. doi: 10.1182/blood-2010-12-32 2891
57. Rowe V, Banovic T, MacDonald KP, Kuns R, Don AL, Morris ES, et al. Host B cells produce IL-10 following TBI and attenuate acute GVHD after allogeneic bone marrow transplantation. *Blood* (2006) 108:2485–92. doi: 10.1182/blood-2006-04-016063
58. Haniffa M, Ginhoux F, Wang XN, Bigley V, Abel M, Dimmick I, et al. Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation. *J Exp Med*. (2009) 206:371–85. doi: 10.1084/jem.20081633
59. Jones SC, Murphy GF, Friedman TM, Korngold R. Importance of minor histocompatibility antigen expression by nonhematopoietic tissues in a CD4+ T cell-mediated graft-versus-host disease model. *J Clin Invest*. (2003) 112:1880–6. doi: 10.1172/JCI19427
60. Vander Lugt B, Khan AA, Hackney JA, Agrawal S, Lesch J, Zhou M, et al. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat Immunol*. (2014) 15:161–7. doi: 10.1038/ni.2795
61. Yao S, Buzo BF, Pham D, Jiang L, Taparowsky EJ, Kaplan MH, et al. Interferon regulatory factor 4 sustains CD8(+) T cell expansion and effector differentiation. *Immunity* (2013) 39:833–45. doi: 10.1016/j.immuni.2013.10.007
62. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol.* (2003) 21:685–711. doi: 10.1146/annurev.immunol.21.120601.141040
63. Xia S, Guo Z, Xu X, Yi H, Wang Q, Cao X. Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory

dendritic cells, maintaining liver tolerance. *Blood* (2008) 112:3175–85. doi: 10.1182/blood-2008-05-159921
64. Mochizuki K, Meng L, Mochizuki I, Tong Q, He S, Liu Y, et al. Programming of donor T cells using allogeneic delta-like ligand 4-positive dendritic cells to reduce GVHD in mice. *Blood* (2016) 127:3270–80. doi: 10.1182/blood-2015-05-644476
65. Meng L, Bai Z, He S, Mochizuki K, Liu Y, Purushe J, et al. The notch ligand DLL4 defines a capability of human dendritic cells in regulating Th1 and Th17 differentiation. *J Immunol.* (2016) 196:1070–80. doi: 10.4049/jimmunol.1501310
66. Mochizuki K, Xie F, He S, Tong Q, Liu Y, Mochizuki I, et al. Delta-like ligand 4 identifies a previously uncharacterized population of inflammatory dendritic cells that plays important roles in eliciting allogeneic T cell responses in mice. *J Immunol.* (2013) 190:3772–82. doi: 10.4049/jimmunol.1202820
67. Ting HA, Schaller MA, de Almeida Nagata DE, Rasky AJ, Maillard IP, Lukacs NW. Notch ligand delta-like 4 promotes regulatory T cell identity in pulmonary viral infection. *J Immunol.* (2017) 198:1492–502. doi: 10.4049/jimmunol.1601654
68. Tran IT, Sandy AR, Carulli AJ, Ebens C, Chung J, Shan GT, et al. Blockade of individual Notch ligands and receptors controls graft-versus-host disease. *J Clin Invest*. (2013) 123:1590–604. doi: 10.1172/JCI 65477
69. Chung J, Ebens CL, Perkey E, Radojcic V, Koch U, Scarpellino L, et al. Fibroblastic niches prime T cell alloimmunity through Delta-like Notch ligands. *J Clin Invest*. (2017) 127:1574–88. doi: 10.1172/JCI89535
70. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science* (2010) 327:291–5. doi: 10.1126/science.1183021
71. Janeway C. Immunogenicity signals 1,2,3 ... and 0. *Immunol Today* (1989) 10:283–6. doi: 10.1016/0167-5699(89)90081-9
72. Krishnaswamy JK, Chu T, Eisenbarth SC. Beyond pattern recognition: NOD-like receptors in dendritic cells. *Trends Immunol*. (2013) 34:224–33. doi: 10.1016/j.it.2012.12.003
73. Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat Rev Immunol*. (2004) 4:469–78. doi: 10.1038/nri1372
74. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* (2006) 124:783–801. doi: 10.1016/j.cell.2006.02.015
75. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol*. (2008) 8:279–89. doi: 10.1038/nri2215
76. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
77. Wilhelm K, Ganesan J, Muller T, Durr C, Grimm M, Beilhack A, et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat Med*. (2010) 16:1434–8. doi: 10.1038/nm.2242
78. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber FC, Guarda G, et al. The Nlrp3 inflammasome regulates acute graft-versus-host disease. *J Exp Med*. (2013) 210:1899–910. doi: 10.1084/jem.20130084
79. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* (2004) 117:515–26. doi: 10.1016/S0092-8674(04)00451-9
80. Mukherjee S, Schaller MA, Neupane R, Kunkel SL, Lukacs NW. Regulation of T cell activation by Notch ligand, DLL4, promotes IL-17 production and Rorc activation. *J Immunol.* (2009) 182:7381–8. doi: 10.4049/jimmunol.0804322
81. Yamaguchi E, Chiba S, Kumano K, Kunisato A, Takahashi T, Takahashi T, et al. Expression of Notch ligands, Jagged1:2 and Delta1 in antigen presenting cells in mice. *Immunol Lett*. (2002) 81:59–64. doi: 10.1016/S0165-2478(01)00326-1
82. Tan AM, Chen HC, Pochard P, Eisenbarth SC, Herrick CA, Bottomly HK. TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen. *J Immunol.* (2010) 184:3535–44. doi: 10.4049/jimmunol.0900340
83. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol*. (2005) 6:769–76. doi: 10.1038/ni1223
84. Schaller MA, Neupane R, Rudd BD, Kunkel SL, Kallal LE, Lincoln P, et al. Notch ligand Delta-like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines. *J Exp Med*. (2007) 204:2925–34. doi: 10.1084/jem.20070661
85. Skokos D, Nussenzweig MC. CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. *J Exp Med*. (2007) 204:1525–31. doi: 10.1084/jem.20062305
86. Spranger S, Frankenberger B, Schendel DJ. NOD/scid IL-2Rg(null) mice: a preclinical model system to evaluate human dendritic cell-based vaccine strategies *in vivo*. *J Transl Med.* (2012) 10:30. doi: 10.1186/1479-5876-10-30
87. Ganguly D, Haak S, Sisirak V, Reizis B. The role of dendritic cells in autoimmunity. *Nat Rev Immunol*. (2013) 13:566–77. doi: 10.1038/nri3477
88. Na IK, Lu SX, Yim NL, Goldberg GL, Tsai J, Rao U, et al. The cytolytic molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *J Clin Invest*. (2010) 120:343–56. doi: 10.1172/JCI39395
89. van den Brink MR, Moore E, Ferrara JL, Burakoff SJ. Graft-versus-host-disease-associated thymic damage results in the appearance of T cell clones with anti-host reactivity. *Transplantation* (2000) 69:446–9. doi: 10.1097/00007890-200002150-00026
90. Zhang Y, Hexner E, Frank D, Emerson SG. CD4+ T cells generated *de novo* from donor hemopoietic stem cells mediate the evolution from acute to chronic graft-versus-host disease. *J Immunol.* (2007) 179:3305–14. doi: 10.4049/jimmunol.179.5.3305
91. Teshima T, Reddy P, Liu C, Williams D, Cooke KR, Ferrara JL. Impaired thymic negative selection causes autoimmune graft-versus-host disease. *Blood* (2003) 102:429–35. doi: 10.1182/blood-2003-01-0266
92. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* (2008) 322:1097–100. doi: 10.1126/science.1164206
93. Murphy KM. Transcriptional control of dendritic cell development. *Adv Immunol*. (2013) 120:239–67. doi: 10.1016/B978-0-12-417028-5.00009-0
94. Price JD, Tarbell KV. The role of dendritic cell subsets and innate immunity in the pathogenesis of type 1 diabetes and other autoimmune diseases. *Front Immunol*. (2015) 6:288. doi: 10.3389/fimmu.2015.00288
95. O'Connell PJ, Li W, Wang Z, Specht SM, Logar AJ, Thomson AW. Immature and mature CD8alpha+ dendritic cells prolong the survival of vascularized heart allografts. *J Immunol.* (2002) 168:143–54. doi: 10.4049/jimmunol.168.1.143
96. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol*. (2005) 6:280–6. doi: 10.1038/ni1165
97. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *Lancet* (2014) 384:1109–17. doi: 10.1016/S0140-6736(14)60958-2
98. Franceschini D, Paroli M, Francavilla V, Videtta M, Morrone S, Labbadia G, et al. PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *J Clin Invest*. (2009) 119:551–64. doi: 10.1172/JCI36604
99. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
100. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* (2007) 27:670–84. doi: 10.1016/j.immuni.2007.09.006
101. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, Carter L, Iwai Y, Yagita H, et al. Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. *J Immunol.* (2003) 171:1272–7. doi: 10.4049/jimmunol.171.3.1272
102. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med*. (2001) 193:839–46. doi: 10.1084/jem.193.7.839
103. Vanasek TL, Khoruts A, Zell T, Mueller DL. Antagonistic roles for CTLA-4 and the mammalian target of rapamycin in the regulation of clonal anergy: enhanced cell cycle progression promotes recall antigen responsiveness. *J Immunol.* (2001) 167:5636–44. doi: 10.4049/jimmunol.167.10.5636

104. Tamura H, Dong H, Zhu G, Sica GL, Flies DB, Tamada K, et al. B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. *Blood* (2001) 97:1809–16. doi: 10.1182/blood.V97.6.1809
105. Beilhack A, Schulz S, Baker J, Beilhack GF, Nishimura R, Baker EM, et al. Prevention of acute graft-versus-host disease by blocking T-cell entry to secondary lymphoid organs. *Blood* (2008) 111:2919–28. doi: 10.1182/blood-2007-09-112789
106. Nguyen VH, Zeiser R, Dasilva DL, Chang DS, Beilhack A, Contag CH, et al. *In vivo* dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood* (2007) 109:2649–56. doi: 10.1182/blood-2006-08-044529
107. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med.* (2003) 9:1144–50. doi: 10.1038/nm915
108. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood* (2010) 116:5738–47. doi: 10.1182/blood-2010-06-287839
109. Leveque-El Mouttie L, Koyama M, Le Texier L, Markey KA, Cheong M, Kuns RD, et al. Corruption of dendritic cell antigen presentation during acute GVHD leads to regulatory T-cell failure and chronic GVHD. *Blood* (2016) 128:794–804. doi: 10.1182/blood-2015-11-680876
110. Reddy V, Iturraspe JA, Tzolas AC, Meier-Kriesche HU, Schold J, Wingard JR. Low dendritic cell count after allogeneic hematopoietic stem cell transplantation predicts relapse, death, and acute graft-versus-host disease. *Blood* (2004) 103:4330–5. doi: 10.1182/blood-2003-09-3325
111. Fearnley DB, Whyte LF, Carnoutsos SA, Cook AH, Hart DN. Monitoring human blood dendritic cell numbers in normal individuals and in stem cell transplantation. *Blood* (1999) 93:728–736.
112. Vakkila J, Thomson AW, Hovi L, Vettenranta K, Saarinen-Pihkala UM. Circulating dendritic cell subset levels after allogeneic stem cell transplantation in children correlate with time post transplant and severity of acute graft-versus-host disease. *Bone Marrow Transplant.* (2005) 35:501–7. doi: 10.1038/sj.bmt.1704827
113. Malard F, Mohty M. New insight for the diagnosis of gastrointestinal acute graft-versus-host disease. *Mediat Inflamm.* (2014) 2014:701013. doi: 10.1155/2014/701013
114. Mohty M, Blaise D, Faucher C, Bardou VJ, Gastaut JA, Viens P, et al. Impact of plasmacytoid dendritic cells on outcome after reduced-intensity conditioning allogeneic stem cell transplantation. *Leukemia* (2005) 19:1–6. doi: 10.1038/sj.leu.2403558
115. Lau J, Sartor M, Bradstock KF, Vuckovic S, Munster DJ, Hart DN. Activated circulating dendritic cells after hematopoietic stem cell transplantation predict acute graft-versus-host disease. *Transplantation* (2007) 83:839–46. doi: 10.1097/01.tp.0000258731.38149.61
116. Wikstrom ME, Fleming P, Kuns RD, Schuster IS, Voigt V, Miller G, et al. Acute GVHD results in a severe DC defect that prevents T-cell priming and leads to fulminant cytomegalovirus disease in mice. *Blood* (2015) 126:1503–14. doi: 10.1182/blood-2015-01-622837
117. Markey KA, Koyama M, Kuns RD, Lineburg KE, Wilson YA, Olver SD, et al. Immune insufficiency during GVHD is due to defective antigen presentation within dendritic cell subsets. *Blood* (2012) 119:5918–30. doi: 10.1182/blood-2011-12-398164
118. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol.* (2005) 23:275–306. doi: 10.1146/annurev.immunol.23.021704.115633
119. Hakki M, Riddell SR, Storek J, Carter RA, Stevens-Ayers T, Sudour P, et al. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. *Blood* (2003) 102:3060–7. doi: 10.1182/blood-2002-11-3472
120. Boeckh M, Leisenring W, Riddell SR, Bowden RA, Huang ML, Myerson D, et al. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. *Blood* (2003) 101:407–14. doi: 10.1182/blood-2002-03-0993
121. Sora F, Antinori A, Piccirillo N, De Luca A, Chiusolo P, Cingolani A, et al. Highly active antiretroviral therapy and allogeneic CD34(+) peripheral blood progenitor cells transplantation in an HIV/HCV coinfected patient with acute myeloid leukemia. *Exp Hematol.* (2002) 30:279–84. doi: 10.1016/S0301-472X(01)00793-7
122. Wherry EJ, Blattman JN, Ahmed R. Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. *J Virol.* (2005) 79:8960–8. doi: 10.1128/JVI.79.14.8960-8968.2005
123. Jamieson BD, Ahmed R. T cell memory. Long-term persistence of virus-specific cytotoxic T cells. *J Exp Med.* (1989) 169:1993–2005. doi: 10.1084/jem.169.6.1993
124. Gilliet M, Boonstra A, Paturel C, Antonenko S, Xu XL, Trinchieri G, et al. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J Exp Med.* (2002) 195:953–8. doi: 10.1084/jem.20020045
125. Watowich SS, Liu YJ. Mechanisms regulating dendritic cell specification and development. *Immunol Rev.* (2010) 238:76–92. doi: 10.1111/j.1600-065X.2010.00949.x
126. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol.* (2015) 15:471–85. doi: 10.1038/nri3865
127. Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseno CG, Iwata A, et al. Transcriptional control of dendritic cell development. *Annu Rev Immunol.* (2016) 34:93–119. doi: 10.1146/annurev-immunol-032713-120204
128. Paul F, Amit I. Plasticity in the transcriptional and epigenetic circuits regulating dendritic cell lineage specification and function. *Curr Opin Immunol.* (2014) 30:1–8. doi: 10.1016/j.coi.2014.04.004
129. Mildner A, Schonheit J, Giladi A, David E, Lara-Astiaso D, Lorenzo-Vivas E, et al. Genomic characterization of murine monocytes reveals C/EBPbeta transcription factor dependence of Ly6C(-) Cells. *Immunity* (2017) 46:849–62 e847.
130. Paul F, Arkin Y, Giladi A, Jaitin DA, Kenigsberg E, Keren-Shaul H, et al. Transcriptional heterogeneity and lineage commitment in myeloid progenitors. *Cell* (2015) 163:1663–77. doi: 10.1016/j.cell.2015.11.013
131. Jaitin DA, Kenigsberg E, Keren-Shaul H, Elefant N, Paul F, Zaretsky I, et al. Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. *Science* (2014) 343:776–9. doi: 10.1126/science.1247651
132. Tian Y, Meng L, Zhang Y. Epigenetic regulation of dendritic cell development and function. *Cancer J.* (2017) 23:302–7. doi: 10.1097/PPO.0000000000000280
133. Shono Y, Ueha S, Wang Y, Abe J, Kurachi M, Matsuno Y, et al. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood* (2010) 115:5401–11. doi: 10.1182/blood-2009-11-253559
134. Anderson KL, Perkin H, Surh CD, Venturini S, Maki RA, Torbett BE. Transcription factor PU.1 is necessary for development of thymic and myeloid progenitor-derived dendritic cells. *J Immunol.* (2000) 164:1855–61. doi: 10.4049/jimmunol.164.4.1855
135. Anderson KL, Smith KA, Conners K, McKercher SR, Maki RA, Torbett BE. Myeloid development is selectively disrupted in PU.1 null mice. *Blood* (1998) 91:3702–10.
136. Paul F, Arkin Y, Giladi A, Jaitin DA, Kenigsberg E, Keren-Shaul H, et al. Transcriptional heterogeneity and lineage commitment in myeloid progenitors. *Cell* (2016) 164:325. doi: 10.1016/j.cell.2015.12.046
137. Salmon H, Idoyaga J, Rahman A, Leboeuf M, Remark R, Jordan S, et al. Expansion and activation of CD103(+) dendritic cell progenitors at the tumor site enhances tumor responses to therapeutic PD-L1 and BRAF inhibition. *Immunity* (2016) 44:924–38. doi: 10.1016/j.immuni.2016.03.012
138. Price JD, Hotta-Iwamura C, Zhao Y, Beauchamp NM, Tarbell KV. DCIR2+ cDC2 DCs and Zbtb32 restore CD4+ T-cell tolerance and inhibit diabetes. *Diabetes* (2015) 64:3521–31. doi: 10.2337/db14-1880
139. Tamura T, Tailor P, Yamaoka K, Kong HJ, Tsujimura H, O'Shea JJ, et al. IFN regulatory factor-4 and−8 govern dendritic cell subset development and their functional diversity. *J Immunol.* (2005) 174:2573–81. doi: 10.4049/jimmunol.174.5.2573

140. Spits H, Couwenberg F, Bakker AQ, Weijer K, Uittenbogaart CH. Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *J Exp Med*. (2000) 192:1775–84. doi: 10.1084/jem.192.12.1775
141. Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B. Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. *Immunity* (2010) 33:905–16. doi: 10.1016/j.immuni.2010.11.023
142. Cisse B, Caton ML, Lehner M, Maeda T, Scheu S, Locksley R, et al. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* (2008) 135:37–48. doi: 10.1016/j.cell.2008.09.016
143. Thomson AW, Ezzelarab MB. Regulatory dendritic cells: profiling, targeting, and therapeutic application. *Curr Opin Organ Transplant*. (2018) 23:538–45. doi: 10.1097/MOT.0000000000000565
144. MacDonald KP, Rowe V, Filippich C, Thomas R, Clouston AD, Welply JK, et al. Donor pretreatment with progenipoietin-1 is superior to granulocyte colony-stimulating factor in preventing graft-versus-host disease after allogeneic stem cell transplantation. *Blood* (2003) 101:2033–42. doi: 10.1182/blood-2002-05-1529
145. Waller EK, Logan BR, Harris WA, Devine SM, Porter DL, Mineishi S, et al. Improved survival after transplantation of more donor plasmacytoid dendritic or naive T cells from unrelated-donor marrow grafts: results from BMTCTN 0201. *J Clin Oncol*. (2014) 32:2365–72. doi: 10.1200/JCO.2013.54.4577
146. Hassan M, Antonova AU, Li JM, Hosoba S, Rupji M, Kowalski J, et al. Flt3L Treatment of bone marrow donors increases graft plasmacytoid dendritic cell content and improves allogeneic transplantation outcomes. *Biol Blood Marrow Transplant*. (2018). doi: 10.1016/j.bbmt.2018.11.029. [Epub ahead of print].
147. Nizzoli G, Krietsch J, Weick A, Steinfelder S, Facciotti F, Gruarin P, et al. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. *Blood* (2013) 122:932–42. doi: 10.1182/blood-2013-04-495424
148. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol*. (2008) 8:594–606. doi: 10.1038/nri2358
149. Chang JT, Wherry EJ, Goldrath AW. Molecular regulation of effector and memory T cell differentiation. *Nat Immunol*. (2014) 15:1104–15. doi: 10.1038/ni.3031
150. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* (2011) 34:2026–32. doi: 10.2337/dc1 1-0472
151. Lee J, Zhou YJ, Ma W, Zhang W, Aljoufi A, Luh T, et al. Lineage specification of human dendritic cells is marked by IRF8 expression in hematopoietic stem cells and multipotent progenitors. *Nat Immunol*. (2017) 18:877–88. doi: 10.1038/ni.3789
152. Helft J, Anjos-Afonso F, van der Veen AG, Chakravarty P, Bonnet D, Reis e Sousa C. Dendritic cell lineage potential in human early hematopoietic progenitors. *Cell Rep*. (2017) 20:529–37. doi: 10.1016/j.celrep.2017.06.075
153. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol*. (2010) 11:585–93. doi: 10.1038/ni.1889

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 January 2019
doi: 10.3389/fimmu.2018.03104

BET Bromodomain Inhibitors Which Permit Treg Function Enable a Combinatorial Strategy to Suppress GVHD in Pre-clinical Allogeneic HSCT

Sabrina N. Copsel[1,2], Casey O. Lightbourn[1], Henry Barreras[1], Ines Lohse[2,3], Dietlinde Wolf[2], Cameron S. Bader[1], John Manov[1], Brandon J. Kale[1], Devangi Shah[1], Shaun P. Brothers[2,3], Victor L. Perez[4], Krishna V. Komanduri[1,2,5], Claes Wahlestedt[2,3] and Robert B. Levy[1,2,4,5]**

[1] Department of Microbiology and Immunology, Miller School of Medicine, University of Miami, Miami, FL, United States, [2] Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, Miami, FL, United States, [3] Center for Therapeutic Innovation and Department of Psychiatry and Behavior Sciences, Miller School of Medicine, University of Miami, Miami, FL, United States, [4] Department of Ophthalmology, Miller School of Medicine, University of Miami, Miami, FL, United States, [5] Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL, United States

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina,
United States

Reviewed by:
Dennis O. Adeegbe,
Moffitt Cancer Center, United States
Jessica Voss,
Northwestern University,
United States

***Correspondence:**
Sabrina N. Copsel
snc49@med.miami.edu
Robert B. Levy
rlevy@med.miami.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: 28 September 2018
Accepted: 17 December 2018
Published: 24 January 2019

Citation:
Copsel SN, Lightbourn CO, Barreras H, Lohse I, Wolf D, Bader CS, Manov J, Kale BJ, Shah D, Brothers SP, Perez VL, Komanduri KV, Wahlestedt C and Levy RB (2019) BET Bromodomain Inhibitors Which Permit Treg Function Enable a Combinatorial Strategy to Suppress GVHD in Pre-clinical Allogeneic HSCT. Front. Immunol. 9:3104. doi: 10.3389/fimmu.2018.03104

A recent approach for limiting production of pro-inflammatory cytokines has been to target bromodomain and extra-terminal (BET) proteins. These epigenetic readers of histone acetylation regulate transcription of genes involved in inflammation, cardiovascular disease, and cancer. Development of BET inhibitors (BETi) has generated enormous interest for their therapeutic potential. Because inflammatory signals and donor T cells promote graft-versus-host disease (GVHD), regulating both pathways could be effective to abrogate this disorder. The objective of the present study was to identify a BETi which did not interfere *in vivo* with $CD4^+FoxP3^+$ regulatory T cell (Treg) expansion and function to utilize together with Tregs following allogeneic hematopoietic stem cell transplantation (aHSCT) to ameliorate GVHD. We have reported that Tregs can be markedly expanded and selectively activated with increased functional capacity by targeting TNFRSF25 and CD25 with TL1A-Ig and low dose IL-2, respectively. Here, mice were treated over 7 days (TL1A-Ig + IL-2) together with BETi. We found that the BETi EP11313 did not decrease frequency/numbers or phenotype of expanded Tregs as well as effector molecules, such as IL-10 and TGF-β. However, BETi JQ1 interfered with Treg expansion and altered subset distribution and phenotype. Notably, in Treg expanded mice, EP11313 diminished tnfa and ifng but not il-2 RNA levels. Remarkably, Treg pSTAT5 expression was not affected by EP11313 supporting the notion that Treg IL-2 signaling remained intact. MHC-mismatched aHSCT (B6→ BALB/c) was performed using *in vivo* expanded donor Tregs with or without EP11313 short-term treatment in the recipient. Early post-transplant, improvement in the splenic and LN CD4/CD8 ratio along with fewer effector cells and high Treg levels in aHSCT recipients treated with expanded Tregs + EP11313 was detected. Interestingly, this group exhibited a significant diminution of GVHD clinical score with less skin and ocular involvement. Finally, using low numbers of highly purified expanded Tregs, improved clinical GVHD scores were observed in EP11313 treated recipients. In total, we conclude that use of this novel combinatorial

strategy can suppress pre-clinical GVHD and posit, *in vivo* EP11313 treatment might be useful combined with Treg expansion therapy for treatment of diseases involving inflammatory responses.

Keywords: Tregs, bromodomain inhibitors, epigenetic regulation, GVHD, TNFRSF25, CD25

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (aHSCT) is utilized as a therapeutic modality for a variety of conditions including genetic disorders, immune deficiency syndromes, and hematologic diseases and malignancies. However, the limiting factor for successful aHSCT is the development of graft-versus-host disease (GVHD). In fact, as many as half of the ∼8,000 aHSCTs performed in the U.S. each year will result in GVHD and therefore new strategies to ameliorate GVHD are needed. GVHD occurs when donor T cells are primed by recipient antigens subsequently eliciting an inflammatory response against the host (1). Acute GVHD is a multi-organ disorder resulting from inflammatory cytokines and donor T cells which primarily damage skin, liver, gastrointestinal tract, and the eye surface (2).

Because GVHD is promoted by donor T cells and inflammatory cytokines, we reason *regulating both* is the most rational strategy to abrogate this complication. Our lab and others have demonstrated that transfer of $CD4^+FoxP3^+$ regulatory T cells (Tregs) is a promising therapy to suppress donor T cells and inhibit GVHD (3–6). Our prior work identified a two-pathway *in vivo* strategy targeting TNFRSF25 and CD25 receptors which elicits a rapid and strong increase in Treg numbers and function (7). In fact, very low numbers of these *in vivo* expanded donor Treg cells demonstrated effective GVHD suppression in recipients following aHSCT (8). Recently, the targeting of bromodomain and extra-terminal (BET) proteins has provided a new strategy for reducing pro-inflammatory cytokine production (9). These readers of histone acetyled lysine residues are involved in transcriptional regulation of many genes involved in human diseases including inflammation, cancer and cardiovascular diseases (10, 11). Recent development of BET inhibitors (BETi) has generated enormous interest for their therapeutic potential (12–14). The BETi I-BET762 and JQ1 showed anti-inflammatory properties by disrupting the expression of pro-inflammatory cytokines (e.g., IL-1β, IL-6, and IL-12) in macrophages and suppressing genes involved in T cell-mediated pro-inflammatory functions (13, 15, 16). A prior study reported that BETi I-BET151 interfered with NF-κb function and diminished cytokine expression in dendritic cells and T cells, altered APC function and decreased experimental GVHD (17). Based on our previous work illustrating the effectiveness of expanded Tregs in ameliorating GVHD, we wanted to ask if BETi could be combined with this cell therapy to augment outcomes of aHSCT. Small biomolecule inhibition of CBP/EP300 bromodomains resulted in diminishment of Treg frequency and differentiation (18). It is notable that STAT5 activation is required for Treg proliferation and function (19, 20). Importantly, although JQ1 was shown to reduce STAT5 function in hematologic cancers and dendritic cells, there is no information regarding this or other BETi effects on (1) the IL-2 signaling pathway via STAT5 in Tregs as well as (2) IL-2 production which is required for Treg survival and their maintenance of suppressive function (21, 22).

The present studies examined if BETi could be combined with Treg cell therapy without interfering with Treg expansion, phenotype and function. We found that the BETi EP11313 did not decrease Treg numbers in treated mice and in Treg expanded mice, EP11313 diminished tnfa and ifng but not il-2 levels in non-Treg cells. Notably, Treg pSTAT5 expression was not affected by EP11313 supporting the notion that Treg IL-2 signaling remained intact. In the presence of this BETi, no alterations in Treg subsets or phenotype markers as well as effector molecules, such as IL-10 and TGF-β were observed. MHC-mismatched aHSCT (donor B6-BALB/c recipient) was performed using *in vivo* expanded donor Tregs with or without EP11313 treatment in the recipient. One week post-transplant we observed significant improvement in the splenic and LN CD4/CD8 ratio along with fewer effector cells and high Treg levels in HSCT recipients treated with expanded Tregs + EP11313. Remarkably, this group exhibited diminished acute GVHD. Finally, using low numbers of highly purified expanded Tregs, we found improved clinical GVHD scores in recipients treated with EP11313. We conclude *in vivo* treatment with selective BETi can be successfully combined with Treg expansion therapy for treatment of diseases involving inflammatory responses.

MATERIALS AND METHODS

Mice and Reagents

The FoxP3 reporter mice on a C57BL/6 background (B6-$FoxP3^{RFP}$) (originally provided by R. Flavell, Yale University, New Haven, CT) (23) and B6-CD45.1 ($H2^b$) mice were bred in our facility. Wild-type BALB/c ($H2^d$) mice were purchased from Taconic (Rensselaer, NY). Mice were used at 6–12 weeks of age and were maintained in pathogen-free conditions at the University of Miami animal facilities. All animal use procedures were approved by the University of Miami Institutional Animal Care and Use Committee. EP11313 (provided by Neomed, Canada) and JQ1 (kindly provided by Dr. James Bradner) were reconstituted in DMSO and further diluted in Tween 80 and saline. The $A20^{luc/YFP}$ cell line (derived from BALB/c mice) was a generous gift of Dr. Robert Negrin (Stanford University).

Antibodies, Reagents, Flow Cytometry, and Cell Sorting

Commercial antibodies for use in flow cytometry were purchased from BD Biosciences (San Jose, CA), Biolegend (San Diego, CA),

or eBioscience (Waltham, MA). Recombinant mouse IL-2 and α-IL-2 monoclonal antibody, clone JES6-5H4, were purchased from eBioscience. IL-2/αIL-2 complex was generated by incubating 1.5/7.5 μg recombinant mouse IL-2 with 8μg JES6-5H4 (∼8,000 IU/injection) for 15 min at room temperature and administered i.p. TL1A-Ig was generated in our laboratory as described previously (24) and administered i.p. at 50 μg/mouse/injection. Single-cell suspensions were prepared from different organs (spleen and lymph nodes). Peripheral blood was collected in heparinized tubes. Peripheral blood mononuclear cells were isolated by standard Ficoll density gradient centrifugation. Next, 10^6 cells were preblocked with anti-mouse CD16/CD32 and stained with different antibody combinations. Intracellular staining was performed according to standard procedures. The following mAbs to the indicated molecules, the fluorescent labels, and their sources were used in this study: CD4, CD8, CD19, CD25, CD44, CD62L, CD103, KLRG1, CD39, CD73, I-COS, Nrp-1, PD-1, CTLA-4, CCR8, Ly-6C, Ki-67, pSTAT5, and Annexin V (**Supplementary Materials and Methods, Table S1**). Flow cytometric analysis was performed on a BD LSR-Fortessa-HTS instrument (BD Biosciences, San Jose, CA) and the analysis was completed using FlowJo software (FlowJo, LLC, Ashland, OR). Splenic and pLN $CD4^-FoxP3^-$, $CD4^+FoxP3^-$, and $CD4^+FoxP3^+$ cells were sorted using a FACS Aria II cell sorter (BD Biosciences) after enrichment of T cells (surface immunoglobulin depletion of B cells).

RNA Isolation, RT-PCR, and Quantitative Real-Time PCR

Total RNA was isolated from unexpanded and expanded Tregs using RNAeasy mini kit following the manufacturer's instructions (Qiagen, Germantown, MD). cDNA was retrotranscribed from 1 μg of total RNA using qScript cDNA Mastermix (Quanta, Beverly, MA). Quantitative real-time PCR was (qPCR) was performed in triplicate using the ABI PRISM 7300 sequence detection system (Applied Biosystems, Whatman, MA) with the specific primers for tnfa, ifng, il-2, il-10, and gapdh (**Supplementary Materials and Methods**, **Table S2**). The PCR mixture contained 7.5 μl of 2X SYBR Green PCR master mix (Applied Biosystems) in a 15 μl final volume. The specificity of each primer set was monitored by analyzing the dissociation curve. The relative mRNA levels of each gene were calculated using the Livak method with GAPDH as the housekeeping gene.

Western Blot

Cells ($0.5–1.0 \times 10^6$) were diluted with Laemmli sample buffer and boiled for 5 min. Proteins were separated by electrophoresis on 4–15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The residual binding sites were blocked with 5% non-fat powdered milk in PBS containing 0.05% Tween 20, and membranes were incubated with anti-TGF-b mAb (0.5 mg/ml; ABCAM, Cambridge, United Kingdom) or anti-actin mAb (0.5 mg/ml; ABCAM) in PBS containing 0.05% Tween 20. All subsequent washes were performed with the same buffer. Reactivity was developed using HRP-coupled secondary polyclonal antibody (1:2,000, Jackson ImmunoResearch) and the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

In vitro Treg Functional Assays

For a standard suppressor assay, $CD4^+FoxP3^-$ splenocytes (10^5) were cultured in 96-well plates and activated with 1 μg soluble anti-CD3 (clone 2C11) antibody in the presence of APCs (5×10^4 T cell depleted splenocytes) and titrating numbers of sorted $CD4^+FoxP3^+$ Tregs. Cultures were incubated for 72 h and pulsed with [^{3}H]-Thymidine (0.5 μCi/well; Perkin Elmer) for the last 10 h. Incorporated isotope was measured by liquid scintillation counting (Micro Beta TriLux counter; Perkin Elmer). For a functional assay, spleen and LN suspensions were activated *in vitro* with 1 μg/ml soluble anti-CD3. After 72 h, proliferating cells were counted by Trypan blue exclusion using the Vi-cell XR cell counter (Beckman Coulter, Brea, CA).

HSCT Experiments

For the HSCT in the major MHC-mismatch model (B6 → BALB/c), female BALB/c mice ($H2^d$) were ablatively conditioned with 8.5 Gy total body irradiation 1 day before transplantation. BM cells were obtained from femurs, tibias, and vertebrae from sex-matched B6-CD45.1 ($H2^b$; Thy1.2) donor animals. A single-cell suspension of marrow cells was prepared by flushing bones with a 21-gauge needle and the cells were filtered through a 100 μm nylon mesh. Donor marrow cells were depleted of T cells via complement mediated lysis using anti–T-cell–specific antibody HO-13-4 (hybridoma supernatant, mouse anti-Thy1.2 IgM; ATCC, Manassas, VA) generously provided by Dr. Bruce Blazar (University of Minnesota), anti-CD4 mAb (clone 72.4), anti-CD8mAb (clone H02.2), and rabbit complement (Cedarlane Laboratories, Burlington, Ontario, Canada). The marrow cells were incubated at 37°C for 45 min, washed twice in RPMI, and resuspended for HSCT. Marrow T cell depletion was routinely >99%. Donor T cells were prepared from spleens or LN obtained from C57BL/6-$FoxP3^{RFP}$-expanded or unexpanded animals. Donor cells were stained for T cells (anti-CD4, clone RM4-5; anti CD8, clone 53-6-7) and adjusted to 1.0×10^6 T cells per mouse before mixing with BM. Recipient mice were injected twice a day with EP11313 10 mg/kg (from day −2 to 4) and underwent transplantation (day 0) with T cell–depleted (TCD) BM (5.5×10^6) and 1.0×10^6 T cells i.v. in a 0.2 ml volume via tail vein injection. GVHD was assessed by monitoring recipients for changes in total body weight, clinical signs, and overall survival. The clinical signs of GVHD were recorded for individual mice. Recipients were scored on a scale from 0 to 2 for 5 clinical parameters (25): weight loss, diarrhea, fur texture, posture, and alopecia and for ocular lid score on a scale from 0 to 4 (26).

Histologic Analysis

Briefly, tissues from animals 4–5 weeks after aHSCT were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) and images were acquired using the Keyence BZ-X700 microscope (Itasca, IL). Slides were scored following a modified system described by Kaplan et al. (27). In

brief, 3–4 parameters were used to compare pathology scores between groups in the skin and the colon.

Cell Survival Assay

Cells were seeded in 384-well micro-titer plates and incubated in a humidified environment at 37°C and 5% CO_2 and cultured for 24 h, followed by incubation with the EP11313, JQ1, iBET762, and iBET151. Individual drugs will be dissolved in 100% DMSO and tested in triplicates starting at a nominal test concentration of 10 μM over a 20,000-fold concentration range to generate dose response curves allowing for calculation of half-maximal and maximal response concentrations. Cell viability was measured by bioluminescence after 72 h of drug exposure and dose response curves were generated for each compound.

Cell Proliferation Assay

In order to monitor cell proliferation in response to treatment with EP11313, cells were seeded in 24-well plates (Corning, NY) at 10^4 cells per well. Cells were treated with 0, 0.05, 0.1, 0.5, 1, and 1.5 μM EP11313 ($n = 4$ per treatment) and proliferation was evaluated using the Incucyte Zoom instrument (Essenbioscience, MI).

Determination of Serum TNF-α Levels

Serum was collected from animals treated *in vivo* with LPS 1 mg/kg and EP11313 10 or 30 mg/kg. TNF-α concentration was determined by Mouse TNF-α ELISA MAX (Biolegend, San Diego, CA), following manufacturer's protocol. Analysis was performed using Benchmark Plus microplate spectrophotometer at 405 OD.

Statistical Analysis

All graphing and statistical analysis were performed using GraphPad Prism (San Diego, CA). Values shown in graphs represent the mean of each group ± SEM. Survival data were analyzed with the Mantel-Cox log-rank test. Non-parametric unpaired two-tailed *t*-test was used for comparisons between two experimental groups, and multiple variable analysis was performed using ANOVA. A *P*-value <0.05 was considered significant. Brackets identifying the groups being compared are presented in each figure where appropriate accompanied by the level of significance or absence of significance (ns).

RESULTS

A Bromodomain Inhibitor (BETi)—EP11313 Does Not Interfere With Treg Expansion, Subsets, and *in vitro* Function

To determine if Treg exposure to BETi *in vivo* impaired homeostatic proliferation of $CD4^+FoxP3^+$ Tregs, C57BL/6-$FoxP3^{RFP}$ (B6-$FoxP3^{RFP}$) mice were administered EP11313 i.p. 2×/day (13 total injections) (**Figure S1A** in **Supplementary Material**). Spleen and lymph node (LN) cell analysis revealed no differences in the frequency of both the $CD4^+$ compartment and Tregs (**Figures S1B**,**C** in **Supplementary Material**). Following Treg isolation via cell sorting (purity > 98%, d*ata not shown*) their *in vitro* functional activity was also not diminished as evidenced by a standard suppressor assay (**Figure S1D** in **Supplementary Material**). Since we employ expanded and proliferating Tregs for use to regulate GVHD (7, 8), we next addressed whether EP11313 altered expansion, subset distribution and function of Tregs undergoing two-pathway *in vivo* expansion following treatment with TL1A-Ig and low dose IL-2. Examination of peripheral (spleen, LN) lymphocytes indicated no change in the relative frequencies of CD4 and CD8 T cells in mice receiving two-pathway stimulation alone or together with EP11313 (**Figures 1A,B**, **S1E** in **Supplementary Material**). Importantly, the frequency and numbers of Treg cells within these two groups were also not altered (**Figures 1C,D**, **S1F** in **Supplementary Material**). Notably, following a 3× increase in EP11313 dose administration the percentage of Tregs within the $CD4^+$ compartment was again not affected (**Figure 1E**). Utilizing Ly6C and CD62-L staining (28, 29), Treg subset evaluation demonstrated no change in the central Tregs ($Ly6C^-$, $CD62L^{hi}$ = cTregs) and effector Tregs ($Ly6C^-$, $CD62L^{lo}$ = eTregs) frequency between animals which had Tregs expanded in the presence or absence of the BETi. We previously found a diminution in the $Ly6C^+$ Treg fraction following two-pathway expansion (8). Here, we also observed the same diminution in this subset in the presence or absence of EP11313 (**Figure 1F**). Lastly, to directly assess the functional activity of the Tregs expanded in the presence and absence of EP11313, spleen and LN cells were removed and immediately stimulated *in vitro* with anti-CD3mAb. The decrease in proliferation by cells from animals following Treg expansion was not significantly different regardless of whether animals also received BETi treatment (equivalent Treg suppressive activity) (**Figure 1G**).

It is well established that BETi possess anti-tumor activity (14). Therefore, to demonstrate activity of the BETs, we assessed tumor cell viability and proliferation using a mouse lymphoma cell line. As anticipated, each BETi examined decreased tumor cell viability and numbers at varying concentrations (**Figure S2** in **Supplementary Material**). Since BETi are also known to inhibit transcription of inflammatory genes, we examined *in vivo* activity by the BETi EP11313. Following injection of two doses of this BETi, LPS was administered and TNF-α serum levels assessed after 1.5 h (**Figure S3A** in **Supplementary Material**). There was a clear decrease which was dose related in the serum levels of this inflammatory cytokine (**Figure S3B** in **Supplementary Material**).

In vivo Administration of the JQ1 Decreases Treg Proliferation and Alters Their Phenotype During Expansion of $CD4^+FoxP3^+$ T Cells

Next, we wanted to address if a prototypic BETi, JQ1 (12) exhibited the same pattern as EP11313 with regard to affecting Treg frequency, proliferation and subset distribution in Tregs undergoing expansion. Groups of B6-$FoxP3^{RFP}$ mice were treated 2×/day (13 total injections, **Figure S1E** in

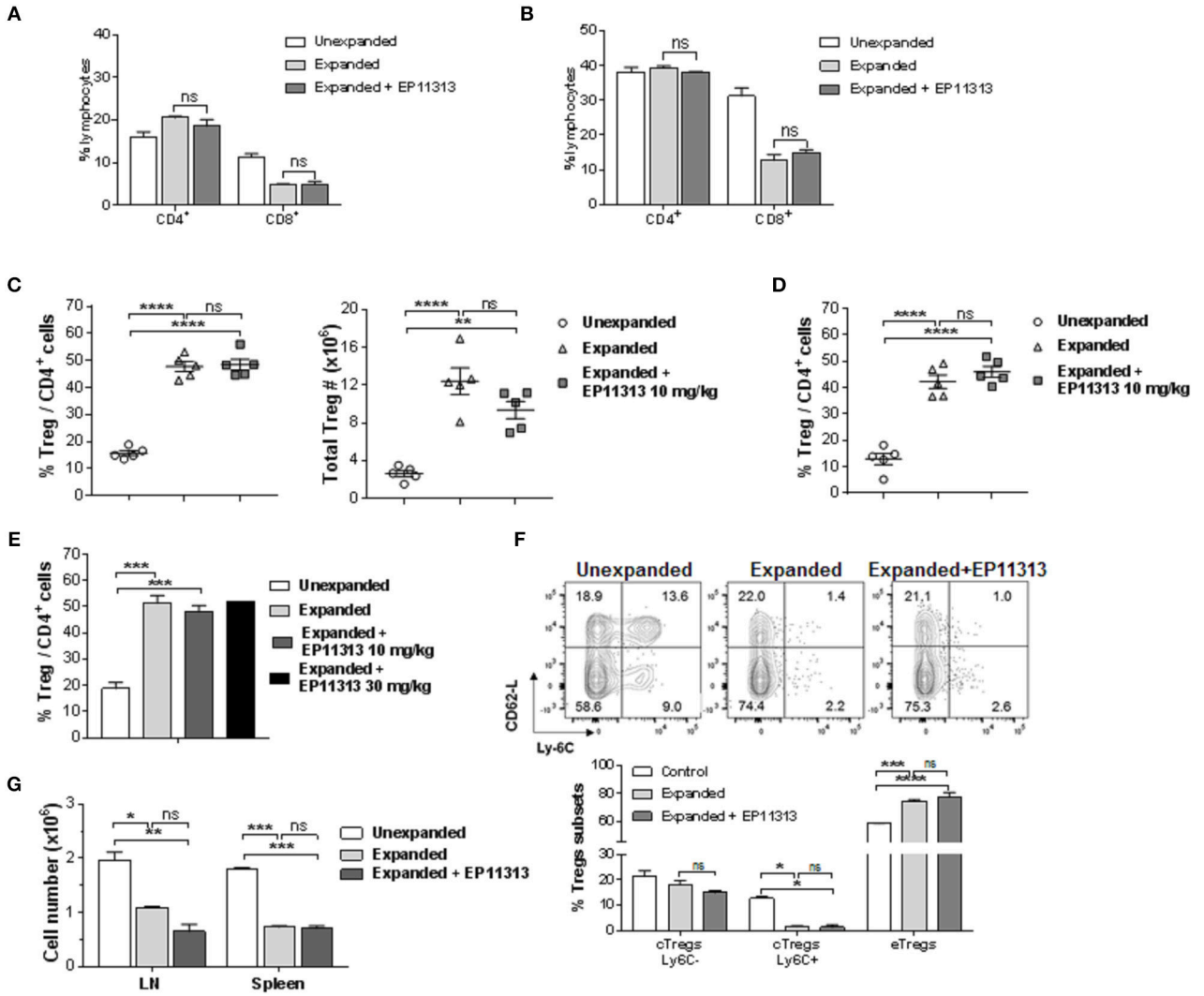


FIGURE 1 | *In vivo* treatment with BETi EP11313 does not interfere with Treg expansion, subset distribution and *in vitro* suppressor function. **(A–G)** Mice were injected i.p. with TL1A-Ig (on days 1–4) and rmIL-2 bound to the anti-IL-2 mAb (JES6-5H4, on days 4 and 6) and EP11313 (10 mg/kg per dose) or administered vehicle on days −1 to 6 (twice per day). Mice were sacrificed on day 7. $CD4^+$ or $CD8^+$ frequency (%) in the spleen **(A)** and peripheral lymph nodes (pLN) **(B)** of mice undergoing Treg expansion (TL1A-Ig + low dose IL-2) in the presence or absence of BETi EP11313. Data representative of three independent experiments ($n = 2$ mice/group). Splenic overall Treg frequency (%) within the CD4 fraction ($CD4^+FoxP3^+/CD4^+$) cells (left) and total numbers splenic Tregs (right) are shown **(C)**. Treg ($CD4^+FoxP3^+$) frequency (%) of total $CD4^+$ cells in pLN **(D)**. Data are pooled from three independent experiments; $n = 5$ mice/group **(C,D)**. Treg frequency (%) of total $CD4^+$ cells in expanded mice treated with EP11313 at 10 or 30 mg/kg **(E)**. Treg subset distribution determined by CD62-L and Ly-6C staining is shown as a representative contour plot (top) and a bar graph of data pooled from two independent experiments (bottom) **(F)**. No significant differences were observed in cTregs $CD62L^{hi}$Ly-$6C^{-or+}$ and eTregs $CD62L^{lo}$Ly-$6C^-$ in the Expanded + EP11313 treated mice vs. Expanded mice **(F)**. Treg expansion leads to a suppressive environment in spleen and LN which is not altered in the presence of BETi EP11313 **(G)**. Cell suspensions of spleen or lymph node cells obtained from indicated mice which underwent expansion treated with EP11313 (or vehicle) or normal, unexpanded mice. The cultures were then stimulated with anti-CD3mAb for 72 h **(G)**. Data are representative of two independent experiments. ns = not significant vs. expanded.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ vs. unexpanded.

Supplementary Material) with 5 mg/kg of JQ1 or 10 mg/kg of EP11313. In contrast to what we observed with EP11313 treatment, exposure of expanding Tregs to JQ1 resulted in a decrease in splenic and LN Treg frequencies (**Figures 2A,B**). A representative dot plot of Treg subsets showed that the cTregs (Ly6C$^-$, $CD62L^{hi}$) fraction was decreased in animals receiving JQ1—but not EP11313—in animals undergoing two-pathway Treg expansion (**Figure 2C**). Following Ki67 staining to assess cell proliferation, we observed that in contrast to EP11313, JQ1 treatment deceased the $Ki67^+$ population within splenic and LN Tregs (**Figures 2D,E**). There was no effect of these BETi's on proliferation of conventional $CD4^+FoxP3^-$ T cells (**Figure S4** in **Supplementary Material**). Lastly, IL-2 induced STAT5 phosphorylation of Tregs *in vitro* was not diminished in the presence of JQ1 or EP11313 (500 nM) (**Figure 2F**).

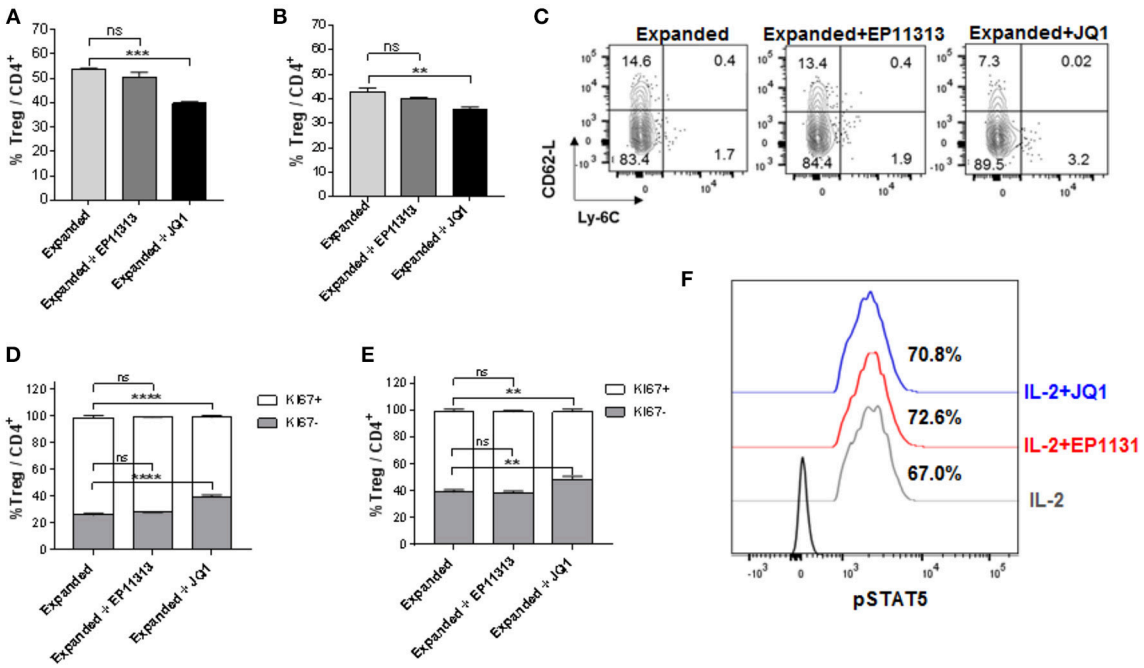


FIGURE 2 | In contrast to BETi EP11313, JQ1 alters Treg frequency, proliferation and subsets with no differences in pSTAT5 expression. **(A–F)** Mice were injected i.p. with TL1A-Ig and rmIL-2 bound to anti-IL-2 mAb as in **Figure 1** and EP11313 (10 mg/kg), JQ1 (5–10 mg/kg) or vehicle (on days −1 to 6). Mice were sacrificed on day 7. *in vivo* treatment with JQ1 significantly decreased overall Treg ($CD4^+FoxP3^+$) frequency (%) of total $CD4^+$ cells in the spleen **(A)** and pLN **(B)**. Representative contour plot of Treg subset distribution determined by CD62L and Ly-6C staining of pLN from mice undergoing Treg expansion treated with EP11313, JQ1, or vehicle. JQ1 treatment diminished cTreg $CD62L^{hi}Ly6C^-$ **(C)**. Treg expanded proliferation was impaired with JQ1 *in vivo* treatment indicated by Ki67 expression in the spleen **(D)** and pLN **(E)**. **(A–E)** All results are representative of two independent experiments $n = 3$ mice/group. Representative histograms of lymph node cells from TL1A-Ig + IL-2 expanded mice treated *in vitro* with 500 nM BETi or vehicle and stimulated with IL-2 10 ng/ml for 15 min **(F)**. ns, not significant. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ vs. expanded.

To more precisely analyze expanded Treg phenotype in the presence of BETi treatment, activation, function and differentiation markers were assessed using mAbs to defined Treg proteins (**Table S1** in **Supplementary Materials and Methods**). Notably, significantly decreased levels of activation and differentiation molecules, specifically ICOS, CD103, PD-1, CD44, and KLRG1 were identified in splenic Tregs undergoing expansion treated with 10 mg/kg of JQ1 (**Figure 3**). Additionally, the Treg functional suppressive mediators CD39, Nrp-1, and CTLA-4 were also diminished in this treated population. Similar results were obtained analyzing LN Tregs (*data not shown*). Notably, in contrast to the findings with JQ1, EP11313 treatment did not alter the expression of any of these phenotypic Treg markers (**Figure 3**).

EP11313 Regulates Inflammatory Cytokines but Spares the Il-2 Pathway and Effector Molecules in Treg Cells

The above findings demonstrate that EP11313 did not interfere with expansion and phenotype of expanding Treg cells. To examine whether treatment with this BETi altered molecules that mediate Treg function, highly purified $CD4^+FoxP3^+$ Tregs were isolated from animals undergoing expansion in the presence or absence of EP11313 treatment (**Figure 4**, **Figure S1E** in **Supplementary Material**). IL-10 RNA and protein levels were not altered in Tregs exposed to this BETi (**Figure 4A**). We also did not detect differences in TGF-β protein levels from sorted Treg populations obtained from the spleen and lymph nodes of treated mice (**Figure 4B**).

Since IL-2 is required for Treg function and survival, we addressed if this cytokine was present in recipients of EP11313 treated mice. Therefore, a Treg negative population (conventional CD4, CD8, NK, macrophage/monocyte and low numbers of contaminating B cells not depleted by sIg treatment) was examined for this cytokine. No differences in the RNA levels of il-2 were identified in these cells from animals undergoing expansion in the presence or absence of this BETi (**Figure 4C**). Since phosphorylation of STAT5 (pSTAT5) is required for IL-2R signaling, levels of this protein were examined within the Treg populations (**Figure 4D**). Importantly, no differences were observed in pSTAT5 expression in Tregs undergoing expansion from BETi treated vs. untreated animals (**Figure 4D**). To validate that EP11313 treatment affected gene transcription in treated animals, RNA from the sorted Treg negative populations was also examined for tnfa and ifng. These inflammatory cytokine RNA were significantly decreased in this population (**Figure 4E**).

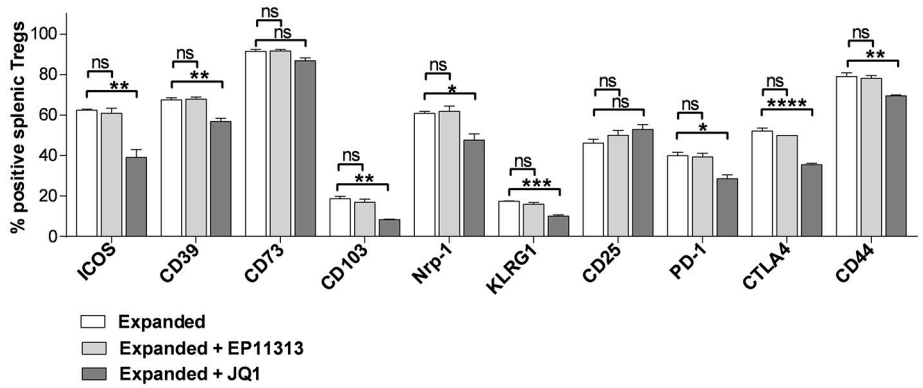


FIGURE 3 | In contrast to BETi EP11313, JQ1 modifies Treg phenotype. Mice were injected i.p. with TL1A-Ig and rmIL-2 bound to anti-IL-2 mAb as in **Figure 1** and EP11313 (10 mg/kg), JQ1 (5–10 mg/kg) or vehicle on days −1 to 6. Mice were sacrificed on day 7. Expression of activation, differentiation (i.e., ICOS, CD103, CD44, KLRG1) and functional (i.e., CD39, CD73, Nrp1, CTLA-4) molecules in splenic Tregs are shown. Data representative of five experiments (Treg expanded) and two independent experiments (expanded plus BETi) $n = 3$ mice/group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ vs. expanded.

Recipients Transplanted With Expanded Donor Tregs and Treated With EP11313 Demonstrate Diminished GVHD Early Post-HSCT

The above findings support the notion that selected BETi can be combined with Treg cells to prevent GVHD following allogeneic-HSCT. Accordingly, we performed a transplant using a fully MHC-mismatched aHSCT model (B6→ BALB/c) (**Figure 5A**). Groups of recipients received unfractionated spleen cells (adjusted to contain 1×10^6 T cells) from Treg expanded donors (TrED) or unexpanded donors (TrUD) in the presence or absence of short-term (Day −2 to 4) EP11313 treatment (**Figure 5A**). Using this protocol, treatment with EP11313 only did not diminish GVHD clinical scoring (**Figure 5A**). As we have reported, recipients of TrED did exhibit decreased GVHD clinical scores and increased survival compared to recipients receiving TrUD (8) (**Figures 5B,C**). Interestingly, the combinatorial strategy of TrED plus EP11313 treatment significantly lowered GVHD scores during the first 3 weeks post-HSCT and did not diminish overall (i.e., 100%) survival (**Figures 5B,C**). To obtain sufficient cell numbers, tissues were pooled from animals 1-week post-HSCT. TrED + EP11313 treatment resulted in increased CD4/CD8 ratios in the spleen and lymph nodes at this time (**Figure 5D**). More apparent in the lymph node T cells, there was a diminishment of the CD4 Teff/mem ($CD44^+CD62L^{lo}$) population and an increase in CD4 T naïve ($CD44^-CD62L^{hi}$) cells from combination (TrED+BETi) treated mice (**Figure 5E**). As anticipated, recipients of TrED had higher levels of Treg cells compared with recipients of TrUD 1-week post-HSCT in the spleen (**Figure 5F**). Consistent with the EP11313 and Treg expansion findings above (**Figures 1–4**), mice receiving the combinatorial (TrED + EP11313) strategy contained similar splenic Treg levels compared with recipients of TrED alone (**Figure 5F**). In this context, our recent studies reported that $<1.75 \times 10^5$ two-pathway expanded donor Tregs were not sufficient to ameliorate GVHD in fully mismatched aHSCT recipients (8). Therefore, transplants were performed using 1×10^5 highly purified donor expanded Tregs (**Figure S5** in **Supplementary Material**) alone or in combination with short-term EP11313 treatment of recipients (**Figure 5G**). The results of two independent pooled transplants demonstrated a significant decrease (up to 3 weeks post-aHSCT) in the clinical GVHD scores between recipients of purified Tregs alone and those receiving the BETi from Days −2 to 4 TrED (**Figure 5H**). No differences in overall survival between these groups was detected (data not shown).

One and two months post-HSCT, clinical, histological and pathology assessments indicated lower ocular adnexa involvement with less clinical lid edema and closure (**Figure 6A**) and decreased skin involvement as assessed by overall thickening and fibrosis (**Figure 6B**). Thymic weight was superior in TrED + EP11313 vs. TrUD + EP11313 and colon length in the former was virtually identical to control BM alone transplanted recipients and significantly greater compared to recipients of TrED alone (**Figure S6** in **Supplementary Material** and **Figure 6C**, respectively). Moreover, histological assessment of the colon 5 weeks post-aHSCT showed mucosal thickening and severe inflammation with villi distortion in recipients of only 1×10^5 purified expanded Tregs. In contrast, colons from mice receiving 1×10^5 expanded Tregs with EP11313 exhibited a mild inflammation and no disruption of villi architecture (**Figure 6D**).

DISCUSSION

The bromodomain and extra-terminal (BET) proteins have a central role in regulating transcription of inflammatory and oncogenic factors and have emerged as attractive druggable targets with therapeutic potential (14, 30). Increasing pre-clinical data, completed and ongoing clinical trials (14) (NCT01943851, NCT01587703, NCT01713582) have demonstrated that BET inhibitors (BETi) possess anti-cancer and anti-inflammatory activity (12, 13, 16, 31). The objective of the present study was to identify a BETi which did not interfere *in vivo* with $CD4^+FoxP3^+$

regulatory T cell (Treg) expansion and function so it could be utilized together with Tregs following aHSCT to ameliorate graft-versus-host disease (GVHD). Notably, *in vitro* analysis of a bromodomain inhibitor of CBP/EP300 reduced human Treg differentiation and suppressive function (18). Interestingly, our studies examining BETi *in vivo* demonstrated that JQ1 interfered with Treg expansion and altered subset distribution and phenotype. In contrast, we found that the BETi EP11313 did not impair the basal (un-manipulated) or the expanded (TL1A + low dose IL-2) Treg compartments. Remarkably, administration of low levels of EP11313 at the time of allogeneic HSCT together with adoptive transfer of expanded Tregs further diminished GVHD.

Regulatory T cells (Tregs) have a critical role in the immune system by maintaining immune homeostasis and preventing occurrence of autoimmune disease (32–34). IL-2 signaling via the high affinity IL-2R (CD25) results in phosphorylation of STAT5 and is necessary for the maintenance and expansion of $CD4^+FoxP3^+$ Tregs (19, 20). Adoptive transfer of regulatory T cells (Tregs) has emerged as a promising therapy for solid organ transplantation, autoimmune diseases and GVHD following aHSCT (3, 4, 6, 35, 36). Our group and others have shown the effectiveness of donor Tregs as a prophylactic strategy to prevent development of GVHD (3–5). We have previously reported that Tregs can be markedly expanded and selectively activated with increased functional capacity by targeting two receptors, i.e., TNFRSF25 and CD25 with TL1A-Ig and low dose IL-2, respectively (7, 8). Additionally, expanded Treg therapy was shown to be as effective as post-transplant cyclophosphamide for GVHD prophylaxis but the former promoted more rapid thymic reconstitution providing earlier recovery of recipient immune function (37). Acute GVHD occurs when donor T cells are primed by recipient antigens subsequently eliciting a rapid inflammatory response ("cytokine storm") in the host.

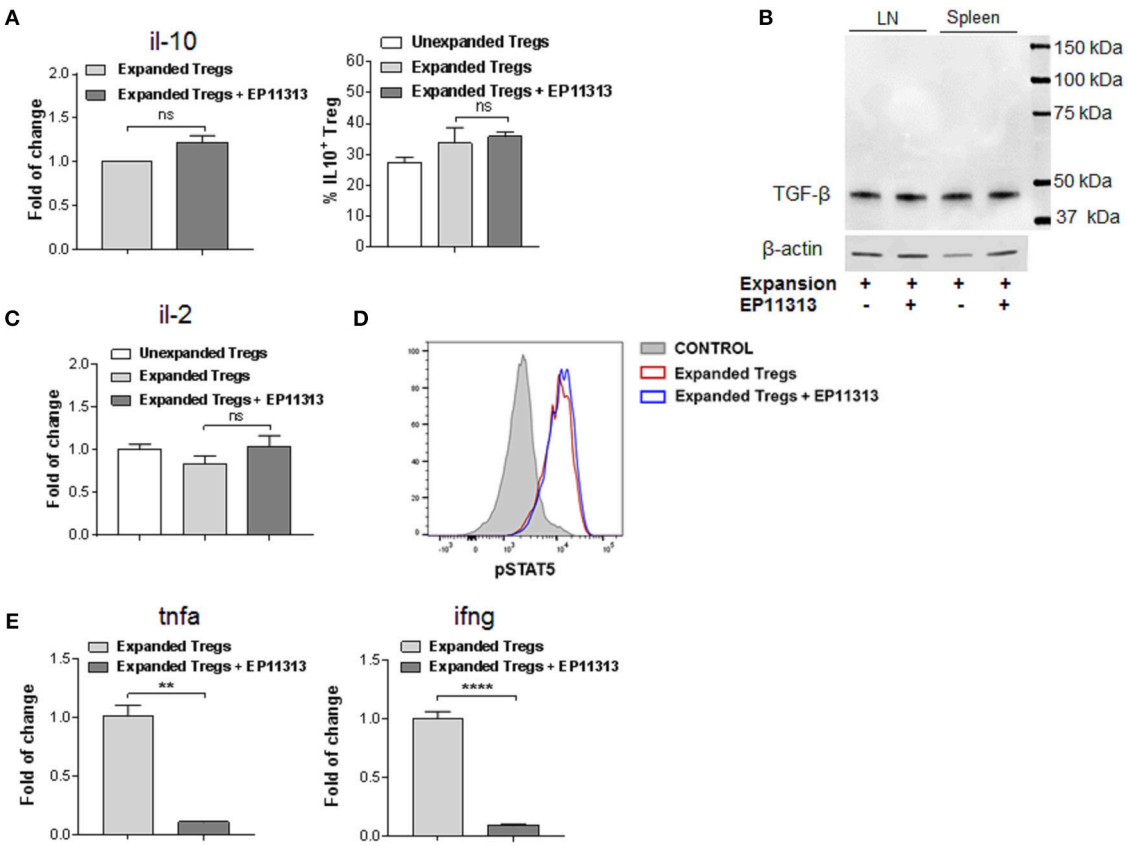


FIGURE 4 | EP11313 regulates inflammatory cytokines but spares the IL-2 pathway and Treg effector molecules. Tregs were expanded with TL1A-Ig + IL-2 in the presence or absence of EP11313 10 mg/kg, mice were sacrificed at day 7 and splenic Tregs ($CD4^+FoxP3^+$) and non-Tregs ($CD4^+FoxP3^-$ and $CD4^-FoxP3^-$) were isolated by FACS **(A–E)**. Quantitative Real-time PCR (qPCR) analysis of il-10 mRNA levels (relative to gapdh) of splenic $CD4^+Foxp3^+$ Tregs sorted from expanded ± EP11313 mice (left). Data are pooled from two independent experiments. IL-10 production by $CD4^+FoxP3^+$ Tregs from LN of expanded and expanded + EP11313 mice after phorbol 12-myristate 13-acetate (1 ng/mL) + ionomycin (1 μM) stimulation for 6 h in the presence of monensin (right) **(A)**. Western Blot analysis of TGF-β levels in sorted Tregs from LN and spleen of mice treated *in vivo* with TL1A-Ig + low dose IL-2 in the presence or absence of EP11313. β-actin was used as a loading control **(B)**. qPCR analysis of il-2 mRNA levels (relative to gapdh) of splenic non-Treg population sorted from unexpanded, expanded or expanded + EP11313 treated mice **(C)**. Representative pSTAT5 staining shown by flow cytometry in $CD4^+FoxP3^+$ Tregs from peripheral blood of expanded ± EP11313 treated B6-$FoxP3^{RFP}$ animals 1 h after final IL-2 injection (3 mice/group) **(D)**. qPCR analysis of tnfa (left) and ifng (right) mRNA levels (relative to gapdh) of splenic non-Treg population sorted from expanded or expanded + EP11313 treated mice **(E)**. Data representative of two independent experiments. ns, not significant. $^{**}p < 0.01$; $^{****}p < 0.0001$.

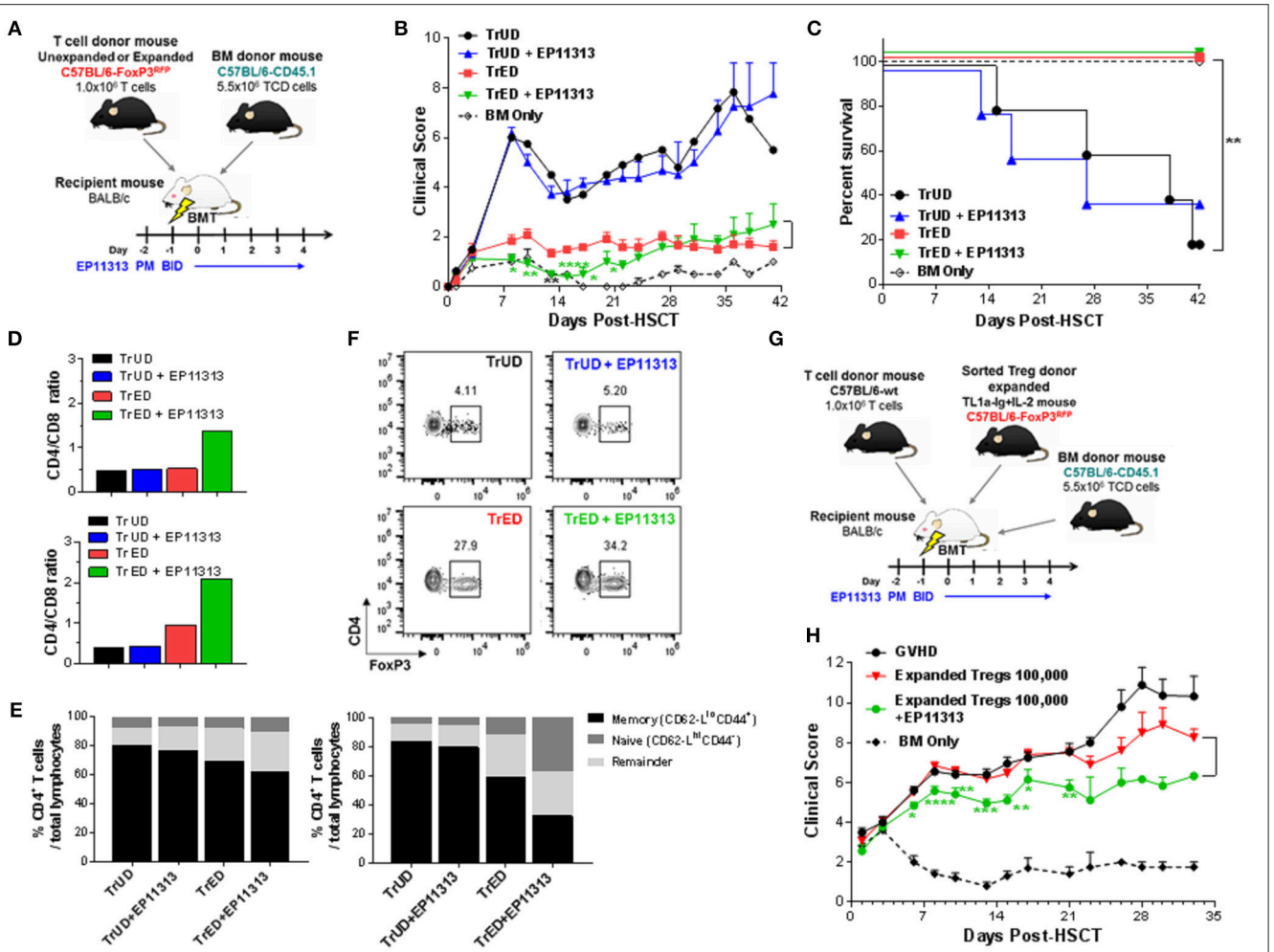


FIGURE 5 | Recipients transplanted with cells from Treg expanded donors (TrED) or low numbers of purified expanded Tregs and treated with EP11313 exhibited diminished acute GVHD after MHC-mismatched aHSCT. An HSCT was performed on Day 0 utilizing a B6 BALB/c donor/recipient mouse model involving a complete MHC mismatch Lethally irradiated (8.5 Gy) BALB/c mice received 5.5 × 10^6 TCD B6-CD45.1 BM cells and spleen cells from expanded (TL1A-Ig + low dose IL-2 = TrED group) or unexpanded B6-FoxP3^{RFP} (= TrUD group) donor mice adjusted to contain 1.0 × 10^6 total T cells. EP11313 10 mg/kg or vehicle were given i.p. from day −2 to 4 post-HSCT **(A–F)**. **(A)** Experimental design of the complete MHC-mismatched aHSCT model used in these studies **(A)**. Clinical GVHD scores (0 = no disease and 10 = severe) **(B)** and survival curves **(C)** are presented (n = 8 mice/group, n = 4 mice/BM Only group). Recipients of TrED and EP11313 demonstrated ameliorated clinical GVHD. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ TrED + EP11313 vs. TrED for clinical score. One week after transplant, the spleens and LNs were evaluated **(D–F)**. Higher CD4/CD8 ratio were found in TrED + EP11313 recipient spleen (top) and LN (bottom) compared to TrED **(D)**. The T cell naïve/memory compartment was analyzed by flow cytometry (CD44/CD62L) in spleen and LN. The naïve compartments of CD4$^+$ cells (CD44$^-$CD62-L^{hi}) in spleen (left) and LN (right) were increased while the T effector/memory (CD44$^+$CD62-L^{lo}) diminished in TrED + EP11313 recipients compared to TrED treated animals **(E)**. Tissues were pooled from 2 to 3 mice/group **(D,E)**. Representative flow cytometry plots of splenic CD4$^+$FoxP3$^+$ Treg frequency of the indicated groups are shown **(F)**. A complete MHC-mismatched aHSCT was performed (as in **A**) transplanting sorted CD4$^+$FoxP3$^+$ expanded Tregs (100,000) together with B6-WT 1 × 10^6 splenic T cells and 5.5 × 10^6 TCD B6-CD45.1 BM cells. The experimental design of the complete MHC-mismatched aHSCT model used in these studies **(G)**. Clinical scores of recipient groups showed decreased scores in mice receiving 100,000 expanded donor Tregs + EP11313 **(H)**. Data is representative of two independent experiments (n = 8 mice/group). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ Expanded Tregs + EP11313 vs. Expanded Tregs for clinical score.

Because GVHD is promoted by inflammatory cytokines and donor T cells, we reasoned regulating both components is a rational strategy to abrogate onset of this disorder. Significant numbers of Tregs are required to inhibit alloreative T effector cells which induce GVHD. Accordingly, development of a successful combinatorial approach must include a BETi which does not interfere with Treg function or proliferation. During Treg expansion induced by TL1A-Ig + IL-2 stimulation, JQ1 treatment impaired their peripheral frequency and altered several key Treg differentiation and functional molecules including ICOS-1, Nrp-1, KLRG-1 as well as, PD-1 and CTLA-4 which were also reported to be reduced in Tregs by CBP/EP300 bromodomain inhibitors (18). While additional experiments are needed, based on JQ1's capacity to inhibit c-myc, is possible based on our data thus far, that this BETi is affecting more proliferative Treg subsets, i.e., cTregs rather than more differentiated eTregs.

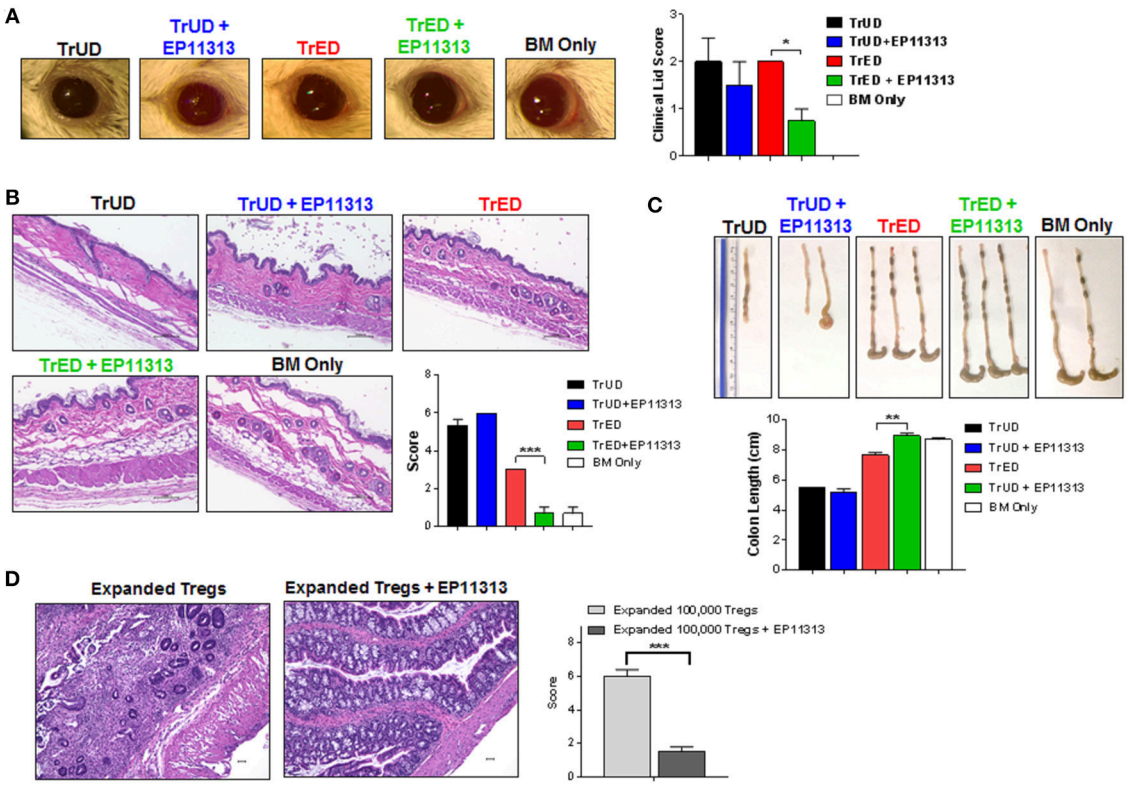


FIGURE 6 | Diminished GVHD in target tissues of recipients treated with EP11313 and TrED. A complete MHC-mismatched aHSCT was performed (as in **Figure 5**) by transplanting 5.5 × 10^6 TCD B6-CD45.1 BM cells and spleen cells from expanded (TL1A-Ig + low dose IL-2: TrED group) or unexpanded B6-FoxP3^{RFP} (TrUD) donor mice adjusted to contain 1.0 × 10^6 total T cells. EP11313 10 mg/kg or vehicle were given i.p. from day −2 to 4 post-HSCT. Four to seven weeks after transplant, **(A)** Representative photographs of clinical ocular differences from the indicated groups (left) and diminished lid scores (right) in TrED + EP11313 treated groups vs. TrED (4 weeks post-transplant) **(A)**. Representative H&E stained sections from skin 5 weeks after aHSCT showed that TrED + EP11313 treatment resulted in more normal architecture with less fibrosis and dermal thickening **(B)**. Pathology scores for these tissues are shown **(B)**. Colon length 7 weeks post-aHSCT was longer in recipients of TrED + EP11313 treatment **(C)**. Representative H&E stained sections from colon 5 weeks after aHSCT of mice treated with 100,000 expanded donor Tregs ± EP11313. Colons from 100,000 expanded Tregs + EP11313 recipients showed mild inflammation and no distortion of the villi compared with colons from 100,000 expanded Tregs alone. Pathology scores for these tissues are shown on the right **(D)**. Magnification 100× for colon and 200× for skin. Values are means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Notably, JQ1 has also been found to inhibit frequency and function of tumor infiltrating Tregs in non-small cell lung cancer (38, 39). However, a non-structurally related BETi, EP11313 did not exhibit this pattern of alteration as no effect on frequency was observed in either these intentionally expanded—or homeostatic Treg compartments. It should be noted that although the ½ lives of both BETi are not different the results found that even in the presence of higher amounts of EP11313 (3X) vs. JQ1 there was no reduction in Treg proliferation by the former. These findings were consistent with the observations that IL-2 production by non-Treg cells was not diminished in EP11313 treated animals. Interestingly, Treg pSTAT5 levels were also not diminished after BETi treatment *in vitro* or *in vivo*. This finding contrasts reported observations that JQ1 inhibited STAT5 phosphorylation and transcriptional activity in monocyte/dendritic cells (22). However, similar to our results, it was previously reported that JQ1 or BRD2 downregulation diminishes STAT5 function through phosphorylation-independent mechanisms in lymphocytic leukemias (21). Although we anticipated that due to Treg proliferation differences in mice treated with either JQ1 or EP11313, STAT5 phosphorylation may have been differentially affected by these BETi's. In contrast to other cell populations, it appears BETi STAT5 regulation of lymphoid lineage populations involves a different mechanism. It was previously reported that STATs can be acetylated under certain conditions (40). Thus, it is possible that STAT5 or proteins involved in this signaling pathway in expanding Tregs or hematologic cancers does not contain acetylated lysine residues which are present following LPS stimulation of dendritic cells (21, 22).

Importantly, using a brief, i.e., 1 week protocol of EP11313 10 mg/kg (a low dose of BETi) as mentioned above, several differentiation and functional molecules (ex. ICOS-1, Nrp-1, PD-1) were not altered in Tregs. Moreover, this regimen of EP11313 did not reduce IL-10 and TGF-β–two key Treg suppressive molecules. As the objective of the present studies was to combine the use of BETi with donor Tregs to more effectively regulate GVHD onset, the findings above support

the strategy of using both EP11313 together with Tregs. A BETi was reported to inhibit cytokine expression and APC function in dendritic cells and to decrease cytokine secretion and T cell expansion *in vivo* (17). Short-term administration of IBET151 early during BMT reduced GVHD severity supporting the notion that inhibiting BET proteins may serve as an approach for preventing GVHD (17). However, we observed that I-BET151 *in vivo* treatment for 1 week markedly reduced B cell levels in the spleen and therefore, would not be useful in the GVHD setting because it might affect recipients' immune reconstitution (data not shown). Importantly, EP11313 possesses distinct pharmacological properties, for example, this BETi is less extruded by P-glycoprotein (expressed on APC and activated T cells) across the cell membrane in comparison with I-BET762 and I-BET151 (MDR/PgP-MDCK efflux ratio BA/AB: 1.3, 27.9, 12.2, respectively, personal observation). EP11313 is therefore more highly retained intracellularly and this persistence may increase the regulation of inflammatory cytokines. Based on these properties together with the above mentioned findings showing that Tregs are not impaired by EP11313, short-term treatment (day −2 to 4) with expanded Tregs was utilized in an aHSCT. This approach was found to diminish early GVHD clinical scores including decreased ocular and skin involvement. Using highly purified donor TL1A-Ig + low dose IL-2 expanded Tregs, this second and more direct assessment of the combinatorial strategy further supported the notion that selective BETi can be used for treatment in combination with adoptive Treg therapy. Although short-term treatment with EP11313 did not enhance overall survival, BETi utilization in pre-clinical tumor models as well as clinical oncology trials have involved long-term (ex. months) administration of higher BETi doses (up to 50 mg/kg), therefore increasing the duration of BETi low dose treatment post-HSCT may further improve recipient outcomes.

It has been demonstrated that adoptive transfer of Tregs can effectively abrogate GVHD while maintaining graft-versus tumor or leukemia/lymphoma (GVT, GVL) (41, 42). In this context, we previously demonstrated using $A20^{luc/YFP}$ cells (murine B cell lymphoma) that transplanting donor TL1A-Ig + IL-2 spleen cells (containing $\sim 4 \times 10^5$ Tregs) GVHD was significantly reduced and GVL was preserved (7). In hematologic malignancies, BETi have demonstrated to possess effective anti-tumor activity by repressing aberrant oncogenic transcription (11, 43, 44). Importantly, here we showed besides GVHD amelioration, a direct effect of BETi EP11313 on A20 $^{luc/YFP}$ cell survival and proliferation. Examination of other mouse tumor cell lines i.e., EL4 (thymoma) and P815 (mastocytoma) indicated the latter was resistant to BETi effect on cell survival so not all tumors are equally sensitive to these compounds (SC, RBL unpublished observations). Our results indicate that JQ1 and EP11313 have similar anti-cancer effects (IC50 = 0.19 and 0.28 μM, respectively) in $A20^{luc/YFP}$ tumor cells. However, these BETi exhibit significantly different biological effects on Tregs, where EP11313 have no interference with Treg proliferation, phenotype and function. We hypothesize that a strategy using expanded Tregs and EP11313 may not impair GVL and could directly inhibit tumor growth. Thus, the overall mechanism proposed involves Treg mediated suppression of donor allo-reactive T cells, BETi blockage of inflammatory cytokines; and direct BETi anti-tumor activity. In total, we posit that selected BETi treatment together with expanded Treg therapy represents a novel and potentially effective combinatorial strategy for ameliorating hematologic cancer and GVHD.

AUTHOR CONTRIBUTIONS

SC designed research studies, discussed data sets, conducted experiments, analyzed data and interpreted data, wrote the manuscript. CL, HB, JM, DS, and BK conducted experiments and analyzed data. IL, DW, and CB performed research, analyzed and interpreted data, and edited the manuscript. SB supported the research. KK discussed Treg studies and supported the research. VLP discussed Treg studies and BETi and supported the research. CW discussed data and manuscript and supported the research. RBL designed research studies, discussed data sets, wrote the paper, supervised and supported the research.

ACKNOWLEDGMENTS

We thank Dr. J. Bradner (Novartis Institutes for Biomedical Research, Cambridge, MA, United States) for kindly providing JQ1. We are grateful to Dr. Oliver Umland and the Sylvester Comprehensive Cancer Center Flow Cytometry Core for excellent assistance with flow cytometry and cell sorting. We are thankful to Dr. Robert Negrin (Stanford, CA) for generously providing the $A20^{luc/YFP}$ cell line. Research support: This work was supported by funds from the Sylvester Comprehensive Cancer Center and the NIH (RO1 EY024484-01), RBL and VLP; Sylvester Comprehensive Cancer Center, RBL and SC; NIH (DA035592, DA035055 and AA023781), Sylvester Cancer Center Molecular shared Resource, and Florida Department of Health (6AZ08 and 7AZ26), CW; NIH (R01NS092671 and R01MH110441), SB; Kalish Family Foundation, KK.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03104/full#supplementary-material

REFERENCES

1. Ferrara JL, Reddy P. Pathophysiology of graft-versus-host disease. *Semin Hematol.* (2006) 43:3–10. doi: 10.1053/j.seminhematol.2005.09.001
2. Zeiser R, Blazar BR. Acute graft-versus-host disease–biologic process, prevention, and therapy. *N Engl J Med.* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
3. Hanash AM, Levy RB. Donor $CD4^+CD25^+$ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell

transplantation. *Blood* (2005) 105:1828–36. doi: 10.1182/blood-2004-08-3213
4. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med.* (2002) 196:389–99. doi: 10.1084/jem.20020399
5. Taylor PA, Lees CJ, Blazar BR. The infusion of *ex vivo* activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* (2002) 99:3493–9. doi: 10.1182/blood.V99.10.3493
6. Heinrichs J, Bastian D, Veerapathran A, Anasetti C, Betts B, Yu XZ. Regulatory T-cell therapy for graft-versus-host disease. *J Immunol Res Ther.* (2016) 1:1–14.
7. Wolf D, Barreras H, Bader CS, Copsel S, Lightbourn CO, Pfeiffer BJ, et al. Marked *in vivo* donor regulatory T cell expansion via interleukin-2 and TL1A-Ig stimulation ameliorates graft-versus-host disease but preserves graft-versus-leukemia in recipients after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2017) 23:757–66. doi: 10.1016/j.bbmt.2017.02.013
8. Copsel S, Wolf D, Kale B, Barreras H, Lightbourn CO, Bader CS, et al. Very low numbers of CD4(+) FoxP3(+) tregs expanded in donors via TL1A-Ig and low-dose IL-2 exhibit a distinct activation/functional profile and suppress GVHD in a preclinical model. *Biol Blood Marrow Transplant.* (2018) 24:1788–94. doi: 10.1016/j.bbmt.2018.04.026
9. Filippakopoulos P, Knapp S. Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat Rev Drug Discov.* (2014) 13:337–56. doi: 10.1038/nrd4286
10. Ferri E, Petosa C, McKenna CE. Bromodomains: structure, function and pharmacology of inhibition. *Biochem Pharmacol.* (2016) 106:1–18. doi: 10.1016/j.bcp.2015.12.005
11. Chaidos A, Caputo V, Karadimitris A. Inhibition of bromodomain and extra-terminal proteins (BET) as a potential therapeutic approach in haematological malignancies: emerging preclinical and clinical evidence. *Ther Adv Hematol.* (2015) 6:128–41. doi: 10.1177/2040620715576662
12. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, et al. Selective inhibition of BET bromodomains. *Nature* (2010) 468:1067–73. doi: 10.1038/nature09504
13. Nicodeme E, Jeffrey KL, Schaefer U, Beinke S, Dewell S, Chung CW, et al. Suppression of inflammation by a synthetic histone mimic. *Nature* (2010) 468:1119–23. doi: 10.1038/nature09589
14. Stathis A, Bertoni F. BET proteins as targets for anticancer treatment. *Cancer Discov.* (2018) 8:24–36. doi: 10.1158/2159-8290.CD-17-0605
15. Bandukwala HS, Gagnon J, Togher S, Greenbaum JA, Lamperti ED, Parr NJ, et al. Selective inhibition of CD4+ T-cell cytokine production and autoimmunity by BET protein and c-Myc inhibitors. *Proc Natl Acad Sci USA.* (2012) 109:14532–7. doi: 10.1073/pnas.1212264109
16. Belkina AC, Nikolajczyk BS, Denis GV. BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses. *J Immunol.* (2013) 190:3670–8. doi: 10.4049/jimmunol.1202838
17. Sun Y, Wang Y, Toubai T, Oravecz-Wilson K, Liu C, Mathewson N, et al. BET bromodomain inhibition suppresses graft-versus-host disease after allogeneic bone marrow transplantation in mice. *Blood* (2015) 125:2724–8. doi: 10.1182/blood-2014-08-598037
18. Ghosh S, Taylor A, Chin M, Huang HR, Conery AR, Mertz JA, et al. Regulatory T cell modulation by CBP/EP300 bromodomain inhibition. *J Biol Chem.* (2016) 291:13014–27. doi: 10.1074/jbc.M115.708560
19. Yu A, Zhu L, Altman NH, Malek TR. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity* (2009) 30:204–17. doi: 10.1016/j.immuni.2008.11.014
20. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol.* (2005) 6:1142–51. doi: 10.1038/ni1263
21. Liu S, Walker SR, Nelson EA, Cerulli R, Xiang M, Toniolo PA, et al. Targeting STAT5 in hematologic malignancies through inhibition of the bromodomain and extra-terminal (BET) bromodomain protein BRD2. *Mol Cancer Ther.* (2014) 13:1194–205. doi: 10.1158/1535-7163.MCT-13-0341
22. Toniolo PA, Liu S, Yeh JE, Moraes-Vieira PM, Walker SR, Vafaizadeh V, et al. Inhibiting STAT5 by the BET bromodomain inhibitor JQ1 disrupts human dendritic cell maturation. *J Immunol.* (2015) 194:3180–90. doi: 10.4049/jimmunol.1401635
23. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci USA.* (2005) 102:5126–31. doi: 10.1073/pnas.0501701102
24. Khan SQ, Tsai MS, Schreiber TH, Wolf D, Deyev VV, Podack ER. Cloning, expression, and functional characterization of TL1A-Ig. *J Immunol.* (2013) 190:1540–50. doi: 10.4049/jimmunol.1201908
25. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J Jr., Crawford JM, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood* (1996) 88:3230–9.
26. Perez VL, Barsam A, Duffort S, Urbieta M, Barreras H, Lightbourn C, et al. Novel scoring criteria for the evaluation of ocular graft-versus-host disease in a preclinical allogeneic hematopoietic stem cell transplantation animal model. *Biol Blood Marrow Transplant.* (2016) 22:1765–72. doi: 10.1016/j.bbmt.2016.07.012
27. Kaplan DH, Anderson BE, McNiff JM, Jain D, Shlomchik MJ, Shlomchik WD. Target antigens determine graft-versus-host disease phenotype. *J Immunol.* (2004) 173:5467–75. doi: 10.4049/jimmunol.173.9.5467
28. Delpoux A, Yakonowsky P, Durand A, Charvet C, Valente M, Pommier A, et al. TCR signaling events are required for maintaining CD4 regulatory T cell numbers and suppressive capacities in the periphery. *J Immunol.* (2014) 193:5914–23. doi: 10.4049/jimmunol.1400477
29. Toomer KH, Yuan X, Yang J, Dee MJ, Yu A, Malek TR. Developmental progression and interrelationship of central and effector regulatory T cell subsets. *J Immunol.* (2016) 196:3665–76. doi: 10.4049/jimmunol.1500595
30. Belkina AC, Denis GV. BET domain co-regulators in obesity, inflammation and cancer. *Nat Rev Cancer* (2012) 12:465–77. doi: 10.1038/nrc3256
31. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci USA.* (2011) 108:16669–74. doi: 10.1073/pnas.1108190108
32. Piccirillo CA, Shevach EM. Naturally-occurring $CD4^+CD25^+$ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin Immunol.* (2004) 16:81–8. doi: 10.1016/j.smim.2003.12.003
33. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol.* (2007) 8:191–7. doi: 10.1038/ni1428
34. Yuan X, Cheng G, Malek TR. The importance of regulatory T-cell heterogeneity in maintaining self-tolerance. *Immunol Rev.* (2014) 259:103–14. doi: 10.1111/imr.12163
35. Tang Q, Bluestone JA, Kang SM. CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. *J Mol Cell Biol.* (2012) 4:11–21. doi: 10.1093/jmcb/mjr047
36. Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Techmanska I, Juscinska J, et al. Administration of $CD4^+CD25^{high}CD127^-$ regulatory T cells preserves beta-cell function in type 1 diabetes in children. *Diabetes Care* (2012) 35:1817–20. doi: 10.2337/dc12-0038
37. Wolf D, Bader CS, Barreras H, Copsel S, Pfeiffer BJ, Lightbourn CO, et al. Superior immune reconstitution using Treg-expanded donor cells versus PTCy treatment in preclinical HSCT models. *JCI Insight* (2018). doi: 10.1172/jci.insight.121717. [Epub ahead of print].
38. Adeegbe DO, Liu Y, Lizotte PH, Kamihara Y, Aref AR, Almonte C, et al. Synergistic immunostimulatory effects and therapeutic benefit of combined histone deacetylase and bromodomain inhibition in non-small cell lung cancer. *Cancer Discov.* (2017) 7 852–67. doi: 10.1158/2159-8290.CD-16-1020
39. Adeegbe DO, Liu Y, Hattersley MM. BET bromodomain inhibition cooperates with PD-1 blockade to facilitate antitumor response in Kras-mutant non-small cell lung cancer. *Cancer Immunol Res.* (2018) 6:1234–45. doi: 10.1158/2326-6066.CIR-18-0077

40. Zhuang S. Regulation of STAT signaling by acetylation. *Cell Signal.* (2013) 25:1924–31. doi: 10.1016/j.cellsig.2013.05.007
41. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. $CD4^+CD25^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med.* (2003) 9:1144–50. doi: 10.1038/nm915
42. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-type specific $CD4^+CD25^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest.* (2003) 112:1688–96. doi: 10.1172/JCI17702
43. Coude MM, Braun T, Berrou J, Dupont M, Bertrand S, Masse A, et al. BET inhibitor OTX015 targets BRD2 and BRD4 and decreases c-MYC in acute leukemia cells. *Oncotarget* (2015) 6:17698–712. doi: 10.18632/oncotarget.4131
44. Chapuy B, McKeown MR, Lin CY, Monti S, Roemer MG, Qi J, et al. Discovery and characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma. *Cancer Cell* (2013) 24:777–90. doi: 10.1016/j.ccr.2013.11.003

Conflict of Interest Statement: RBL is a scientific advisory board member of Heat Biologics and a consultant for Heat Biologics and Pelican Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 January 2019
doi: 10.3389/fimmu.2018.03186

Blockade of TNFα to Improve Human CD34+ Cell Repopulating Activity in Allogeneic Stem Cell Transplantation

*Vitalyi Senyuk, Pritesh Patel, Nadim Mahmud and Damiano Rondelli**

Division of Hematology/Oncology, Department of Medicine and UI Cancer Center, University of Illinois at Chicago, Chicago, IL, United States

Early release of TNFα after hematopoietic stem cell transplantation (HSCT) correlates with development of acute graft-vs.-host disease (GVHD). Here we tested the effect of TNFα and alloreactive T cells on early hematopoietic HSC genotype and function. Addition of TNFα (10 ng/ml) in liquid cultures with CD34+ cells for 6–72 h resulted in the downregulation of genes associated with stem cell activity, such as DNMT3A, DNMT3B, TET1, TET2, SOX2, NANOG, and OCT4, whereas no significant effect was observed on DNMT1 and GATA2 expression. These findings were reversed by using an anti-TNFα antibody. Similar gene downregulation was observed when CD34+ cells were co-cultured with alloreactive T cells CD34+ cells for 48–72 h, and this effect was partially prevented by rapamycin and an anti-TNFα antibody. CD34+ cells pre-incubated with TNFα for 48 h and transplanted in irradiated NOD-SCID γ^{null} (NSG) mice showed a reduced myeloid engraftment compared to control mice. By using a xenograft model recently developed in our lab, we co-transplanted CD34+ cells and allogeneic T lymphocytes at 1:0.1 ratio in one group that also received etanercept (TNFα inhibitor) at 100 μg intra-peritoneum (i.p.) on days −1,+1,+3,+5 post-HSCT, and in the control group. At 6 weeks post-transplant, mice that received etanercept had a significantly higher number of marrow huCD45+CD34+CD38- early stem cells ($p = 0.03$) and a reduced number of huCD45+CD3+ splenic T cells ($p = 0.04$) compared to controls. The repopulating activity of marrow cells from mice treated with etanercept vs. controls was tested in secondary transplants. Although the overall engraftment was similar in the two groups, CD34+ cells isolated from recipients of marrow from the etanercept group showed a significantly greater expression of stem cell-associated genes and a higher number of CD45+CD34+CD38- cells than in controls ($p = 0.03$). Our findings suggest that early TNFα increase post-transplant can affect long-term stem cell engraftment, and that blockade of TNFα early after transplant may limit a cytokine-mediated suppressive effect on repopulating stem cell function.

Keywords: CD34, TNFα, transplantation, GVHD, T cells, repopulating activity

OPEN ACCESS

Edited by:
Brian Christopher Betts, University of Minnesota Twin Cities, United States

Reviewed by:
Hung D. Nguyen, Medical University of South Carolina, United States
Lohith Gowda, Yale University, United States

****Correspondence:***
Damiano Rondelli
drond@uic.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *16 October 2018*
Accepted: *31 December 2018*
Published: *22 January 2019*

Citation:
Senyuk V, Patel P, Mahmud N and Rondelli D (2019) Blockade of TNFα to Improve Human CD34+ Cell Repopulating Activity in Allogeneic Stem Cell Transplantation. Front. Immunol. 9:3186. doi: 10.3389/fimmu.2018.03186

INTRODUCTION

The engraftment of donor hematopoietic stem cells (HSC) after transplantation requires a profound immunosuppression of the host to prevent the risk of rejection. However, the immunosuppression of the host also favors the expansion of donor immune cells that can target non-hematopoietic tissues and cause graft-vs.-host disease (GVHD) (1). The immunologic events determining GVHD start immediately after transplant and are largely based on an initial release of pro-inflammatory cytokines caused by the effect of chemotherapy/radiotherapy and antigen presenting cell: T cell interaction (1, 2). In fact, patients undergoing an allogeneic HSC transplant (HSCT) from HLA matched donors receive GVHD prophylaxis starting from the time of transplant and, in case of transplant from HLA haploidentical donors, with high doses of cyclophosphamide administered on days +3 and + 4 after transplant (3).

TNFα is a pro-inflammatory cytokine released particularly by donor T cells upon transplantation, and has previously been demonstrated to play a key role in the initial immune process leading to GVHD, both facilitating the activation of antigen presenting cells (APC) and the expansion of alloreactive T cells (4, 5). Moreover, detection of an increased serum level of TNFα receptor 1 in patients within the first week after transplantation is one of the biomarkers predicting future development of GVHD (6).

Previous findings from our lab demonstrated that TNFα mediates a direct effect of T cells on a subset of CD34+ cells hematopoietic progenitors, inducing their differentiation into the monocytic/dendritic lineage and increasing their direct as well as indirect antigen presenting function (7). Here we tested the question whether early hematopoietic stem cells with repopulating stem cell activity could also be a target of TNFα, thus hypothesizing that stem cell engraftment after transplantation could be affected by TNFα. We analyzed the *in vitro* effect of TNFα, as well as of allogeneic T cells, on CD34+ cell expression of genes regulating DNA methylation or pluripotency, such as DNMT1, DNMT3A, DNMT3B, NANOG, OCT4, SOX2 (8, 9). Then, we utilized a xenograft transplant (10) model to study the *in-vivo* effect of TNFα on HSC and the role of a TNFα inhibitor after co-transplantation of CD34+ and allogeneic T cells. The results shown here suggest that TNFα can affect early HSC and that blockade of TNFα may preserve a pool of stem cells with repopulating activity. Based on these findings, new therapeutic strategies may be tested to better protect stem cell engraftment after allogeneic transplantation.

MATERIALS AND METHODS

Cell Separation

Healthy donor G-CSF mobilized peripheral blood stem cells (PBSC) from AllCells (Alameda, CA) and PB cells from healthy volunteers were utilized in this study. Mononuclear cells (MNC), $CD34^+$ cells and CD3+ T cells were purified as previously described (10). Isolated CD34+, or T cell samples were acquired on a FACS Calibur™ (Becton Dickinson) and analyzed using the Cell Quest ™ software (Becton Dickinson), and showed, on average, >95% cell purity.

Flow Cytometry

Fluorescein isthiocyanate (FITC), or phycoerythrin (PE), or peridin chlorophyll protein (PerCP), conjugated mAbs (CD45, CD34, CD38, CD33, CD3) or isotype controls (Becton-Dickinson, San Jose', CA) were employed. Stained cells were washed twice in PBS and sample acquisition and analysis was performed within 2 h on a FACSCaliburTM (Becton Dickinson).

Co-cultures of CD34+ and T Cells

Purified human CD34+ cells (1–2 x 10^5 cells) were co-cultured with human allogeneic T cells at 1:0.1, or 1:2 ratio in round-bottomed 96-well plates for 48–72 h at 37°C in a 5% CO2 humidified atmosphere, as previously described. In selected experiments, CD34+ cells and T cells were cultured in the presence of the following molecules described: TNFα, Rapamycin, Cyclosporin A (Sigma-Aldrich (St. Louis, MO), Mycophenolate Motefil (Cayman Chemical Company, Ann Arbor, MI), Abatacept (Bristol Meyers Squibb, New York, NY), rabbit anti-thymocyte globulin (rATG, Thymoglobulin, Genzyme, Cambridge, MA), anti-TNFα antibody (AF-210-NA) from R&D Systems (Minneapolis, MN).

qRT-PCR

CD34+ cells re-isolated on human CD34+ MicroBead Kit UltraPure (Miltenyi Biotec, Bergisch Gladbach, Germany) after MLC or after transplantation were used for total RNA extraction with TRIzol reagent (Life Technologies Corporation, Grand Island, NY). RNA was transcribed into cDNA with SuperScript® III First-Strand Synthesis SuperMix (Life Technologies Corporation, Grand Island, NY) and analyzed with SYBR green (Applied Biosystems, Inc., Grand Island, NY) on the 7500 FAST Real Time PCR detection system (Applied Biosystems, Inc., Grand Island, NY). The human primers used are: ACTB, forward: 5-ggacttcgagcaagagatgg-3′, reverse: 5′-agc actcgtgttggcgtacag-3′; DNMT1, forward: 5′-tgctgaagcctccga gat-3′, reverse: 5′-ttctgttaagctgtctctttcca-3′; DNMT3A, forward: 5′-tacttccagagcttcagggc-3′, reverse: 5′-attccttctcacaacccgc-3; DNMT3B, forward: 5′-gagattcgcgagcccag-3′, reverse: 5′-tctcca ttgagatgcctggt-3′; TET1, forward: 5′-gagggaaaagaagcccaaag-3′, reverse: 5′-tcttccccatgaccacatct-3′; TET2, forward: 5′-agaaaag ggaaaggagagcg-3′, reverse: 5′-gagagggtgtgctgctgaat-3; TET3, forward: 5′-gccggtcaatggtgctagag-3′, reverse: 5′-cggttgaaggtt tcatagagcc-3′; NANOG, forward: 5′-gatttgtgggcctgaagaaa-3′, reverse: 5′-cagggctgtcctgaataagc-3′; OCT4, forward: 5′-gtggag gaagctgacaacaa-3′, reverse: 5′-ggttctcgatactggttcgc-3; SOX2, forward: 5′-aaccccaagatgcaccaactc-3′, reverse: 5′-gcttagcctcgtcga tgaac-3,. GATA2, forward: 5′- cacaagatgaatgggcagaa−3′, reverse: 5′- acaatttgcacaacaggtgc−3′.

TNFα Blockade

In vitro TNFα blockade was tested in MLC assays with anti-TNFα antibody (AF-210-NA). In titration experiment, we tested 0.1 μg/ml, 0.5 μg/ml and 1 μg/ml of anti-TNFα antibody, and in selected experiments at 5 μg/ml. The tested anti-TNFα/

TNFα excess range (10x−100x) covers whole possible TNFα neutralization range, according to the manufacturer's guide. *In vivo* TNFα blockade was tested in NSG mice co-transplanted with CD34+ and allogeneic T cells at 1:0.1 ratio by injecting etanercept (Enbrel, Immunex Corporation, Thousand Oaks, CA) intra-peritoneum (i.p.).

Transplantation

Immunodeficient nonobese diabetic/*ltsz-scid*/*scid* (NOD/SCID) IL2 receptor gamma chain knockout mice (NSG) were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in a strict barrier environment. The study was approved by UIC Animal Care Committee and performed in accordance with national guidelines of laboratory animal care. Human CD34+ cells were initially incubated with or without TNFα (10 ng/ml) *in-vitro* for 48 h, re-isolated immune-magnetically using a human CD34+ MicroBead Kit UltraPure (Miltenyi Biotec, Bergisch Gladbach, Germany) and 2×10^5 cells were transplanted intravenously (i.v.) into sublethally irradiated (300 cGy) NSG mice as previously described (10). In a second set of experiments, purified CD34+ cells (2×10^5/mouse) were mixed at 1:0.1 ratio with allogeneic T cells and then injected i.v. into sublethally irradiated (300 cGy) NSG mice. A group of mice was also injected intra-peritoneum (i.p.) with etanercept at 100 μg/mouse on days: −1, +1, +3 and +5 post-transplant ($n = 10$ mice/group). At day 42 post-transplant, marrow cells were collected to analyze engraftment, as well as to perform secondary transplants. To this purpose, marrow cells obtained after primary transplant were depleted of human T cells on a Miltenyi column after incubating the cells with MicroBeads conjugated to monoclonal anti-human CD3 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Then secondary NSG mice were transplanted with 5×10^6 T cell depleted marrow cells/mouse from the etanercept and control groups ($n = 5$ mice/group). Forty-two days following second transplant, mice were sacrificed and analyzed for stem cell engraftment in the marrow andspleen, as previously described (10). Human cell engraftment was assessed by measuring the expression of huCD45 marker. Analysis of all markers on marrow or spleen cells was performed on gated huCD45+ cells.

Statistical Analysis

Student *t*-test was performed to compare 2 series of data. Statistical tests were performed by using Graph Pad Prism version 7.0 (GraphPad Inc., San Diego, CA).

RESULTS

TNFα-mediated Downregulation of Early Stem Cell Gene Expression in CD34+ Cells

It has been previously demonstrated that TNFα induces the differentiation of a subset of CD34+ cells committed to the monocytic-dendritic lineage within 3–5 days of liquid culture (7). To test whether also early hematopoietic progenitors can be targeted by the effect of TNFα, we incubated human CD34+ cells with or without TNFα at 10 ng/ml for 6–72 h and then extracted their mRNA to measure the expression of genes associated with self-renewal or pluripotent stem cell activity, such as: GATA2, DNMT1, DNMT3A, DNMT3B, TET1, TET2, TET3, NANOG, SOX2, and OCT4 (**Figure 1A**). In 5 separate experiments, GATA2 expression was not affected by TNFα at any time point (data not shown). On the contrary, all the other genes were significantly downregulated at either one or all three time points, compared to control experiments without TNFα. The low expression of DNMT3A, DNMT3B, TET2, NANOG, SOX2, and OCT4 at 24 and 72 h suggested that genes regulating stem cell proliferation can be rapidly downregulated by TNFα in CD34+ cells. We then incubated the CD34+ cells with TNFα and increasing doses of a blocking anti-TNFα antibody for 72 h. The expression of all the genes previously downregulated was restored to the level of control CD34+ in the presence of higher doses of the blocking antibody (**Figure 1B**). In control experiments, the anti-TNFa antibody alone did not affect gene expression on CD34+ cells (data unshown). These findings showed that TNFα directly modified the expression of multiple genes in CD34+ cells.

T Cell-mediated Downregulation of Early Stem Cell Gene Expression in CD34+ Cells

We previously demonstrated that allogeneic T cells induce a rapid differentiation of a subset of CD34+ cells into monocytic/dentritic cells, mostly mediated by TNFα (7). Based on the observation above that TNFα downregulated the expression of genes associated with DNA methylation and pluripotent stem cell activity, we tested whether alloreactive T cells can induce a similar effect. We incubated CD34+ cells with allogeneic CD3+ T cells at 1:0 (control), 1:0.1 or 1:2 ratio as previously described (10). After 72 h, CD34+ cells were immunomagnetically re-purified and their mRNA was isolated to assess the gene expression. In the presence of low number of T cells (1:0.1 ratio), only DNMT3A, TET1, and NANOG expression was decreased, whereas at higher concentration of T cells (1:2 ratio) all the genes tested were downregulated with the exception of DNMT1 (**Figure 2**) and GATA2 (not shown). These findings suggested that alloreactive T cells may affect the expression of some of the genes associated with early stem cell activity in CD34+ cells.

Variable Effect of Standard Immunosuppressive Agents on T Cell-mediated Epigenetic Changes in CD34+ Cells

Since we observed that alloreactive T cells rapidly affect the expression of genes that could regulate stem cell long term engraftment, we investigated on whether an anti-TNFα antibody or immunosuppressive molecules commonly used as GVHD prophylaxis could prevent T cell effect on CD34+ cells. We performed 72 h liquid cultures with CD34+ and allogeneic T cells at 1:2 ratio or CD34+ alone, then we added standard immunosuppressive agents such as cyclosporine A (1 μg/ml), rapamycin (1 μg /ml), mycophenolate mofetil (0.5 μg/ml), abatacept (100 μg /ml), thymoglobulin (100 μg /ml) or an anti-TNFα antibody (5 μg/ml) (**Figure 3**). After 72 h the CD34+ cells were immunomagnetically re-isolated in order to extract

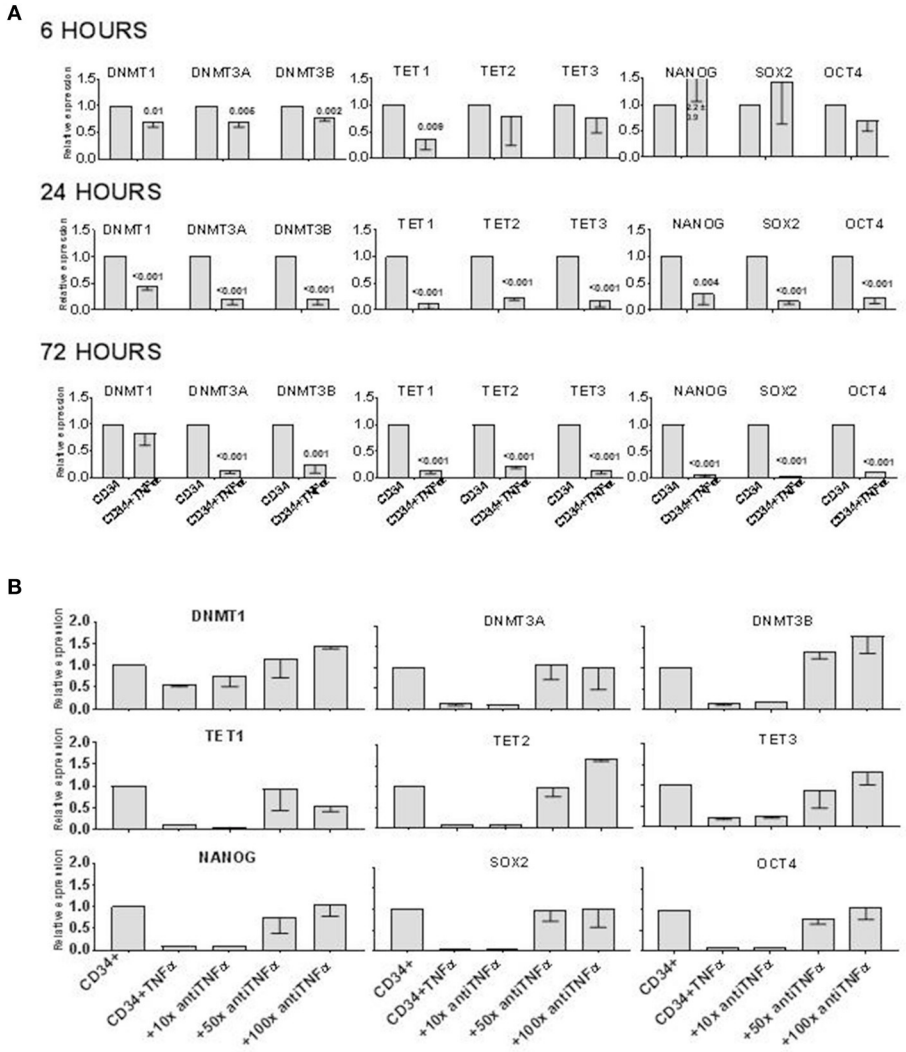


FIGURE 1 | TNFα downregulates epigenetics- and pluripotency-related genes in CD34+ cells *in vitro*. **(A)** CD34+ cells were incubated *in vitro* ± TNFα (10 ng/ml) for 6–72 h, before analyzing expression of TET1, TET2, TET3, DNMT1, DNMT3A, DBMT3B, NANOG, SOX2, OCT4 genes by qRT PCR. Gene expression in CD34+ cells alone was arbitrary taken as 1 for each gene. ACTB expression was used as normalization control. The data is shown as mean values of 4 separate experiments and standard deviation bars are included. Differences in the expression of each gene in cells treated with TNFα and controls were calculated by *t*-test and *p*-values are shown. **(B)** The effect of TNFα on gene expression in CD34+ cells was prevented by adding an anti-TNFα antibody to liquid culture with CD34+ cells. The antibody (1:100 = 1 μg/ml) was added at 10x, or 50x, or 100x excess of TNFα (by mass) to CD34+ cells cultured alone or with TNFα for 72 h before qRT PCR analysis. Gene expression in CD34+ cells alone was arbitrary taken as 1 for each gene. ACTB expression was used as normalization control. The data is shown as mean values of 4 separate experiments and standard deviation bars are included.

the mRNA and test gene expression. Although none of the immunosuppressive drugs, or the anti-TNFα antibody, could completely prevent the T cell-mediated gene downregulation in CD34+ cells, a trend for a greater activity in preserving epigenetic and pluripotency gene expression was observed with rapamycin and anti-TNFα antibody, suggesting possible combination studies.

Short Exposure to TNFα Reduces CD34+ Cell Engraftment After Transplant

In order to test whether TNFα may affect the repopulating activity of CD34+ cells, initial experiments were designed to transplant CD34+ cells into NSG mice after short exposure to TNFα. Liquid cultures of CD34+ cells with or without TNFα at 10 ng/ml concentration were carried out for 48 h. Analysis of gene expression (not shown) confirmed downregulation of genes associated with early stem cell activity, as described above. CD34+ cells pretreated with TNFα for 48 h, or untreated CD34+ cells as control, were washed and then transplanted into sublethally irradiated NSG mice at 1×10^5 CD34+ cells/mice ($n = 5$ mice per group). Five to 6 weeks after transplant the mice were sacrificed and stem cell engraftment was measured in the marrow and spleen by flow cytometry. Mice transplanted with CD34+ cells pretreated with TNFα had a lower bone

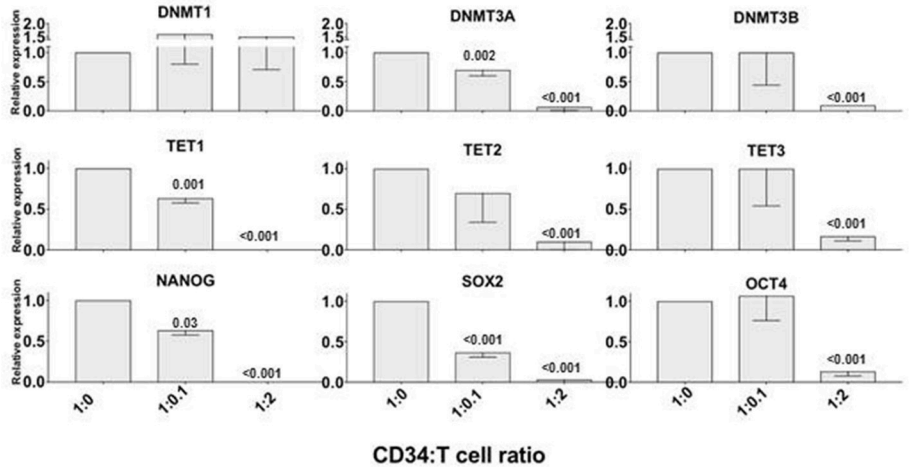


FIGURE 2 | Alloreactive T cells rapidly downregulate epigenetics- and pluripotency-related genes in CD34+ cells. Purified CD34+ cells were co-cultured with allogeneic CD3+ T cells at 1:0 (control), 1:0.1 and 1:2 ratios for 72 h; then were re-isolated immunomagnetically before analyzing the expression of TET1, TET2, TET3, DNMT1, DNMT3A, DBMT3B, NANOG, SOX2, OCT4 genes by qRT PCR. Gene expression in CD34+ cells cultured without T cells was arbitrary taken as 1 for each gene. ACTB expression was used as normalization control. The data is shown as mean values of 3 separate experiments and standard deviation bars are included. Differences in the expression of each gene in CD34+ cells co-cultured with T cells vs. control were calculated by *t*-test and significant *p*-values are shown.

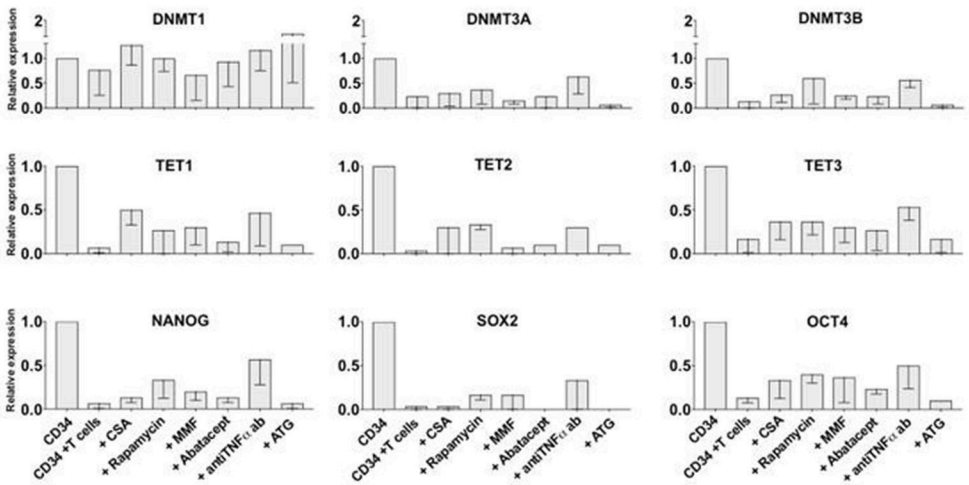


FIGURE 3 | Partial effect of standard immunosuppressive agents in preventing T cell-mediated epigenetic and pluripotency gene downregulation in CD34+ cells *in-vitro*. CD34+ and allogeneic T cells were co-cultured at 1:2 ratio for 72 h with or without one of the following standard immunosuppressive molecules: cyclosporin A, rapamycin, mycophenolate mofetil, abatacept, anti-TNFα antibody, ATG. CD34+ cells were then re-isolated immunomagnetically and analyzed by qRT PCR. Gene expression in CD34+ cells cultured without T cells as control was arbitrary taken as 1 for each gene. ACTB expression was used as normalization control. The data is shown as mean values of 3 separate experiments and standard deviation bars are included.

marrow stem cell engraftment compared to controls, shown in a representative example in **Figure 4A**, and documented as lower percentage and absolute number of huCD45+ cells (**Figure 4B**). We also analyzed whether exposure of CD34+ cells to TNFα would affect the pool of marrow CD34+ cells after transplant, possibly responsible for long term engraftment. The absolute number of CD34+ cells was found significantly lower in the marrow of mice transplanted with TNFα-pretreated CD34+ cells (**Figure 4C**). These findings are consistent with the hypothesis that a brief exposure to TNFα may impair the engraftment ability of CD34+ cells.

Etanercept Preserves the Engraftment of CD34+CD38- Cells After Co-transplantation With Allogeneic T Cells

We recently observed that co-transplantation of CD34+ and allogeneic T cells at 1:0.1 ratio in a xenograft model results in low stem cell engraftment and in the expansion of T cells (10). Here we adopted this model to test whether *in-vivo* blockade of TNFα would improve the engraftment of CD34+ cells by reducing the effect of alloreactivity. Two groups of NSG mice were co-transplanted with CD34+ cells and T lymphocytes at 1:0.1 ratio

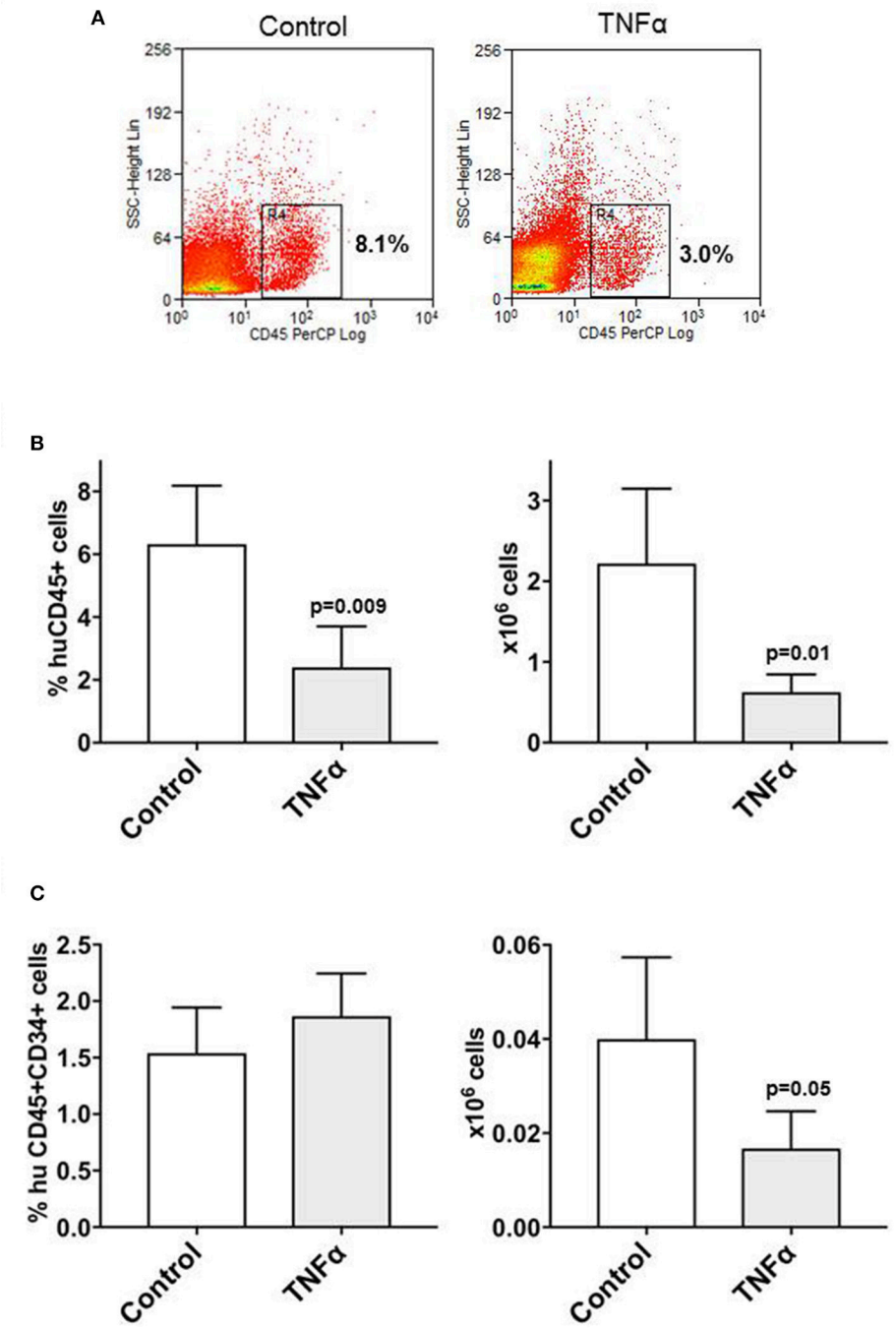


FIGURE 4 | Pre-exposure of CD34+ cells to TNFα reduces stem cell engraftment in NSG mice**.** Purified CD34+ cells were initially cultured *in-vitro* with/without TNFα (10 ng/ml) for 48 h and then 2 sets of sublethally irradiated NSG mice ($n = 5$ each) were transplanted. Five weeks transplantation, the mice were sacrificed and cells isolated from the bone marrow were stained with anti-huCD45 and anti-CD34 antibodies. The reduced engraftment of human CD45+ cells in mice transplanted with CD34+ cells pre-treated with TNFα is shown in one representative example of cytofluorimetric analysis from each group of mice **(A)**. After transplant, the average percentage and absolute number of huCD45+ cells detected in the marrow of mice pre-treated with TNFα were significantly lower than in control animals **(B)**, as well as the absolute number of marrow huCD45+CD34+ cells **(C)**. Differences between groups were analyzed by *t*-test and significant *p*-values are shown.

and one of these groups was injected i.p. with etanercept at 100 μg on days −1, +1, +3 and + 5 post transplant. Six weeks after transplant, the marrow of mice treated with etanercept and those in the control group showed similar percentages of huCD45+ cells and human myeloid CD33+ cells (**Figure 5A**). Instead, the percentage and absolute number of early hematopoietic

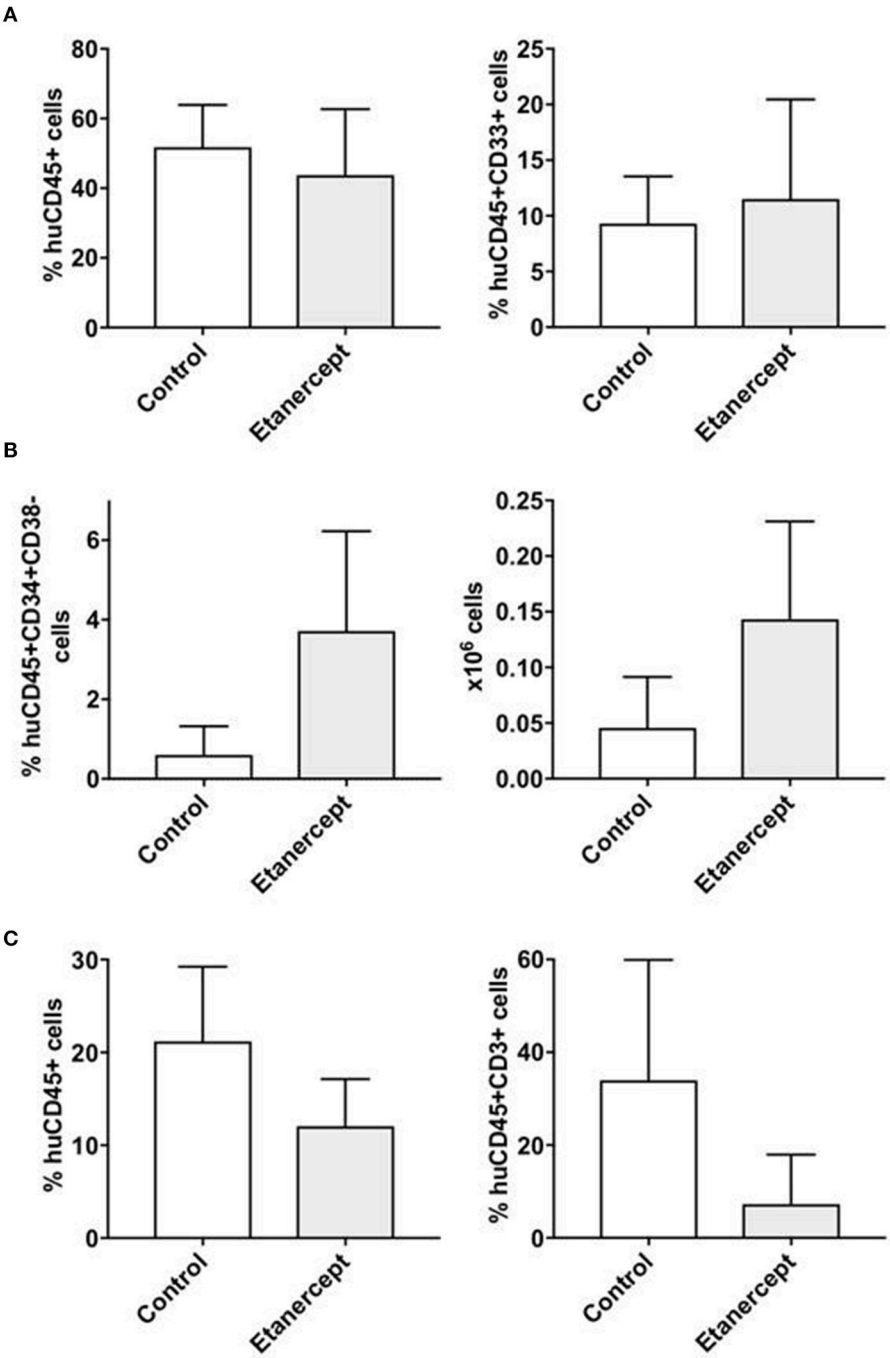


FIGURE 5 | Peri-transplant treatment with etanercept improves the persistence of CD34+CD38- early hematopoietic progenitors after co-transplantation of CD34+ and allogeneic T cells in NSG mice. NSG mice were sublethaly irradiated and co-transplanted with CD34+ cells (2×10^5/mouse) and allogeneic T cells at 1:0.1 ratio. One group of mice ($n = 10$) were also injected i.p. with TNFα inhibitor etanercept on days: −1, +1, +3 and +5 following transplantation, while the control group ($n = 10$) did not receive etanercept. Six weeks after transplantation, the mice were sacrificed and bone marrow and spleen cells were isolated and analyzed by flow cytometry to detect the percentage and the absolute number of human **(A)** myeloid (CD45+CD33+) and **(B)** lymphoid T (CD45+CD3+) cells, as well as for **(C)** early hematopoietic progenitors (CD45+CD34+CD38-) cells. The results are represented as mean values and standard deviation bars are shown. Differences between groups were analyzed by *t*-test and significant *p*-values are shown.

progenitors identified as CD45+CD34+CD38- cells were significantly higher in the etanercept group ($p = 0.03$), compared to control mice (**Figure 5B**). In addition, mice treated with etanercept showed a significantly lower expansion of T cells in the spleen, as demonstrated by a lower percentage of CD45+ and CD45+CD3+ cells in the spleen (**Figure 5C**).

These findings suggested that early blockade of TNFα after transplant may preserve the pool of early hematopoietic stem cells. To test whether these cells also had a greater repopulating stem cell activity, a secondary transplant with marrow cells obtained from the etanercept and control groups was performed. Secondary NSG mice were transplanted with equivalent number of marrow cells collected from primary transplants and 6 week later (total 12 weeks) the marrow was analyzed for myeloid engraftment and persistence of early progenitor cells. Recipients of marrow cells from the etanercept and control groups had comparable percentages of CD45+ and CD45+CD33+ myeloid cells after secondary transplant (not shown). However, a higher percentage and absolute number of CD34+CD38- cells were detected in recipients of marrow cells from the etanercept group (**Figure 6A**). Finally, CD34+ cells from both the etanercept and control groups were re-isolated after transplant. Because of the small absolute number of CD34+ cells, we could not analyze each single mouse separately and marrows from either group were pooled before CD34+ cell selection and RNA extraction. The results of this experiment (**Figure 6B**) showed a 4–8 fold increase of gene expression in the etanercept group compared to mice that had not received etanercept not during primary transplant. Persistence of high expression of pluripotent genes in CD34+ cells from the etanercept group after secondary transplant did not correlate with a skiewed differentiation capacity since myeloid engraftment was comparable in the two groups. These data are consistent with initial hypothesis that release of TNFα immediately after transplant may affect long-term reconstitution of hematopoietic progenitors.

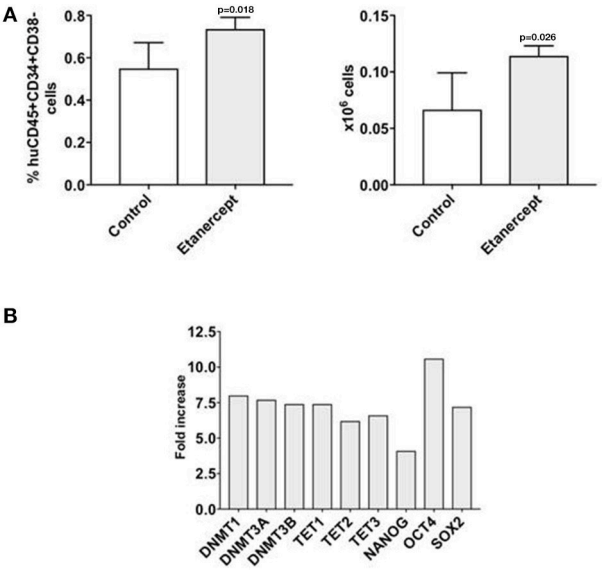


FIGURE 6 | Early treatment with etanercept in primary transplant preserves the pool of CD34+CD38- hematopoietic progenitors after secondary transplant. Bone marrow cells were isolated from NSG mice 42 days after primary transplant with CD34+ and allogeneic T cells at 1:0.1 ratio, with/without treatment with etanercept. A fixed amount of 5×10^6 T cell depleted marrow cells/mouse were re-transplanted in a secondary transplant in new NSG mice. After 42 more days, mice were sacrificed and bone marrow were isolated and analyzed by flow cytometry **(A)** to detect the percentage and the absolute number of human early hematopoietic progenitors (CD45+CD34+CD38-) cells. The results are represented as mean values and standard deviation bars are shown. Differences between groups were analyzed by *t*-test and significant *p*-values are shown. Another fraction of marrow cells were utilized to analyze the gene expression on CD34+ cells **(B)**. Because of the limited number of CD34+ cells, marrow cells from each group had to be pooled together to be re-isolated and analyzed by qRT PCR. In this experiment CD34+ cells from controls (untreated animals) were arbitrary taken as 1 for each gene. ACTB expression was used as normalization control. The results are shown as fold-increase of expression for each gene analyzed in the CD34+ cells isolated after secondary transplant in the etanercept group vs. control.

DISCUSSION

This study demonstrates that TNFα and alloreactive T cells rapidly affect human early hematopoietic precursors by downregulating the expression of genes associated with self-renewal and pluripotent stem cell activity, and affecting the engraftment of repopulating HSC *in-vivo*. A protective effect on repopulating HSC was elicited by blockade of TNFα in a xenograft model of co-transplantation of CD34+ and allogeneic T cells.

The role of TNFα in the development of GVHD has been extensively studied both in experimental models and in the clinical setting (1, 2, 4, 11–13). In this latter, it was also demonstrated that increased serum levels of TNFR1 on day 7 after transplant were shown to predict patients who then developed GVHD (6, 14–16). TNFα receptors (TNFR1 and TNFR2) were previously detected also on CD34+ hematopoietic progenitors (17, 18). Stimulation of TNF receptors with TNFα was then shown to upregulate the expression of interferon ɤ (IFNɤR) and FasL receptors, thus increasing their susceptibility to inhibitory effects of TNFα or IFNɤ. However, although some experimental models suggested a negative effect of TNFα in regulating normal hematopoiesis *in-vivo* (19, 20), other studies observed an impaired long-term hematopoietic reconstitution in mice lacking TNFR1 (21). We previously showed that committed CD34+ progenitors can stimulate allogeneic T cell responses *in-vitro* (22, 23) via B7:CD28 costimulation, and that ∼70% of PB CD34+ cells upregulated CD40 costimulatory molecule and increased their immunostimulatory activity following 24 h exposure to TNFα (24). Endogenous TNFα released in co-cultures of CD34+ and allogeneic T cells was then demonstrated to mediate the rapid differentiation of a subset of CD34+ cells into monocytic/dendritic cells, whereas co-transplantation of CD34+ and T cells into NOD/SCID mice resulted in increased differentiation of mature dendritic cells (7). Our current study shows for the first time that within 6–72 h in liquid culture, TNFα downregulated the expression of some of the genes regulating stem cell methylation, such as DNMT3A/B, or TET1, TET2, TET3, and other transcription factor genes, such as NANOG, SOX2, or OCT4 that are associated with pluripotent status of stem cells (8, 9). Since it has previously reported that: *de novo* DNA methylation through DNMT3A/B activity is essential for stem cell long term reconstitution activity (25, 26); concomitant reduction of DNMT3A and TET-mediated DNA

methylation can impair both self-renewal and differentiation of hematopoietic stem cells (27); and transcription factors such as OCT4, SOX2, and NANOG contribute to maintaining the pluripotency properties of early stem cells (9), it is conceivable that the effect of TNFα may target the epigenetic regulation of early CD34+ cells capable of post-transplant long-term hematopoietic reconstitution. Based on these observations on the downregulation of some of the genes affecting DNA methylation concomitantly with a reduced engraftment ability of HSC, new studies can be started to address the causality of each gene dysregulation and CD34+ cell post-transplant engraftment capacity. Because the same genes downregulated by TNFα were also rapidly downregulated *in-vitro* by allogeneic T cells, the cytokine storm caused by alloreactive T cells in the early phase post-transplant may affect CD34+ cell function either through TNFα alone, or through a combination of multiple soluble factors. Indeed, the T cell mediated epigenetic changes on CD34+ cells were only partially prevented by an anti-TNFα antibody. Interestingly, when we tested the effect of immunosuppressive molecules that are commonly used to prevent GVHD, such as cyclosporine A, rapamycin, mycophenolate mofetil, thymoglobulin, abatacept, or an anti-TNFα antibody in co-cultures of CD34+ and allogeneic T cells, none of them could completely prevent gene downregulation, and only rapamycin and anti-TNFα ab showed some partial protective effect. Future studies will test the effect of combination of these two agents. Consistent with *in-vitro* data, transplantation of CD34+ cells exposed to TNFα for 48 h resulted in a lower engraftment. In addition, by utilizing a xenograft model of co-transplantation of CD34+ and allogeneic T cells recently developed in our lab (10), we showed that injection of etanercept in the first week after transplant maintained a greater pool of CD34+CD38- hematopoietic progenitors after both primary and secondary transplant. These findings are consistent with a recent study where the authors transplanted high numbers of human umbilical cord (UC) cells into NSG mice and assessed the engraftment after only 4 weeks, because of xenogeneic GVHD limiting mice survival. In this study, they demonstrated that UC T cells produced high levels of multiple cytokines, including TNFα, which directly impaired stem cell engraftment (28). Although they did not test the repopulating stem cell activity in secondary transplants, they clearly showed a negative effect of T cell-derived TNFα on short and long-term hematopoietic stem cell subsets by inducing stem cell apoptosis, and proved that stem cell engraftment in mice transplanted with UC grafts plus etanercept was comparable to those receiving T cell-depleted grafts. Similarly, we demonstrated that in mice transplanted with CD34+ and allogeneic T cells TNFα blockade with etanercept in the first week of transplant allowed a greater engraftment of CD34+CD38- cells, not only in primary but also in secondary transplants. This could be due to limiting T cell expansion and preventing a TNFα-mediated epigenetic dysregulation of the physiologic balance of stem cell self-renewal and differentiation activity of CD34+ cells. This hypothesis was indirectly supported by our observation of higher expression of DNMTs, TETs and pluripotency transcription factor genes in CD34+ cells obtained from the etanercept group after secondary transplants.

All these findings expand the knowledge of an early effect of TNFα on the human hematopoietic system after allogeneic stem cell transplantation. In a clinical setting, it is conceivable that the pool of newly transplanted stem cells may be partially affected by the cytokines released by alloreactive T cells. Depending on the overall number of stem cells transplanted into a patient, a small loss of stem cell function may not be clinically relevant since the graft may have an abundant number of residual stem cells to guarantee the engraftment. However, patients fully engrafted with donor cells and yet experiencing late post-transplant cytopenias (29), or those with cytopenias in the context of GVHD, could be affected by a T cell-mediated anti-stem cell reactivity. This could be identified as a cytokine-mediated graft-vs.-marrow disease. TNFα blockade was previously proven to ameliorate acute GVHD in combination with other immunosuppressive drugs (30–32). Our findings could prompt new studies testing etanercept in the context of GVHD prophylaxis, not only to better prevent GVHD, but also to protect donor HSC from the detrimental effect of TNFα and facilitate long-term engraftment.

AUTHOR CONTRIBUTIONS

VS, PP, and DR contributed to the study design. VS, NM, and PP performed all the experiments, VS and DR analyzed the data. VS and DR wrote of the manuscript. All the authors assisted in the critical review of the manuscript and approved the final version of the manuscript for submission.

ACKNOWLEDGMENTS

The study was partially supported by a grant from the Perry Family Foundation; a grant from the Michael Reese Research & Education Foundation; and a grant from the Thorek Memorial Foundation to DR.

REFERENCES

1. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
2. Antin JH, Ferrara JL. Cytokine dysregulation and acute graft-versus-host disease. *Blood* (1992) 80:2964–8.
3. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant.* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
4. Levine JE. Implications of TNF-alpha in the pathogenesis and management of GVHD. *Int J Hematol.* (2011) 93:571–7. doi: 10.1007/s12185-011-0803-1
5. Holler E, Kolb HJ, Moller A, Kempeni J, Liesenfeld S, Pechumer H, et al. Increased serum levels of tumor necrosis factor alpha precede major complications of bone marrow transplantation. *Blood* (1990) 75:1011–6.
6. Levine JE, Braun TM, Harris AC, Holler E, Taylor A, Miller H, et al. Transplant clinical trials: a prognostic score for acute graft-versus-host disease

based on biomarkers: a multicentre study. *Lancet Haematol.* (2015) 2:e21–9. doi: 10.1016/S2352-3026(14)00035-0
7. Abbasian J, Mahmud D, Mahmud N, Chunduri S, Araki H, Reddy P, et al. Allogeneic T cells induce rapid CD34+ cell differentiation into CD11c+CD86+ cells with direct and indirect antigen-presenting function. *Blood* (2006) 108:203–8. doi: 10.1182/blood-2005-11-4330
8. An J, Rao A, Ko M. TET family dioxygenases and DNA demethylation in stem cells and cancers. *Exp Mol Med.* (2017) 49:e323. doi: 10.1038/emm.2017.5
9. Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* (2008) 132:567–82. doi: 10.1016/j.cell.2008.01.015
10. Oh AL, Mahmud D, Nicolini B, Mahmud N, Senyuk V, Patel PR, et al. T cell-mediated rejection of human CD34(+) cells is prevented by costimulatory blockade in a xenograft model. *Biol Blood Marrow Transplant.* (2017) 23:2048–56. doi: 10.1016/j.bbmt.2017.08.009
11. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol.* (2007) 25:139–70. doi: 10.1146/annurev.immunol.25.022106.141606
12. Cooke KR, Hill GR, Crawford JM, Bungard D, Brinson YS, Delmonte J Jr, et al. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. *J Clin Invest*. (1998) 102:1882–91. doi: 10.1172/JCI4285
13. Schmaltz C, Alpdogan O, Muriglan SJ, Kappel BJ, Rotolo JA, Ricchetti ET, et al. Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood* (2003)101:2440–5. doi: 10.1182/blood-2002-07-2109
14. Kitko CL, Paczesny S, Yanik G, Braun T, Jones D, Whitfield J, et al. Plasma elevations of tumor necrosis factor-receptor-1 at day 7 postallogeneic transplant correlate with graft-versus-host disease severity and overall survival in pediatric patients. *Biol Blood Marrow Transplant.* (2008) 14:759–65. doi: 10.1016/j.bbmt.2008.04.002
15. Choi SW, Kitko CL, Braun T, Paczesny S, Yanik G, Mineishi S, et al. Change in plasma tumor necrosis factor receptor 1 levels in the first week after myeloablative allogeneic transplantation correlates with severity and incidence of GVHD and survival. *Blood* (2008) 112:1539–42. doi: 10.1182/blood-2008-02-138867
16. Hartwell MJ, Ozbek U, Holler E, Renteria AS, Major-Monfried H, Reddy P, et al. An early-biomarker algorithm predicts lethal graft-versus-host disease and survival. *JCI Insight* (2017) 2:e89798. doi: 10.1172/jci.insight.89798
17. Sato T, Selleri C, Anderson S, Young NS, Maciejewski JP. Expression and modulation of cellular receptors for interferon-gamma, tumour necrosis factor, and Fas on human bone marrow CD34+ cells. *Br J Haematol*. (1997) 97:356–65. doi: 10.1046/j.1365-2141.1997.562704.x
18. Pronk CJ, Veiby OP, Bryder D, Jacobsen SE. Tumor necrosis factor restricts hematopoietic stem cell activity in mice: involvement of two distinct receptors. *J Exp Med.* (2011) 208:1563–70. doi: 10.1084/jem.20110752
19. Bryder D, Ramsfjell V, Dybedal I, Theilgaard-Monch K, Hogerkorp CM, Adolfsson J, et al. Self-renewal of multipotent long-term repopulating hematopoietic stem cells is negatively regulated by Fas and tumor necrosis factor receptor activation. *J Exp Med.* (2001) 194:941–52. doi: 10.1084/jem.194.7.941
20. Dybedal I, Bryder D, Fossum A, Rusten LS, Jacobsen SE. Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. *Blood* (2001) 98:1782–91. doi: 10.1182/blood.V98.6.1782
21. Rebel VI, Hartnett S, Hill GR, Lazo-Kallanian SB, Ferrara JL, Sieff CA. Essential role for the p55 tumor necrosis factor receptor in regulating hematopoiesis at a stem cell level. *J Exp Med*. (1999) 190:1493–504. doi: 10.1084/jem.190.10.1493
22. Rondelli D, Andrews RG, Hansen JA, Ryncarz R, Faerber MA, Anasetti C. Alloantigen presenting function of normal human CD34+ hematopoietic cells. *Blood* (1996) 88:2619–75.
23. Rondelli D, Anasetti C, Fortuna A, Ratta M, Arpinati M, Bandini G, et al. T cell alloreactivity induced by normal G-CSF-mobilized CD34+ blood cells. *Bone Marrow Transplant.* (1998) 21:1183–91. doi: 10.1038/sj.bmt.1701259
24. Rondelli D, Lemoli RM, Ratta M, Fogli M, Re F, Curti A, et al. Rapid induction of CD40 on a subset of granulocyte colony-stimulating factor-mobilized CD34(+) blood cells identifies myeloid committed progenitors and permits selection of nonimmunogenic CD40(-) progenitor cells. *Blood* (1999) 94:2293–300.
25. Tadokoro Y, Ema H, Okano M, Li E, Nakauchi H. *De novo* DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *J Exp Med.* (2007) 204:715–22. doi: 10.1084/jem.20060750
26. Challen GA, Sun D, Mayle A, Jeong M, Luo M, Rodriguez B, et al. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. *Cell Stem Cell* (2014) 15:350–64. doi: 10.1016/j.stem.2014.06.018
27. Zhang X, Su J, Jeong M, Ko M, Huang Y, Park HJ,et al. DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. *Nat Genet.* (2016) 48:1014–23. doi: 10.1038/ng.3610
28. Wang W, Fujii H, Kim HJ, Hermans K, Usenko T, Xie S, et al. Enhanced human hematopoietic stem and progenitor cell engraftment by blocking donor T cell-mediated TNF alpha signaling. *Sci Transl Med.* (2017) 9:eaag3214.. doi: 10.1126/scitranslmed.aag3214
29. Klyuchnikov E, El-Cheikh J, Sputtek A, Lioznov M, Calmels B, Furst S, et al. CD34(+)-selected stem cell boost without further conditioning for poor graft function after allogeneic stem cell transplantation in patients with hematological malignancies. *Biol Blood Marrow Transplant.* (2014) 20:382–6. doi: 10.1016/j.bbmt.2013.11.034
30. Levine JE, Paczesny S, Mineishi S, Braun T, Choi SW, Hutchinson RJ, et al. Etanercept plus methylprednisolone as initial therapy for acute graft-versus-host disease. *Blood* (2008) 111:2470–5. doi: 10.1182/blood-2007-09-112987
31. Kennedy GA, Butler J, Western R, Morton J, Durrant S, Hill GR. Combination antithymocyte globulin and soluble TNFalpha inhibitor (etanercept) +/- mycophenolate mofetil for treatment of steroid refractory acute graft-versus-host disease. *Bone Marrow Transplant.* (2006) 37:1143–7. doi: 10.1038/sj.bmt.1705380
32. Alousi AM, Weisdorf DJ, Logan BR, Bolanos-Meade J, Carter S, Difronzo N, et al. Transplant clinical trials, Etanercept, mycophenolate, denileukin, or pentostatin plus corticosteroids for acute graft-versus-host disease: a randomized phase 2 trial from the blood and marrow transplant clinical trials network. *Blood* (2009) 114:511–7. doi: 10.1182/blood-2009-03-212290

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 December 2018
doi: 10.3389/fimmu.2018.03041

Strategies for Enhancing and Preserving Anti-leukemia Effects Without Aggravating Graft-Versus-Host Disease

Ying-Jun Chang[1], *Xiang-Yu Zhao*[1] *and Xiao-Jun Huang*[1,2]*

[1] Peking University People's Hospital & Peking University Institute of Hematology, Beijing Key Laboratory of Hematopoietic Stem Cell Transplantation, Beijing, China, [2] Peking-Tsinghua Center for Life Sciences, Beijing, China

Allogeneic stem cell transplantation (allo-SCT) is a curable method for the treatment of hematological malignancies. In the past two decades, the establishment of haploidentical transplant modalities make "everyone has a donor" become a reality. However, graft-versus-host disease (GVHD) and relapse remain the major two causes of death either in the human leukocyte antigen (HLA)-matched transplant or haploidentical transplant settings, both of which restrict the improvement of transplant outcomes. Preclinical mice model showed that both donor-derived T cells and natural killer (NK) cells play important role in the pathogenesis of GVHD and the effects of graft-versus-leukemia (GVL). Hence, understanding the immune mechanisms of GVHD and GVL would provide potential strategies for the control of leukemia relapse without aggravating GVHD. The purpose of the current review is to summarize the biology of GVHD and GVL responses in preclinical models and to discuss potential novel therapeutic strategies to reduce the relapse rate after allo-SCT. We will also review the approaches, including optimal donor selection and, conditioning regimens, donor lymphocyte infusion, BCR/ABL-specific CTL, and chimeric antigen receptor-modified T cells, which have been successfully used in the clinic to enhance and preserve anti-leukemia activity, especially GVL effects, without aggravating GVHD or alleviate GVHD.

Keywords: allogeneic stem cell transplantation, graft-versus-leukemia, graft-versus-host disease, relapse, G-CSF

OPEN ACCESS

Edited by:
Brian Christopher Betts, University of Minnesota Twin Cities, United States

Reviewed by:
Sergio Querol, Banc de Sang i Teixits, Spain
Luisa Sisinni, Hospital de la Santa Creu i Sant Pau, Spain

***Correspondence:**
Xiao-Jun Huang
huangxiaojun@bjmu.edu.cn

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *01 October 2018*
Accepted: *10 December 2018*
Published: *21 December 2018*

Citation:
Chang Y-J, Zhao X-Y and Huang X-J (2018) Strategies for Enhancing and Preserving Anti-leukemia Effects Without Aggravating Graft-Versus-Host Disease. Front. Immunol. 9:3041. doi: 10.3389/fimmu.2018.03041

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) remains a potentially curative therapeutic strategy for hematological malignancies (1–4). Currently, for patients who require transplantation, but have no related or unrelated donors with matching human-leukocyte antigen (HLA), haploidentical HSCT is an alternative modality, allowing everyone to have a donor (5, 6). Allo-HSCT benefits these malignancies due to a graft-versus-leukemia (GVL) effect that is mainly mediated by donor-derived alloreactive T cells and/or natural killer (NK) cells (7–12). However, T cells are also responsible for acute and/or chronic graft versus-host disease (GVHD), which leads to significant morbidity and mortality (13–15). Although the depletion of T cells from allografts alleviates GVHD either in human leukocyte antigen (HLA)-matched transplant settings or in HLA-haploidentical transplant modalities, removal of these cells results in increased graft failure and increased rates of leukemia relapse (16). Unfortunately, the immunosuppressive agents used for

the prophylaxis and treatment of GVHD can also reduce the beneficial GVL effects. Therefore, the separation of GVL effects from GVHD remains the "holy grail" of allo-HSCT (17–20), which is urgently needed to allow a more effective therapy for hematological malignancies.

The challenge for the separation of GVL effects from GVHD is attributed to the underlying similarity of the alloreactive T responses between the two processes (4, 21, 22). In the past 20 years, great efforts have been made by researchers to elucidate specific distinguishing immune mechanisms of GVL vs. GVHD (23–25). In addition, a number of preclinical experiments have been performed to identify approaches that could be successfully used to separate GVL effects from GVHD (26–41). Clinically, a series of strategies, including donor selection, allograft engineering, adoptive immune cell infusion, and pharmacological agents have been established (42–44). More recently, the use of chimeric antigen receptor T (CAR-T) cells that target tumor cells with a limited capacity for GVHD induction, have been identified for enhancing GVL effects without aggravating GVHD (45–47). Previously, several reviews have been published related to the separation of GVL from GVHD (48–51) The present review briefly summarizes the underlying mechanisms related to GVHD and GVL effects, mainly focusing on recent advances in strategies for enhancing and preserving anti-leukemia activity without aggravating GVHD, especially approaches aimed at the separation of GVL effects from GVHD in preclinical mouse models and in the clinic.

MECHANISMS RELEVANT TO GVHD AND GVL EFFECTS

The pathophysiology of acute GVHD had been reviewed by several researchers (13–15), beginning with the activation of host antigen-presenting cells (APCs) by damage-associated molecular patterns and/or pathogen-associated molecular patterns expressed on damaged tissues. Activated host APCs then present host antigens to donor T cells, leading to alloactivation and inflammatory cytokine release, for example Interferon-γ (IFN-γ) and lipopolysaccharides (LPS). These inflammatory cytokines then recruit and induce the proliferation of additional immune effector cells, including Th1, Th2, Th17, neutrophils, and macrophages, which cause tissue injury and inflammation in a reaction that overwhelms any tolerance-promoting response from immune suppressor cells, such as regulatory T cells (Treg) (52), regulatory B cells (Breg) (22), mesenchymal stem cells (MSC) (25), and myeloid-derived suppressor cells (MDSC) (53).

The mechanisms underlying GVL are of interest (8, 54), as both T cells, natural killer (NK) cells, and cytokines, such as IFN-γ and tumor necrosis factor-α, possess anti-leukemia activity. Two molecular path ways, including perforin and Fas, are mainly used by T cell to mediate cytotoxicity. $CD3^+CD4^+$ T cells utilize the Fas pathway and $CD3^+CD8^+$ T cells use both, while NK cells employ the perforin pathway. Interestingly, all of these cells also play an antileukemia role via cytokine release. Recently, more attention has been focused on the role of γδT cells (55), and iNKT cells in GVL effects. The target antigens for alloreactive T cells include major histocompatibility complex (MHC) and multiplex immunohistochemistry (miHC), or leukemia-associated antigens (56). The importance of MHC and miHC antigens in GVL is underlined by the close association between GVHD and GVL, although selective miHC antigens are considered to be attractive targets for anti-leukemia immunotherapy. More recently, a number of studies have demonstrated that the overall balance between regulatory cells, including Treg, MDSC, and effector cells might be related to the extent of organ damage in GVHD settings and the effects of GVL in anti-leukemia settings (**Figures 1**–**3**) (21, 22, 25, 52, 53, 57).

STRATEGIES FOR ENHANCING AND PRESERVING ANTI-LEUKEMIA EFFECTS WITHOUT AGGRAVATING GVHD IN PRECLINICAL MODELS

Several strategies, such as the use of cytokines (59), selectively depletion of alloreactive T cells, regulatory immune cells (60, 61), and pharmacological agents, such as bortezomib and azacitidine (AZA), have been investigated to enhance and preserve the anti-leukemia effects without aggravating GVHD after allo-HSCT (**Table 1**) (31, 72, 73).

Cytokines

Granulocyte colony-stimulating factor (G-CSF) is widely used during transplantation to mobilize hemopoietic stem cells, which is also a mediator of T cell tolerance (74, 75). Using a murine leukemia model, several researchers have demonstrated that G-CSF mobilization of peripheral blood stem cell transplantation could maintain GVL effects through T cells via a perforin-dependent pathway and/or NKTs and prevent GVHD by reducing systemic levels of LPS and TNF-α as well as inducing a type 2 cytokine profile, $CD34^+$ monocyte, and tolerogenic APCs (19, 76). Subsequent studies have shown that allografts mobilized by G-CSF analogs, such as pegylated G-CSF and progenipoietins (engineered chimeric G-CSF and Flt-3L protein), have marked tolerogenic properties that reside in the T cell and APC compartments. Additionally, mobilization with G-CSF analogs allows the concurrent enhancement of NKT cell numbers and activities, promoting host DC activation and subsequent CD8-dependent GVL effects while promoting the generation of Tregs to prevent CD4-dependent GVHD.

Except for G-CSF and its anlogs, other cytokines (77), including KGF, IL-11, IL-18 (28), IL-35 (62), and interleukin-12/23p40 (78), can also be used to separate GVL effects from GVHD in animal models. Moreover, the roles in GVHD of IL-21 and IL-22, two proinflammatory cytokines produced by Th17 cells, have been assessed in several studies (79–81). Couturier et al. (59) and Hanash et al. (79), respectively, demonstrated that IL-22 deficiency in donor T cells and abrogation of donor T-cell IL-21 signaling, could alleviate murine acute GVHD mortality while sparing the GVL effects. Hartung et al. (82) indicated that allografts mobilized by G-CSF plus stem cell factor exerted significantly enhanced antileukemic activity compared with those harvested after treatment with G-CSF

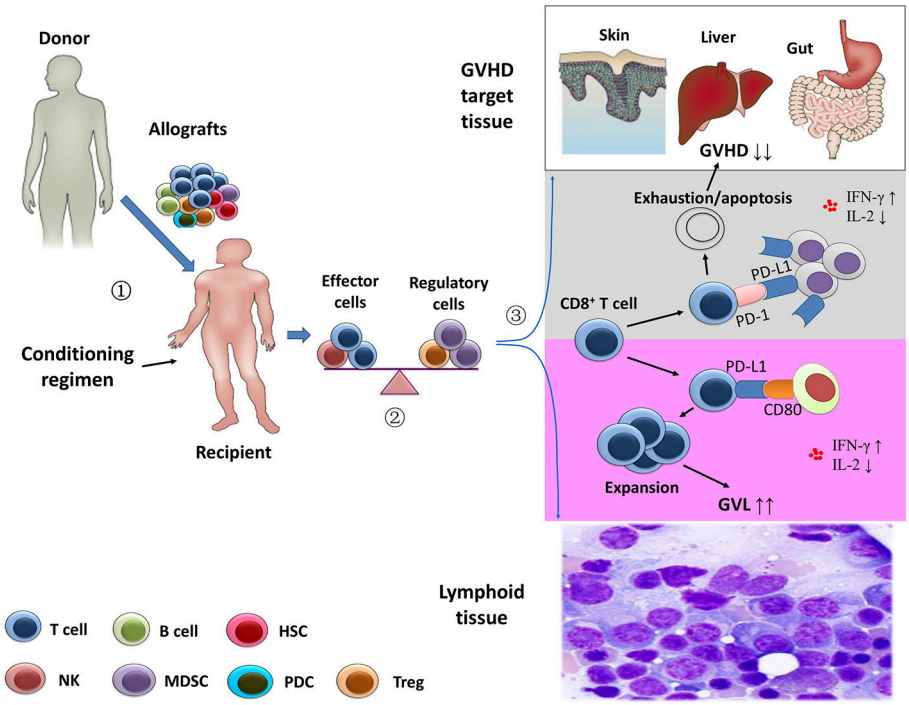


FIGURE 1 | Separation of the graft-versus-leukemia effects from graft-versus-host disease. ① Stem cell harvests obtained from healthy donor were infused in the recipients after the conditioning regimen. ② After transplantation, the balance between effector immune cells and regulatory immune cells might contribute to the prevention of graft-versus-host disease (GVHD) and the anti-leukemia activity. ③ Using animal models, Ni et al. (58) showed that the depletion of $CD4^+$ T cells following allogeneic stem cell transplantation significantly increased systemic levels of interferon-γ and decreased interleukin-2. In GVHD-targeted tissues, $CD4^+$ T cell depletion enhanced the interaction of PD-L1/PD-1 interactions between $CD8^+$ T cells and cells of GVHD-targeted tissues, leading to exhaustion and apoptosis of host-attacking $CD8^+$ T cells (gray area). However, the profiles of the cytokines might promote expansion of $CD8^+$ T cells via PD-L1/CD80 interactions in lymphoid tissue, resulting in an enhanced anti-leukemia capacity (purple area) (21).

alone, suggesting that a combination of different cytokines may be a better strategy for the separation of GVL effects from GVHD.

Depletion of Alloreactive Cells

To investigate the subsets of T cells that were effector cells with anti-leukemia effects without causing GVHD, a murine transplant model of chronic phase chronic myelogenous leukemia was generated. Zheng et al. (63) found that $CD4^+CD62L^-CD44^+CD25^-$ effector memory T cells ($CD4^+$ TEMs), but not naïve T cells (T_N), unprimed to recipient cells mediated GVL without causing GVHD, because they retained key cytolytic functions but lacked other features that are pivotal for initiating GVHD. In another study, Chen et al. (83) reported that sorted $CD45RB^+CD62L^+CD44^+$ central memory T cells (T_{CM}, a mix of $CD4^+$ and $CD8^+$ cells) did not cause GVHD in a fully MHC-mismatched transplant mouse model. However, using the same model as Chen et al. (83), Zheng et al. (64) demonstrated that highly purified $CD8^+$ TCM induced GVHD, albeit less severe than that induced by T_N. However, $CD8^+$ TCM also contribute to GVL.

More recently, using multiple GVHD models (two murine allogeneic HCT models and a human → mouse xenogeneic HCT model), Ni et al. (58) showed that $CD4^+$ T cell depletion increased serum IFN-γ levels, leading to an upregulation of PD-L1 in recipient tissues and donor $CD8^+$ T cells. In GVHD target tissues, they also found that increased PD-L1/PD-1 interactions between recipient tissues and donor $CD8^+$ T cells led to T cell exhaustion and apoptosis, thereby preventing GVHD. In lymphoid tissues, enhanced PD-L1/CD80 interactions between $CD8^+$ T cells augmented T cell survival and expansion and preserved the GVL response. In summary, the data reported by Ni et al. (58) suggested that the separation of GVL effects from GVHD could be ascribed to the PD-L1–mediated effect on $CD8^+$ T cells depending on whether $CD4^+$ T cells were present, the nature of the interacting partner expressed by $CD8^+$ T cells, and the tissue microenvironment (**Figure 1**) (19, 21).

In the clinic, depletion of TN from stem cell allografts has been successfully used to reduce the incidence of chronic GVHD, while preserving the transfer of functional T cell memory (84). Overall, these results suggest that depletion of alloreactive T cells may represent a promising method to preserve GVL effects with decreasing or without causing GVHD.

Adoptive Transfer of Effective Immune Cells

Adoptive transfer of effective cells represents another strategy for the separation of GVL effects from GVHD. Olson et al. (85)

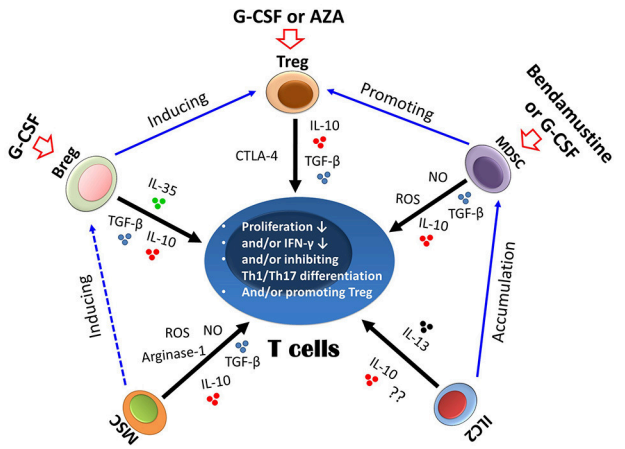


FIGURE 2 | Suppressive mechanism of regulatory immune cells on T cells. Different regulatory cells could suppress T cells either via cytokines, such as IL-10 and TGF-β, or via other molecules, such as arginase-1 and reactive oxygen species (ROS) (indicated by black arrows). The biological interactions between different regulatory cells are indicated by blue arrows. Regulatory immune cells could also be induced by a number of approaches, such as granulocyte colony-stimulating factor (G-CSF), azacitidine (AZA), and bendamustine (indicated by red arrows). Treg, regulatory T cells; Bregs, regulatory B cells; MSCs, mesenchymal stem cells; MDSCs, myeloid-derived suppressor cells; ILC2, group 2 innate lymphoid cells.

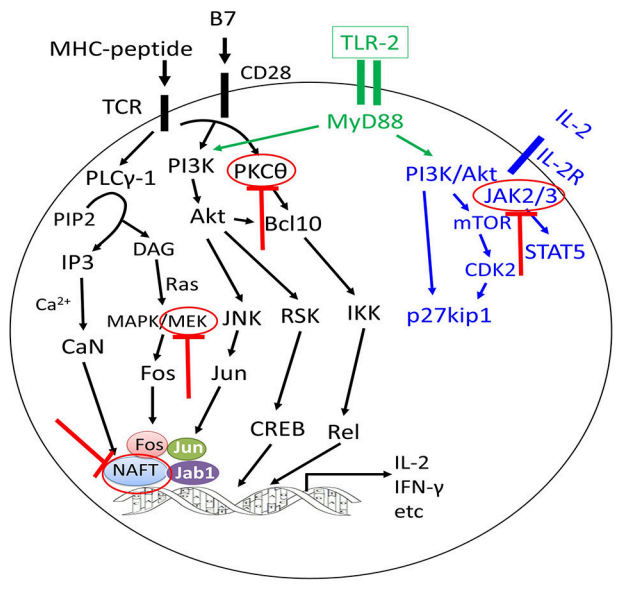


FIGURE 3 | Approaches to separate GVL effects from GVHD using inhibitors targeting different signaling pathways of T cells. The graft-versus-leukemia effects could be enhanced or preserved by targeting different signaling pathways of T cells without aggravating graft-versus-host disease (GVHD) or with alleviation of GVHD (highlighted by red colors).

demonstrated that donor T cells exhibited reduced proliferation, CD25 expression, and IFN-γ production in the presence of NK cells. In addition, activated NK cells mediated direct lysis of reisolated GVHD-inducing T cells *in vitro*, both of which lead to the alleviation of GVHD. In addition, the GVL effects were maintained in the presence of NK cells. Using mismatched hematopoietic transplant models, Ruggeri et al. (86) demonstrated, for the first time, that donor-versus-recipient NK cell alloreactivity could eliminate leukemia relapse by killing host lymphohematopoietic cells, and protect patients against GVHD by eliminating recipient-type APCs. The effects of NK cells in enhancing anti-leukemia activity and mitigating GVHD has also confirmed by other researchers. Ghosh et al. (87) reported that adoptively transferred donor-type unsorted TRAIL$^+$ T cells could potentially enhance the curative potential of allo-HSCT by increasing the GVT response via fratricide of alloactivated T cells and suppressing GVHD through limiting alloreactive T cell expansion.

Recently, CAR T-cells have been shown to possess a novel adoptive immune therapy (40, 88). Both allogeneic and syngeneic CAR T cells show initial expansion as effector T cells. Jacoby et al. (88) found that, in a mouse model, CAR-mediated acute GVHD was only observed in the presence of leukemia, suggesting that CAR-target interactions induced GVHD. Additionally, Ghosh et al. (40) demonstrated that allogeneic donor CD19-specific CD28z CAR T cells could promote anti-lymphoma activity by non-alloreactive cells, which retained activity against CD19$^+$ targets, with minimal GVHD by exhaustion and eventual deletion of the alloreactive CAR-T cells. They also reported that first-generation and 4-1BB-costimulated CARs increased GVHD. Overall, the data obtained from the mouse models suggest that CAR T cells could be used to enhance the anti-leukemia response, although its' effects on GVHD remain controversial.

Regulatory Immune Cells

Regulatory cell subsets, including Tregs, Bregs, MDSCs, and MSCs, may not only control immune homeostasis, but they also reduce detrimental T cell responses to foreign antigens. In 2003, Edinger et al. (52) observed that, in a mouse model, CD4$^+$CD25$^+$ Tregs could suppress the early expansion of alloreactive donor T cells, their IL-2-receptor alpha-chain expression and their capacity to induce GVHD without compomising their GVT effects, mediated primarily by the perforin lysis pathway of T conv cells. Interestingly, recipient-type specific Tregs could also control GVHD while favoring immune reconstitution and maintaining GVL effects. (89) In addition, Zheng et al. (90) reported that *ex vivo*-induced CD8^{hi} Tregs controlled GVHD in an allospecific manner by reducing alloreactive T cell proliferation as well as decreasing inflammatory cytokine and chemokine secretion within target organs through a CTLA-4-dependent mechanism in humanized mice. Currently reported data in the literatures suggest that Tregs might be the most important regulatory cells in preventing GVHD (4) through a series of approaches, including aurora A/JAK2 inhibition (91, 92), selective TNFR2 activation (93), DR3 signaling modulation (94), activated protein C signals (95), and IL-2 (96), which can be used to alleviate GVHD through a Tregs-dependent mechanism.

MDSCs are a heterogeneous group of immature immunosuppressive cells of the myeloid lineage, which can induce immunosuppressive cells such as Tregs and skew macrophages toward a proinflammatory type 2 phenotype via

TABLE 1 | Representative approaches for the separation of GVHD and GVL in preclinical models.

Strategies	Authors,yr	Approaches	Mechanisms	References
Cytokines	Teshima et al., 1999	Interleukin-11	IL-11 selectively inhibited CD4-mediated GVHD, while retaining both CD4- and CD8-mediated GVL.	(41)
	Couturier et al., 2013	IL-22	The absence of T-cell-derived IL-22 led to a reduction of inflammatory CD8 T cells and an expansion of Treg cells in lymphoid organs as well as a reduction of inflammatory mediators both systemically and in aGVHD target organs, both of which resulted in decreased aGVHD severity without compromising GVL effects.	(59)
	Liu et al., 2015	IL-35	IL-35 expression leads to the Treg expansion and suppression of Th1 cytokine production, which alleviates aGVHD and retains GVL effects.	(62)
	Banovic et al., 2009	Multipeg-G-CSF	Multipeg–G-CSF could modulate immune function, characterized by the generation of regulatory myelogenous and T cell populations and Th2 differentiation, as well as improve GVL via activation of invariant natural killer (iNK) T cells and enhancement of CTL function.	(24)
	Morris et al., 2005	Potent G-CSF analogs	Mobilization with potent G-CSF analogs thus allowed concurrent enhancement of NKT cell numbers and activities, promoting host DC activation and subsequent CD8-dependent GVL effects while promoting the generation of regulatory T cells to prevent CD4-dependent GVHD.	(19)
Depletion of alloreactive cells	Zheng et al., 2008	Naïve $CD4^+$ T cells	TEMs did not induce high systemic levels of TNF-α and IFN-γ in recipients, as did TNs. In ddition, a greater fraction of TNs produced IFN-γ. GVL mediated by CD4+ TNs was intact even when both perforin- and FasL-mediated killing were prevented.	(63, 64)
Adoptive transfer of immune cells	Ghosh et al., 2017	CAR-T cells	Allogeneic donor CD19-specific CD28z CAR T cells promote anti-lymphoma activity, with minimal GVHD.	(40)
	Song et al., 2018	NK cells	IL-12/15/18-preactivated NK cells predominantly mediated the lysis of donor allo-reactive T cells to inhibit aGVHD without promising GVL effects.	(20)
Regulatory immune cells	Sato et al., 2003	Regulatory DCs	Allogeneic regulatory DC regulation of the cytotoxic activity of transplanted $CD8^+$ T cells, which failed to cause acute GVHD, might be sufficient to cause an efficient GVL effect.	(61)
	Heinrichs et al., 2016	Tregs	Harnessing the unique differences between alloreactive $CD4^+$ and $CD8^+$ iTregs could create an optimal iTreg therapy for GVHD prevention with maintained GVL responses.	(57)
	Li et al., 2014	MSCs	Directing the migration of MSCs by CCR7 from their broad battle field (inflammatory organs) to the modulatory center (SLOs) of immune response could attenuate GvHD while preserving the GvL effect.	(25, 27)
	Highfill et al., 2010	MDSCs	MDSCs generated in the presence of IL-13 could inhibit GVHD, migrate to sites of allopriming, and limit the activation and proliferation of donor T cells, but they did not diminish the GVL effect of donor T cells.	(53)
	Darlak et al., 2013	pDCs	Enrichment of pDCs might augment GVL without increasing GVHD is through the production of IFN-α and/or IL-13 by pDCs.	(60)
Signaling pathway	Vaeth et al., 2015	Nuclear factor of activated T cells	Ablation of NFAT1, NFAT2, or a combination of both resulted in ameliorated GVHD due to reduced proliferation, target tissue homing, and impaired effector function of allogenic donor T cells. In addition, the beneficial antitumor activities were largely preserved in NFAT-deficient effector T cells.	(65)
	Haarberg et al., 2013	Inhibition of PKCα and PKCθ	Inhibition of PKCα and PKCθ impaired donor T-cell proliferation, migration, and chemokine/cytokine production and significantly decreased GVHD, but spared T-cell cytotoxic function and GVL effects.	(66)
	Schutt et al., 2018	Inhibition of the IRE-1α/XBP-1 pathway	Inhibition of the IRE-1a/XBP-1 pathway regulated B-cell activation and function and prevented the development of cGVHD while preserving GVL.	(67)
	Itamura et al., 2016	RAS/MEK/ERK pathway	MEK inhibitors affected human T cells in a memory stage-dependent manner, i.e., they selectively inhibited naive and central memory T cells while sparing effector memory T cells.	(68)
Pharmacological agents	Sun et al., 2004	Proteasome inhibitor	Bortezomib might rapidly induce the preferential deletion of the very high-affinity alloreactive T cells, thus allowing expansion of the remaining T cells that maintain GVT responses yet have a reduced potential for promoting GVHD.	(38)
	Strokes et al., 2016	Bendamustine	BEN alleviated GVHD via enhancing MDSC suppressive function without promising GVL effects.	(69)
	Choi et al., 2010	Azacitidine	AzaC could mitigate GVHD while preserving GVL by peripheral conversion of alloreactive effector T cells into $FOXP3^+$ Tregs and epigenetic modulation of genes downstream of Foxp3 required for the suppressor function of Tregs.	(70)

(Continued)

TABLE 1 | Continued

Strategies	Authors,yr	Approaches	Mechanisms	References
	Ehx et al., 2017	Azacitidine	AZA significantly decreased human T-cell proliferation as well as IFN-γ and TNF-α serum levels, and it reduced the expression of GRANZYME B and PERFORIN 1 by cytotoxic T cells, leading to prevention of GVHD without compromising GVL effects.	(71)
Others	Ghosh et al., 2013	Promyelocytic leukemia zinc finger	PLZF-TG T cells mediated less GVHD due to Fas-mediated fratricidal regulation and the biphenotypic TH1/TH2 response leading to limited alloreactive expansion, and an intact GVT activity.	(72)
	Marcondes et al., 2014	a-1-antitrypsin	Treatment of transplant donors with human AAT resulted in an increase in IL-10 messenger RNA and $CD8^+CD11c^+CD205^+$MHC II^+DCs, and the prevention or attenuation of acute GVHD in the recipients. The GVL effect was maintained or even enhanced with AAT treatment of the donor, mediated by an expanded population of $NK1.1^+$, $CD49B^+$, $CD122^+$, and $CD335^+$ NKG2D-expressing NK cells.	(35)
	Wu et al., 2015	MicroRNA-17-92	Blockade of miR-17 or miR-19b in this cluster significantly inhibited alloreactive T-cell expansion and IFN-γ production, and it prolonged survival in recipients afflicted with GVHD while preserving the GVL effect.	(73)

GVL, graft-versus-leukemia; GVHD, graft-versus-host disease; Tregs, regulatory T cells; aGVHD, acute GVHD; G-CSF, granulocyte colony-stimulating factor; DCs, dendritic cells; TEM, effector memory T cell; TN, naïve T cells; IFN-γ, interferon-γ; CAR-T, chimeric antigen receptor T; MSCs, mesenchymal stem cells; MDSCs, myeloid-derived suppressive cells; pDCs, plasmoid dendritic cells; TNF-α, tumor necrosis factor-α

IL-10 production. MDSCs can also suppress T-cells via arginase-1, NO, reactive oxygen species, heme oxygenase-1, TFG-β and IL-10, as well as promote Tregs. Highfill et al. (53) found that MDSCs generated in the presence of IL-13 could inhibit GVHD, migrate to sites of allopriming, and limit the activation and proliferation of donor T cells as well as induce a type 2 T cell response that was indispensable for GVHD prevention, but they did not diminish the GVL effect of donor T cells.

Another type of regulatory immune cells is the MSCs, which can inhibit the activation, proliferation, and function of T cells via arginase-1, NO, reactive oxygen species, chemokines, TGF-β, and IL-10. Interestingly, *in vivo* experiment have shown that MSCs are actively induced to undergo perforin-dependent apoptosis by recipient phagocytes that produced indoleamine 2,3-dioxygenase, which was essential to initiate MSC-induced immunosuppression (97). Directing the migration of MSCs by CCR7 from their broad battle field (inflammatory organs) to the modulatory center of the immune response could attenuate GVHD by exerting immunosuppressive effects on T cells, while preserving GVL effects by sparing the NK cell activity that contributes to GVL effects (25, 98).

Bregs can suppress immunopathology by prohibiting the expansion of pathogenic T cells and other pro-inflammatory lymphocytes through the production of IL-10, IL-35, and TGF-β (99). Our group showed that, in the acute GVHD mouse model, cotransplantation of Bregs prevented onset by inhibiting Th1 and Th17 differentiation and expanding regulatory T cells. In the GVL mouse model, Bregs contributed to the suppression of acute GVHD but had no adverse effects on GVL activity (22).

Excluding the abovementioned regulatory cells, group 2 innate lymphoid cells (ILC2) make up a large portion of the ILC population, which can polarize T cells to Th2 cells by secreting IL-4, and macrophages or DCs to an macrophage 2 or type 2 chemokine-secreting phenotype by secreting IL-13, respectively (100). ILC2 can alleviate GVHD by reducing donor Th1 and Th17 cells as well as accumulating MDSCs mediated by IL-13. Moreover, ILC2 do not inhibit the GVL response (101).

In summary, these preclinical studies suggest that cotransplantation or adoptive transfer of regulatory cells could be successfully used to alleviate GVHD without compromising the GVL effects. Therefore, pilot studies are warranted to evaluate the safety and feasibility of these regulatory cells in preventing and/or treating GVHD as well as preserving GVL effects in clinic.

Signaling Pathways

Several signaling pathways have been demonstrated to be correlated with T cell function. Janus kinases (JAKs) are intracellular signaling components of many type I/II cytokines (102, 103). There are 4 members of the JAK family that regulate the development and function of immune cells, including DCs, macrophages, T cells, B cells, and neutrophils, of which JAK1, JAK2, and JAK3 may be most relevant for the pathophysiology of GVHD (51). In murine models of GVHD and leukemia or lymphoma relapse, treatment with ruxolitinib reduced GVHD in the skin, liver, and gastrointestinal organs while preserving GVL activity, leading to improved survival (44, 104, 105). Betts et al. (91) found that the transfer of $JAK2^{-/-}$ donor T cells to allogeneic recipients led to attenuate GVHD by inhibiting Th1 differentiation, promoting Th2 polarization, and increasing and/or stabilizing $CD8^+$ iTreg, yet it maintained GVL effects (106). In addition, pacritinib, a multikinase inhibitor with potent activity against JAK2, could significantly reduce GVHD and xenogeneic skin graft rejection in distinct rodent models and maintain donor anti-tumor immunity. Overall, these data suggest that JAK inhibition or other compounds, such as TG101348 (92), represents a new and potentially clinically relevant approach to separate GVL effects from GVHD.

Excluding JAKs, increasing data have demonstrated that targeting signaling pathways, such as the PKCα and PKCθ (66),

MEK (68), NFAT (65), and IRE-1a/XBP-1 pathway (67), ikaros (107), toll-like receptor/myeloid differentiation factor 88 (108), DR3 signaling (94), and activated protein C signals (95), might provide strategies for alleviating GVHD, while enhancing or without compromising the GVL effects.

Pharmacological Agents

The roles played by biological agents in the separation of GVL effects from GVHD have been investigated in animal models (38, 71). Sun et al. (38) demonstrated that bortezomib might rapidly induce the preferential deletion of very high-affinity alloreactive T cells, thus allowing for expansion of the remaining T cells to maintain GVT responses yet with a reduced potential for promoting GVHD. Ehx et al. (71) found that AZA significantly decreased human T-cell proliferation as well as IFN-γ and TNF-α serum levels, and it reduced the expression of GRANZYME B and PERFORIN 1 by cytotoxic T cells, leading to the prevention of GVHD. AZA could also induce the expression of tumor antigens by AML cells, leading to the generation of donor-derived tumor specific cytotoxic T cells, which have been demonstrated to prevent AML relapse (70). In addition, Stokes et al. (69) reported that bendamustine could alleviate GVHD by enhancing MDSC suppressive function without compromising GVL effects.

Caballero-Velázquez et al. (30) showed that the combination of sirolimus and bortezomib synergistically inhibited both the activation and proliferation of stimulated T cells. Additionally, the production of Th1 cytokines (IFN γ, IL-2, and TNF-α) was significantly inhibited. This effect was due, at least in part, to the inhibition of Erk and Akt phosphorylation. *In vivo*, the combination reduced the risk of GVHD without hampering GVL effects, as shown in mice that received GVHD prophylaxis with sirolimus plus bortezomib infused with tumor WEHI cells plus C57BL/6 donor BM and splenocytes. Overall, this study suggests a synergistic effect of the combination different pharmacological agents to prevent GVHD while maintaining the GVL effect.

In summary, experiment results from mouse models suggest that effective and regulatory immune cells play a key role in separation of GVL effects from GVHD. The approaches explored in preclinical settings have demonstrated, for example, that cytokines or inhibitors targeting signaling pathways of T cells might enhance and/or preserve ant-leukemia effects without compromising GVHD through regulating the functions of effective and regulatory immune cells (**Figures 2**, **3**).

STRATEGIES FOR ENHANCING AND PRESERVING ANTI-LEUKEMIA EFFECTS WITHOUT AGGRAVATING GVHD IN THE CLINIC

Several approaches, including donor selection, conditioning regimens, graft engineering and adoptive transfusion of immune cells, have been successfully used in the clinic to separate GVL effects from GVHD before and after allo-HSCT (**Figure 4**).

Donor Selection

In unrelated donor transplantation settings, Kawase et al. (109) suggested that donor selection made in consideration of these results might allow the separation of GVL from acute GVHD in patients with AML, ALL, or those with chronic myeloid leukemia (CML), especially in HLA-DPB1 mismatch combinations. Fleischhauer et al. (110) further demonstrated that avoidance of an unrelated donor with a non-permissive T-cell-epitope mismatch at HLA-DPB1 might contribute to a lower risk of mortality. In cord blood transplant modality, HLA-DPB1 mismatch was also associated with a significant reduction of leukemia relapse (HR 0.61, $P = 0.001$), and no significant effect of HLA-DPB1 mismatch was observed on the risk of acute GVHD, engraftment or mortality (111). Laghmouchi et al. (112) suggested that the allo-HLA-DP-specific T cell repertoire contained T cells with restricted recognition of hematopoietic cells, which might contribute to specific GVL effector reactivity without coincident GVHD (112).

In T cell depleted haplo-SCT settings, Ruggeri et al. (86) showed that increased NK cell alloreactivity in humans, based on the "missing self" model, was associated with a decreased CIR and improved survival in patients with AML but not in patients with ALL. In contrast, Huang et al. (113) following the Beijing Protocol, demonstrated that host MHC class I could determine NK cell responses. The functional recovery of donor-derived NK cells was higher in recipients that expressed ligands for donor inhibitory KIRs, and a high functional NK recovery correlated with better relapse control (114). In haplo-SCT with PT/Cy settings, Shimoni et al. (115) also demonstrated a trend toward higher relapse rates in patients with KIR ligand mismatching (HR 1.36, $P = 0.09$) in a total group of 444 acute leukemia patients. This trend was observed in patients with AML (HR 1.48, $P = 0.07$) but not in those with ALL (HR 0.95, $P = 0.88$).

In summary, these data suggest that donor selection according to HLA-DPB1 mismatch, NK cell alloreactivity, and other variables (116–119), could represent a strategy for the separation of GVL effects and GVHD, although further studies are still needed.

Conditioning Regimen

More recently retrospective registry studies and some, but not all, prospective randomized trials have demonstrated increased relapse rates in recipients of an RIC compared with an MAC regimen in patients with AML and MDS who underwent allo-HSCT (120, 121). However, these finding remain controversial (3). In a multicenter randomized controlled trial (122), 178 HR-AML patients received haplo-HSCT with conditioning regimens involving recombinant human G-CSF or non-rhG-CSF. The cumulative incidences of acute GVHD, chronic GVHD, transplantation-related toxicity, and infectious complications appeared to be equivalent. The 2-year probabilities of LFS and OS in the G-CSF-priming and non-rhG-CSF-priming groups were 55.1 vs. 32.6% ($P < 0.01$) and 59.6 vs. 34.8% ($P < 0.01$), respectively. This study suggests that the G-CSF-priming conditioning regimen is an acceptable choice for HR-AML patients, which may lead to partially separation of GVL effects from GVHD.

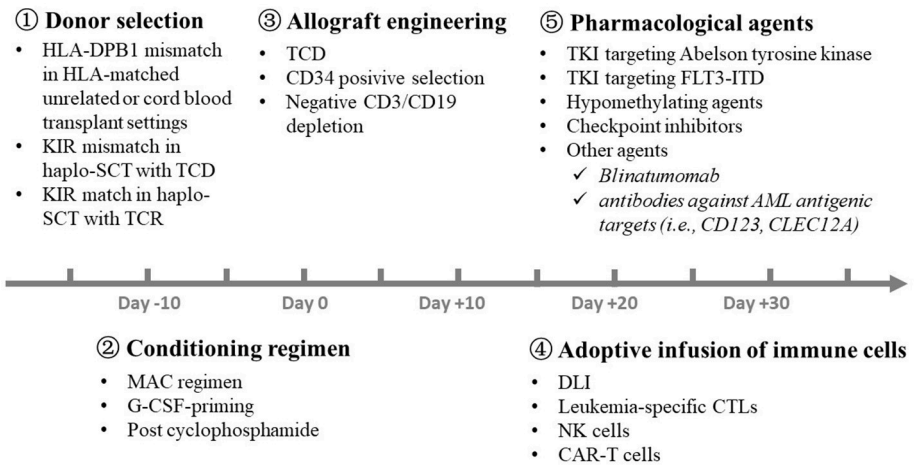


FIGURE 4 | Strategies for the separation of GVL effects from GVHD in the clinic. A number of approaches, including ①donor selection, ②conditioning regimen, ③graft engineering, ④adoptive transfusion of immune cells, and ⑤ pharmacological agents, have been successfully used in the clinic to separate GVL effects from GVHD at different time point before and after allo-HSCT. GVL, graft-versus-leukemia; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; SCT, stem cell transplantation; TCD, T cell depletion; TCR, T cell replete; MAC, myeloablative regimen; G-CSF, granulocyte colony-stimulating factor; DLI, donor lymphocyte infusion; NK, natural killer; CAR-T, chimeric antigen receptor T; TKI, Tyrosine kinase inhibitor; AML, acute myeloid leukemia.

Overall, considering the central importance of regimen in determinng leukemia relapse risk based on the biological characteristics of disease and pretransplantation minimal residual disease (MRD), there remains an urgent need for randomized comparisons of different conditioning regimens to separate GVL from GVHD.

Allograft Engineering

In a single-arm clinical trial, 35 cases patients with high-risk leukemia received naïve T cell-depleted G-PBSCs following a myeloablative conditioning regimen. GVHD prevention includes tacrolimus immunosuppression alone. Bleakley et al. (42) reported that all the cases engrafted. GVHD in these patients was universally corticosteroid responsive, although the incidence of aGVHD was not reduced. Chronic GVHD was remarkably infrequent (9%) compared with historical rates of ∼50% with T cell-replete grafts. Memory T cells in the graft resulted in rapid T cell recovery and the transfer of protective virus-specific immunity. No excessive rates of infection or relapse occurred, and the OS was 78% at 2 years. These results suggest that the depletion of naïve T cells from allografts not only reduces the incidence of cGVHD but also preserves the transfer of functional T cell memory.

To decrease the incidence of GVHD in haploidentical allograft settings, the Perugia group established a protocol that includes TCD and a graft containing a mega-dose of highly purified $CD34^+$ cells (average 10×10^6/kg body weight), which is administered following a myeloablative conditioning regimen (86). This protocol ensures a high engraftment rate, despite the HLA barrier, without triggering GVHD. However, the benefit (the absence of GVHD) from this CD34 selected haplotype transplant approach is offset by a very slow immune recovery due to the small number of T cells infused and the ATG application, which result in high rates of opportunistic infections, such as viral and fungal infections, leading to a high TRM. To accelerate immune recovery, the Perugia group demonstrated, for the first time, that the adoptive transfer of Tregs promotes lymphoid reconstitution and improves immunity to opportunistic pathogens without weakening the GVL effects in the TCD haploidentical setting (16). This finding suggests that the adoptive transfer of gene modified T cells and/or pathogen-specific T cells may be needed to improve clinical outcomes. In a phase II study, researchers from Germany found that haplo-SCT with a negative CD3/CD19 depletion and reduced intensity conditioning allowed for a successful transplantation in an older, heavily pretreated patient population (16). The estimated 2-event free survival was 25%. The incidence of grade II-IV aGVHD was 46%, and the incidence of cGVHD was 18%. Therefore, new strategies are needed to further establish novel strategies for the separation of GVL effects and GVHD.

Luznik et al. (123) summarized that *in vivo* cyclophosphamide posttransplantation (PT/Cy) could induce the destruction of peripheral, alloantigen-reactive T cells, while a relative resistance of donor Teff/memory T cells to PT/Cy, as demonstrated in mice, might contribute to the overall reconstitution of peripheral T-cell pools and immune competence over the long term. These results suggest that *in vivo* allograft engineering with PT/Cy represents a novel method for GVL and GVHD separation, and it has been widely used in haploidentical and HLA-matched sibling donor transplant settings (6, 124).

Adoptive Transfusion of Immune Cells

Currently, adoptive transfusion of immune cells, such as donor lymphocyte infusion (DLI), cytotoxic T lymphocyte (CTLs), NK cells, and CAR-T, had been successfully used to separate GVL effects from GVHD.

DLI

In 1990, Kolb et al. (125) first reported sustained remission after DLI in patients with CML who relapsed after allo-HSCT. Since then, DLI had become the mainstay allogeneic cellular therapy. The NCI recommendations list DLI as the routinely considered method for patients who relapsed after allo-HSCT and do not have GVHD (126). Based on immune tolerance induced by G-CSF, such as the ability to polarize T cells from the Th1 to the Th2 phenotype and the hyporesponsiveness of T cells, Huang's group established a modified DLI protocol (127) that includes the following: (i) the use of G-CSF mobilized peripheral blood stem cell harvests (G-PBSCs) instead of a steady lymphocyte infusion; (ii) the introduction of short-term immune suppressive agents, including cyclosporine A (CSA) or methotrexate (MTX), to further decrease the incidence of GVHD. Impressively, the feasibility and efficacy of the modified DLI were confirmed either for treatment or prevention of relapse after haploidentical HSCT (127). Our group also demonstrated that MRD-directed DLI could significantly decreased the relapse rate without aggravating GVHD (128). The use of DLI was also demonstrated in patients who underwent haploidentical HSCT with PT/Cy (129).

Recently, Nikiforow et al. (130) undertook a phase I study of DLI depleted of $CD25^+$ T cells in 21 patients with hematologic malignancies who had relapsed after allo-HSCT. Two dose levels were administered: 1×10^7 ($n = 6$) and 3×10^7 $CD3^+$ cells/kg ($n = 15$). A median 2.3 log-depletion of Tregs was achieved. Seven subjects (33%) developed clinically significant GVHD by 1 year, including one patient who died. At dose level 1, five subjects had progressive disease and one had stable disease. At dose level 2, nine subjects (60%) achieved or maintained responses (8 CR, 1 PR), including seven with active disease at the time of infusion. A shorter period between relapse and infusion was associated with the response at dose level 2 ($P = 0.016$). The 1-year survival rate was 53% among patients treated with dose level 2. Four of eight subjects with AML remained in remission at 1 year. When compared to unmodified DLI in 14 contemporaneous patients meeting study eligibility, CD25/Treg depletion was associated with a better response rate and improved EFS.

Overall, the available data suggest that DLI represents a widely used approach in prophylactic, pre-emptive therapy and therapy for relapse either in HLA-matched HSCT or in haploidentical transplant settings. Furthermore, CD25/Treg-depleted DLI appears to be feasible and capable of inducing GVL effects without excessive GVHD (131), although multicenter, prospective study are warranted to confirm the results.

Leukemia Specific CTLs

Researchers from Italy have investigated the feasibility of expanding/priming p190BCR-ABL–specific T cells *in vitro* by stimulation with DCs pulsed with p190BCR-ABL peptides derived from the BCR-ABL junctional region and alternative splicing, and of adoptively administering them to patients with relapsed disease (132). Three patients were enrolled in this study. Patient 1 was a 61-year-old man experiencing a second molecular recurrence after matched unrelated donor (MUD) alloHSCT and unmanipulated DLIs. Patient 2 was a 30-year-old man diagnosed with Ph^+ ALLwith hyperleukocytosis and central nervous system (CNS) involvement, experiencing his third hematologic relapse (BM blast 66%, F317L mutation) after MUD-HSCT, DLI, and rescue therapy with nilotinib. Patient 3 was a 62-year-old woman diagnosed with Ph^+ ALL with CNS involvement, showing persistent molecular disease (last MRD before T-cell therapy 0.1% BCR-ABL/ABL) after induction, maintenance chemotherapy, and prolonged TKI treatment. She was not eligible for alloHSCT due to comorbidities. The results showed no postinfusion toxicity, except for a grade II skin GVHD in the patient who was treated for hematologic relapse. All patients achieved a molecular or hematologic CR after T-cell therapy, upon emergence of p190BCR-ABL-specific T cells in the BM. These results demonstrate that p190BCR-ABL-specific CTLs are capable of controlling treatment-refractory Ph^+ ALL *in vivo*, and they support the development of adoptive immunotherapeutic approaches with BCR-ABL CTLs in Ph^+ ALL. Therefore, further studies including large sample sizes are needed to confirm the abovementioned results.

Excuding BCR-ABL CTLs, the anti-leukemia effects of WT1 specific CTL were also observed in 11 relapsed or high-risk leukemia patients who underwent allo-HSCT (133). Chapuis et al. (133) found that $CD8^+$ transferred T cells with a memory phenotype could be detected after long-term follow-up. An approach to generate multi-TAA-specific CTLs using peptide libraries of 15-mer peptides overlapping by 11 amino acids spanning the whole amino acid sequence of a target antigen was developed by Weber et al. (134) They also showed that TAAmix-specific CTLs could inhibit the colony formation of leukemia blasts. In summary, leukemia-specific CTLs might be a promising method for enhancing anti-leukemia activity.

NK Cells

The role played by NK cells in anti-leukemia activity had been fully investigated in allo-HSCT settings. In a dose-escalation study, Choi et al. (43) showed that, when given 2–3 weeks after haploidentical HSCT, donor-derived NK cells were well-tolerated at a median total dose of 2.0×10^8 cells/kg. In a phase I study, the safety of haploidentical third-party NK cell infusion was further confirmed in 21 patients with high-risk myeloid malignancies who received a preparative regimen with busulfan and fludarabine followed by infusion of IL-2-activated NK cells with a dose ranging from 0.02 to 8.32 $\times 10^6$/kg. Lee et al. (135) demonstrated that five patients were alive, and 5 and 11 cases had died from transplant-related causes and relapse, respectively. Among the total patients, only 5 cases developed a maximum acute GVHD of grade 2, and 2 cases grade 3 GVHD. These results indicated that the infusion of third-party NK cells was well-tolerated and did not increase the rate of GVHD after allo-HSCT. Ciurea et al. (136) initiated a phase 1 dose-escalation study of membrane-bound interleukin 21-expanded donor NK cells infused before and after haploidentical HSCT for high-risk myeloid malignancies. NK cells were infused on days −2, +7, and +28 posttransplant. All NK expansions achieved the required cell number, and 11 of 13 patients enrolled received all 3 planned NK-cell doses (1×10^5/kg to 1×10^8/kg per dose). No infusional reactions or dose-limiting toxicities occurred. All patients were engrafted with donor cells. Seven patients (54%)

developed grade I-II acute GVHD (aGVHD), and no patients developed grade III-IV aGVHD or chronic GVHD. All other patients were alive and in remission at the last follow-up (median, 14.7 months). Overall, this trial demonstrated the production feasibility and safety of infusing high doses of *ex vivo*-expanded NK cells after haploidentical HSCT without adverse effects, increased GVHD, or higher mortality, which was associated with significantly improved NK-cell numbers and function, fewer viral infections, and a low relapse rate posttransplant. Further study deomonstrated that $CD56^+$ donor cell infusion after PT/Cy and short-course cyclosporine were feasible with prompt engraftment, rapid reconstitution of $CD4^+$ T, Tregs and NK cells and a reduced incidence of relapse and acute GVHD (137).

CAR-T Cells

Researchers from Peking University described six ALL patients with no response to modified DLI who received one and two infusions of CAR T cells from haplo-HSCT donors. Five patients (83.33%) achieved MRD-negative remission; one patient was discharged without evaluation after developing severe thrombotic microangiopathies (46, 47, 138). More recently, Anwer et al. (45) performed a systemic review, including 72 patients from seven studies who were treated with donor-derived CAR T cells. The authors reported that the use of donor-derived CAR T cell for relapse prophylaxis, MRD clearance or salvage from relapse is therefore highly effective, and the risk of GVHD flare is very low.

In summary, donor-derived CAR T-cell infusion seems to be an effective and safe alternative method for relapsed B-ALL after haplo-HSCT (47). Therefore, with the definition of multiple antigen targets, such as CD7, CD38. CD138, FLT-3, and B-cell maturation antigen, CAR-T cell could be increasingly used for anti-hematological malignancies.

PHARMACOLOGICAL AGENTS

Tyrosine Kinase Inhibitor (TKI) Targeting Abelson Tyrosine Kinase

Currently, few patients with CML will receive allo-HSCT. Therefore, the use of TKI after transplantation mainly focuses on cases with Ph-positive ALL (139–141). Chen et al. (142) reported that 14 patients who were positive for BCR-ABL1 expression, received imatinib therapy after allo-HSCT. Eight patients became BCR-ABL1-negative at 1 month after imatinib therapy, and only two patients died from hematological relapse. In the nonimatinib-treated group, six of 20 patients relapsed, and five of these patients died from hematological relapse. Here, recommendations for the use of TKIs according to the pre- and post-transplant MRD status by the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation are provided as follows (143).

First, for cases with positive pre-MRD, but negative posttransplantation MRD (post-MRD), prophylactic TKI should be administered according to the pretransplantation mutation status, or observation only. If positive post-MRD is detected, imatinib or another TKI can be administered according to the mutation status. If MRD reoccurs within 3 months after transplantation or at a high level, a 2nd generation TKI should be given.

Second, for cases with positive post-MRD not considering the status of pre-MRD, TKI is administered according to mutation status or using 2nd generation TKI.

Third, for cases with both negative pre-MRD and negative post-MRD, prophylactic TKI or observation, if positive post-MRD is detected, imatinib or another TKI can be administered according to the mutation status. If MRD reoccurrs within 3 months after transplantation or at a high level, a 2nd generation TKI should be given.

TKIs Targeting FLT3-ITD

A number of FLT3 TKIs have been or are being investigated in allo-HSCT settings for FLT3-ITD AML, including sorafenib (144, 145), midostaurin, quizartinib, crenolanib, and gilteritinib (144–149). The mechanism of action of TKIs targeting FLT3 may not only involved in direct tumor cell killing, but also in increased interleukin-15, leadings to an increase in $CD8^+CD107a^+IFN\text{-}\gamma^+$ T cells with features of longevity (high levels of Bcl-2 and reduced PD-1 levels), which could eradicate leukemia in secondary recipients (146). More recently, Xuan et al. (147) performed a study that enrolled a total of 144 patients with FLT3-ITD AML undergoing allo-HSCT. Depending on whether they were receiving sorafenib before transplantation or sorafenib maintenance after transplantation, patients were divided into 4 groups: patients receiving sorafenib before transplantation (group A; $n = 36$), patients receiving sorafenib after transplantation (group B; $n = 32$), patients receiving sorafenib both before and after transplantation (group C; $n = 26$), and patients receiving sorafenib neither before nor after transplantation (group D; $n = 50$). Xuan et al. (147) showed that the 3-year relapse rates were 22.2, 18.8, 15.8, and 46.1% for groups A, B, C, and D, respectively ($P = 0.006$). The 3-year LFS rates were 69.4, 78.1, 80.4, and 34.8%, respectively ($P < 0.001$). A multivariate analysis revealed that sorafenib before transplantation, sorafenib maintenance after transplantation, and their combined application were protective factors for a lower relapse rate and longer LFS, respectively.

More recent studies have shown that targeting the FLT3-ITD driver mutation with a highly potent and selective FLT3 inhibitor, such as quizartinib, is a promising clinical strategy to help improve clinical outcomes in patients with relapsed or refractory AML (148, 149). Therefore, further studies are needed to investigate the effectiveness of these agents in allo-HSCT settings, especially for the separation of GVL from GVHD.

Hypomethylating Agents

Hypomethylating agents are used as treatments for relapse and may also be used in pre-emptive interventions after allo-HSCT (150–154). In a phase 1 study enrolling 27 patients with AML post allo-HSCT. Goodyear et al. (155) showed that azacitidine (AZA) both augmented the expansion of regulatory T cells and induced cytotoxic $CD8^+$ T-cell responses to several tumor antigens, and leading to hopes that it might facilitate successful cultivation of the GVL response without inducing significant GVHD. In a multicenter retrospective study, Craddock et al.

(156) investigated the tolerability and activity of AZA in 181 patients who relapsed after an allograft for AML ($n = 116$) or MDS ($n = 65$). Sixty-nine patients received additional DLI. Forty-six of 157 (25%) assessable patients responded to AZA therapy: 24 (15%) achieved a CR and 22 a PR. In patients who achieved a CR, the 2-year overall survival was 48 vs. 12% for the whole population. The authors suggested that AZA represents an important new therapy in select patients with AML/MDS who relapse after allo-HSCT, thus warranting prospective studies. Moreover, the combination of sorafenib, AZA, and DLI represent a novel direction for the treatment or prevention of relapse without aggravating GVHD after allo-HSCT (151, 157).

Recently, Schroeder et al. (158) retrospectively analyzed data obtained for 36 patients with hematological ($n = 35$) or molecular relapse ($n = 1$) of AML ($n = 29$), or MDS ($n = 7$). Decitabine (DAC) was the first salvage therapy in 16 patients (44%), whereas 20 patients (56%) had previously received 1–5 lines of salvage therapy, including 16 cases who had been treated with AZA. In 22 patients (61%), a median of 2 DLI per patient (range, 1–5) was administered in addition to DAC. As a result, the overall response rate was 25%, including 6 CR (17%) and 3 PR (8%). Three patients within the first-line group achieved CR, while 3 patients receiving DAC as second-line treatment reached CR, including 2 patients with previous AZA failure. The median duration of CR was 10 months (range, 2–33), and none of the patients have relapsed to date. The incidence of acute and chronic GVHD was 19 and 5% (158). These data suggest that DAC may be an alternative to AZA or even a second choice after AZA failure. In summary, hypomethylating agents used alone or in combination with DLI might represent promising approaches for the separation of GVL from GVHD in the clinic.

Checkpoint Inhibitors

The relapse of hematological malignancies after allo-HSCT can be mediated by high levels of checkpoint receptors, including PD-1 and CTLA-4, on donor derived effective T cells and high expression of cognate ligands on residual leukemia cells (159, 160). In a phase 1 study, Bashey et al. (161). showed that a single dose of ipilimumab (between 0.1 and 3.0 mg/kg) for patients with malignancies who relapsed after allo-HSCT did not seem to cause clinically significant GVHD and achieved responses in 3 patients with lymphoid malignancies. In a subsequent phase 1/2 study, ipilimumab was started at 3 mg/kg but could be escalated to 10 mg/kg (162). Although no objective responses were observed in six patients who received ipilimumab treatment at 3 mg/kg, a total of 13 patients presented a decrease in tumor burden among 22 patients treated at a dose of 10 mg/kg, with four responses persisting for >1 year. Impressively, four patients with extramedullary AML and one patient with smoldering MDS that developed into AML had a complete response. These data suggest a particular sensitivity of AML to ipilimumab treatment after allo-HSCT. Davids et al. (162) also observed that responders showed a reduction of $CD4^+$ regulatory T cells with an increase in conventional T cells in peripheral blood as well as an increase in $CD62L^-$ effector memory T cells.

A phase 2 investigator-initiated trial enrolled patients with lymphoid malignancies who relapsed after allogeneic HSCT ($n = 10$) and high-risk patients after autologous HSCT ($n = 7$) (163). Both cases received 10 mg of oral lenalidomide daily for 21 days followed by intravenous ipilimumab at 3 mg/kg body weight. The regimen was repeated 4 weeks later for a total of 4 treatments. Khouri et al. (163) demonstrated that 4 of 10 patients in the allogeneic group had complete responses and 3 partial responses. The disease in 6 of 7 patients in the autologous group remains in remission. The authors suggested that the responses might be related to a 2- to 3 -fold increases in inducible $ICOS^+CD4^+FoxP3^-$ T cells number.

In summary, checkpoint inhibitor used alone or in combination with other methods, such as immunomodulatory agents (163) and CAR-T cells (164), could be promising approaches for the treatment or prevention of relapse after transplantation without aggravating GVHD, although further studies are warranted for confirmation.

Other Agents

Several other novel agents (165), including histone deacetylase inhibitor (panobinostat), and monoclonal antibodies, such as blinatumomab (a novel bispecific CD19-directed CD3 T-cell engager), as well as antibodies against AML antigenic targets (i.e., CD123, CLEC12A), have been or are currently being investigated for the prevention and treatment of relapse in patients with hematological malignancies who have undergone allo-HSCT. Therefore, further prospective studies are warranted to select optimal methods that are currently available for killing leukemia cells without leading to GVHD.

FUTURE DIRECTIONS

In the past two decades, increasing evidence supports the notion that GVL effects could be, at least partially, separated from GVHD both in animal models and in the clinic. Recently, Fanning et al. (18) have demonstrated that Vβ spectratyping can identify T cells involved in antihost and antitumor reactivity and that tumor presensitization can aid in the separation of GVHD and GVL responses. However, no studies have demonstrated the successful use of this technique for separating GVL effects from GVHD in patients who have undergone allo-HSCT. In addition, several other questions remain to be answered in the future. First, although preclinical experiments have demonstrated the feasibility of a number of strategies for enhancing or preserving anti-leukemia activity without compromising GVHD, planned prospective studies are required to evaluate the clinical efficacy and to move these approaches from preclinical research to the standard-of care. Second, it remains uncertain whether the available methods for inducing anti-leukemia activity without causing GVHD can be successfully used in different transplant modalities, especially haploidentical allografts. Third, little is known about the immunological mechanisms underlying the separation of GVL effects from GVHD. Therefore, further studies are imperative.

In summary, with the elucidation of the immune mechanisms of both GVL effects and GVHD, the advances in the establishment of novel approaches for the prevention and/or

treatment of leukemia relapse and GVHD, as well as the evaluation of these new methods based on prospective clinical trials, an increasing number of patients will benefit from the successful separation of GVL effects from GVHD, ultimately leading to superior survival.

AUTHOR CONTRIBUTIONS

X-JH designed the study. Y-JC and X-YZ collected data and drafted the manuscript. All authors contributed to data interpretation, manuscript preparation, and approval of the final version.

FUNDING

This work was supported by the National Key Research and Development Program of China (no. 2017YFA0104500), the Foundation for Innovative Research Groups of the National Natural Science Foundation of China (81621001), and the Beijing Natural Science Foundation (7162196).

ACKNOWLEDGMENTS

We would also like to thank American journal experts (https://www.aje.com/) for assistance in editing this manuscript.

REFERENCES

1. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med*. (2006) 354:1813–26. doi: 10.1056/NEJMra052638
2. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, et al. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood* (2015) 125:3956–62. doi: 10.1182/blood-2015-02-627786
3. Fasslrinner F, Schetelig J, Burchert A, Kramer M, Trenschel R, Hegenbart U, et al. Long-term efficacy of reduced-intensity versus myeloablative conditioning before allogeneic haemopoietic cell transplantation in patients with acute myeloid leukaemia in first complete remission: retrospective follow-up of an open-label, randomised phase 3 trial. *Lancet Haematol*. (2018) 5:e161–9. doi: 10.1016/S2352-3026(18)30022-X
4. Blazar BR, MacDonald KPA, Hill GR. Immune regulatory cell infusion for graft-versus-host disease prevention and therapy. *Blood* (2018) 131:2651–60. doi: 10.1182/blood-2017-11-785865
5. Chang YJ, Huang XJ. Haploidentical stem cell transplantation: anti-thymocyte globulin-based experience. *Semin Hematol*. (2016) 53:82–9. doi: 10.1053/j.seminhematol.2016.01.004
6. Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol*. (2016) 13:10–24. doi: 10.1038/nrclinonc.2015.128
7. Negrin RS. Graft-versus-host disease versus graft-versus-leukemia. Hematology *Am Soc Hematol Educ Program* (2015) 2015:225–30. doi: 10.1182/asheducation-2015.1.225
8. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. *Nat Rev Cancer* (2004) 4:371–80. doi: 10.1038/nrc1365
9. Dickinson AM, Norden J, Li S, Hromadnikova I, Schmid C, Schmetzer H, et al. Graft-versus-Leukemia effect following hematopoietic stem cell transplantation for leukemia. *Front Immunol*. (2017) 8:496. doi: 10.3389/fimmu.2017.00496
10. Falkenburg JHF, Jedema I. Graft versus tumor effects and why people relapse. Hematology *Am Soc Hematol Educ Program* (2017) 2017:693–8. doi: 10.1182/asheducation-2017.1.693
11. Locatelli F, Pende D, Falco M, Della Chiesa M, Moretta A, Moretta L. NK cells mediate a crucial graft-versus-leukemia effect in Haploidentical-HSCT to cure high-risk acute leukemia. *Trends Immunol*. (2018) 39:577–90. doi: 10.1016/j.it.2018.04.009
12. Cooley S, Parham P, Miller JS. Strategies to activate NK cells to prevent relapse and induce remission following hematopoietic stem cell transplantation. *Blood* (2018) 131:1053–62. doi: 10.1182/blood-2017-08-752170
13. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
14. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. *N Engl J Med*. (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
15. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. *Blood* (2014) 124:363–73. doi: 10.1182/blood-2014-01-514786
16. Martelli MF, Di Ianni M, Ruggeri L, Pierini A, Falzetti F, Carotti A, et al. "Designed" grafts for HLA-haploidentical stem cell transplantation. *Blood* (2014) 123:967–73. doi: 10.1182/blood-2013-10-531764
17. Li J, Heinrichs J, Haarberg K, Semple K, Veerapathran A, Liu C, et al. HY-specific induced regulatory T cells display high specificity and efficacy in the prevention of acute graft-versus-host disease. *J Immunol*. (2015) 195:717–25. doi: 10.4049/jimmunol.1401250
18. Fanning SL, Zilberberg J, Stein J, Vazzana K, Berger SA, Korngold R, et al. Unraveling graft-versus-host disease and graft-versus-leukemia responses using TCR Vβ spectratype analysis in a murine bone marrow transplantation model. *J Immunol*. (2013) 190:447–57. doi: 10.4049/jimmunol.1201641
19. Morris ES, MacDonald KP, Rowe V, Banovic T, Kuns RD, Don AL, et al. NKT cell-dependent leukemia eradication following stem cell mobilization with potent G-CSF analogs. *J Clin Invest*. (2005) 115:3093–103. doi: 10.1172/JCI25249
20. Song Y, Hu B, Liu Y, Jin Z, Zhang Y, Lin D, et al. IL-12/IL-18-preactivated donor NK cells enhance GVL effects and mitigate GvHD after allogeneic hematopoietic stem cell transplantation. *Eur J Immunol*. (2018) 48:670–82. doi: 10.1002/eji.201747177
21. Brennan TV, Yang Y. PD-L1 serves as a double agent in separating GVL from GVHD. *J Clin Invest*. (2017) 127:1627–30. doi: 10.1172/JCI94196
22. Hu Y, He GL, Zhao XY, Zhao XS, Wang Y, Xu LP, et al. Regulatory B cells promote graft-versus-host disease prevention and maintain graft-versus-leukemia activity following allogeneic bone marrow transplantation. *Oncoimmunology* (2017) 6:e1284721. doi: 10.1080/2162402X.2017.1284721
23. Okiyama N, Furumoto Y, Villarroel VA, Linton JT, Tsai WL, Gutermuth J, et al. Reversal of CD8 T-cell-mediated mucocutaneous graft-versus-host-like disease by the JAK inhibitor tofacitinib. *J Invest Dermatol*. (2014) 134:992–1000. doi: 10.1038/jid.2013.476
24. Banovic T, MacDonald KP, Markey KA, Morris ES, Kuns RD, Varelias A, et al. Donor treatment with a multipegylated G-CSF maximizes graft-versus-leukemia effects. *Biol Blood Marrow Transplant*. (2009) 15:126–30. doi: 10.1016/j.bbmt.2008.11.019
25. Li H, Jiang Y, Jiang X, Guo X, Ning H, Li Y, et al. CCR7 guides migration of mesenchymal stem cell to secondary lymphoid organs: a novel approach to separate GvHD from GvL effect. *Stem Cells* (2014) 32:1890–903. doi: 10.1002/stem.1656
26. Montagna D, Daudt L, Locatelli F, Montini E, Turin I, Lisini D, et al. Single-cell cloning of human, donor-derived antileukemia T-cell lines for in vitro separation of graft-versus-leukemia effect from graft-versus-host reaction. *Cancer Res* (2006) 66:7310–6. doi: 10.1158/0008-5472.CAN-06-0591
27. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, West ML, Burgents JE, Blazar BR, et al. Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. *Blood* (2010) 115:4914–22. doi: 10.1182/blood-2009-08-239848
28. Reddy P, Teshima T, Hildebrandt G, Williams DL, Liu C, Cooke KR, et al. Pretreatment of donors with interleukin-18 attenuates acute graft-versus-host disease via STAT6 and preserves graft-versus-leukemia effects. *Blood* (2003) 101:2877–85. doi: 10.1182/blood-2002-08-2566
29. Long J, Chang L, Shen Y, Gao WH, Wu YN, Dou HB, et al. Valproic acid ameliorates graft-versus-host disease by downregulating Th1 and Th17 cells. *J Immunol*. (2015) 195:1849–57. doi: 10.4049/jimmunol.1500578

30. Caballero-Velázquez T, Sánchez-Abarca LI, Gutierrez-Cosio S, Blanco B, Calderon C, Herrero C, et al. The novel combination of sirolimus and bortezomib prevents graft-versus-host disease but maintains the graft-versus-leukemia effect after allogeneic transplantation. *Haematologica* (2012) 97:1329–37. doi: 10.3324/haematol.2011.058677
31. Wu Y, Heinrichs J, Bastian D, Fu J, Nguyen H, Schutt S, et al. MicroRNA-17-92 controls T-cell responses in graft-versus-host disease and leukemia relapse in mice. *Blood* (2015) 126:1314–23. doi: 10.1182/blood-2015-02-627356
32. Krijanovski OI, Hill GR, Cooke KR, Teshima T, Crawford JM, Brinson YS, et al. Keratinocyte growth factor separates graft-versus-leukemia effects from graft-versus-host disease. *Blood* (1999) 94:825–31.
33. Zeng Y, Stokes J, Hahn S, Hoffman E, Katsanis E. Activated MHC-mismatched T helper-1 lymphocyte infusion enhances GvL with limited GvHD. *Bone Marrow Transplant*. (2014) 49:1076–83. doi: 10.1038/bmt.2014.91
34. Fricke S, Hilger N, Fricke C, Schönfelder U, Behre G, Ruschpler P, et al. Prevention of graft-versus-host-disease with preserved graft-versus-leukemia-effect by *ex vivo* and *in vivo* modulation of CD4(+) T-cells. *Cell Mol Life Sci*. (2014) 71:2135–48. doi: 10.1007/s00018-013-1476-0
35. Marcondes AM, Karoopongse E, Lesnikova M, Margineantu D, Welte T, Dinarello CA, et al. α-1-Antitrypsin (AAT)-modified donor cells suppress GVHD but enhance the GVL effect: a role for mitochondrial bioenergetics. *Blood* (2014) 124:2881–91. doi: 10.1182/blood-2014-04-570440
36. Lu Y, Giver CR, Sharma A, Li JM, Darlak KA, Owens LM, et al. IFN-γ and indoleamine 2,3-dioxygenase signaling between donor dendritic cells and T cells regulates graft versus host and graft versus leukemia activity. *Blood* (2012) 119:1075–85. doi: 10.1182/blood-2010-12-322891
37. Wang H, Asavaroengchai W, Yeap BY, Wang MG, Wang S, Sykes M, et al. Paradoxical effects of IFN-gamma in graft-versus-host disease reflect promotion of lymphohematopoietic graft-versus-host reactions and inhibition of epithelial tissue injury. *Blood* (2009) 113:3612–9. doi: 10.1182/blood-2008-07-168419
38. Sun K, Welniak LA, Panoskaltsis-Mortari A, O'Shaughnessy MJ, Liu H, Barao I, et al. Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. *Proc Natl Acad Sci USA*. (2004) 101:8120–5. doi: 10.1073/pnas.0401563101
39. Karimi MA, Bryson JL, Richman LP, Fesnak AD, Leichner TM, Satake A, et al. NKG2D expression by CD8+ T cells contributes to GVHD and GVT effects in a murine model of allogeneic HSCT. *Blood* (2015) 125:3655–63. doi: 10.1182/blood-2015-02-629006
40. Ghosh A, Smith M, James SE, Davila ML, Velardi E, Argyropoulos KV, et al. Donor CD19 CAR T cells exert potent graft-versus-lymphoma activity with diminished graft-versus-host activity. *Nat Med*. (2017) 23:242–9. doi: 10.1038/nm.4258
41. Teshima T, Hill GR, Pan L, Brinson YS, van den Brink MR, Cooke KR, et al. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. *J Clin Invest*. (1999) 104:317–25. doi: 10.1172/JCI7111
42. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest*. (2015) 125:2677–89. doi: 10.1172/JCI81229
43. Choi I, Yoon SR, Park SY, Kim H, Jung SJ, Jang YJ, et al. Donor-derived natural killer cells infused after human leukocyte antigen-haploidentical hematopoietic cell transplantation: a dose-escalation study. *Biol Blood Marrow Transplant*. (2014) 20:696–704. doi: 10.1016/j.bbmt.2014.01.031
44. Choi J, Cooper ML, Staser K, Ashami K, Vij KR, Wang B, et al. Baricitinib-induced blockade of interferon gamma receptor and interleukin-6 receptor for the prevention and treatment of graft-versus-host disease. *Leukemia* (2018) 32:2483–94. doi: 10.1038/s41375-018-0123-z
45. Anwer F, Shaukat AA, Zahid U, Husnain M, McBride A, Persky D, et al. Donor origin CAR T cells: graft versus malignancy effect without GVHD, a systematic review. *Immunotherapy* (2017) 9:123–30. doi: 10.2217/imt-2016-0127
46. Kochenderfer JN, Dudley ME, Carpenter RO, Kassim SH, Rose JJ, Telford WG, et al. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood* (2013) 122:4129–39. doi: 10.1182/blood-2013-08-519413
47. Park JH, Rivière I, Gonen M, Wang X, Sénéchal B, Curran KJ, et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med*. (2018) 378:449–59. doi: 10.1056/NEJMoa1709919
48. Morris ES, MacDonald KP, Hill GR. Stem cell mobilization with G-CSF analogs: a rational approach to separate GVHD and GVL? *Blood* (2006) 107:3430–5. doi: 10.1182/blood-2005-10-4299
49. Stenger EO, Turnquist HR, Mapara MY, Thomson AW. Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity. *Blood* (2012) 119:5088–103. doi: 10.1182/blood-2011-11-364091
50. Warren EH, Deeg HJ. Dissecting graft-versus-leukemia from graft-versus-host-disease using novel strategies. *Tissue Antigens* (2013) 81:183–93. doi: 10.1111/tan.12090
51. Schroeder MA, Choi J, Staser K, DiPersio JF. The role of janus kinase signaling in graft-versus-host disease and graft versus leukemia. *Biol Blood Marrow Transplant*. (2018) 24:1125–34. doi: 10.1016/j.bbmt.2017.12.797
52. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med*. (2003) 9:1144–50. doi: 10.1038/nm915
53. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood* (2010) 116:5738–47. doi: 10.1182/blood-2010-06-287839
54. Mavroudis D, Barrett J. The graft-versus-leukemia effect. *Curr Opin Hematol*. (1996) 3:423–9. doi: 10.1097/00062752-199603060-00005
55. Handgretinger R, Schilbach K. The potential role of gammadelta T cells after allogeneic HCT for leukemia. *Blood* (2018) 131:1063–72. doi: 10.1182/blood-2017-08-752162
56. Zilberberg J, Feinman R, Korngold R. Strategies for the identification of T cell-recognized tumor antigens in hematological malignancies for improved graft-versus-tumor responses after allogeneic blood and marrow transplantation. *Biol Blood Marrow Transplant*. (2015) 21:1000–7. doi: 10.1016/j.bbmt.2014.11.001
57. Heinrichs J, Li J, Nguyen H, Wu Y, Bastian D, Daethanasanmak A, et al. CD8(+) Tregs promote GVHD prevention and overcome the impaired GVL effect mediated by CD4(+) Tregs in mice. *Oncoimmunology* (2016) 5:e1146842. doi: 10.1080/2162402X.2016.1146842
58. Ni X, Song Q, Cassady K, Deng R, Jin H, Zhang M, et al. PD-L1 interacts with CD80 to regulate graft-versus-leukemia activity of donor CD8+ T cells. *J Clin Invest*. (2017) 127:1960–77. doi: 10.1172/JCI91138
59. Couturier M, Lamarthée B, Arbez J, Renauld JC, Bossard C, Malard F, et al. IL-22 deficiency in donor T cells attenuates murine acute graft-versus-host disease mortality while sparing the graft-versus-leukemia effect. *Leukemia* (2013) 27:1527–37. doi: 10.1038/leu.2013.39
60. Darlak KA, Wang Y, Li JM, Harris WA, Owens LM, Waller EK. Enrichment of IL-12-producing plasmacytoid dendritic cells in donor bone marrow grafts enhances graft-versus-leukemia activity in allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. (2013) 19:1331–9. doi: 10.1016/j.bbmt.2013.06.016
61. Sato K, Yamashita N, Yamashita N, Baba M, Matsuyama T. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity* (2003) 18:367–79. doi: 10.1016/S1074-7613(03)00055-4
62. Liu Y, Wu Y, Wang Y, Cai Y, Hu B, Bao G, et al. IL-35 mitigates murine acute graft-versus-host disease with retention of graft-versus-leukemia effects. *Leukemia* (2015) 29:939–46. doi: 10.1038/leu.2014.310
63. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, et al. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood* (2008) 111:2476–84. doi: 10.1182/blood-2007-08-109678
64. Zheng H, Matte-Martone C, Jain D, McNiff J, Shlomchik WD. Central memory CD8+ T cells induce graft-versus-host disease and mediate graft-versus-leukemia. *J Immunol*. (2009) 182:5938–48. doi: 10.4049/jimmunol.0802212

65. Vaeth M, Bäuerlein CA, Pusch T, Findeis J, Chopra M, Mottok A, et al. Selective NFAT targeting in T cells ameliorates GvHD while maintaining antitumor activity. *Proc Natl Acad Sci USA*. (2015) 112:1125–30. doi: 10.1073/pnas.1409290112
66. Haarberg KM, Li J, Heinrichs J, Wang D, Liu C, Bronk CC, et al. Pharmacologic inhibition of PKCalpha and PKCtheta prevents GVHD while preserving GVL activity in mice. *Blood* (2013) 122:2500–11. doi: 10.1182/blood-2012-12-471938
67. Schutt SD, Wu Y, Tang CH, Bastian D, Nguyen H, Sofi MH, et al. Inhibition of the IRE-1α/XBP-1 pathway prevents chronic GVHD and preserves the GVL effect in mice. *Blood Adv*. (2018) 2:414–27. doi: 10.1182/bloodadvances.2017009068
68. Itamura H, Shindo T, Tawara I, Kubota Y, Kariya R, Okada S, et al. The MEK inhibitor trametinib separates murine graft-versus-host disease from graft-versus-tumor effects. *JCI Insight* (2016) 1:e86331. doi: 10.1172/jci.insight.86331
69. Stokes J, Hoffman EA, Zeng Y, Larmonier N, Katsanis E. Post-transplant bendamustine reduces GvHD while preserving GvL in experimental haploidentical bone marrow transplantation. *Br J Haematol*. (2016) 174:102–16. doi: 10.1111/bjh.14034
70. Choi J, Ritchey J, Prior JL, Holt M, Shannon WD, Deych E, et al. *In vivo* administration of hypomethylating agents mitigate graft-versus-host disease without sacrificing graft-versus-leukemia. *Blood* (2010) 116:129–39. doi: 10.1182/blood-2009-12-257253
71. Ehx G, Fransolet G, de Leval L, D'Hondt S, Lucas S, Hannon M, et al. Azacytidine prevents experimental xenogeneic graft-versus-host disease without abrogating graft-versus-leukemia effects. *Oncoimmunology* (2017) 6:e1314425. doi: 10.1080/2162402X.2017.1314425
72. Ghosh A, Holland AM, Dogan Y, Yim NL, Rao UK, Young LF, et al. PLZF confers effector functions to donor T cells that preserve graft-versus-tumor effects while attenuating GVHD. *Cancer Res*. (2013) 73:4687–96. doi: 10.1158/0008-5472.CAN-12-4699
73. Wu Y, Schutt S, Paz K, Zhang M, Flynn RP, Bastian D, et al. MicroRNA-17-92 is required for T-cell and B-cell pathogenicity in chronic graft-versus-host disease in mice. *Blood* (2018) 131:1974–86. doi: 10.1182/blood-2017-06-789321
74. Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol*. (2005) 175:7085–91. doi: 10.4049/jimmunol.175.11.7085
75. Perobelli SM, Mercadante AC, Galvani RG, Gonçalves-Silva T, Alves AP, Pereira-Neves A, et al. G-CSF-induced suppressor IL-10+ neutrophils promote regulatory T cells that inhibit graft-versus-host disease in a long-lasting and specific way. *J Immunol*. (2016) 197:3725–34. doi: 10.4049/jimmunol.1502023
76. Pan L, Teshima T, Hill GR, Bungard D, Brinson YS, Reddy VS, et al. Granulocyte colony-stimulating factor-mobilized allogeneic stem cell transplantation maintains graft-versus-leukemia effects through a perforin-dependent pathway while preventing graft-versus-host disease. *Blood* (1999) 93:4071–8.
77. Wang H, Yang YG. The complex and central role of interferon-γ in graft-versus-host disease and graft-versus-tumor activity. *Immunol Rev*. (2014) 258:30–44. doi: 10.1111/imr.12151
78. Wu Y, Bastian D, Schutt S, Nguyen H, Fu J, Heinrichs J, et al.. Essential role of interleukin-12/23p40 in the development of graft-versus-host disease in mice. *Biol Blood Marrow Transplant*. (2015) 21:1195–204. doi: 10.1016/j.bbmt.2015.03.016
79. Hanash AM, Kappel LW, Yim NL, Nejat RA, Goldberg GL, Smith OM, et al. Abrogation of donor T-cell IL-21 signaling leads to tissue-specific modulation of immunity and separation of GVHD from GVL. *Blood* (2011) 118:446–55. doi: 10.1182/blood-2010-07-294785
80. Pan B, Xia F, Wu Y, Zhang F, Lu Z, Fu R, et al. Recipient-derived IL-22 alleviates murine acute graft-versus-host disease in association with reduced activation of antigen presenting cells. *Cytokine* (2018) 111: 33–40. doi: 10.1016/j.cyto.2018.08.010
81. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity* (2012) 37:339–50. doi: 10.1016/j.immuni.2012.05.028
82. Hartung G, Uharek L, Zeis M, Glass B, Steinmann J, Dreger P, et al. Superior antileukemic activity of murine peripheral blood progenitor cell (PBPC) grafts mobilized by G-CSF and stem cell factor (SCF) as compared to G-CSF alone. *Bone Marrow Transplant*. (1998) 21(Suppl. 3):S16–20.
83. Chen BJ, Deoliveira D, Cui X, Le NT, Son J, Whitesides JF, et al. Inability of memory T cells to induce graft-versus-host disease is a result of an abortive alloresponse. *Blood* (2007) 109:3115–23. doi: 10.1182/blood-2006-04-016410
84. Bleakley M, Turtle CJ, Riddell SR. Augmentation of anti-tumor immunity by adoptive T-cell transfer after allogeneic hematopoietic stem cell transplantation. *Expert Rev Hematol*. (2014) 5:409–25. doi: 10.1586/ehm.12.28
85. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* (2010) 115:4293–301. doi: 10.1182/blood-2009-05-222190
86. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* (2002) 295:2097–100. doi: 10.1126/science.1068440
87. Ghosh A, Dogan Y, Moroz M, Holland AM, Yim NL, Rao UK, et al. Adoptively transferred TRAIL+ T cells suppress GVHD and augment antitumor activity. *J Clin Invest*. (2013) 123:2654–62. doi: 10.1172/JCI66301
88. Jacoby E, Yang Y, Qin H, Chien CD, Kochenderfer JN, Fry TJ. Murine allogeneic CD19 CAR T cells harbor potent antileukemic activity but have the potential to mediate lethal GVHD. *Blood* (2016) 127:1361–70. doi: 10.1182/blood-2015-08-664250
89. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest*. (2003) 112:1688–96. doi: 10.1172/JCI17702
90. Zheng J, Liu Y, Liu Y, Liu M, Xiang Z, Lam KT, et al. Human CD8+ regulatory T cells inhibit GVHD and preserve general immunity in humanized mice. *Sci Transl Med*. (2013) 5:168ra9. doi: 10.1126/scitranslmed.3004943
91. Betts BC, Bastian D, Iamsawat S, Nguyen H, Heinrichs JL, Wu Y, et al. Targeting JAK2 reduces GVHD and xenograft rejection through regulation of T cell differentiation. *Proc Natl Acad Sci USA*. (2018) 115:1582–7. doi: 10.1073/pnas.1712452115
92. Betts BC, Veerapathran A, Pidala J, Yang H, Horna P, Walton K, et al. Targeting Aurora kinase A and JAK2 prevents GVHD while maintaining Treg and antitumor CTL function. *Sci Transl Med*. (2017) 9:eaai8269. doi: 10.1126/scitranslmed.aai8269
93. Chopra M, Biehl M, Steinfatt T, Brandl A, Kums J, Amich J, et al. Exogenous TNFR2 activation protects from acute GvHD via host T reg cell expansion. *J Exp Med*. (2016) 213:1881–900. doi: 10.1084/jem.20151563
94. Nishikii H, Kim BS, Yokoyama Y, Chen Y, Baker J, Pierini A, et al. DR3 signaling modulates the function of Foxp3+ regulatory T cells and the severity of acute graft-versus-host disease. *Blood* (2016) 128:2846–58. doi: 10.1182/blood-2016-06-723783
95. Ranjan S, Goihl A, Kohli S, Gadi I, Pierau M, Shahzad K, et al. Activated protein C protects from GvHD via PAR2/PAR3 signalling in regulatory T-cells. *Nat Commun*. (2017) 8:311. doi: 10.1038/s41467-017-00169-4
96. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. (2011) 365:2055–66. doi: 10.1056/NEJMoa1108188
97. Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, et al. Apoptosis in mesenchymal stromal cells induces *in vivo* recipient-mediated immunomodulation. *Sci Transl Med*. (2017) 9:eaam7828. doi: 10.1126/scitranslmed.aam7828
98. Auletta JJ, Eid SK, Wuttisarnwattana P, Silva I, Metheny L, Keller MD, et al. Human mesenchymal stromal cells attenuate graft-versus-host disease and maintain graft-versus-leukemia activity following experimental allogeneic bone marrow transplantation. *Stem Cells* (2015) 33:601–14. doi: 10.1002/stem.1867
99. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity* (2015) 42:607–12. doi: 10.1016/j.immuni.2015.04.005

100. Colonna M. Innate lymphoid cells: diversity, plasticity, and unique functions in immunity. *Immunity* (2018) 48:1104–17. doi: 10.1016/j.immuni.2018.05.013
101. Bruce DW, Stefanski HE, Vincent BG, Dant TA, Reisdorf S, Bommiasamy H, et al. Type 2 innate lymphoid cells treat and prevent acute gastrointestinal graft-versus-host disease. *J Clin Invest*. (2017) 127:1813–25. doi: 10.1172/JCI91816
102. MacDonald KP, Blazar BR, Hill GR. Cytokine mediators of chronic graft-versus-host disease. *J Clin Invest*. (2017) 127:2452–63. doi: 10.1172/JCI90593
103. Schwartz DM, Bonelli M, Gadina M, O'Shea JJ. Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. *Nat Rev Rheumatol*. (2016) 12:25–36. doi: 10.1038/nrrheum.2015.167
104. Carniti C, Gimondi S, Vendramin A, Recordati C, Confalonieri D, Bermema A, et al. Pharmacologic inhibition of JAK1/JAK2 signaling reduces experimental murine acute GVHD while preserving GVT effects. *Clin Cancer Res.* (2015) 21:3740–9. doi: 10.1158/1078-0432.CCR-14-2758
105. Choi J, Cooper ML, Alahmari B, Ritchey J, Collins L, Holt M, et al. Pharmacologic blockade of JAK1/JAK2 reduces GvHD and preserves the graft-versus-leukemia effect. *PLoS ONE* (2014) 9:e109799. doi: 10.1371/journal.pone.0109799
106. Iamsawat S, Daenthanasanmak A, Voss JH, Nguyen H, Bastian D, Liu C, et al. Stabilization of Foxp3 by targeting JAK2 enhances efficacy of CD8 induced regulatory T cells in the prevention of graft-versus-host disease. *J Immunol*. (2018) 201:2812–23. doi: 10.4049/jimmunol.1800793
107. Toubai T, Guoqing H, Rossi C, Mathewson N, Oravecz-Wilson K, Cummings E, et al. Ikaros deficiency in host hematopoietic cells separates GVL from GVHD after experimental allogeneic hematopoietic cell transplantation. *Oncoimmunology* (2015) 4:e1016699. doi: 10.1080/2162402X.2015.1016699
108. Lim JY, Ryu DB, Lee SE, Park G, Choi EY, Min CK. Differential effect of MyD88 signal in donor T cells on graft-versus-leukemia effect and graft-versus-host disease after experimental allogeneic stem cell transplantation. *Mol Cells* (2015) 38:966–74. doi: 10.14348/molcells.2015.0158
109. Kawase T, Matsuo K, Kashiwase K, Inoko H, Saji H, Ogawa S, et al. HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. *Blood* (2009) 113:2851–8. doi: 10.1182/blood-2008-08-171934
110. Fleischhauer K, Shaw BE, Gooley T, Malkki M, Bardy P, Bignon JD, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. *Lancet Oncol*. (2012) 13:366–74. doi: 10.1016/S1470-2045(12)70004-9
111. Yabe T, Azuma F, Kashiwase K, Matsumoto K, Orihara T, Yabe H, et al. HLA-DPB1 mismatch induces a graft-versus-leukemia effect without severe acute GVHD after single-unit umbilical cord blood transplantation. *Leukemia* (2018) 32:168–75. doi: 10.1038/leu.2017.202
112. Laghmouchi A, Hoogstraten C, Van Balen P, Falkenburg JHF, Jedema I. The allo-HLA-DP restricted T cell repertoire provoked by allogeneic dendritic cells contains T cells that show restricted recognition of hematopoietic cells including primary malignant cells. *Haematologica* (2018). doi: 10.3324/haematol.2018.193680. [Epub ahead of print].
113. Huang XJ, Zhao XY, Liu DH, Liu KY, Xu LP. Deleterious effects of KIR ligand incompatibility on clinical outcomes in haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion. *Leukemia* (2007) 21:848–51. doi: 10.1038/sj.leu.2404566
114. Zhao XY, Chang YJ, Zhao XS, Xu LP, Zhang XH, Liu KY, et al. Recipient expression of ligands for donor inhibitory KIRs enhances NK-cell function to control leukemic relapse after haploidentical transplantation. *Eur J Immunol*. (2015) 45:2396–408. doi: 10.1002/eji.201445057
115. Shimoni A, Labopin M, Lorentino F, Van Lint MT, Koc Y, Gülbas Z, et al. Killer cell immunoglobulin-like receptor ligand mismatching and outcome after haploidentical transplantation with post-transplant cyclophosphamide. *Leukemia* (2018). doi: 10.1038/s41375-018-0170-5. [Epub ahead of print].
116. Chang YJ, Luznik L, Fuchs EJ, Huang XJ. How do we choose the best donor for T-cell-replete, HLA-haploidentical transplantation? *J Hematol Oncol*. (2016) 9: 35. doi: 10.1186/s13045-016-0265-2
117. Escudero A, Martínez-Romera I, Fernández L, Valentín J, González-Vicent M, Vicario JL, et al. Donor KIR genotype impacts on clinical outcome after T cell-depleted HLA matched related allogeneic transplantation for high-risk pediatric leukemia patients. *Biol Blood Marrow Transplant*. (2018) 24:2493–500. doi: 10.1016/j.bbmt.2018.08.009
118. Fleischhauer K, Hsu KC, Shaw BE. Prevention of relapse after allogeneic hematopoietic cell transplantation by donor and cell source selection. *Bone Marrow Transplant*. (2018) 53:1498–507. doi: 10.1038/s41409-018-0218-1
119. Widman A, Reshef R. Precision in donor selection: identifying ideal stem-cell donors through their T cells. *Exp Hematol*. (2016) 44:1020–3. doi: 10.1016/j.exphem.2016.07.013
120. Kröger N, Iacobelli S, Franke GN, Platzbecker U, Uddin R, Hübel K, et al. Dose-reduced versus standard conditioning followed by allogeneic stem-cell transplantation for patients with myelodysplastic syndrome: a prospective randomized phase III study of the EBMT (RICMAC Trial). *J Clin Oncol*. (2017) 35:2157–64. doi: 10.1200/JCO.2016.70.7349
121. Scott BL, Pasquini MC, Logan BR, Wu J, Devine SM, Porter DL, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. (2017) 35:1154–61. doi: 10.1200/JCO.2016.70.7091
122. Gao L, Wen Q, Chen X, Liu Y, Zhang C, Gao L, et al. Effects of priming with recombinant human granulocyte colony-stimulating factor on conditioning regimen for high-risk acute myeloid leukemia patients undergoing human leukocyte antigen-haploidentical hematopoietic stem cell transplantation: a multicenter randomized controlled study in southwest China. *Biol Blood Marrow Transplant*. (2014) 20:1932–9. doi: 10.1016/j.bbmt.2014.08.001
123. Luznik L, O'Donnell PV, Fuchs EJ. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical bone marrow transplantation. *Semin Oncol*. (2012) 39:683–93. doi: 10.1053/j.seminoncol.2012.09.005
124. Romee R, Cooley S, Berrien-Elliott MM, Westervelt P, Verneris MR, Wagner JE, et al. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood* (2018) 131:2515–27. doi: 10.1182/blood-2017-12-823757
125. Kolb HJ, Mittermüller J, Clemm C, Holler E, Ledderose G, Brehm G, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood* (1990) 76:2462–5.
126. de Lima M, Porter DL, Battiwalla M, Bishop MR, Giralt SA, Hardy NM, et al. Proceedings from the National Cancer Institute's Second International Workshop on the Biology, Prevention, and Treatment of Relapse After Hematopoietic Stem Cell Transplantation: part III. Prevention and treatment of relapse after allogeneic transplantation. *Biol Blood Marrow Transplant*. (2014) 20:4–13. doi: 10.1016/j.bbmt.2013.08.012
127. Chang YJ, Huang XJ. Donor lymphocyte infusions for relapse after allogeneic transplantation: when, if and for whom? *Blood Rev*. (2013) 27:55–62. doi: 10.1016/j.blre.2012.11.002
128. Yan CH, Liu DH, Liu KY, Xu LP, Liu YR, Chen H, et al. Risk stratification-directed donor lymphocyte infusion could reduce relapse of standard-risk acute leukemia patients after allogeneic hematopoietic stem cell transplantation. *Blood* (2012) 119:3256–62. doi: 10.1182/blood-2011-09-380386
129. Goldsmith SR, Slade M, DiPersio JF, Westervelt P, Schroeder MA, Gao F, et al. Donor-lymphocyte infusion following haploidentical hematopoietic cell transplantation with peripheral blood stem cell grafts and PTCy. *Bone Marrow Transplant*. (2017) 52:1623–8. doi: 10.1038/bmt.2017.193
130. Nikiforow S, Kim HT, Daley H, Reynolds C, Jones KT, Armand P, et al. A phase I study of CD25/regulatory T-cell-depleted donor lymphocyte infusion for relapse after allogeneic stem cell transplantation. *Haematologica* (2016) 101:1251–9. doi: 10.3324/haematol.2015.141176
131. Di Ianni M, Olioso P, Giancola R, Santarone S, Natale A, Papalinetti G, et al. Treg-protected donor lymphocyte infusions: a new tool to address the graft-versus-leukemia effect in the absence of graft-versus-host disease in patients relapsed after HSCT. *Int J Hematol*. (2017) 106:860–4. doi: 10.1007/s12185-017-2292-3
132. Comoli P, Basso S, Riva G, Barozzi P, Guido I, Gurrado A, et al. BCR-ABL-specific T-cell therapy in Ph+ ALL patients on tyrosine-kinase inhibitors. *Blood* (2017) 129:582–6. doi: 10.1182/blood-2016-07-731091
133. Chapuis AG, Ragnarsson GB, Nguyen HN, Chaney CN, Pufnock JS, Schmitt TM, et al. Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med*. (2013) 5:174ra27. doi: 10.1126/scitranslmed.3004916

134. Weber G, Gerdemann U, Caruana I, Savoldo B, Hensel NF, Rabin KR, et al. Generation of multi-leukemia antigen-specific T cells to enhance the graft-versus-leukemia effect after allogeneic stem cell transplant. *Leukemia* (2013) 27:1538–47. doi: 10.1038/leu.2013.66
135. Lee DA, Denman CJ, Rondon G, Woodworth G, Chen J, Fisher T, et al. Haploidentical natural killer cells infused before allogeneic stem cell transplantation for myeloid malignancies: a phase I trial. *Biol Blood Marrow Transplant*. (2016) 22:1290–8. doi: 10.1016/j.bbmt.2016.04.009
136. Ciurea SO, Schafer JR, Bassett R, Denman CJ, Cao K, Willis D, et al. Phase 1 clinical trial using mbIL21 *ex vivo*-expanded donor-derived NK cells after haploidentical transplantation. *Blood* (2017) 130:1857–68. doi: 10.1182/blood-2017-05-785659
137. Jaiswal SR, Zaman S, Nedunchezhian M, Chakrabarti A, Bhakuni P, Ahmed M, et al. CD56-enriched donor cell infusion after post-transplantation cyclophosphamide for haploidentical transplantation of advanced myeloid malignancies is associated with prompt reconstitution of mature natural killer cells and regulatory T cells with reduced incidence of acute graft versus host disease: a pilot study. *Cytotherapy* (2017) 19:531–42. doi: 10.1016/j.jcyt.2016.12.006
138. Chen Y, Cheng Y, Suo P, Yan C, Wang Y, Chen Y, et al. Donor-derived CD19-targeted T cell infusion induces minimal residual disease-negative remission in relapsed B-cell acute lymphoblastic leukaemia with no response to donor lymphocyte infusions after haploidentical haematopoietic stem cell transplantation. *Br J Haematol*. (2017) 179:598–605. doi: 10.1111/bjh.14923
139. Carpenter PA, Snyder DS, Flowers ME, Sanders JE, Gooley TA, Martin PJ, et al. Prophylactic administration of imatinib after hematopoietic cell transplantation for high-risk Philadelphia chromosome-positive leukemia. *Blood* (2007) 109:2791–3. doi: 10.1182/blood-2006-04-019836
140. Brissot E, Labopin M, Beckers MM, Socié G, Rambaldi A, Volin L, et al. Tyrosine kinase inhibitors improve long-term outcome of allogeneic hematopoietic stem cell transplantation for adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia. *Haematologica* (2015) 100:392–9. doi: 10.3324/haematol.2014.116954
141. Pfeifer H, Wassmann B, Bethge W, Dengler J, Bornhäuser M, Stadler M, et al. Randomized comparison of prophylactic and minimal residual disease-triggered imatinib after allogeneic stem cell transplantation for BCR-ABL1-positive acute lymphoblastic leukemia. *Leukemia* (2013) 27:1254–62. doi: 10.1038/leu.2012.352
142. Chen H, Liu KY, Xu LP, Liu DH, Chen YH, Zhao XY, et al. Administration of imatinib after allogeneic hematopoietic stem cell transplantation may improve disease-free survival for patients with Philadelphia chromosome-positive acute lymphobla stic leukemia. *J Hematol Oncol*. (2012) 5: 29. doi: 10.1186/1756-8722-5-29
143. Giebel S, Czyz A, Ottmann O, Baron F, Brissot E, Ciceri F, et al. Use of tyrosine kinase inhibitors to prevent relapse after allogeneic hematopoietic stem cell transplantation for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia: a position statement of the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Cancer* (2016) 122:2941–51. doi: 10.1002/cncr.30130
144. Brunner AM, Li S, Fathi AT, Wadleigh M, Ho VT, Collier K, et al. Haematopoietic cell transplantation with and without sorafenib maintenance for patients with FLT3-ITD acute myeloid leukaemia in first complete remission. *Br J Haematol*. (2016) 175:496–504. doi: 10.1111/bjh.14260
145. Battipaglia G, Ruggeri A, Massoud R, El Cheikh J, Jestin M, Antar A, et al. Efficacy and feasibility of sorafenib as a maintenance agent after allogeneic hematopoietic stem cell transplantation for Fms-like tyrosine kinase 3-mutated acute myeloid leukemia. *Cancer* (2017) 123:2867–74. doi: 10.1002/cncr.30680
146. Mathew NR, Baumgartner F, Braun L, O'Sullivan D, Thomas S, Waterhouse M, et al. Sorafenib promotes graft-versus-leukemia activity in mice and humans through IL-15 production in FLT3-ITD-mutant leukemia cells. *Nat Med*. (2018) 24:282–91. doi: 10.1038/nm.4484
147. Xuan L, Wang Y, Huang F, Jiang E, Deng L, Wu B, et al. Effect of sorafenib on the outcomes of patients with FLT3-ITD acute myeloid leukemia undergoing allogeneic hematopoietic stem cell transplantation. *Cancer* (2018) 124:1954–63. doi: 10.1002/cncr.31295
148. Cortes JE, Tallman MS, Schiller GJ, Trone D, Gammon G, Goldberg SL, et al. Phase 2b study of two dosing regimens of quizartinib monotherapy in FLT3-ITD mutated, relapsed or refractory AML. *Blood* (2018) 132:598–607. doi: 10.1182/blood-2018-01-821629
149. Cortes J, Perl AE, Döhner H, Kantarjian H, Martinelli G, Kovacsovics T, et al. Quizartinib, an FLT3 inhibitor, as monotherapy in patients with relapsed or refractory acute myeloid leukaemia: an open-label, multicentre, single-arm, phase 2 trial. *Lancet Oncol*. (2018) 19:889–903. doi: 10.1016/S1470-2045(18)30240-7
150. Waespe N, Van Den Akker M, Klaassen RJ, Lieberman L, Irwin MS, Ali SS, et al. Response to treatment with azacitidine in children with advanced myelodysplastic syndrome prior to hematopoietic stem cell transplantation. *Haematologica* (2016) 101:1508–15. doi: 10.3324/haematol.2016.145821
151. Ishikawa T, Fujii N, Imada M, Aoe M, Meguri Y, Inomata T, et al. Graft-versus-leukemia effect with a WT1-specific T-cell response induced by azacitidine and donor lymphocyte infusions after allogeneic hematopoietic stem cell transplantation. *Cytotherapy* (2017) 19:514–20. doi: 10.1016/j.jcyt.2016.12.007
152. Cooper ML, Choi J, Karpova D, Vij K, Ritchey J, Schroeder MA, et al. Azacitidine mitigates graft-versus-host disease via differential effects on the proliferation of T effectors and natural regulatory T cells *in vivo*. *J Immunol*. (2017) 198:3746–54. doi: 10.4049/jimmunol.1502399
153. El-Cheikh J, Massoud R, Fares E, Kreidieh N, Mahfouz R, Charafeddine M, et al. Low-dose 5-azacytidine as preventive therapy for relapse of AML and MDS following allogeneic HCT. *Bone Marrow Transplant*. (2017) 52:918–21. doi: 10.1038/bmt.2017.31
154. de Lima M, Oran B, Champlin RE, Papadopoulos EB, Giralt SA, Scott BL, et al. CC-486 maintenance after stem cell transplantation in patients with acute myeloid leukemia or myelodysplastic syndromes. *Biol Blood Marrow Transplant*. (2018) 24:2017–24. doi: 10.1016/j.bbmt.2018.06.016
155. Goodyear OC, Dennis M, Jilani NY, Loke J, Siddique S, Ryan G, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood* (2012) 119:3361-9. doi: 10.1182/blood-2011-09-377044
156. Craddock C, Labopin M, Robin M, Finke J, Chevallier P, Yakoub-Agha I, et al. Clinical activity of azacitidine in patients who relapse after allogeneic stem cell transplantation for acute myeloid leukemia. *Haematologica* (2016) 101:879–83. doi: 10.3324/haematol.2015.140996
157. Rautenberg C, Nachtkamp K, Dienst A, Schmidt PV, Heyn C, Kondakci M, et al. Sorafenib and azacitidine as salvage therapy for relapse of FLT3-ITD mutated AML after allo-SCT. *Eur J Haematol*. (2017) 98:348–54. doi: 10.1111/ejh.12832
158. Schroeder T, Rautenberg C, Krüger W, Platzbecker U, Bug G, Steinmann J, et al. Treatment of relapsed AML and MDS after allogeneic stem cell transplantation with decitabine and DLI-a retrospective multicenter analysis on behalf of the German Cooperative Transplant Study Group. *Ann Hematol*. (2018) 97:335–42. doi: 10.1007/s00277-017-3185-5
159. Liu L, Chang YJ, Xu LP, Zhang XH, Wang Y, Liu KY, et al. T cell exhaustion characterized by compromised MHC class I and II restricted cytotoxic activity associates with acute B lymphoblastic leukemia relapse after allogeneic hematopoietic stem cell transplantation. *Clin Immunol*. (2018) 190:32–40. doi: 10.1016/j.clim.2018.02.009
160. Liu L, Chang YJ, Xu LP, Zhang XH, Wang Y, Liu KY, et al. Reversal of T cell exhaustion by the first donor lymphocyte infusion is associated with the persistently effective antileukemic responses in patients with relapsed AML after Allo-HSCT. *Biol Blood Marrow Transplant*. (2018) 24:1350–9. doi: 10.1016/j.bbmt.2018.03.030
161. Bashey A, Medina B, Corringham S, Pasek M, Carrier E, Vrooman L, et al. CTLA4 blockade with ipilimumab to treat relapse of malignancy

after allogeneic hematopoietic cell transplantation. *Blood* (2009) 113:1581–8. doi: 10.1182/blood-2008-07-168468

162. Davids MS, Kim HT, Bachireddy P, Costello c, Liguori R, Savel A, et al. Ipilimumab for patients with relapse after allogeneic transplantation. *N Engl J Med*. (2016) 375:143–53. doi: 10.1056/NEJMoa1601202
163. Khouri IF, Fernandez Curbelo I, Turturro F, Jabbour EJ, Milton DR, Bassett RL, et al. Ipilimumab plus Lenalidomide after allogeneic and autologous stem cell transplantation for patients with lymphoid malignancies. *Clin Cancer Res*. (2018) 24:1011–8. doi: 10.1158/1078-0432.CCR-17-2777
164. Rafiq S, Yeku OO, Jackson HJ, Purdon TJ, van Leeuwen DG, Drakes DJ, et al. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy *in vivo*. *Nat Biotechnol*. (2018) 36:847–56. doi: 10.1038/nbt.4195
165. Bug G, Burchert A, Wagner EM, Kröger N, Berg T, Güller S, et al. Phase I/II study of the deacetylase inhibitor panobinostat after allogeneic stem cell transplantation in patients with high-risk MDS or AML (PANOBEST trial). *Leukemia* (2017) 31:2523–5. doi: 10.1038/leu.2017.242

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 December 2018
doi: 10.3389/fimmu.2018.03061

Regulation of GVHD and GVL Activity via PD-L1 Interaction With PD-1 and CD80

Kaniel Cassady[1,2,3], *Paul J. Martin*[4,5] *and Defu Zeng*[1,2,3]*

[1] Irell and Manella Graduate School of Biological Sciences of City of Hope, Duarte, CA, United States, [2] Department of Hematology/Hematopoietic Cell Transplantation, Beckman Research Institute at City of Hope National Medical Center, Duarte, CA, United States, [3] Diabetes and Metabolism Research Institute, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA, United States, [4] Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA, United States, [5] Department of Medicine, University of Washington, Seattle, WA, United States

Allogeneic hematopoietic cell transplantation (HCT) is a curative therapy for hematological malignancies (i.e. leukemia and lymphoma), because graft-versus-leukemia (GVL) activity mediated by alloreactive T cells can eliminate residual malignant cells and prevent relapse. However, the same alloreactive T cells also mediate a severe side effect, graft-versus-host disease (GVHD), and prevention of GVHD while preserving GVL activity remains an elusive goal. The immune checkpoint molecule PD-L1 and its interaction with PD-1 receptor in regulating cancer immunity is under intensive and wide-spread study, but knowledge about this interaction in regulating GVHD and GVL activity is very limited. In this review, we summarize the literature exploring how PD-L1 interaction with its receptors PD-1 and CD80 regulate GVHD and GVL activities, how PD-L1 signaling regulates T cell metabolic profiles, and how a differential role of PD-L1 interaction with PD-1, CD80 or both may provide a novel avenue to prevent GVHD while preserving strong GVL effects.

Keywords: HCT, GVHD, alloreactive, T cell, PD-L1, PD-1, CD80

OPEN ACCESS

Edited by:
Brian Christopher Betts, University of Minnesota Twin Cities, United States

Reviewed by:
Philippe Saas, INSERM U1098 Interactions Hôte-Greffon-Tumeur & Ingénierie Cellulaire et Génique, France
Benedetto Bruno, Università degli Studi di Torino, Italy

***Correspondence:**
Defu Zeng
DZeng@coh.org

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *28 September 2018*
Accepted: *10 December 2018*
Published: *21 December 2018*

Citation:
Cassady K, Martin PJ and Zeng D (2018) Regulation of GVHD and GVL Activity via PD-L1 Interaction With PD-1 and CD80. Front. Immunol. 9:3061. doi: 10.3389/fimmu.2018.03061

INTRODUCTION

Hematopoietic cell transplantation (HCT) provides curative therapy for hematological malignancies, such as lymphoma and leukemia, owing to graft-vs.-leukemia/lymphoma (GVL) effects mediated by alloreactive T cells (1–5). However, the same alloreactive T cells also mediate a severe side effect, called graft-vs.-host disease (GVHD). Prevention of GVHD while preserving GVL effect remains a long-sought and elusive goal.

Alloreactive T cells are activated by host antigen presenting cells (APCs) in lymphoid tissues early after HCT, and they then migrate to GVHD target tissues that were initially damaged by conditioning regimen to cause tissue inflammation and GVHD (6, 7). Acute GVHD is characterized by systemic lymphocyte infiltration and uncontrolled inflammation involving the release of damage-associated molecular pattern (DAMP) factors in target organs such as the gut, liver, lung, and skin (8–12). Chronic GVHD is a chronic autoimmune systemic syndrome characterized by moderate lymphocytic inflammation and fibrosis (13–16). Chronic GVHD and acute GVHD both affect the gut, liver, and skin, but chronic GVHD differs from acute GVHD in attacking other target tissue such as salivary and lacrimal glands and resembles a systemic auto-immune disease, like lupus, with dysregulated tolerance mechanisms (17–19).

GVHD pathogenesis is regulated by multiple factors, such as T regulatory $CD4^+$ cells (Treg), donor APCs, B regulatory cells (Breg), myeloid-derived suppressor cells (MDSC), and immune checkpoints including interactions of programmed death ligand 1 (PD-L1) with programmed death-1 (PD-1) and CD80 (20–26). Although immune checkpoint regulation of tumor immunity is under intensive study (27), immune checkpoint regulation of GVHD and GVL effects, and especially their regulation by PD-L1 interaction with CD80, is not yet fully understood. In this review, we focus on how PD-L1 interaction with PD-1 and CD80 differentially regulates auto- and allo-immunity. We also discuss how these interactions can separate GVL activity from GVHD in preclinical animal models and highlight the recent clinical application and challenges of PD-L1/PD-1 blockade after HCT for augmenting GVL activity.

PROGRAMMED DEATH LIGAND-1 (PD-L1)-MEDIATED SIGNALING PATHWAYS

PD-L1, (also known as CD274 or B7H1), is a member of the B7 family of immuno-coinhibitory and costimulatory molecules and functions as an immune checkpoint via its interaction with its receptors PD-1 and CD80 (also known as B7.1). PD-L1 is an Ig-V like transmembrane protein constitutively expressed by hematopoietic cells such as T, B and dendritic cells and by parenchymal cells in response to cytokine (i.e., IFN-γ) induction. PD-L1 was co-discovered in 2000 by two groups who reported it to have conflicting functions in the regulation of T cell activation, proliferation and apoptosis (28, 29). The Chen group first discovered and characterized the function of PD-L1 via cloning a homolog related to the B7.1 and B7.2 gene sequence, sequencing it and creating an expression plasmid containing the extracellular portion of PD-L1 in frame with the Fc portion of murine IgG2a (29). They found that costimulation of purified murine T cells in the presence of anti-CD3 and PD-L1-Ig led to an increase in T cell proliferation and IL-10 production, with moderate increase in IL-2 production—indicating a co-stimulatory signal mediated by the PD-L1 (29). On the other hand, costimulation of T cells with PD-L1-Ig produced by the Sharpe group led to a reduction in T cell proliferation and reduction in IL-10 production, indicating a coinhibitory signal mediated by PD-L1 (28).

Early *in vivo* studies on the role of PD-L1 were also conflicting. Tissue-specific transgenic expression of PD-L1 under the insulin promoter in islet beta cells augmented the rejection of islet grafts, which was associated with increased proliferation and reduced apoptosis of infiltrating $CD8^+$ T cells (30). However, in a cardiac allograft model, treatment with PD-L1-Ig was associated with prolonged allograft survival and reduced lymphocytic infiltrate in the graft (7). Further characterization of the interactions of PD-L1/PD-1 and PD-L1/CD80 in unraveling the dual properties of the PD-L1-mediated signaling pathways are described below.

PD-L1/PD-1 Signaling Pathway

The role of PD-L1 in regulating the immune response has been best characterized via its interaction with its dominant receptor PD-1, also termed Pdcd1 (6, 7, 23, 24). PD-1 is a monomeric co-inhibitory receptor that was originally identified in the 2B4.11 T cell hybridoma cell line as being upregulated upon induction of activation-induced apoptosis following stimulation with PMA and ionomycin (21). PD-1 expressed by activated T cells upon stimulation, is localized to the immunological synapse near the TCR and functions to attenuate T cell adaptive immune responses by inhibiting T cell proliferation and inducing T cell exhaustion, anergy, and apoptosis (6, 7). The importance of PD-1 in maintaining peripheral tolerance was highlighted by the generation of $PD\text{-}1^{-/-}$ mice that develop Lupus-like arthritis and glomerulonephritis. Peripheral T and B cells from these mice exhibit hyper-reactivity upon stimulation (27, 31). The primary intracellular molecular mechanism responsible for PD-1 attenuation of the T cell response is attributed to the function of the immunoreceptor tyrosine-based inhibitory motif (ITIM) located in the cytoplasmic tail of PD-1 (7, 32). PD-L1/PD-1 ligation induces phosphorylation of this ITIM and recruits the protein-tyrosine phosphatases SHP1/2, in a TCR-stimulation dependent manner (33). Due to the proximity of the PD-1 cytoplasmic tail in the synapse to the TCR phosphorylation signaling cascade, SHP-1/2 phosphatase localization to PD-1 leads to dephosphorylation of TCR downstream signaling molecules, such as PI3K, ZAP70, and PTEN (34, 35). Collectively, dephosphorylation of this cascade leads to cell-cycle arrest, reduction in T cell proliferation/expansion and exhaustion/apoptosis, which can be reversed via PD-L1/PD-1 blockade to restore T cell function (36–38).

More recently, work by the Boussiotis group (39) has described a link between PD-L1/PD-1 signaling in the regulation of T cell metabolism by restricting nutrient uptake and utilization to inhibit T cell function (discussed below). Taken together, the PD-L1/PD-1 pathway inhibits the TCR signaling cascade to dampen the T cell immune response to maintain peripheral T cell tolerance.

PD-L1/CD80 Signaling Pathway

In addition to interacting with PD-1, PD-L1 binds to and signals through a second receptor, CD80 (B7.1, B7-1). CD80, a member of the B7-super family, is a dimeric transmembrane protein, is constitutively expressed by T cells and is further upregulated upon T cell activation (22). Generally recognized for its function as a costimulatory ligand (along with CD86) for CD28, CD80 was first identified as a receptor on T cells for PD-L1 and was characterized by its ability to bidirectionally inhibit T cell responses (40, 41). The sites on PD-L1 that bind, respectively, to CD80 and PD-1 partially overlap, and the affinity of PD-L1 for CD80 is ∼3-fold lower than its affinity for PD-1 (41). Using beads coated with anti-CD3 and CD80-Ig fusion protein or human IgG-Fc as a control, the authors stimulated $CTLA4^{-/-}$ $CD28^{-/-}$ T cells (T cells deficient for the two known binding partners of CD80). Under these conditions, costimulation with CD80-Ig decreased the proliferation of double-deficient T cells,

indicating that CD80 can signal through PD-L1 expressed by T cells to inhibit proliferation (41). Furthermore, using beads coated with anti-CD3 and PD-L1-Ig fusion protein or human IgG-Fc as a control, the authors stimulated WT T cells and PD-$1^{-/-}$ T cells. Under these conditions, costimulation with PD-L1-Ig decreased the proliferation of PD-$1^{-/-}$ T cells, indicated that PD-L1 can signal through CD80 expressed by T cells to inhibit proliferation (41). Taken together, these results suggest a bi-directional inhibitory signal mediated by PD-L1/CD80 interaction.

in vivo studies using an anti-PD-L1 mAb that specifically blocks PD-L1/CD80 interaction while preserving PD-L1/PD-1 interaction have established PD-L1/CD80 "reverse signaling" into T cells as being pro-tolerogenic. In a murine model of immunization, blockade of PD-L1/CD80 interaction led to increased expansion and reduced induction of T cell anergy during the contraction phase following immunization (42). Moreover, in models of both Type-1 diabetes (T1D) and cardiac allograft transplantation, blockade of PD-L1/CD80 led to the increased production of proinflammatory cytokines by T cells and exacerbated T1D and graft rejection, respectively (43, 44).

On the other hand, agonistic anti-CD80 mAb interaction with naïve $CD4^+$ T cells induced an increase in intracellular $Ca2^+$ influx and led to phosphorylation of the Th1-promoting transcription factor T-bet (pT-bet). Phosphorylated T-bet localized to the *Ifng* locus and subsequently increased IFN-γ expression (45). Therefore, the impact of signaling mediated by PD-L1/CD80 interaction on T cell activation and tolerance induction requires further studies.

PD-L1/CD28 Co-stimulatory Signaling Pathway Crosstalk

In addition to the direct effects of PD-L1 on T cell activation, expansion, and apoptosis exerted through its receptors PD-1 and CD80, PD-L1 signaling also exhibits cross-talk with another T cell co-stimulatory pathway, the CD28 signaling pathway. This canonical signaling pathway functions to complement the signals received via ligation of the TCR to activate the NF-kappaB transcriptional pathway and augment T cell survival and proliferation (46). Two groups recently co-discovered that the functional blockade of the PD-L1/PD-1 signaling pathway, which promotes anti-viral and anti-tumor immunity, is contingent on interruption of the co-stimulatory signaling cascade received from CD28 (47, 48). However, the interplay between PD-L1/CD80 pathway and CD28 pathway was not investigated. Additionally, the effect of interactions between the PD-L1 signaling pathway and CD28 on alloreactive T cells remains unexplored.

PD-L1/PD-1 INTERACTION IN REGULATING ACUTE GVHD

The PD-L1-mediated signaling pathway serves as a critical immunological checkpoint of the alloreactive T cell response after HCT in both animal models and humans. Early studies performed by Blazar et al. demonstrated that the PD-L1/PD-1 interactions following HCT are critical for preventing GVHD (20). Following HCT (GVHD murine model: C57BL/6→B10.BR), the investigators treated HCT recipients with either a blocking PD-L1-IgG2a protein or anti-PD-1 mAb (clone J43). Recipients treated with either method of PD-L1/PD-1 blockade exhibited increased clinical signs of GVHD and mortality compared to control IgG-treated recipients (20). Additionally, recipients of PD-$1^{-/-}$ donor T cells showed increased mortality after HCT compared to recipients of WT donor T cells, indicating a critical role for PD-1 in preventing pathogenic effects of alloreactive T cells (20).

Because PD-1 interacts with a second ligand, PD-L2, to mediate coinhibitory signals to T cells (49), additional work by the Blazar group showed that the PD-L1/PD-1 signaling pathway dominates the PD-L2/PD-1 signaling pathway in the regulation of alloreactive T cell pathogenesis during GVHD (50). Hematopoietic cells upregulate expression of both PD-L2 and PD-L1 after HCT, but only PD-L1 is broadly expressed by parenchymal cells in host GVHD target tissues. Blockade of PD-L1/PD-1 interaction, but not PD-L2/PD-1 interaction can prevent GVHD after HCT (50). Together, these studies identified PD-L1/PD-1 interaction as having a dominant role in regulating alloreactive T cell expansion and pathogenesis to prevent GVHD after HCT.

PD-L1/CD80 INTERACTION IN REGULATING ACUTE GVHD

Our group has carried out experiments to elucidate how PD-L1/CD80 interaction impacts PD-L1/PD-1 interaction. First, we found that PD-L1/CD80 interaction augments the expansion of donor natural regulatory $CD4^+$ T cells (nTregs) after HCT in a minor MHC-mismatched model of GVHD (DBA/2→BALB/c) (22). Survival of WT donor-derived nTregs after HCT was lower in PD-$L1^{-/-}$ recipients than in WT recipients, which was associated with increased severity of GVHD (22). Moreover, treatment of HCT recipients with either anti-PD-1 mAb or anti-PD-L1 mAb (clone 43H12 which specifically blocks PD-L1/CD80 interaction), demonstrated that PD-L1/CD80 interaction but not PD-L1/PD-1 interaction is responsible for the augmentation of donor nTreg cell survival after HCT (22). Finally, neutralization of IFN-γ, a potent inducer of PD-L1 expression in APCs, led to a reduction in PD-L1 expression by host APCs, a reduction in the number of peripheral nTreg cells and increased severity of GVHD (22).

In a subsequent study, our group also characterized the effect of host parenchymal cell PD-L1 expression on donor alloreactive $CD8^+$ T cell pathogenesis in GVHD target tissues after HCT (51). First, we observed that anti-CD3-conditioning prevent induction of acute GVHD, and this effect depends on host-tissue expression of PD-L1. In three murine models of GVHD, we found that increased parenchymal expression of PD-L1 was inversely correlated with the severity of GVHD in the colon, liver, lung and skin (51). Moreover, hepatocyte specific expression of PD-L1-Fc protein induced via hydrodynamic injection of

plasmids encoding PD-L1-Fc led to hepatocyte expression of PD-L1, high levels of serum PD-L1, reduced numbers of $CD8^+$ T cells infiltrating the liver, and resolution of GVHD (51).

Although this study demonstrated a clear requirement for PD-L1 expression by parenchymal cells in order to tolerize T cells infiltrating the liver, but the distinct contributions of PD-L1/PD-1 vs. PD-L1/CD80 interactions remained unclear. We subsequently evaluated the separate contributions of the two PD-L1-mediated signaling pathways in regulating GVHD induced by both $CD4^+$ and $CD8^+$ T cells. As depicted in the diagram **(Figure 1)**, first, using MHC-mismatched C57BL/6 donors and BALB/c recipients, we showed that PD-$1^{-/-}$ donor alloreactive $CD4^+$ T cells had decreased proliferation and apoptosis when transferred into PD-$L1^{-/-}$ hosts compared WT hosts (25). PD-$L1^{-/-}$ recipients of PD-$1^{-/-}$ T cells also exhibited a reduction in the severity of GVHD when compared to WT recipients, indicating that the interaction of recipient PD-L1 with CD80 expressed by donor $CD4^+$ T cells in the absence of PD-1 increases their expansion, survival and pathogenesis (25). This phenomenon was recapitulated by blocking PD-L1/CD80 interaction on PD-$1^{-/-}$ T cells *in vivo*. In contrast, treatment of allogeneic recipients of PD-$1^{+/+}$ $CD4^+$ T cells with anti-PD-L1 43H12 mAb (anti-PD-L1 mAb which specifically blocks PD-L1/CD80 interaction) on day 5 after HCT led to a reduction in proliferation and apoptosis, leading to an augmentation of donor T cell survival and an augmentation of GVHD (25). Finally, *in vivo* expression of PD-L1-Fc protein via hydrodynamic injection augmented PD-$1^{+/+}$ $CD4^+$ T cell proliferation and apoptosis leading to an amelioration of GVHD (25). Taken together, these results demonstrate the dual nature of PD-L1/CD80 signaling pathway: PD-L1/CD80 interaction augments T cell proliferation; this interaction also augments activation induced apoptosis mediated by PD-L1/PD-1 interaction. Thus, PD-L1 interaction with PD-1 and CD80 *simultaneously* is required to effectively ameliorate alloreactive T cell-mediated GVHD.

DIFFERENTIAL PD-L1/PD-1 AND PD-L1/CD80 INTERACTIONS SEPARATE GVL EFFECTS FROM GVHD

More recent studies investigating the role of PD-L1 expression on donor lymphoid cells and host tissue following HCT have provided further insight into the complexity of PD-L1-mediated signaling pathways. Before HCT, donor T cells express low levels of PD-L1. PD-L1 expression is upregulated on allogeneic but not syngeneic murine donor T cells within 5 days after HCT. PD-L1 is induced within hours after stimulation of human $CD4^+$ and $CD8^+$ T cells *in vitro* (52). Additionally, genetic ablation of *Pdl1,* the gene that encodes PD-L1, in donor T cells led to a reduction in the severity of GVHD in mice, which was associated with reduced production of proinflammatory cytokines (IFN-γ, TNF-α) and reduced expression of gut homing and chemokine receptors (52). PD-$L1^{-/-}$ donor T cells also exhibited reduced proliferation and yield in lymphoid tissues after HCT, together with increased apoptosis associated with increased expression of FasL and reduced expression of the anti-apoptotic protein Bcl-xL (52).

Our group recently performed a thorough examination of the role of donor T cell expression vs. host parenchymal expression of PD-L1 on the separation of GVHD from GVL activity (26). Using both murine and xeno-GVHD models in which selective depletion of $CD4^+$ T cells early after HCT can prevent GVHD while preserving GVL activity, we investigated the differential expression of both PD-L1 and its receptors on donor T cells and in target tissues on the development of GVHD. After $CD4^+$ T depletion, IFN-γ produced by donor $CD8^+$ T cells upregulated expression of PD-L1 by both host tissues and donor $CD8^+$ T cells. The relative expression of PD-1 and CD80 by donor $CD8^+$ T cells depends on their location. Donor $CD8^+$ T cells in the lymphoid tissues had preferential expression of CD80, while those in GVHD target tissues had preferential expression of PD-1. The dominant interaction of PD-L1 with CD80 in lymphoid tissues promoted donor $CD8^+$ T proliferation and survival, thus preserving GVL effects (26). On the other hand, the dominant interaction of PD-L1 with PD-1 on donor $CD8^+$ T cells in GVHD-target tissues promoted tolerance through induction of apoptosis, anergy, and exhaustion of $CD8^+$ infiltrating T cells (26). Therefore, the outcome of PD-L1-mediated signaling on GVHD and GVL effect depends on the microenvironment and on T cell expression of CD80 and PD-1.

PD-L1 PATHWAY REGULATION OF ALLOREACTIVE T CELL METABOLISM

As in all cells, immune cells require energy to execute cellular functions, such as survival, proliferation, and cytokine secretion (53). This required energy is provided as adenosine triphosphate (ATP) by several metabolic pathways. The first is glycolysis, which involves the conversion of glucose to pyruvate in the cytosol. The second pathway is the tricarboxylic acid (TCA) cycle (also called the Krebs cycle), which donates electrons to the electron transport chain located in the mitochondria to fuel oxidative phosphorylation (OXPHOS) or respiration. This OXPHOS process generates ATP in the mitochondria. Other substrates, such as fatty acids via β-oxidation (also called fatty acid oxidation [FAO]), can replenish the TCA cycle to fuel OXPHOS. Moreover, the preferential use of glycolysis vs. OXPHOS depends on oxygen availability (53).

T cell proliferation and rapid expansion is a highly dynamic process that taxes intracellular bioenergetics (54). Much like the characteristic "Warburg Effect" observed in malignant cells, the activation and proliferation of T cells requires an immediate switch from a quiescent metabolic state that relies on OXPHOS to an active metabolic state that relies on glycolysis to support rapid flux of ATP, production of biosynthetic intermediates, and synthesis of macromolecules for the process of cell division (55–57). During resolution of the immune response, a subset of T cells augment mitochondrial capacity and revert to multi-substrate OXPHOS, thereby forming memory T cells to provide long-term adaptive immunity (56, 58).

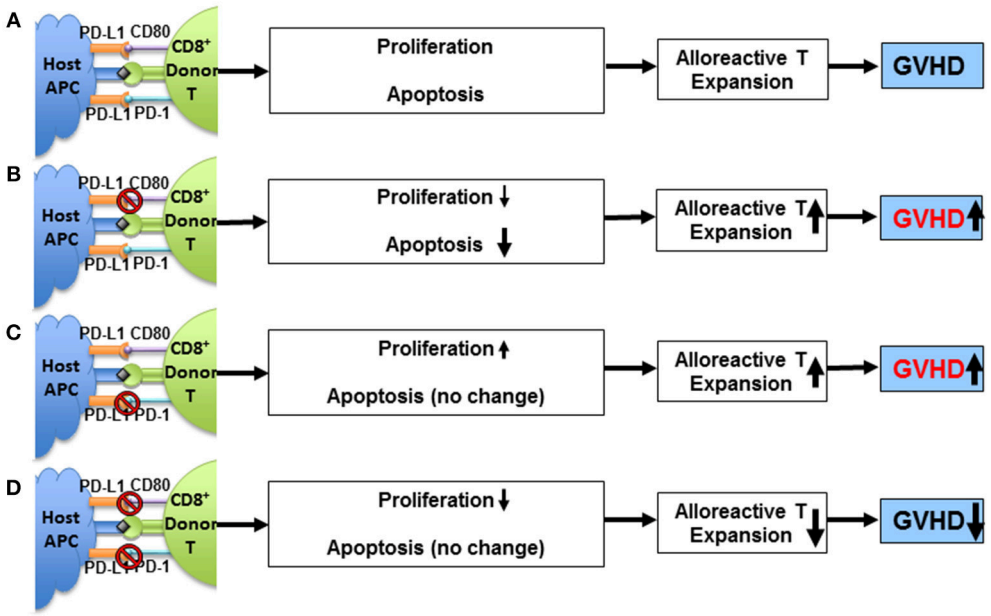


FIGURE 1 | Impact of PD-L1/CD80 interaction on T cell proliferation, PD-1-dependent T cell apoptosis, and GVHD severity. **(A)** Activated alloreactive Tcon cells upregulate expression of PD-1 and CD80 that interact with PD-L1 expressed by host tissue cells (e.g., APCs) after allogeneic HCT. The interactions cannot prevent induction of GVHD. **(B)** Blockade of PD-L1/CD80 interaction reduces alloreactive Tcon cell proliferation and apoptosis, and reduction of apoptosis outweighs reduction of proliferation, resulting in expansion of alloreactive Tcon cells and worsening GVHD. **(C)** In the absence of PD-1 (i.e., lack of PD-L1/PD-1 interaction), PD-L1/CD80 interaction *per se* augments alloreactive Tcon cell proliferation with no impact on apoptosis, resulting in expansion of alloreactive T cells and worsening GVHD. **(D)** In the absence of PD-1, blockade of PD-L1/CD80 interaction reduces Tcon cell proliferation and reduces Tcon expansion and ameliorates GVHD. Adapted from Deng et al. (25).

In the context of HCT and GVHD, the kinetic metabolic phenotype of alloreactive T cells remains controversial. Consistent with the canonical paradigm of T cell metabolism during activation and expansion is the recent observation by Rathmell et al. that deletion of Glut1, the primary glucose transporter expressed by T cells, reduces the severity of alloreactive T cell mediated GVHD (59). The reduced severity of GVHD was associated with the failure of Glut1$^{-/-}$ T cells, especially CD4^{+} T cells, to expand *in vivo* (59). Additionally, a thorough follow-up study by the Yu group tracked the metabolic profile of alloreactive T cells after HCT (60). In comparison to syngeneic T cells, alloactivated T cells isolated from recipients with GVHD exhibit a switch from fatty acid oxidation (FAO) in the mitochondria to glycolysis over time after HCT. This change occurred without a subsequent reversion to FAO over time, suggesting that these cells must exhibit an increased reliance on glutaminolysis and the pentose phosphate (PPP) pathway for macromolecule biosynthesis in the absence of the tricarboxylic acid (TCA) cycle (60). In this model, inhibition of glycolysis, either by inhibiting mTorc1 (a key molecular regulator of glycolysis) or 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) (a rate limiting step in the glycolytic pathway), reduced the number of alloactivated Th1 cells and ameliorated GVHD (60).

These observations were contradicted by results reported by Byersdorfer et al. who showed that increased Glut1 expression and the switch to glycolysis after alloactivation is merely an *in vitro* phenomenon. Glut1 expression by T cells remained quite static after HCT, while fatty acid uptake and OXPHOS increased in alloactivated T cells by 7 days after HCT (61, 62). Moreover, inhibition of fatty acid uptake by treatment with the irreversible carnitine palmitoyl transerase I (CPT1A; the rate limiting enzyme of fatty acid transport into the mitochondria) inhibitor etomoxir (Etx) induced T cell apoptosis and reduced GVHD severity (61, 63). Finally, an early increase in both glycolysis and OXPHOS in alloactivated T cells has been reported by groups working with Blazar groups (50, 63).

While each of these models has shown some promise in the manipulation of T cell bioenergetics for GVHD prevention, the specific metabolic programs utilized by alloactivated T cells after HCT remain controversial, and a definitive and effective regimen for targeting alloreactive T cell metabolism for the prevention of GVHD remains elusive.

Of interest, Boussiotis et al. recently showed that ligation of PD-1 on human CD4^{+} T cells *in vitro* via PD-L1-Ig stimulation led to upregulation of CPT1A expression with increased fatty acid uptake and enhanced OXPHOS activity, accompanied by downregulation of Glut1 expression and glycolysis via inhibition of the PI3K/AKT/mTOR pathway (39). Consistently, *in vivo* blockade of PD-L1 signaling using anti-PD-L1 mAb (clone 10F.9G2) that blocks both PD-L1/CD80 and PD-L1/PD-1 interactions augmented GVHD and led to an increase in

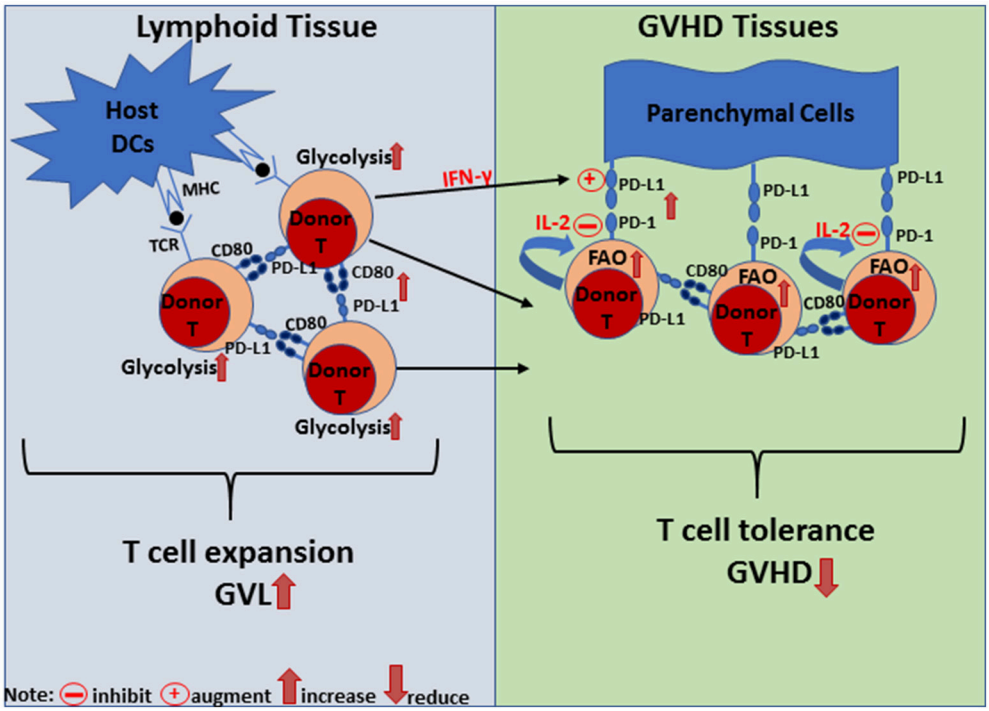


FIGURE 2 | Diagram of PD-L1-mediated signaling regulation of T cell metabolism, proliferation and tolerance in lymphoid and GVHD target tissues. In lymphoid tissue, PD-L1/CD80 interaction is dominant and leads to T cell expansion to promote GVL effects. Signaling either through PD-L1 or CD80 via DC:T or T:T interactions leads to increased glycolysis, proliferation, expansion, and IFN-gamma production. In GVHD target tissues, PD-L1 expressed by parenchymal cells interacts with PD-1 expressed by donor T cells and induces their anergy, exhaustion or apoptosis (depending on the microenvironment) to establish T cell tolerance and prevent GVHD. IFN-gamma produced by donor T cells can augment PD-L1 expression and PD-L1/PD-1 interaction reprograms donor T cells toward a quiescent metabolic phenotype. On the other hand, autocrine or paracrine IL-2 signaling can overcome PD-L1/PD-1 interaction to maintain donor T cell effector function.

Glut1 expression and glycolysis with a subsequent reduction in mitochondrial respiration (OXPHOS) (64). In contrast, it was also reported that *in vivo* after HCT, alloreactive T cells transplanted into PD-L1$^{-/-}$ recipients exhibited accelerated expansion and pathogenesis; in this situation, absence of host PD-L1 was associated with an increase in both glycolysis and OXPHOS in proliferating donor T cells during expansion (50). Specifically, donor T cells from PD-L1$^{-/-}$ recipients exhibited an increase in Glut1 expression, lactate production (indicative of increased glycolysis), oxygen consumption [indicative of increased utilization of O2 in the electron transport chain (ETC)], mitochondrial membrane potential and ROS production (both indicators of increased mitochondrial respiration) (50). In contrast, the same group reported that PD-L1 deficiency in donor T cells reduces acute GVHD with reduced aerobic glycolysis, oxidative phosphorylation, and fatty acid metabolism in the spleen (52).

The observations with PD-L1$^{-/-}$ recipients suggest that in the absence of host PD-L1, PD-L1 interactions with PD-1 and CD80 among donor cells may modulate donor T cell metabolism. In addition, PD-L1/CD80 interaction may play an important role in augmenting donor cell expansion in lymphoid tissues. We recently demonstrated that PD-L1 expressed by alloactivated donor T cells augment donor T cell expansion in lymphoid tissue through T cell-T cell interaction to augment GVL (26). Importantly, this expansion required donor PD-L1/CD80 interaction (26). Whether and how PD-L1/CD80 interactions among donor T cells affects alloactivated donor T cell metabolism needs to be further addressed.

SUMMARY

Current literature indicates that regulation of GVHD and GVL activity of alloreactive T cells via PD-L1 interaction with PD-1 and CD80 depends on both the tissue compartment and effector cell composition. As depicted in **(Figure 2)**, in the lymphoid tissues, donor cells express higher levels of CD80 and PD-L1 but low levels of PD-1. Here, PD-L1 interaction can augment donor T cell expansion, effector function, and GVL activity. This expansion event may be associated with a PD-L1/CD80 dependent increase in the glycolysis of effector T cells. In contrast, donor T cells express higher levels of PD-1 and lower levels of CD80 in GVHD target tissues, and at the same time, host-tissues express high levels of PD-L1. Here, the simultaneous PD-L1 interaction with PD-1 and CD80 can lead to infiltrating T cell anergy/exhaustion and apoptosis. This PD-L1/PD-1 dominant environment may promote the reversion to a quiescent metabolic phenotype (OXPHOS) in T cells to support reduction in effector T cell function. It should be noted, however, that this mechanism is effective in preventing GVHD only in

the absence of donor $CD4^+$ T cells or lack of IL-2, because IL-2 from $CD4^+$ T cells can help $CD8^+$ T cells or help $CD4^+$ T cells through autocrine signaling to become resistant against PD-L1-mediated anergy, exhaustion, and apoptosis, perhaps through the promotion of glycolysis (26).

That the 2018 Nobel Prize Award in Medicine was in part awarded to Tasuko Honjo for the discovery of PD-1 highlights the attention that this pathway has achieved abroad and in the clinic for treating cancer. Unfortunately, while much success has been realized with regards to the treatment of cancer with anti-PD-1 mAbs, there are significant side-effects to the non-specific activation of the adaptive immune response (65, 66). Of particular interest to our studies and this review is patient outcome after treatment of relapsed hematological malignancies following HCT (67, 68). Multiple groups have now documented the use of anti-PD-1 mAbs post-HCT for the treatment of relapsed hematological malignancies, such as Hodgkin's lymphoma (67, 69, 70). While outcomes have been favorable regarding cancer-free progression, anti-PD-1 treatment after HCT is associated with the induction of alloimmunity in the form of T cell-mediated acute graft-vs.-host disease (aGVHD) (67, 69). Thus, while the PD-L1/PD-1 pathway is a master regulator of immune-tolerance and can be taken advantage of for the augmentation of anti-tumor immunity, clinical transplantation demonstrates that it is a double-edged sword in that it is also required for the maintenance of T cell tolerance to prevent autoimmunity in cancer patients as well as alloimmunity following HCT. This is consistent with our preclinical observation that PD-L1 checkpoint enforcement, but not blockade, can prevent GVHD after HCT while preserving GVL activity (25, 26).

In future studies, it will be important to determine how PD-L1 interaction with PD-1 or CD80 alone or together regulates donor T cell metabolism. Reagents that regulate T cell metabolism (e.g., 2-DG, ETX, etc.), inhibit IL-2 activity or augment PD-L1/PD1 interactions in GVHD target tissues could tolerize infiltrating T cells while maintaining the function of T cells in lymphoid tissues, such that GVHD could be effectively prevented while strong GVL activity is preserved.

AUTHOR CONTRIBUTIONS

KC wrote the manuscript. PM and DZ revised the manuscript.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant R01 AI066008 and R01 CA228465 (to DZ).

REFERENCES

1. Appelbaum FR. Haematopoietic cell transplantation as immunotherapy. *Nature* (2001) 411:385–9. doi: 10.1038/35077251
2. Reddy P, Maeda Y, Liu C, Krijanovski OI, Korngold R, Ferrara JL. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nat Med.* (2005) 11:1244–9. doi: 10.1038/nm1309
3. Fefer A. Graft-vs.-tumor responses. In: Blume KG, Forman SJ, Appelbaum FR, editors, *Thomas' Hematopoietic Cell Transplantation*. Malden, MA: Blackwell Publishing Ltd. (2007). p. 369–79.
4. Riddell SR. The graft-versus-leukemia effect–breaking the black box open. *Biol Blood Marrow Transplant.* (2008) 14:2–3. doi: 10.1016/j.bbmt.2007.10.004
5. Schroeder MA, Dipersio JF. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech.* (2011) 4:318–33. doi: 10.1242/dmm.006668
6. Koyama M, Kuns RD, Olver SD, Raffelt NC, Wilson YA, Don AL, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat Med.* (2011) 18:135–42. doi: 10.1038/nm.2597
7. Li H, Demetris AJ, Mcniff J, Matte-Martone C, Tan HS, Rothstein DM, et al. Profound depletion of host conventional dendritic cells, plasmacytoid dendritic cells, and B cells does not prevent graft-versus-host disease induction. *J Immunol.* (2012) 188:3804–11. doi: 10.4049/jimmunol.1102795
8. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* (1997) 90:3204–13.
9. Chakraverty R, Cote D, Buchli J, Cotter P, Hsu R, Zhao G, et al. An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T cells to peripheral tissues. *J Exp Med.* (2006) 203:2021–31. doi: 10.1084/jem.20060376
10. Cutler C, Antin JH. Manifestations and treatment of acute graft-versus-host disease. In: Appelbaum FR, Forman SJ, Negrin RS, Blume KG, editors, *Thomas' Hematopoietic Cell Transplantation*. Hoboken, NJ: Wiley-Blackwell (2009). p. 1287–303. doi: 10.1002/9781444303537.ch86
11. Korngold R, Antin J. Biology and management of acute graft-versus-host disease. In: Bishop MR, editor. *Hematopoietic Stem Cell Transplantation*. New York, NY: Springer US (2009). p. 257–75. doi: 10.1007/978-0-387-78580-6_11
12. Zeiser R, Blazar BR. Acute graft-versus-host disease - biologic process, prevention, and therapy. *N Engl J Med.* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
13. Lee SJ, Vogelsang G, Flowers ME. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* (2003) 9:215–33. doi: 10.1053/bbmt.2003.50026
14. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant.* (2005) 11:945–56. doi: 10.1016/j.bbmt.2005.09.004
15. Pavletic SZ, Vogelsang GB. Chronic graft-versus-host disease: clinical manifestations and therapy. In: Appelbaum FR, Forman SJ, Negrin RS, Blume KG, editors. *Thomas' Hematopoietic Cell Transplantation*. 4th ed. Hoboken, NJ: Blackwell Publishing (2009). p. 1304–24.
16. Schultz KR. Pathophysiology of chronic graft versus host disease. In: Vogelsang GB, Pavletic SZ, editors. *Chronic Graft versus Host Disease: Interdisciplinary Management*. New York, NY: Cambridge University Press (2009). p. 17–30. doi: 10.1017/CBO9780511576751.004
17. Rouquette-Gally AM, Boyeldieu D, Prost AC, Gluckman E. Autoimmunity after allogeneic bone marrow transplantation. A study of 53 long-term-surviving patients. *Transplantation* (1988) 46:238–40. doi: 10.1097/00007890-198808000-00010
18. Levy S, Nagler A, Okon S, Marmary Y. Parotid salivary gland dysfunction in chronic graft-versus-host disease (cGVHD): a longitudinal study in a mouse model. *Bone Marrow Transplant.* (2000) 25:1073–8. doi: 10.1038/sj.bmt.1702383
19. Zeiser R, Blazar BR. Pathophysiology of chronic graft-versus-host disease and therapeutic targets. *N Engl J Med.* (2017) 377:2565–79. doi: 10.1056/NEJMra1703472
20. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, Carter L, Iwai Y, Yagita H, et al. Blockade of programmed death-1 engagement accelerates

graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. *J Immunol.* (2003) 171:1272–7. doi: 10.4049/jimmunol.171.3.1272
21. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1–dependent mechanism that is up-regulated by interleukin-13. *Blood* (2010) 116:5738–47. doi: 10.1182/blood-2010-06-287839
22. Yi T, Li X, Yao S, Wang L, Chen Y, Zhao D, et al. Host APCs augment *in vivo* expansion of donor natural regulatory T cells via B7H1/B7.1 in allogeneic recipients. *J. Immunol.* (2011) 186:2739–49. doi: 10.4049/jimmunol.1002939
23. Beres A, Drobyski W. The role of regulatory t cells in the biology of graft versus host disease. *Front Immunol.* (2013) 4:163. doi: 10.3389/fimmu.2013.00163
24. De Masson A, Bouaziz JD, Le Buanec H, Robin M, O'meara A, Parquet N, et al. CD24(hi)CD27(+) and plasmablast-like regulatory B cells in human chronic graft-versus-host disease. *Blood* (2015) 125:1830–9. doi: 10.1182/blood-2014-09-599159
25. Deng R, Cassady K, Li X, Yao S, Zhang M, Racine J, et al. B7H1/CD80 interaction augments pd-1-dependent t cell apoptosis and ameliorates graft-versus-host disease. *J Immunol.* (2015) 194:560–74. doi: 10.4049/jimmunol.1402157
26. Ni X, Song Q, Cassady K, Deng R, Jin H, Zhang M, et al. PD-L1 interacts with CD80 to regulate graft-versus-leukemia activity of donor CD8+ T cells. *J Clin Invest.* (2017) 127:1960–77. doi: 10.1172/JCI91138
27. Baumeister SH, Freeman GJ, Dranoff G, Sharpe AH. Coinhibitory pathways in immunotherapy for cancer. *Annu Rev Immunol.* (2016) 34:539–73. doi: 10.1146/annurev-immunol-032414-112049
28. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the Pd-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) 192:1027–34. doi: 10.1084/jem.192.7.1027
29. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med.* (1999) 5:1365–9. doi: 10.1038/70932
30. Subudhi SK, Zhou P, Yerian LM, Chin RK, Lo JC, Anders RA, et al. Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection. *J Clin Invest.* (2004) 113:694–700. doi: 10.1172/JCI19210
31. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* (1999) 11:141–51. doi: 10.1016/S1074-7613(00)80089-8
32. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev.* (2009) 229:114–25. doi: 10.1111/j.1600-065X.2009.00767.x
33. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol.* (2004) 173:945–54. doi: 10.4049/jimmunol.173.2.945
34. Patsoukis N, Brown J, Petkova V, Liu F, Li L, Boussiotis VA. Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation. *Sci Signal.* (2012) 5:ra46. doi: 10.1126/scisignal.2002796
35. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med.* (2012) 209:1201–17. doi: 10.1084/jem.20112741
36. Fuse S, Tsai CY, Molloy MJ, Allie SR, Zhang W, Yagita H, et al. Recall responses by helpless memory CD8+ T cells are restricted by the up-regulation of PD-1. *J Immunol.* (2009) 182:4244–54. doi: 10.4049/jimmunol.0802041
37. Patsoukis N, Sari D, Boussiotis VA. PD-1 inhibits T cell proliferation by upregulating p27 and p15 and suppressing Cdc25A. *Cell Cycle* (2012) 11:4305–9. doi: 10.4161/cc.22135
38. Odorizzi PM, Pauken KE, Paley MA, Sharpe A, Wherry EJ. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8+ T cells. *J Exp Med.* (2015) 212:1125–37. doi: 10.1084/jem.20142237
39. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, et al. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nat Commun.* (2015) 6:6692. doi: 10.1038/ncomms7692
40. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol.* (2002) 2:116–26. doi: 10.1038/nri727
41. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* (2007) 27:111–22. doi: 10.1016/j.immuni.2007.05.016
42. Park JJ, Omiya R, Matsumura Y, Sakoda Y, Kuramasu A, Augustine MM, et al. B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. *Blood* (2010) 116:1291–8. doi: 10.1182/blood-2010-01-265975
43. Paterson AM, Brown KE, Keir ME, Vanguri VK, Riella LV, Chandraker A, et al. The programmed death-1 ligand 1:B7-1 pathway restrains diabetogenic effector T cells *in vivo*. *J Immunol.* (2011) 187:1097–105. doi: 10.4049/jimmunol.1003496
44. Yang J, Riella LV, Chock S, Liu T, Zhao X, Yuan X, et al. The novel costimulatory programmed death ligand 1/B7.1 pathway is functional in inhibiting alloimmune responses *in vivo*. *J Immunol.* (2011) 187:1113–9. doi: 10.4049/jimmunol.1100056
45. Podojil JR, Miller SD. Cross-linking of CD80 on CD4+ T cells activates a calcium-dependent signaling pathway. *J Immunol.* (2009) 182:766–73. doi: 10.4049/jimmunol.182.2.766
46. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 Costimulation: from mechanism to therapy. *Immunity* (2016) 44:973–88. doi: 10.1016/j.immuni.2016.04.020
47. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) 355:1428–33. doi: 10.1126/science.aaf1292
48. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science* (2017) 355:1423–7. doi: 10.1126/science.aaf0683
49. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol.* (2001) 2:261–8. doi: 10.1038/85330
50. Saha A, Aoyama K, Taylor PA, Koehn BH, Veenstra RG, Panoskaltsis-Mortari A, et al. Host programmed death ligand 1 is dominant over programmed death ligand 2 expression in regulating graft-versus-host disease lethality. *Blood* (2013) 122:3062–73. doi: 10.1182/blood-2013-05-500801
51. Li X, Deng R, He W, Liu C, Wang M, Young J, et al. Loss of B7-H1 expression by recipient parenchymal cells leads to expansion of infiltrating donor CD8+ T cells and persistence of graft-versus-host disease. *J Immunol.* (2012) 188:724–34. doi: 10.4049/jimmunol.1102630
52. Saha A, O'connor RS, Thangavelu G, Lovitch SB, Dandamudi DB, Wilson CB, et al. Programmed death ligand-1 expression on donor T cells drives graft-versus-host disease lethality. *J Clin Invest.* (2016) 126:2642–60. doi: 10.1172/JCI85796
53. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity* (2013) 38:633–43. doi: 10.1016/j.immuni.2013.04.005
54. Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. *J Leukoc Biol.* (2008) 84:949–57. doi: 10.1189/jlb.0108024
55. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol.* (2005) 5:844–52. doi: 10.1038/nri1710
56. Van Der Windt GJ, Pearce EL. Metabolic switching and fuel choice during T-cell differentiation and memory development. *Immunol Rev.* (2012) 249:27–42. doi: 10.1111/j.1600-065X.2012.01150.x
57. Maciver NJ, Michalek RD, Rathmell JC. Metabolic regulation of T lymphocytes. *Annu Rev Immunol.* (2013) 31:259–83. doi: 10.1146/annurev-immunol-032712-095956
58. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, et al. Mitochondrial dynamics controls t cell fate through metabolic programming. *Cell* 166:63–76. doi: 10.1016/j.cell.2016.05.035
59. MacIntyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, et al. The glucose transporter Glut1 is selectively essential for

CD4 T cell activation and effector function. *Cell Metab.* (2014) 20:61–72. doi: 10.1016/j.cmet.2014.05.004
60. Nguyen HD, Chatterjee S, Haarberg KMK, Wu Y, Bastian D, Heinrichs J, et al. Metabolic reprogramming of alloantigen-activated T cells after hematopoietic cell transplantation. *J Clin Invest.* (2016) 126:1337–52. doi: 10.1172/JCI82587
61. Byersdorfer CA, Tkachev V, Opipari AW, Goodell S, Swanson J, Sandquist S, et al. Effector T cells require fatty acid metabolism during murine graft-versus-host disease. *Blood* (2013) 122:3230–7. doi: 10.1182/blood-2013-04-495515
62. Glick GD, Rossignol R, Lyssiotis CA, Wahl D, Lesch C, Sanchez B, et al. Anaplerotic metabolism of alloreactive T cells provides a metabolic approach to treat graft-versus-host disease. *J Pharmacol Exp Ther.* (2014) 351:298–307. doi: 10.1124/jpet.114.218099
63. Gatza E, Wahl DR, Opipari AW, Sundberg TB, Reddy P, Liu C, et al. Manipulating the bioenergetics of alloreactive T cells causes their selective apoptosis and arrests graft-versus-host disease. *Sci Transl Med.* (2011) 3:67ra68. doi: 10.1126/scitranslmed.3001975
64. Tkachev V, Goodell S, Opipari AW, Hao LY, Franchi L, Glick GD, et al. Programmed death-1 controls T cell survival by regulating oxidative metabolism. *J Immunol.* (2015) 194:5789–800. doi: 10.4049/jimmunol.1402180
65. Naidoo J, Page DB, Li BT, Connell LC, Schindler K, Lacouture ME, et al. Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies. *Ann Oncol.* (2015) 26:2375–91. doi: 10.1093/annonc/mdv383
66. De Velasco G, Je Y, Bosse D, Awad MM, Ott PA, Moreira RB, et al. Comprehensive meta-analysis of key immune-related adverse events from CTLA-4 and PD-1/PD-L1 inhibitors in cancer patients. *Cancer Immunol Res.* (2017) 5:312–8. doi: 10.1158/2326-6066.CIR-16-0237
67. Haverkos BM, Abbott D, Hamadani M, Armand P, Flowers ME, Merryman R, et al. PD-1 blockade for relapsed lymphoma post-allogeneic hematopoietic cell transplant: high response rate but frequent GVHD. *Blood* (2017) 130:221–8. doi: 10.1182/blood-2017-01-761346
68. Little RF, Streicher H. Checking up on checkpoint inhibitors. *Blood* (2017) 130:107–8. doi: 10.1182/blood-2017-05-783696
69. Godfrey J, Bishop MR, Syed S, Hyjek E, Kline J. PD-1 blockade induces remissions in relapsed classical Hodgkin lymphoma following allogeneic hematopoietic stem cell transplantation. *J Immunother Cancer* (2017) 5:11. doi: 10.1186/s40425-017-0211-z
70. Herbaux C, Gauthier J, Brice P, Drumez E, Ysebaert L, Doyen H, et al. Efficacy and tolerability of nivolumab after allogeneic transplantation for relapsed Hodgkin lymphoma. *Blood* (2017) 129:2471–8. doi: 10.1182/blood-2016-11-749556

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 December 2018
doi: 10.3389/fimmu.2018.03003

The Role of Co-stimulatory/Co-inhibitory Signals in Graft-vs.-Host Disease

Sandeep Kumar[1†], *Nicholas D. Leigh*[1†] *and Xuefang Cao*[1,2*]

[1] *Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States,* [2] *Department of Microbiology and Immunology, Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland, Baltimore, MD, United States*

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina, United States

Reviewed by:
Luca Castagna,
Humanitas Research Hospital, Italy
Marco Andreani,
Bambino Gesù Ospedale Pediatrico (IRCCS), Italy

***Correspondence:**
Xuefang Cao
xuefangcao@som.umaryland.edu

†Present Address:
Sandeep Kumar,
Division of Surgical Oncology, Department of Surgery, University of Illinois at Chicago, Chicago, IL, United States
Nicholas D. Leigh,
Department of Orthopedic Surgery, Harvard Medical School Brigham, Women's Hospital, Boston, MA, United States

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *09 August 2018*
Accepted: *05 December 2018*
Published: *21 December 2018*

Citation:
Kumar S, Leigh ND and Cao X (2018) The Role of Co-stimulatory/Co-inhibitory Signals in Graft-vs.-Host Disease. Front. Immunol. 9:3003. doi: 10.3389/fimmu.2018.03003

Allogeneic hematopoietic cell transplantation (allo-HCT) is an effective immunotherapeutic approach for various hematologic and immunologic ailments. Despite the beneficial impact of allo-HCT, its adverse effects cause severe health concerns. After transplantation, recognition of host cells as foreign entities by donor T cells induces graft-vs.-host disease (GVHD). Activation, proliferation and trafficking of donor T cells to target organs and tissues are critical steps in the pathogenesis of GVHD. T cell activation is a synergistic process of T cell receptor (TCR) recognition of major histocompatibility complex (MHC)-anchored antigen and co-stimulatory/co-inhibitory signaling in the presence of cytokines. Most of the currently used therapeutic regimens for GVHD are based on inhibiting the allogeneic T cell response or T-cell depletion (TCD). However, the immunosuppressive drugs and TCD hamper the therapeutic potential of allo-HCT, resulting in attenuated graft-vs.-leukemia (GVL) effect as well as increased vulnerability to infection. In view of the drawback of overbroad immunosuppression, co-stimulatory, and co-inhibitory molecules are plausible targets for selective modulation of T cell activation and function that can improve the effectiveness of allo-HCT. Therefore, this review collates existing knowledge of T cell co-stimulation and co-inhibition with current research that may have the potential to provide novel approaches to cure GVHD without sacrificing the beneficial effects of allo-HCT.

Keywords: allogeneic hematopoietic cell transplantation (allo-HCT), graft-vs.-host disease (GVHD), graft -vs.-leukemia (GVL) effect, T cells, co-stimulation/co-inhibition

INTRODUCTION

Hematopoietic cell transplantation (HCT) is a proven therapeutic approach for patients suffering from various hematologic and immunologic diseases (1). HCT is an immunotherapy procedure during which a healthy donor provides hematopoietic cells including stem and progenitor cells to a diseased host. Transplantation of donor-derived cells is an attempt to re-establish hematopoietic and immunological activities in the host. HCT is effective for a number of diseases including various hematologic malignancies, non-cancerous diseases including aplastic anemia, thalassemia, sickle cell anemia, and severe combined immunodeficiency (2). Apart from hematologic malignancies such as leukemia, lymphoma, and myeloma, HCT showed positive yet limited effects in patients taking chemotherapeutic regimens for solid tumors. For example, uses of HCT with anticancer regimens were reported to be effective in germ cell tumors, soft tissue sarcomas, and neuroblastoma (3).

Based on the use of conditioning regimens, HCT can be categorized into myeloablative and non-myeloablative transplantation. In myeloablative transplantation, patients usually go through a high dose of chemotherapy or radiation exposure prior to HCT while non-myeloablative transplantation is a reduced intensity transplantation procedure that is performed after less intensive chemotherapy (4). Based on human leukocyte antigen (HLA) phenotyping, HCT can be categorized into allogeneic HCT (allo-HCT) and syngeneic HCT (syn-HCT). Allo-HCT is a potentially curative procedure, for a variety of health concerns including cancerous and non-cancerous conditions (5). Based on HLA phenotyping and source of donor hematopoietic cells, allo-HCT can be further divided into match related, match unrelated and haploidentical HCT. In match related allo-HCT, the donor is a biological family member that bears 10 major HLAs identical to the host. In match unrelated allo-HCT, the donor is genetically unrelated yet possesses 10 major HLAs identical to the host. Haploidentical donor is typically a family member with HLAs half-matched to the host. All of these major types of allo-HCT have demonstrated beneficial effects in patients. However, a major limitation is the prevalence of adverse effects in the host known as graft- vs.-host disease (GVHD), which remains a major cause of morbidity and mortality in allo-HCT patients (6). In allo-HCT, donor immune cells recognize the host as a foreign entity and subsequently attack and damage normal host tissues and organs. Based on the occurrence timeline and target organs, GVHD is divided into acute GVHD (aGVHD) and chronic GVHD (cGVHD). Prevalence of aGVHD is associated with factors including differences in HLA phenotypes (7), previous pregnancy of donor and advanced age of either the donor or the recipient (8). Prevalence of cGVHD can occur due to HLA-mismatched donor or from an HLA-matched unrelated donor (9), or patients that may have already experienced aGVHD (10). Patients with aGVHD who fail to respond adequately to corticosteroids are known as steroid resistant (SR) and require salvage treatment, with anti-T cell antibodies being the most commonly utilized group of agents (11). These currently practiced clinical strategies have shown limited success in controlling GVHD. Therefore, novel approaches including those targeting T cell activation are being vigorously pursued in order to cure GVHD.

Donor-derived T cells play a central role in GVHD (12). The inflammatory cytokines produced by allo-reactive effector $CD4^+$ and $CD8^+$ T cells are involved in the pathogenesis of GVHD (13). Cytokines produced by the conditioned host cells and donor T cells contribute to allo-reactive T cell activation, proliferation and trafficking to the target organs including skin, gut and liver (14). Aside from soluble cytokines, direct contact-dependent cytotoxic damage of host cells by donor T cells also contributes to the pathology of GVHD (15, 16). Once activated, allo-reactive effector T cells migrate to target tissues, where they deliver their destructive potential mediated by Fas ligand, TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), perforin, granzymes (Gzm), and IFN-γ that lead to apoptosis in epithelial target cells (17). Later, allo-reactive memory T cells are generated and cause persistent host tissue injury (18). The damage inflicted by donor T cells provides the rationale for T cell depletion (TCD), which has been shown to be effective in the prevention of GVHD (19). However, overall TCD eliminates all T cell subsets and leads to defective immunity that results in a disease prone host. Therefore, there is an urgent need for a therapeutic approach that attenuates allo-reactive T cell function without compromising overall T cell immunity.

T cell activation is a complex yet highly regulated process. Binding of T cell receptor (TCR) to the major histocompatibility complex (MHC)-anchored antigen peptides is the first step of T cell activation. However, this activation is not optimal in the absence of co-stimulatory signals. Co-stimulatory molecules potentially regulate various functions of T cells including activation, proliferation, differentiation and survival. Common examples of co-stimulatory molecules are CD28, ICOS, CD40, CD30, CD27, OX40, and 4-1BB. Optimal activation of T cells comprises various inter-cellular and intra-cellular events including engagement of TCR, recruitment of tyrosine kinases to TCR complex, subsequent signal transduction into the nucleus and initiation of transcription and translation. On the other hand, negative regulation of T cell activation is mediated by co-inhibitory signals such as CTLA-4, PD-1, TIM-3, and LAG-3 (20). During GVHD development, co-stimulatory/co-inhibitory molecules are involved in the functional alloreactivity of immune cells which is associated with up-regulated expressions and activities of several co-stimulatory/co-inhibitory signals (21). For example, expressions of co-stimulatory molecules CD134 (OX40) and CD154 (CD40 Ligand) are up-regulated on $CD4^+$ and $CD8^+$ T cells in aGVHD patients (21). In this review, we explore various components of co-stimulatory/co-inhibitory pathways in the setting of allo-HCT and aim to illuminate their potential roles in GVHD. This review also attempts to discuss co-stimulatory/co-inhibitory molecules that can be targeted as potential therapeutic options for GVHD.

RELEVANCE OF T CELL CO-STIMULATION/CO-INHIBITION MOLECULES IN GVHD

A cascade of cellular and molecular interactions are responsible for T cell activation, differentiation, migration, and effector function during GVHD. In addition to TCR-mediated "signal one," co-stimulatory molecules provide "signal two" that is essential to fully activate T cells while avoiding anergy (22). In the form of "signal two," the involvement of various molecular pathways can lead to positive as well as negative regulation of T cell function. Therefore, they have been

Abbreviations: Allo-HCT, allogeneic hematopoietic cell transplantation; BMT, bone marrow transplantation; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF, transforming growth factor; GVHD, graft-vs.-host disease; GzmB, granzyme B; MLN, mesenteric lymph node; TCD, T cell depletion; MHC, major histocompatibility complex; TCR, T cell receptor; PD-1, programmed cell death protein 1; CTLA-4 or CD152, cytotoxic T-lymphocyte-associated protein 4; MoAbs, monoclonal antibodies; DC, dendritic cells; APCs, antigen presenting cells; CD, cluster of differentiation; Treg, regulatory T cell; iNKT, invariant NKT; ITAMs, immunoreceptor tyrosine-based activation motifs.

classified as *co-stimulatory* and *co-inhibitory* signals. The majority of co-stimulatory/co-inhibitory molecules belong to either immunoglobulin superfamily (Ig-SF) or TNF receptor superfamily (TNFR-SF). Both of these receptor families are integral in T cell regulation and are dynamically and temporally regulated. In addition, there are several other co-stimulatory molecules that are different in structure and functions when compared to Ig-SF and TNFR-SF. One example is the nectin and nectin-like co-stimulatory family. Here we summarize the roles of various co-stimulatory/co-inhibitory molecules in the pathogenesis of GVHD.

Ig-SF Co-signaling Molecules

Many Ig-SF members have been thoroughly studied for their involvement in the activation, tolerance, and functionality of T cells. The best known Ig-SF members include CD28, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), B7-1 (CD80), B7-2 (CD86), inducible co-stimulator (ICOS), B7-H2, and programmed cell death protein 1 (PD-1), B7-H1 (PD-L1), and lymphocyte-activation gene 3 (LAG-3) (23). Here, we will discuss their roles in the context of GVHD.

Because CD28-mediated co-stimulation has an important role in the initiation and maintenance of T cell response, several studies were carried out to explore whether CD28 is critical for the development of GVHD. These studies demonstrated that CD28 is involved in GVHD and the severity of GVHD could be decreased by the administration of agents that block CD28 function (24, 25). Beneficial outcomes in GVHD due to the interruption of CD80/CD28 axis are well-established (24). Using anti-B7-1 (also known as CD80) plus anti-B7-2 (also known as CD86) monoclonal antibodies, it was demonstrated that B7-1 expression on donor T cells is critical for maximal GVHD lethality induced by either $CD8^+$ or $CD4^+$ T cells (24). This outcome was later corroborated by another approach advocating antisense gene therapy targeting B7-1 that resulted in diminished rejection of allogeneic graft (26). Another notable finding is that a CD28 superagonist has the ability to decrease GVHD via increasing immunosuppressive T regulatory (Treg) cells (27). This further emphasizes the complexity of modulating co-stimulation in GVHD. However, this finding will unlikely be clinically applicable due to the catastrophic clinical trial with CD28 superagonist (28, 29).

ICOS (CD278) is a member of Ig-SF expressed on activated T cells that contributes to the induction of GVHD in the absence of B7/CD28 co-stimulation (30). Blocking of CD28 and ICOS while sparing CTLA-4 represents a promising approach to abrogate pathogenic T cell response following allo-HCT (30). It was reported that interaction between B7-related protein-1 (B7RP-1) and ICOS is important because blockade of this interaction suppresses allo-reactive T cells and reduces lethal aGVHD (31). However, a surprising result was that ICOS played differential roles in $CD4^+$ and $CD8^+$ T cell-mediated GVHD (32). ICOS deficiency was found to increase $CD8^+$ T cell mediated GVHD, while it played the expected role in $CD4^+$ T cells—that is, decreased GVHD with ICOS deficiency. Intercellular adhesion molecule (ICAM) is also a member of Ig-SF that binds to lymphocyte function-associated antigen 1 (LFA1) receptor. Blocking of CD28/B7 and LFA1/ICAM pathways can effectively prevent GVHD in MHC-mismatched mouse models (33).

In contrast to these co-stimulatory Ig-SF members, there are several Ig-SF members that induce inhibitory effects on T cell activation and function. CTLA-4 possesses similar structure to CD28. Due to this structural similarity, CTLA-4 acts as a competitor to CD28 (34, 35). An intriguing study demonstrated that lethality of aGVHD is highly dependent on CD28/CTLA-4 competition (34). Use of CTLA4-Ig has been found to improve survival rate in mice suffering from GVHD (36). Since $CD4^+CD25^+$ Treg T cells constitutively express CTLA-4 and activated T cells express B7-1 and B7-2, interaction between CTLA-4 and B7-1/B7-2 among T cells may represent an important mechanism for suppression (37). Also, donor Treg cells interact with host APCs via B7-1/B7-H1 but not PD-1/B7-H1 axis that augments donor Treg survival and expansion (38). B7-H4 is another co-inhibitory molecule that has shown an important role in GVHD (39). B7-H4 expressed on human bone marrow-derived mesenchymal stem cells inhibits T cell activation and proliferation via induction of cell cycle arrest and inhibition of NF-κB nuclear translocation (39). PD-1 (CD279) is another co-signaling molecule that induces an inhibitory effect on T cell activation and proliferation. PD-1 has also demonstrated an important role in the suppression of GVHD (40). PD-1H is a subtype of PD-1 that was recently identified as an Ig-SF co-inhibitory molecule, with a study showing that a single injection of PD-1H agonistic MoAb protects mice from GVHD (40). LAG-3, also known as CD223, is a type I transmembrane protein with four extracellular Ig-like domains. Elimination of LAG-3 signaling resulted in increased GVHD (41).

In summary, most of these studies concluded that blocking Ig-SF co-stimulatory molecules had the ability to decrease GVHD, while abrogation of Ig-SF co-inhibitory signaling increases GVHD. However, complicated and differential roles were reported regarding the function of some receptor/ligand pairs on various T cell subsets. Further studies are required for exploitation of these molecules as therapeutic targets.

TNFR-SF Co-signaling Molecules

TNFR-SF is another extensively studied co-signaling receptor super family involved in T cell activation. TNFR-SF members bind to TNF-SF ligands and mediate their action via downstream signaling molecules including TNFRSF1A-associated death domain (TRADD), TNF receptor associated factors (TRAF), TNF-dependent recruitment of the protein kinase (RIP) and Fas-associated protein with death domain (FADD) (42). TNFR-SF members include TNFR1, TNFR2, OX40, CD40, Fas, decoy receptor 1, 2, and 3, CD27, CD30, 4-1BB, death receptor 3 (DR3), DR4, DR5, and DR6, receptor activator of nuclear factor κB (RANK), osteoprotegerin (OPG), TNF-like weak inducer of apoptosis (TWEAK) receptor, trans membrane activator and CAML interactor (TACI), herpes virus entry mediator (HVEM), glucocorticoid-induced TNF receptor (GITR), orphan receptor in the TNF family (TAJ), and receptor expressed in lymphoid tissues (RELT). Here, we will discuss how various TNFR-SF members are involved in GVHD.

OX40 (also known as CD134) was reported as an option to attenuate GVHD aggravation because it has negative baseline expression yet rapid upregulation after activation (43). OX40 is an activation-induced co-stimulatory molecule, expressed by activated $CD4^+$, $CD8^+$ T cells and Treg cells after TCR ligation (44, 45). OX40L (also known as CD134L) is the binding target for OX40. APCs including DCs, B cells, and macrophages express OX40L on their surface. CD40 or LPS stimuli are important for the activation of OX40/OX40L axis (44, 45). Several studies suggest a potential role of OX40/OX40L axis in GVHD using various approaches such as antagonistic anti-OX40L MoAb or OX40KO donor or OX40LKO host mice (44–46). The depletion of OX40 from allogeneic graft has been found to suppress GVHD severity without hampering GVL effect or immunity against infectious pathogens (43). Although both $CD4^+$ and $CD8^+$ T cells demonstrate elevated expression of OX40, major OX40 effect was observed in $CD4^+$ T cell-mediated GVHD. Of note, the OX40/OX40L axis is not directly linked to CD28/B7 pathway (46, 47). Interestingly, triggering the OX40/OX40L axis on $CD4^+CD25^+$ Treg cells may block their suppressive function (45). A similar study suggests that OX40 blockade might be crucial to optimize the use of Treg cells to prevent GVHD (48).

CD137 (TNFRSF9), commonly known as 4-1BB, is an inducible type I membrane protein of TNFR-SF (49). 4-1BB plays an important role in co-stimulation of $CD4^+$ and $CD8^+$ T cells following antigenic or mitogenic activation (50). $CD8^+$ T cells are more responsive to early activation and proliferative signals trigged by the TCR and 4-1BB, while the function of the 4-1BB/4-1BBL axis is reciprocal to the CD28/B7 co-stimulatory signals (49). Several studies suggest that 4-1BB plays an important role in GVHD (49, 51, 52). In a murine model of aGVHD, it was observed that administration of epitope-specific anti-4-1BB MoAb increases host-reactive cytotoxic T cell population (49). In addition, *in vitro* exposure of donor T cells to 4-1BBL MoAb may attenuate GVHD (51).

CD40 is a member of TNFR-SF expressed mainly on APCs that binds to CD154 (also known as CD40L) expressed on T cells (53). Blockade of CD40/CD40L was found to decrease T cell-mediated GVHD (54, 55). CD40L deficiency can be exploited for GVHD management (56). In a murine model of cGVHD, an agonistic CD40 moAb prevented donor $CD8^+$ T cell anergy. Subsequently activated donor $CD8^+$ T cells deleted host $CD4^+$ T cells and host B cells involved in autoantibody production, leading to decreased cGVHD (57). Furthermore, activated donor $CD8^+$ T cells induced full engraftment of donor hematopoietic cells and exhibited an increased GVL effect (57). In addition, simultaneous use of CD40 and CD28 antagonists has shown a benefit in the attenuation of aGVHD (58). Both CD40-activated B cells and immature DCs can function as professional APCs to induce antigen-specific Treg cells (59). However, CD40-activated B cells are more potent in expanding Treg cells which is more efficient in attenuating GVHD (59). Together, these studies suggest a therapeutic potential for targeting CD40 in GVHD management.

CD27 receptor is an important member of TNFR-SF that is required for the generation and long-term maintenance of T cell immunity (60). It binds to ligand CD70, and plays a key role in regulating B cell activation and immunoglobulin synthesis (60). A study carried out with CD27 knockout mice reveals that CD27 is essential in $CD4^+$ and $CD8^+$ T cell activation and memory formation (61). A clinical study showed that patients who developed cGVHD had proportional increase in $CD27^+$ B cells in the first year after HSCT (62). However, our recent studies with mouse models demonstrate that CD27/CD70 pathway surprisingly provides immunosuppressive signaling during GVHD. The absence of CD27/CD70 signaling in both donor T cells and the host significantly increases T cell expansion and effector function, which subsequently leads to increased GVHD lethality (63, 64). Another surprising and complex result was found with GITR, with $GITR^{-/-}$ $CD4^+$ T cells mediating increased GVHD vs. WT controls and $GITR^{-/-}$ $CD8^+$ T cells showing decreased alloreactivity (65).

Taken together, members of TNFR-SF have demonstrated significant therapeutic potential in GVHD. However, results also show that these co-signaling molecules form a complex system. Further methodical and extensive study is needed to fully delineate the roles for these co-stimulatory and co-inhibitory molecules in GVHD.

Other Co-signaling Molecules

Apart from the two major co-stimulatory/co-inhibitory super families, there are several other co-stimulatory molecules that are structurally or functionally different. Nectin and nectin-like (Necl) molecules are immunoglobulin like type I trans membrane glycoproteins that possess property of Ca++ independent cellular adhesion (66). The known members of this family are Nectin-1, Nectin-2, Nectin-3 and Nectin-4, Nectin-5, Necl-1, Necl-2, Necl-3, Necl-4, and Necl-5.

Thus far, literature is not abundant for the role of Nectins or Necls in GVHD. Nectin-2 is a ligand for DNAX accessory molecule (DNAM-1, also known as CD226) and involved in NK and T cell-mediated cytotoxicity (67). DNAM-1 is involved in regulating NK cell IFN-γ production and cytotoxicity against various cancer and infected cells (68). NK cells suppress GVHD by attenuating activation of alloreactive T cells without hampering GVL effect (69). Thus, manipulating Nectins and Necls on NK and T cells may represent a novel approach to manage GVHD. In support of this rationale, absence of DNAM-1 on the donor graft attenuates GVHD in MHC-mismatched and MHC-matched allo-HCT, whereas it is not critical for GVL effect against CD155 (another DNAM-1 ligand)-expressing and CD155 non-expressing leukemias. In addition, absence of DNAM-1 promotes the expansion and suppressive function of Treg cells after allo-HCT (70).

Cytotoxic and regulatory T cell molecule (CRTAM) is a member of Necls family. It is a MHC I-restricted T cell associated molecule and its expression is restricted to activated NKT and $CD8^+$ T cells (71, 72). Interestingly, $CD4^+$ T cells that express CRTAM upon activation gained the characteristics of $CD8^+$ T cells. Further analysis of $CRTAM^+CD4^+$ T cells revealed IFN-γ production, expressions of CTL-related genes like eomesodermin, GzmB, and perforin, and cytotoxic function. Furthermore, $CRTAM^+$ T cells traffic to mucosal tissues and inflammatory sites where they release IFN-γ and deliver

cytotoxic activity (73). These features would make it interesting to study the possible involvement of CRTAM in GVHD.

Leukocyte immunoglobulin (Ig)-like receptors (LILRs) or immunoglobulin-like transcript (ILT) family or CD85 genes are a family of inhibitory and stimulatory receptors (74). Several members from this family have been discovered including ILT2, ILT3, and ILT5. ILT2 is expressed on activated T cells and may function to shut down T cell activation, culminating in T cell death or induction of anergy (75). The expression of HLA-G (an ILT2 ligand) during allogeneic recognition is associated with better graft acceptance (76). The interaction of HLA-G with ILT2 is associated with immunosuppressive mechanisms that require expansion of myeloid-derived suppressor cells (MDSCs). Induction of MDSCs by ILT2/HLA-G axis can prevent allograft rejection (77). Another member of ILT family, ILT3, is crucial for the tolerogenic activity acquired by DCs exposed to allogeneic antigen-specific $CD8^+$ T suppressor cells (78). A derivative of ILT3, ILT3-Fc, can serve as a potent immune regulatory agent that attenuates allograft rejection in humanized NOD/SCID mice by induction of $CD8^+$ T suppressor cells (79). A recent study suggests that a subset of allo-HCT recipients generate antibodies directed to surface molecules of DCs, in particular ILT5. The ILT5-specific antibodies can mediate depletion of ILT5-bearing cells. ILT5 expression has been observed in some leukemic cells, indicating that it might be a target for GVL effect (80).

T cell immunoglobulin mucin (Tim) family members regulate immune responses, autoimmunity, and allergy (81). Members of Tim family have also been reported to be involved in GVHD (82, 83). Tim-3 is a member of Tim family expressed on Th1 cells. Tim-3 binds to galectin-9 and negatively regulates Th1 response. During immune homeostasis, Tim-3 interaction with galectin-9 leads to the deletion of $Tim\text{-}3^+$ T cells. Tim-3 is up-regulated on activated T cells during GVHD (84). Blockade of Tim-3/galectin-9 interaction by infusion of a Tim-3-Ig fusion protein or Tim-3 knockout in donor T cells increases T cell proliferation and GVHD lethality (84). Inhibition of Tim-3 in aGVHD augments the activation of effector T cells expressing IFN-γ or exerting cytotoxic activity (82). A proteomic study also identifies increased levels of soluble Tim-3 in plasma of subjects with mid-gut and upper-gut GVHD (83).

Taken together, other co-signaling molecules besides Ig-SF and TNFR-SR may be important regulators of T cell function during allo-HCT. These molecules add complexities to T cell co-signaling that we need to comprehensively study to explore their therapeutic potential in GVHD.

CO-SIGNALING RECEPTOR SIGNAL TRANSDUCTION PATHWAYS IN GVHD

T cell activation is triggered by two signals (TCR/MHC and co-stimulatory) in the presence of cytokines. Activation of TCR and subsequent engagement of CD4 or CD8 co-receptor induce the recruitment of tyrosine phosphatase CD45 (85), which dephosphorylates Src family tyrosine kinases FYN and lymphocyte protein tyrosine kinase (LCK) (86). Activation of LCK resulted in phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) on CD3 in the TCR complex (86). Phosphorylation of ITAMs activates zeta-chain associated protein kinase-70 (ZAP-70). Activation of ZAP-70 leads to phosphorylation of ZAP-70 substrates including adapters like SLP76 and inducible T cell kinase (ITK). ITK phosphorylates phospholipase C γ1 (PLCγ1) that leads to the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) and second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG activation leads to subsequent activation of PKC-θ that induces MAPK/ERK pathways and ultimately leads to the activation of transcription factor NF-κB (86). IP3 causes release of Ca++ from the endoplasmic reticulum that promotes influx of external Ca++ into the cells due to the formation of Ca++ dependent channel (87, 88). Then Ca++ binds to calmodulin and activates calcineurin (a phosphatase) that up-regulates transcription of IL-2 through NFAT (87, 88). These signaling events set in motion various immune responses including antibody production, activation of phagocytic cells and direct cell killing (87, 88). In this section we will describe how various co-stimulatory/co-inhibitory signal transduction pathways contribute to GVHD.

The CD28/B7 pathway is highly important in the pathogenesis of GVHD. Several studies were performed to understand the role of CD28/B7 axis in signal transduction (89–91). The patterns of tyrosine phosphorylation in T cells triggered by CD28 interaction with B7-1 and B7-2 are identical, but different from the tyrosine phosphorylation induced by TCR-MHC interaction (89). The major difference is adapter protein Grb2 that is regulated by TCR both *in vivo* and *in vitro* whereas *in vivo* study reveals no apparent regulation of Grb2 complex in response to B7-1 or B7-2 (89). The other unique protein is adaptor protein p62 that is phosphorylated in B7-1 and B7-2 signaling but not in TCR signaling (89). B7-1 and B7-2 do not activate Raf-1/ERK2 cascade in MAP kinase pathway. Instead, B7-1 and B7-2 cooperate with intracellular Ca++ increase and PKC activation to stimulate Jun kinases (90). CD28 binding to B7 contributes to setting the level of TCR-induced phosphorylated LAT for recruiting signaling complexes, while CD28 signaling further boosts multiple pathways by facilitating PLCγ1 activation (91). A recent work revealed that CD28 and ITK signaling regulate the trafficking of self-reactive T cells to target tissues in an autoimmune disease model, and pharmacological inhibition of ITK prevented this trafficking (92). Another co-stimulatory molecule, ICOS potentially activates PI3K pathway (93). ICOS signal transduction has been studied in GVHD. It was observed that PI3K-independent ICOS signaling mechanisms contribute to T cell co-stimulation during GVHD. Interestingly, $CD4^+$ T cells preferably transduce via ICOS/PI3K signaling pathway, whereas $CD8^+$ T cells induce GVHD in a PI3K-independent ICOS signaling mechanism (93). The OX40/OX40L axis induces activation of PLC signal transduction pathway (94). Interestingly, *in vivo* blockade of OX40/OX40L axis inhibited GVHD via a mechanism that did not require CD28 signaling, Stat-4, or Stat-6 signaling (47). CD27 is another member of TNFR-SF co-stimulatory family that is involved in GVHD. The CD27/CD70 axis transduces signals leading to the activation of NF-κB and

MAPK8/JNK signaling via TRAF2 and TRAF5 (61). However, in the setting GVHD, literature is still inadequate on how these signal transduction pathways regulate allogeneic T cell response.

On the other hand, co-inhibitory molecules are highly important in immune regulation during GVHD. CTLA-4 ligation has been reported to downregulate activity of transcription factors including AP-1, NFAT, and NF-kB in activated $CD4^+$ T cells. Reduced DNA-binding by AP-1 and NFAT complexes in the nucleus was observed due to CTLA-4 ligation (95). PD-1 signaling attenuates various steps of T cell signaling by TCR including phosphorylation of ZAP70 and PKCθ activation (96). CTL-4 ligation induces inhibitory effect on AKT but not on PI3K activation (97). In contrast, PD-1 signaling inhibits PI3K activity (96). These results support the current model of T cell co-stimulation vs. co-inhibition in which CD28 signaling promotes GVHD whereas CTLA-4 signaling inhibits GVHD (98). Interestingly of note, it appears that the inducible co-stimulatory ICOS and the co-inhibitory PD-1 may converge on PI3K to modulate T cell response.

Altogether, these co-signaling receptor signal transduction pathways may play a very important role in GVHD but further studies are required to exploit these pathways for more effective therapeutic intervention.

THERAPEUTIC REGIMENS IN GVHD MANAGEMENT

Currently, a spectrum of therapeutic regimens are available to treat GVHD. Several drugs are used before and after allo-HCT to suppress allogeneic immune response. For examples, glucocorticoids including methylprednisolone and prednisone are commonly used and can effectively control GVHD in some patients. Mycophenolate mofetil, cyclosporine, and methotrexate (MTX) are also used to manage GVHD (99). Methylprednisolone or prednisone has also been used in combinations with other drugs including cyclosporine and MTX to control aGVHD (100). Other regiments including antithymocyte globulin, denileukin diftitox, infliximab, sirolimus, and tacrolimus are available now or are in clinical trials as supplemental drugs to standard treatment. However, steroid resistance (SR) in GVHD patients has been a rising concern. Therefore, studies are underway to investigate therapeutic options that can ameliorate GVHD in SR patients. Several examples of such drugs are daclizumab, etanercept, extracorporeal photopheresis, infliximab, pentostatin, rituximab, tacrolimus, thalidomide, and imatinib mesylate. Although these drugs are effective to control GVHD to a certain extent, detrimental side effects are still common and serious. These side effects include diarrhea, nausea, infection, diabetes, psychosis, insomnia, anemia, renal dysfunctions, neurotoxicity, hypertension, infusion reactions, hepatitis reactivation, hypertriglyceridemia, renal insufficiency, and cytopenia (101). Considering these severe side effects caused by the current therapeutic regimens, there is an urgent need for novel therapeutic interventions with minimal toxicity in GVHD. In response to this need, clinical trials are being carried out targeting co-signaling molecules to prevent or treat GVHD. Several ongoing trails are listed as a summary in **Table 1.**

RECENT ADVANCEMENTS IN ALLO-HCT

Allo-HCT studies have been very productive in recent years, with discovery of new drug targets and diagnostic approaches. One example is aurora kinase A (AURKA). This kinase is associated with cellular division and proliferation and its defective form is associated with cancer. A recent study carried out a comprehensive elucidation of T cell transcriptome in non-human primate aGVHD. Results suggest that AURKA can be a potential target for preventing GVHD (102). Another newly introduced therapeutic target is soluble suppression of tumorigenicity 2 (sST2). The main function of sST2 is to sequester IL-33. As a result, IL-33 is not available to membrane bound ST2 (mST2) on Th2 cells and $ST2^+FoxP3^+$ Treg cells. Blocking of ST2 in peritransplant period attenuated GVHD severity and lethality (103).

A proper diagnosis is crucial for GVHD management. Recently, increased serum ferritin levels in allo-HCT patients have been correlated with GVHD, suggesting that ferritin can serve as a diagnosis marker in combination with other laboratory markers (104). HMGB1 is a mediator of inflammation that plays an important role in Treg/Th17 homeostasis. HMGB1 expression is reported to be positively correlated with aGVHD severity and may therefore also serve as diagnostic marker (105). In addition, several microRNAs may serve as biomarkers as GVHD. For example, the main function of miR-181a is modulation of T cell function via downregulation of IFN-γ. Interestingly, the level of miR-181a reduces significantly prior to the onset of aGVHD and its reduction seems to indicate the severity of aGVHD (106). Significant levels of another microRNA, miR-586, were observed

TABLE 1 | Currently ongoing clinical trials involving co-stimulatory or co-inhibitory signals.

Status	Study title	Co-signaling molecules involved
Active, not recruiting	Adoptive immunotherapy with activated marrow infiltrating lymphocytes and cyclophosphamide graft-vs.-host disease prophylaxis in patients with relapse of hematologic malignancies after allogeneic hematopoietic cell transplantation	Using anti-CD3/CD28 activated lymphocytes as treatment of relapse after allo-HCT for patients with hematologic malignancies
Recruiting	Abatacept for GVHD prophylaxis after hematopoietic stem cell transplantation for pediatric sickle cell disease	Co-inhibitory abatacept (CTLA4-Ig) added to standard GVHD prophylaxis regimen
Not yet recruiting	CD40-L blockade for prevention of acute graft-vs.-host disease	CD40-L blockade for prevention of GVHD
Active, not recruiting	Bridging Pediatric and Adult Biomarkers in graft-vs.-host disease	ST2 as a predictive biomarker for GVHD diagnosis and prognosis

in plasma at day 7 post allo-HCT and miR-586 could be a potential biomarker for predicting aGVHD and may also be targeted for GVHD management (107).

Due to serious side effects of the currently practiced regimens such as MMF or MTX, new therapeutic targets with less side effects are being vigorously pursued. For example, a study carried out on cyclosporine A based GVHD prophylaxis with enteric-coated mycophenolate sodium instead of MMF or MTX reported to reduce GVHD with less side effects (108). Another major problem in GVHD management is drug resistance, especially, SR. Therefore, a study carried out to test genetic disruption of the glucocorticoid receptor gene. This study provides clinical protocols for producing and administering high-purity genetically-engineered virus-specific T cells that are resistant to the suppressive effect of corticosteroids (109). In addition, Ruxolitinib, a JAK1/2 inhibitor has been found effective in SR patients with aGVHD and cGVHD (110). The αβTCR is highly important in the pathogenesis of GVHD because it is the primary signal for activating T cells. The humanized MoAb of GZ-αβTCR attenuates the function T cells, suppresses clinical signs of GVHD and increases the survival of patients (111). It is most desirable that allo-HCT may cause high GVL effect and negligible GVHD. A recent study has shown the importance of cytolytic T cells in the enhancement of GVL response. The adoptive transfer of naïve donor-derived $CD8^+$ cytolytic T cells has evolved as a promising strategy to improve GVL effect (112).

In conclusion, the recent development in GVHD therapy and diagnosis has opened a new dimension of innovative strategies

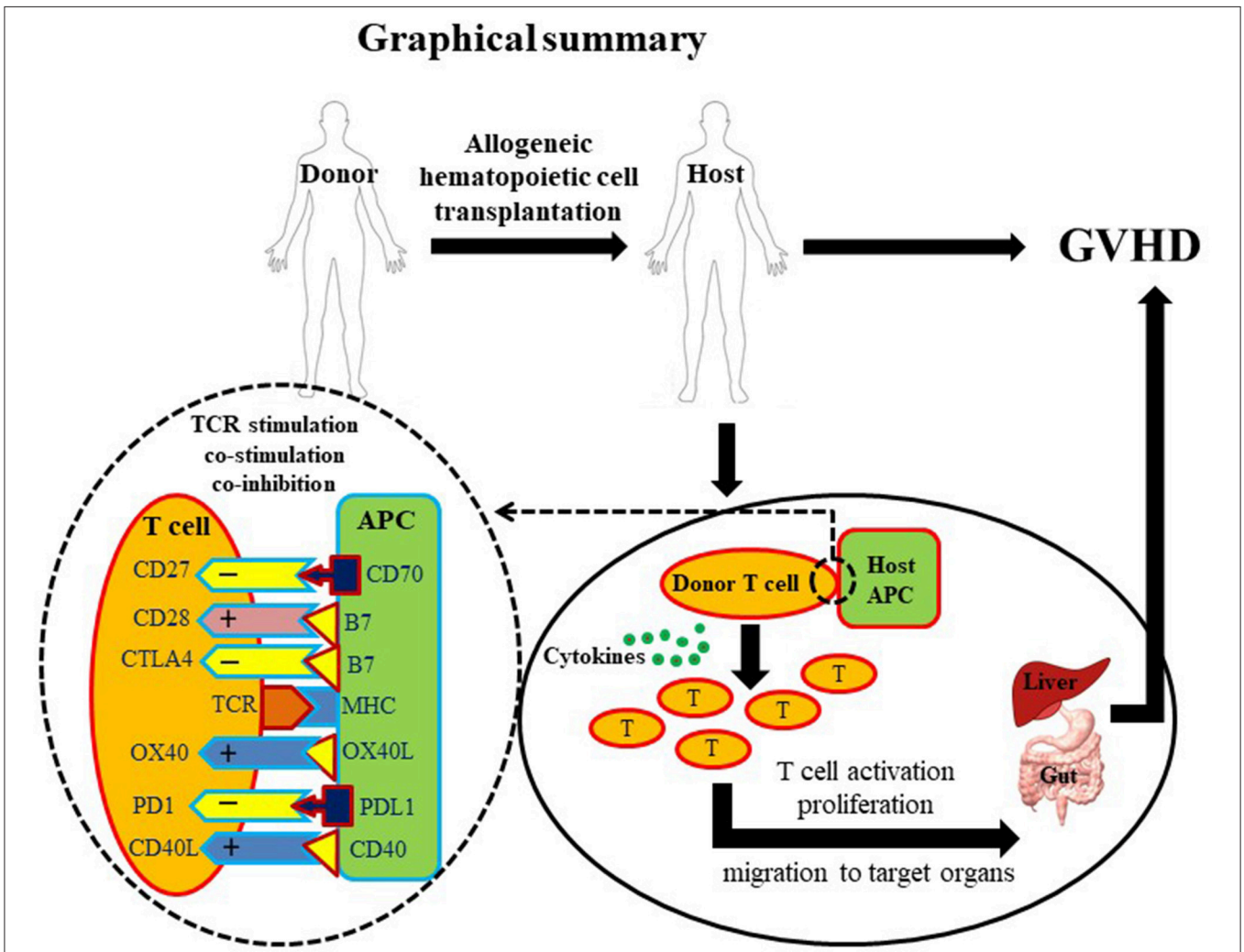


FIGURE 1 | Graphical summary. A diagrammatic illustration of GVHD occurrence post allo-HCT. Briefly, a healthy donor provides hematopoietic cells to a diseased host. Post-transplantation, donor-derived T cells activate, proliferate and migrate to target tissues. T cell activation requires three basic signals: first, T cell receptor/MHC signal; second, co-stimulatory signal; and third, cytokines. Due to the critical role of T cells in GVHD pathogenesis, T cell depletion (TCD) and suppression account for most established therapeutic approaches. However, overall T cell depletion and suppression may give rise to a disease prone host. Co-stimulatory molecules (depicted with + signs) are highly important for optimal activation of T cells. In contrast, co-inhibitory molecules (depicted with – signs) are important to down-regulate T cell activation preventing excessive immune response. Innovative manipulation of co-stimulatory/co-inhibitory signals may represent a more specific approach to control GVHD.

toward a potential cure for the adverse side effects of allo-HCT. However, further study is required to bring these novel strategies to the bedside.

FUTURE PERSPECTIVES

Allo-HCT has demonstrated a beneficial impact for patients suffering from various health ailments. However, prevalence of GVHD either acute or chronic poses a severe health concern and remains a major obstacle for more successful application of allo-HCT. Although a number of therapeutic options are currently available to manage GVHD, these regimens have serious adverse effects. Due to the central role of T cells in GVHD pathogenesis, most of the therapeutic regimens are targeted at TCD and T cell suppression. However, because T cells are essential in tumor immunity and infection control, these strategies of overall T cell suppression undesirably compromise host health. Therefore, more specific modulation of T cell function is required for successful GVHD treatment. In this setting, further elucidation of how co-stimulatory and co-inhibitory molecules modulate allogeneic immune response may reveal feasible targets that can bring beneficial outcomes. Of note, recent mouse models and human studies have demonstrated that intestinal microbiota are involved in inducing GVHD and one potential mechanism is influencing reconstitution of various T cell subsets after allo-HCT (113–115). It remains to be determined whether microbiota may modulate T cell co-signaling during allo-HCT.

Past studies have clearly demonstrated the important roles of several co-stimulatory/co-inhibitory molecules in GVGD, including CD28, CTLA-4, PD-1, OX40, and CD27. However, many other molecules are yet to be studied for their impact in allo-HCT. For example, leukocyte-associated Ig-like receptor (LAIR) molecules belong to the Ig-SF that contain one Ig-like domain and two cytoplasmic ITIM domains. These LAIR molecules may function as an inhibitory receptor on NK cells, T cells, B cells, monocytes, DCs and most thymocytes (116). CD96, also known as Tactile (T cell activation, increased late expression) is expressed on $CD4^+$ and $CD8^+$ T cells, NK cells and also present on selected B cells (117). Human CD96 interacts with nectin and nectin-like proteins and regulates NK cell function (118). CD160 is found on a subpopulation of cytolytic T cells and NK cells and functions as a broad specificity receptor for MHC I and associated molecules. The binding of CD160 to MHC I resulted in inhibition of cell-mediated cytotoxicity by $CD8^+$ T cells and NK cells (119). Considering the roles of T cells and NK cells in GVHD, these molecules could be involved in the pathogenesis of GVHD. CD200, also known as OX-2, is another Ig-SF membrane glycoprotein primarily expressed on myeloid lineage and inhibits myeloid cell activity (120). The involvement of myeloid cells in allo-HCT suggests that CD200 may play a role in GVHD. The CD300 family of molecules, also known as IREM (immune receptor expressed by myeloid cells), possess paired activating and inhibitory receptor functions and recognize lipids exposed on the outer leaflet of plasma membrane of dead and activated cells (121). Their ability to tune leukocyte function and immune responses suggests potential involvement in GVHD. The butyrophilin (BTN) and BTN like (BTNL) co-stimulatory family members are structurally similar to B7 family but are functionally different (122). BTN or BTNL family members are involved in immune regulation but their role in GVHD is yet to be explored. The signaling lymphocyte activation molecule (SLAM or CD150) family is a subset of the CD2 family of receptors that can either promote or inhibit the function of primary activating receptors (123). How SLAM is involved in GVHD is unknown. Taken these examples together, the roles and mechanisms by which many of these less studied co-signaling molecules regulate GVHD are largely undefined. Many concerted studies are needed to determine whether these molecules can serve as potential therapeutic options for successful treatment of GVHD.

CONCLUDING REMARK

In summary, the potential benefits of allo-HCT are offset by the incidence of GVHD. The current therapeutics based on TCD or T cell suppression are partially effective to control GVHD but carry serious side effects. Co-stimulatory and co-inhibitory pathways involved in T cell function have shown substantial significance in GVHD pathogenesis (**Figure 1**). Further intensive and extensive exploration of these pathways is needed before these potential therapeutic targets become new clinical options to cure GVHD without causing severe side effects.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by NIH Grant R01 HL135325 (to XC).

ACKNOWLEDGMENTS

This work attempts to comprehend most studies on the co-stimulatory/co-inhibitory pathways relevant to GVHD. However, we apologize for not being able to be comprehensive in including all publications on this topic due to limitation of space.

REFERENCES

1. Peters C, Steward CG. Hematopoietic cell transplantation for inherited metabolic diseases: an overview of outcomes and practice guidelines. *Bone Marrow Transplant*. (2003) 31:229–39. doi: 10.1038/sj.bmt.1703839
2. Barriga F, Ramírez P, Wietstruck A, Rojas N. Hematopoietic stem cell transplantation: clinical use and perspectives. *Biol Res*. (2012) 45:307–16. doi: 10.4067/S0716-97602012000300012
3. Gratwohl A, Baldomero H, Demirer T, Rosti G, Dini G, Ladenstein R, et al. Hematopoetic stem cell transplantation for solid tumors in Europe. *Ann Oncol*. (2004) 15:653–60. doi: 10.1093/annonc/mdh142

4. Sandmaier BM, Mackinnon S, Childs RW. Reduced intensity conditioning for allogeneic hematopoietic cell transplantation: current perspectives. *Biol Blood Marrow Transplant*. (2007) 13 (Suppl. 1):87–97. doi: 10.1016/j.bbmt.2006.10.015
5. Gyurkocza B, Rezvani A, Storb RF. Allogeneic hematopoietic cell transplantation: the state of the art. *Expert Rev. Hematol*. (2010) 3:285–99. doi: 10.1586/ehm.10.21
6. Ball LM, Egeler RM. Acute GvHD: pathogenesis and classification. *Bone Marrow Transplant*. (2008) 41:S58–64. doi: 10.1038/bmt.2008.56
7. Petersdorf EW. The major histocompatibility complex: a model for understanding graft-versus-host disease. *Blood* (2013) 122:1863–72. doi: 10.1182/blood-2013-05-355982
8. Kollman C, Howe CW, Anasetti C, Antin JH, Davies SM, Filipovich AH, et al. Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood* (2001) 98:2043–51. doi: 10.1182/blood.V98.7.2043
9. Petersdorf EW. Role of major histocompatibility complex variation in graft-versus-host disease after hematopoietic cell transplantation. *F1000Res* (2017) 6:617. doi: 10.12688/f1000research.10990.1
10. Ratanatharathorn V, Ayash L, Lazarus HM, Fu J, Uberti JP. Chronic graft-versus-host disease: clinical manifestation and therapy. *Bone Marrow Transplant* (2001) 28:121–9. doi: 10.1038/sj.bmt.1703111
11. McCaul KG, Nevill TJ, Barnett MJ, Toze CL, Currie CJ, Sutherland HJ, et al. Treatment of steroid-resistant acute graft-versus-host disease with rabbit antithymocyte globulin. *J Hematother Stem Cell Res*. (2000) 9:367–74. doi: 10.1089/15258160050079470
12. Truitt RL, Atasoylu AA. Contribution of CD4+ and CD8+ T cells to graft-versus-host disease and graft-versus-leukemia reactivity after transplantation of MHC-compatible bone marrow. *Bone Marrow Transplant*. (1991) 8:51–8.
13. Zhao K, Ruan S, Yin L, Zhao D, Chen C, Pan B, et al. Dynamic regulation of effector IFN-gamma-producing and IL-17-producing T cell subsets in the development of acute graft-versus-host disease. *Mol Med Rep*. (2015) 13:1395–403. doi: 10.3892/mmr.2015.4638
14. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, et al. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood* (2009) 114:3101–12. doi: 10.1182/blood-2009-05-219402
15. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *J Clin Invest*. (1997) 100:904–11. doi: 10.1172/JCI 119606
16. Graubert TA, Russell JH, Ley TJ. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. *Blood* (1996) 87:1232–7.
17. Ramirez-Montagut T, Chow A, Kochman AA, Smith OM, Suh D, Sindhi H, et al. IFN-gamma and Fas ligand are required for graft-versus-tumor activity against renal cell carcinoma in the absence of lethal graft-versus-host disease. *J Immunol*. (2007) 179:1669–80. doi: 10.4049/jimmunol.179.3.1669
18. Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Alloreactive memory T cells are responsible for the persistence of graft-versus-host disease. *J Immunol*. (2005) 174:3051–8. doi: 10.4049/jimmunol.174.5.3051
19. Weiss L, Lubin I, Factorowich I, Lapidot Z, Reich S, Reisner Y, et al. Effective graft-versus-leukemia effects independent of graft-versus-host disease after T cell-depleted allogeneic bone marrow transplantation in a murine model of B cell leukemia/lymphoma. Role of cell therapy and recombinant IL-2. *J Immunol*. (1994) 153:2562–7.
20. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annu Rev Immunol*. (2009) 27:591–619. doi: 10.1146/annurev.immunol.021908.132706
21. Paz Morante M, Briones J, Canto E, Sabzevari H, Martino R, Sierra J, et al. Activation-associated phenotype of CD3 T cells in acute graft-versus-host disease. *Clin Exp Immunol*. (2006) 145:36–43. doi: 10.1111/j.1365-2249.2006.03104.x
22. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. (2013) 13:227–42. doi: 10.1038/nri3405
23. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. (2002) 2:116–26. doi: 10.1038/nri727
24. Blazar BR, Taylor PA, Boyer MW, Panoskaltsis-Mortari A, Allison JP, et al. (1997). CD28/B7 interactions are required for sustaining the graft-versus-leukemia effect of delayed post-bone marrow transplantation splenocyte infusion in murine recipients of myeloid or lymphoid leukemia cells. *J Immunol* 159, 3460–3473.
25. Yu XZ, Martin PJ, Anasetti C. Role of CD28 in acute graft-versus-host disease. *Blood* (1998) 92:2963–70.
26. Liu C, Pan S, Jiang H, Sun X. Gene transfer of antisense B7.1 attenuates acute rejection against splenic allografts in rats. *Transplant Proc*. (2007) 39:3391–5. doi: 10.1016/j.transproceed.2007.08.094
27. Beyersdorf N, Ding X, Hünig T, Kerkau T. Superagonistic CD28 stimulation of allogeneic T cells protects from acute graft-versus-host disease. *Blood* (2009) 114:4575–82. doi: 10.1182/blood-2009-04-218248
28. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med*. (2006) 355:1018–28. doi: 10.1056/NEJMoa063842
29. Eastwood D, Findlay L, Poole S, Bird C, Wadhwa M, Moore M, et al. Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. *Br J Pharmacol*. (2010) 161:512–26. doi: 10.1111/j.1476-5381.2010.00922.x
30. Li J, Semple K, Suh WK, Liu C, Chen F, Blazar BR, et al. Roles of CD28, CTLA4, and inducible costimulator in acute graft-versus-host disease in mice. *Biol Blood Marrow Transplant*. (2011) 17:962–9. doi: 10.1016/j.bbmt.2011.01.018
31. Fujimura J, Takeda K, Kaduka Y, Saito M, Akiba H, Yagita H, et al. Contribution of B7RP-1/ICOS co-stimulation to lethal acute GVHD. *Pediatr Transplant*. (2010) 14:540–8. doi: 10.1111/j.1399-3046.2009.01279.x
32. Yu XZ, Liang Y, Nurieva RI, Guo F, Anasetti C, Dong C. Opposing effects of ICOS on graft-versus-host disease mediated by CD4 and CD8 T cells. *J Immunol*. (2006) 176:7394–401. doi: 10.4049/jimmunol.176.12.7394
33. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Gray GS, Vallera DA. Coblockade of the LFA1:ICAM and CD28/CTLA4:B7 pathways is a highly effective means of preventing acute lethal graft-versus-host disease induced by fully major histocompatibility complex-disparate donor grafts. *Blood* (1995) 85:2607–18.
34. Wallace PM, Johnson JS, MacMaster JF, Kennedy KA, Gladstone P, Linsley PS. CTLA4Ig treatment ameliorates the lethality of murine graft-versus-host disease across major histocompatibility complex barriers. *Transplantation* (1994) 58:602–10. doi: 10.1097/00007890-199409150-00013
35. Kumar S, Verma AK, Das M, Dwivedi PD. A molecular insight of CTLA-4 in food allergy. *Immunol Lett*. (2013) 149:101–9. doi: 10.1016/j.imlet.2012.12.003
36. Blazar BR, Taylor PA, Linsley PS, Vallera DA. *In vivo* blockade of CD28/CTLA4: B7/BB1 interaction with CTLA4-Ig reduces lethal murine graft-versus-host disease across the major histocompatibility complex barrier in mice. *Blood* (1994) 83:3815–25.
37. May KF, Chang X, Zhang H, Lute KD, Zhou P, Kocak E, et al. B7-deficient autoreactive T cells are highly susceptible to suppression by CD4(+)CD25(+) regulatory T cells. *J Immunol*. (2007) 178:1542–52. doi: 10.4049/jimmunol.178.3.1542
38. Yi T, Li X, Yao S, Wang L, Chen Y, Zhao D, et al. Host APCs augment *in vivo* expansion of donor natural regulatory T cells via B7H1/B7.1 in allogeneic recipients. *J Immunol*. (2011) 186:2739–49. doi: 10.4049/jimmunol.1002939
39. Xue Q, Luan XY, Gu YZ, Wu HY, Zhang GB, Yu GH, et al. The negative co-signaling molecule b7-h4 is expressed by human bone marrow-derived mesenchymal stem cells and mediates its T-cell modulatory activity. *Stem Cells Dev*. (2010) 19:27–38. doi: 10.1089/scd.2009.0076
40. Flies DB, Higuchi T, Chen L. Mechanistic assessment of PD-1H coinhibitory receptor-induced T cell tolerance to allogeneic antigens. *J Immunol*. (2015) 194:5294–304. doi: 10.4049/jimmunol.1402648
41. Sega EI, Leveson-Gower DB, Florek M, Schneidawind D, Luong RH, Negrin RS. Role of lymphocyte activation gene-3 (Lag-3) in conventional and regulatory T cell function in allogeneic transplantation. *PLoS ONE* (2014) 9:e86551. doi: 10.1371/journal.pone.0086551
42. Wajant H, Henkler F, Scheurich P. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors,

kinases and their regulators. *Cell Signal* (2001) 13:389–400. doi: 10.1016/S0898-6568(01)00160-7
43. Ge X, Brown J, Sykes M, Boussiotis VA. CD134-allodepletion allows selective elimination of alloreactive human T cells without loss of virus-specific and leukemia-specific effectors. *Biol Blood Marrow Transplant*. (2008) 14:518–30. doi: 10.1016/j.bbmt.2008.02.010
44. Miura Y, Thoburn CJ, Bright EC, Arai S, Hess AD. Regulation of OX40 gene expression in graft-versus-host disease. *Transplant Proc*. (2005) 37:57–61. doi: 10.1016/j.transproceed.2005.01.014
45. Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, Colombo MP. Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood* (2005) 105:2845–51. doi: 10.1182/blood-2004-07-2959
46. Tsukada N, Akiba H, Kobata T, Aizawa Y, Yagita H, Okumura K. Blockade of CD134 (OX40)-CD134L interaction ameliorates lethal acute graft-versus-host disease in a murine model of allogeneic bone marrow transplantation. *Blood* (2000) 95:2434–9.
47. Blazar BR, Sharpe AH, Chen AI, Panoskaltsis-Mortari A, Lees C, Akiba H, et al. Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogeneic bone marrow transplant recipients. *Blood* (2003) 101:3741–8. doi: 10.1182/blood-2002-10-3048
48. Sanchez J, Casaño J, Alvarez MA, Roman-Gomez J, Martin C, Martinez F, et al. Kinetic of regulatory CD25high and activated CD134+ (OX40) T lymphocytes during acute and chronic graft-versus-host disease after allogeneic bone marrow transplantation. *Br J Haematol*. (2004) 126:697–703. doi: 10.1111/j.1365-2141.2004.05108.x
49. Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, et al. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses. *J Exp Med*. (1997) 186:47–55. doi: 10.1084/jem.186.1.47
50. Melero I, Shuford WW, Newby SA, Aruffo A, Ledbetter JA, Hellström KE, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat Med*. (1997) 3:682–5.
51. Xu K, Li C, Pan X, Du B. Study of relieving graft-versus-host disease by blocking CD137-CD137 ligand costimulatory pathway *in vitro*. *Int J Hematol*. (2007) 86:84–90. doi: 10.1532/IJH97.A10613
52. Shah NN, Baird K, Delbrook CP, Fleisher TA, Kohler ME, Rampertaap S, et al. Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. *Blood* (2015) 125:784–92. doi: 10.1182/blood-2014-07-592881
53. Ma DY, Clark EA. The role of CD40 and CD154/CD40L in dendritic cells. *Semin Immunol*. (2009) 21:265–72. doi: 10.1016/j.smim.2009.05.010
54. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Buhlman J, Xu J, Flavell RA, et al. Blockade of CD40 ligand-CD40 interaction impairs CD4+ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and function after bone marrow transplantation. *J Immunol*. (1997) 158:29–39.
55. Blazar BR, Taylor PA, Noelle RJ, Vallera DA. CD4(+) T cells tolerized *ex vivo* to host alloantigen by anti-CD40 ligand (CD40L:CD154) antibody lose their graft-versus-host disease lethality capacity but retain nominal antigen responses. *J Clin Invest*. (1998) 102:473–82. doi: 10.1172/JCI3741
56. Allewelt H, Martin PL, Szabolcs P, Chao N, Buckley R, Parikh S. Hematopoietic stem cell transplantation for CD40 ligand deficiency: single institution experience. *Pediatr Blood Cancer* (2015) 62:2216–22. doi: 10.1002/pbc.25711
57. Kim J, Park K, Kim HJ, Kim J, Kim HA, Jung D, et al. Breaking of CD8+ T cell tolerance through *in vivo* ligation of CD40 results in inhibition of chronic graft-versus-host disease and complete donor cell engraftment. *J Immunol*. (2008) 181:7380–9. doi: 10.4049/jimmunol.181.10.7380
58. Saito K, Sakurai J, Ohata J, Kohsaka T, Hashimoto H, Okumura K, et al. Involvement of CD40 ligand-CD40 and CTLA4-B7 pathways in murine acute graft-versus-host disease induced by allogeneic T cells lacking CD28. *J Immunol*. (1998) 160:4225–31.
59. Zheng J, Liu Y, Lau YL, Tu W. CD40-activated B cells are more potent than immature dendritic cells to induce and expand CD4(+) regulatory T cells. *Cell Mol Immunol*. (2010) 7:44–50. doi: 10.1038/cmi.2009.103
60. Prasad KV, Ao Z, Yoon Y, Wu MX, Rizk M, Jacquot S, et al. CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. *Proc Natl Acad Sci USA*. (1997) 94:6346–51. doi: 10.1073/pnas.94.12.6346
61. Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol*. (2000) 1:433–40. doi: 10.1038/80877
62. Sarantopoulos S, Stevenson KE, Kim HT, Cutler CS, Bhuiya NS, Schowalter M, et al. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood* (2009) 113:3865–74. doi: 10.1182/blood-2008-09-177840
63. Leigh ND, O'Neill RE, Du W, Chen C, Qiu J, Ashwell JD, et al. Host-derived CD70 suppresses murine graft-versus-host disease by limiting donor T cell expansion and effector function. *J Immunol*. (2017) 199:336–47. doi: 10.4049/jimmunol.1502181
64. O'Neill RE, Du W, Mohammadpour H, Alqassim E, Qiu J, Chen G, et al. T cell-derived CD70 delivers an immune checkpoint function in inflammatory T cell responses. *J Immunol*. (2017) 199:3700–10. doi: 10.4049/jimmunol.1700380
65. Muriglan SJ, Ramirez-Montagut T, Alpdogan O, Van Huystee TW, Eng JM, Hubbard VM, et al. GITR activation induces an opposite effect on alloreactive CD4(+) and CD8(+) T cells in graft-versus-host disease. *J Exp Med*. (2004) 200:149–57. doi: 10.1084/jem.20040116
66. Takai Y, Miyoshi J, Ikeda W, Ogita H. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nat Rev Mol Cell Biol*. (2008) 9:603–15. doi: 10.1038/nrm2457
67. Tahara-Hanaoka S, Shibuya K, Onoda Y, Zhang H, Yamazaki S, Miyamoto A, et al. Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int Immunol*. (2004) 16:533–8. doi: 10.1093/intimm/dxh059
68. Chan CJ, Andrews DM, Smyth MJ. Receptors that interact with nectin and nectin-like proteins in the immunosurveillance and immunotherapy of cancer. *Curr Opin Immunol*. (2012) 24:246–51. doi: 10.1016/j.coi.2012.01.009
69. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* (2010) 115:4293–301. doi: 10.1182/blood-2009-05-222190
70. Koyama M, Kuns RD, Olver SD, Lineburg KE, Lor M, Teal BE, et al. Promoting regulation via the inhibition of DNAM-1 after transplantation. *Blood* (2013) 121:3511–20. doi: 10.1182/blood-2012-07-444026
71. Kennedy J, Vicari AP, Saylor V, Zurawski SM, Copeland NG, Gilbert DJ, et al. A molecular analysis of NKT cells: identification of a class-I restricted T cell-associated molecule (CRTAM). *J Leukoc Biol*. (2000) 67:725–34. doi: 10.1002/jlb.67.5.725
72. Patiño-Lopez G, Hevezi P, Lee J, Willhite D, Verge GM, Lechner SM, et al. Human class-I restricted T cell associated molecule is highly expressed in the cerebellum and is a marker for activated NKT and CD8+ T lymphocytes. *J Neuroimmunol*. (2006) 171:145–55. doi: 10.1016/j.jneuroim.2005.09.017
73. Takeuchi A, Badr Mel S, Miyauchi K, Ishihara C, Onishi R, Guo Z, et al. CRTAM determines the CD4+ cytotoxic T lymphocyte lineage. *J Exp Med*. (2016) 213:123–38. doi: 10.1084/jem.20150519
74. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. *Tissue Antigens* (2004) 64:215–25. doi: 10.1111/j.0001-2815.2004.00290.x
75. Zhang Y, Lu N, Xue Y, Zhang M, Li Y, Si Y, et al. Expression of immunoglobulin-like transcript (ILT)2 and ILT3 in human gastric cancer and its clinical significance. *Mol Med Rep*. (2012) 5:910–6. doi: 10.3892/mmr.2012.744
76. Créput C, Durrbach A, Menier C, Guettier C, Samuel D, Dausset J, et al. Human leukocyte antigen-G (HLA-G) expression in biliary epithelial cells is associated with allograft acceptance in liver-kidney transplantation. *J Hepatol*. (2003) 39:587–94.
77. Zhang W, Liang S, Wu J, Horuzsko A. Human inhibitory receptor immunoglobulin-like transcript 2 amplifies CD11b+Gr1+ myeloid-derived suppressor cells that promote long-term survival of allografts. *Transplantation* (2008) 86:1125–34. doi: 10.1097/TP.0b013e318186fccd
78. Kim-Schulze S, Scotto L, Vlad G, Piazza F, Lin H, Liu Z, et al. Recombinant Ig-like transcript 3-Fc modulates T cell responses via induction of Th

anergy and differentiation of CD8+ T suppressor cells. *J Immunol*. (2006) 176:2790–8. doi: 10.4049/jimmunol.176.5.2790
79. Vlad G, D'Agati VD, Zhang QY, Liu Z, Ho EK, Mohanakumar T, et al. Immunoglobulin-like transcript 3-Fc suppresses T-cell responses to allogeneic human islet transplants in hu-NOD/SCID mice. *Diabetes* (2008) 57:1878–86. doi: 10.2337/db08-0054
80. Pfistershammer K, Lawitschka A, Klauser C, Leitner J, Weigl R, Heemskerk MH, et al. Allogeneic disparities in immunoglobulin-like transcript 5 induce potent antibody responses in hematopoietic stem cell transplant recipients. *Blood* (2009) 114:2323–32. doi: 10.1182/blood-2008-10-183814
81. Santiago C, Ballesteros A, Tami C, Martínez-Muñoz L, Kaplan GG, Casasnovas JM. Structures of T Cell immunoglobulin mucin receptors 1 and 2 reveal mechanisms for regulation of immune responses by the TIM receptor family. *Immunity* (2007) 26:299–310. doi: 10.1016/j.immuni.2007.01.014
82. Oikawa T, Kamimura Y, Akiba H, Yagita H, Okumura K, Takahashi H, et al. Preferential involvement of Tim-3 in the regulation of hepatic CD8+ T cells in murine acute graft-versus-host disease. *J Immunol*. (2006) 177:4281–7. doi: 10.4049/jimmunol.177.7.4281
83. Hansen JA, Hanash SM, Tabellini L, Baik C, Lawler RL, Grogan BM, et al. A novel soluble form of Tim-3 associated with severe graft-versus-host disease. *Biol Blood Marrow Transplant*. (2013) 19:1323–30. doi: 10.1016/j.bbmt.2013.06.011
84. Veenstra RG, Taylor PA, Zhou Q, Panoskaltsis-Mortari A, Hirashima M, Flynn R, et al. Contrasting acute graft-versus-host disease effects of Tim-3/galectin-9 pathway blockade dependent upon the presence of donor regulatory T cells. *Blood* (2012) 120:682–90. doi: 10.1182/blood-2011-10-387977
85. Julius M, Maroun CR, Haughn L. Distinct roles for CD4 and CD8 as co-receptors in antigen receptor signalling. *Immunol Today* (1993) 14:177–83. doi: 10.1016/0167-5699(93)90282-P
86. Denny MF, Patai B, Straus DB. Differential T-cell antigen receptor signaling mediated by the Src family kinases Lck and Fyn. *Mol Cell Biol*. (2000) 20:1426–35. doi: 10.1128/MCB.20.4.1426-1435.2000
87. Cantrell DA. T-cell antigen receptor signal transduction. *Immunology* (2002) 105:369–74. doi: 10.1046/j.1365-2567.2002.01391.x
88. Myung PS, Boerthe NJ, Koretzky GA. Adapter proteins in lymphocyte antigen-receptor signaling. *Curr Opin Immunol*. (2000) 12:256–66. doi: 10.1016/S0952-7915(00)00085-6
89. Nunès JA, Truneh A, Olive D, Cantrell DA. Signal transduction by CD28 costimulatory receptor on T cells. B7-1 and B7-2 regulation of tyrosine kinase adaptor molecules. *J Biol Chem*. (1996) 271:1591–8.
90. Nunes JA, Battifora M, Woodgett JR, Truneh A, Olive D, Cantrell DA. CD28 signal transduction pathways. A comparison of B7-1 and B7-2 regulation of the map kinases: ERK2 and Jun kinases. *Mol Immunol*. (1996) 33:63–70. doi: 10.1016/0161-5890(95)00121-2
91. Michel F, Attal-Bonnefoy G, Mangino G, Mise-Omata S, Acuto O. CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. *Immunity* (2001) 15:935–45. doi: 10.1016/S1074-7613(01)00244-8
92. Jain N, Miu B, Jiang JK, McKinstry KK, Prince A, Swain SL, et al. CD28 and ITK signals regulate autoreactive T cell trafficking. *Nat Med*. (2013) 19:1632–7. doi: 10.1038/nm.3393
93. Li J, Heinrichs J, Leconte J, Haarberg K, Semple K, Liu C, et al. Phosphatidylinositol 3-kinase-independent signaling pathways contribute to ICOS-mediated T cell costimulation in acute graft-versus-host disease in mice. *J Immunol*. (2013) 191:200–7. doi: 10.4049/jimmunol.1203485
94. Yan J, Wang C, Du R, Liu P, Chen G. OX40-OX40 ligand interaction may activate phospholipase C signal transduction pathway in human umbilical vein endothelial cells. *Chem Biol Interact*. (2009) 180:460–4. doi: 10.1016/j.cbi.2009.04.009
95. McCoy KD, Le Gros G. The role of CTLA-4 in the regulation of T cell immune responses. *Immunol Cell Biol* (1999) 77:1–10. doi: 10.1046/j.1440-1711.1999.00795.x
96. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett*. (2004) 574:37–41. doi: 10.1016/j.febslet.2004.07.083
97. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol*. (2005) 25:9543–53. doi: 10.1128/MCB.25.21.9543-9553.2005
98. Yu XZ, Bidwell SJ, Martin PJ, Anasetti C. CD28-specific antibody prevents graft-versus-host disease in mice. *J Immunol*. (2000) 164:4564–8. doi: 10.4049/jimmunol.164.9.4564
99. Hamilton BK, Rybicki L, Dean R, Majhail NS, Haddad H, Abounader D, et al. Cyclosporine in combination with mycophenolate mofetil versus methotrexate for graft versus host disease prevention in myeloablative HLA-identical sibling donor allogeneic hematopoietic cell transplantation. *Am J Hematol*. (2015) 90:144–8. doi: 10.1002/ajh.23882
100. Yau JC, LeMaistre CF, Zagars GK, Williams LA, Meneghetti CM, Luke DR, et al. Methylprednisolone, cyclosporine and methotrexate for prophylaxis of acute graft-versus-host disease. *Bone Marrow Transplant*. (1990) 5:269–72.
101. Olivieri J, Coluzzi S, Attolico I, Olivieri A. Tirosin kinase inhibitors in chronic graft versus host disease: from bench to bedside. *Sci World J*. (2011) 11:1908–31. doi: 10.1100/2011/924954
102. Furlan SN, Watkins B, Tkachev V, Flynn R, Cooley S, Ramakrishnan S, et al. Transcriptome analysis of GVHD reveals aurora kinase A as a targetable pathway for disease prevention. *Sci Transl Med*. (2015) 7:315ra191. doi: 10.1126/scitranslmed.aad3231
103. Zhang XH, Zhou Y, Zhang JM, Zhou SY, Wang M, Feng R, et al. IL-35 inhibits acute graft-versus-host disease in a mouse model. *Int Immunopharmacol*. (2015) 29:383–92. doi: 10.1016/j.intimp.2015.10.025
104. Döring M, Cabanillas Stanchi KM, Feucht J, Queudeville M, Teltschik HM, Lang P, et al. Ferritin as an early marker of graft rejection after allogeneic hematopoietic stem cell transplantation in pediatric patients. *Ann Hematol*. (2016) 95:311–23. doi: 10.1007/s00277-015-2560-3.
105. Xu YJ, Li L, Chen Y, Fu B, Wu DS, Li XL, et al. Role of HMGB1 in regulation of STAT3 expression in CD4(+) T cells from patients with aGVHD after allogeneic hematopoietic stem cell transplantation. *Clin Immunol*. (2015) 161:278–83. doi: 10.1016/j.clim.2015.08.012
106. Sang W, Zhang C, Zhang D, Wang Y, Sun C, Niu M, et al. MicroRNA-181a, a potential diagnosis marker, alleviates acute graft versus host disease by regulating IFN-gamma production. *Am J Hematol*. (2015) 90:998–1007. doi: 10.1002/ajh.24136
107. Wang Y, Zhao X, Ye X, Luo H, Zhao T, Diao Y, et al. Plasma microRNA-586 is a new biomarker for acute graft-versus-host disease. *Ann Hematol*. (2015) 94:1505–14. doi: 10.1007/s00277-015-2414-z
108. Weber T, Niestadtkötter J, Wienke A, Müller-Tidow C, Müller LP. Enteric-coated mycophenolate sodium containing GvHD-prophylaxis reduces GvHD rate after allogeneic HSCT. *Eur J Haematol*. (2015) 97:232–8. doi: 10.1111/ejh.12710.
109. Menger L, Gouble A, Marzolini MA, Pachnio A, Bergerhoff K, Henry JY, et al. TALEN-mediated genetic inactivation of the glucocorticoid receptor in cytomegalovirus-specific T cells. *Blood* (2015) 126:2781–9. doi: 10.1182/blood-2015-08-664755
110. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in corticosteroid-refractory graft-versus-host disease after allogeneic stem cell transplantation: a multicenter survey. *Leukemia* (2015) 29:2062–8. doi: 10.1038/leu.2015.212
111. Blank G, Welker C, Sipos B, Sonntag K, Müller F, Eckert F, et al. Preemptive administration of human alphabeta T cell receptor-targeting monoclonal antibody GZ-alphabetaTCR potently abrogates aggressive graft-versus-host disease *in vivo*. *Ann Hematol*. (2015) 94:1907–19. doi: 10.1007/s00277-015-2471-3
112. Distler E, Albrecht J, Brunk A, Khan S, Schnürer E, Frey M, et al. Patient-individualized CD8(+) cytolytic T-cell therapy effectively combats minimal residual leukemia in immunodeficient mice. *Int J Cancer* (2016) 138:1256–68. doi: 10.1002/ijc.29854
113. Han L, Jin H, Zhou L, Zhang X, Fan Z, Dai M, et al. Intestinal Microbiota at Engraftment Influence Acute Graft-Versus-Host Disease via the Treg/Th17 Balance in Allo-HSCT Recipients. *Front Immunol*. (2018) 9:669. doi: 10.3389/fimmu.2018.00669
114. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following

allogeneic bone marrow transplantation. *J Exp Med*. (2012) 209:903–11. doi: 10.1084/jem.20112408

115. Simms-Waldrip TR, Sunkersett G, Coughlin LA, Savani MR, Arana C, Kim J, et al. Antibiotic-induced depletion of anti-inflammatory clostridia is associated with the development of graft-versus-host disease in pediatric stem cell transplantation patients. *Biol Blood Marrow Transplant* (2017) 23:820–9. doi: 10.1016/j.bbmt.2017.02.004
116. van der Vuurst de Vries, A. R., Clevers, H., Logtenberg, T., and Meyaard, L. (1999). Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur J Immunol*. 29, 3160–3167.
117. Bernhardt G. TACTILE becomes tangible: CD96 discloses its inhibitory peculiarities. *Nat Immunol*. (2014) 15:406–8. doi: 10.1038/ni.2855
118. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat Immunol*. (2014) 15:431–8. doi: 10.1038/ni.2850
119. Giustiniani J, Marie-Cardine A, Bensussan A. A soluble form of the MHC class I-specific CD160 receptor is released from human activated NK lymphocytes and inhibits cell-mediated cytotoxicity. *J Immunol*. (2007) 178:1293–300. doi: 10.4049/jimmunol.178.3.1293
120. Barclay AN, Wright GJ, Brooke G, Brown MH. CD200 and membrane protein interactions in the control of myeloid cells. *Trends Immunol*. (2002) 23:285–90. doi: 10.1016/S1471-4906(02)02223-8
121. Borrego F. The CD300 molecules: an emerging family of regulators of the immune system. *Blood* (2013) 121:1951–60. doi: 10.1182/blood-2012-09-435057
122. De Libero G, Lau SY, Mori L. Phosphoantigen presentation to TCR gammadelta cells, a conundrum getting less gray zones. *Front Immunol*. (2014) 5:679. doi: 10.3389/fimmu.2014.00679
123. Veillette A. SLAM-family receptors: immune regulators with or without SAP-family adaptors. *Cold Spring Harb Perspect Biol*. (2010) 2:a002469. doi: 10.1101/cshperspect.a002469

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 19 December 2018
doi: 10.3389/fimmu.2018.02979

Cytotoxic Pathways in Allogeneic Hematopoietic Cell Transplantation

Wei Du[1†] *and Xuefang Cao*[1,2*]

[1] Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [2] Department of Microbiology and Immunology, Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland, Baltimore, MD, United States

Allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially curative treatment for hematologic malignancies, and other hematologic and immunologic diseases. Donor-derived immune cells identify and attack cancer cells in the patient producing a unique graft-vs.-tumor (GVT) effect. This beneficial response renders allo-HCT one of the most effective forms of tumor immunotherapy. However, alloreactive donor T cells can damage normal host cells thereby causing graft-vs.-host disease (GVHD), which results in substantial morbidity and mortality. To date, GVHD remains as the major obstacle for more successful application of allo-HCT. Of special significance in this context are a number of cytotoxic pathways that are involved in GVHD and GVT response as well as donor cell engraftment. In this review, we summarize progress in the investigation of these cytotoxic pathways, including Fas/Fas ligand (FasL), perforin/granzyme, and cytokine pathways. Many studies have delineated their distinct operating mechanisms and how they are involved in the complex cellular interactions amongst donor, host, tumor, and infectious pathogens. Driven by progressing elucidation of their contributions in immune reconstitution and regulation, various interventional strategies targeting these pathways have entered translational stages with aims to improve the effectiveness of allo-HCT.

Keywords: allogeneic hematopoietic cell transplantation (allo-HCT), graft-vs.-host disease (GVHD), graft-vs.-tumor (GVT) effect, cytotoxic pathways, the Fas/Fas ligand (FasL) system, the perforin/granzyme pathway, cytokines

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina, United States

Reviewed by:
Ralf Dressel,
University Medical Center Göttingen, Germany
Tomomi Toubai,
Yamagata University, Japan

****Correspondence:***
Xuefang Cao
xuefangcao@som.umaryland.edu

†Present Address:
Wei Du,
Department of Pathology, School of Medicine, Virginia Commonwealth University, Richmond, VA, United States

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *05 September 2018*
Accepted: *04 December 2018*
Published: *19 December 2018*

Citation:
Du W and Cao X (2018) Cytotoxic Pathways in Allogeneic Hematopoietic Cell Transplantation. Front. Immunol. 9:2979. doi: 10.3389/fimmu.2018.02979

INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially curative treatment for leukemia, lymphoma, and other hematologic malignancies. It is also an effective therapy for some non-malignant diseases, such as aplastic anemia, immunodeficiencies, and autoimmune diseases (1, 2). In allo-HCT, donors and recipients must have at least partially matched human leukocyte antigen (HLA) genotype to ensure engraftment and decrease the possibility and severity of graft-vs.-host disease (GVHD) (3). After the recipients are treated with conditioning regimens that include high-dose chemotherapy or combined with radiotherapy, donor bone marrow cells or peripheral blood stem cell (PBSCs) mobilized by granulocyte colony-stimulating factor (G-CSF) are infused to the recipients. The cells in the allogeneic graft, which include hematological stem cells and pre-existing immune cells, are not only important for re-establishing the hematological system,

but also critical for reconstitution of immunity against tumor and infectious pathogens (4). In case of malignant diseases, donor immune cells are able to attack and eradicate residue malignant cells. This unique immune response has been defined as the graft-vs.-tumor (GVT) effect (5). However, the development of GVHD may limit the success of allo-HCT, which results from donor allogeneic T cells damaging normal recipient tissues as foreign (4, 6, 7). Acute GVHD may develop within a few weeks after allo-HCT, characterized by damage to susceptible organs, causing skin lesion, liver dysfunction, and diarrhea. Chronic GVHD occurs later leading to further damage to connective tissue, respiratory tract, and exocrine glands. Multiple modalities, including T cell depletion (TCD), immunosuppressive agents and different conditioning regimens, have being utilized to prevent or treat GVHD. Nevertheless, these strategies are not always effective, and may adversely cause infection, cancer relapse, or secondary malignancies (4, 6). Therefore, the "holy grail" of allo-HCT remains the separation of the adverse GVHD from the desired GVT effect.

It has been established that many types of donor-derived immune cells, such as different subsets of T cells (4, 8), B cells (9, 10), and NK cells (11, 12) are involved in mediating GVHD and GVT effect. Donor-derived T cells remain the main player for both GVHD and GVT response. Simply depleting T cells from the allo-graft could successfully prevent GVHD (13), but increases the risk of cancer relapse (14). Most of the therapeutic approaches for GVHD are targeting T cells, such as T cell modulation in different stages of transplantation (15, 16), co-stimulatory and co-inhibitory modulation (17–21), and targeting cytokines produced by T cells (22–24). The most practiced GVHD therapy still use glucocorticoids that have strong and broad anti-inflammatory effects including suppression of T cell-mediated cytotoxicity (25).

Both $CD4^+$ and $CD8^+$ T cells can cause GVHD (26). At the molecular level, a number of pathways have been described for allogeneic T cell-mediated cytotoxicity, including Fas/Fas ligand (FasL), perforin/granzymes, and cytokines such as tumor necrosis factor α (TNFα), interferon γ (IFNγ), and TNF-related apoptosis-inducing ligand (TRAIL) (27–29) **(Figure 1).** Many studies have examined these pathways in allo-HCT. Interestingly, most of these T cell-derived cytotoxic molecules can affect both target cells and T cells themselves, while different T cell subsets (e.g., CTLs vs. Tregs) can use the same molecule to perform distinct functions thereby causing different impact on GVHD and GVT response (28, 30–32). In principle, the Fas/FasL pathway has been reported to function mainly in $CD4^+$ T cell-mediated GVHD, while the perforin/granzyme pathway is essential in $CD8^+$ T cell-mediated GVHD (33). In addition, many reports have demonstrated the importance of cytokines in regulating GVHD and GVT effects (34–39). In this review, we provide updates for research progress and treatment strategies targeting these cytotoxic pathways.

Abbreviations: Allo-HCT, allogeneic hematopoietic cell transplantation; GVHD, graft-vs.-host disease; GVT, graft-vs.-tumor effect; TCD, T cell depletion; MHC, major histocompatibility complex; TCR, T cell receptor; FasL, Fas ligand; GzmA, granzyme A; GzmB, granzyme B; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand.

Fas/FasL PATHWAY IN ALLO-HCT

Fas, also known as CD95, belongs to TNF receptor superfamily and is expressed in multiple organs, playing a crucial role in extrinsic programmed cell death. FasL, also known as TNFL6, is predominantly expressed on activated T cells, macrophage, and neutrophils. Fas is a type I transmembrane receptor protein, existing as a homotrimer. Once engaged by FasL, Fas will trigger the formation of the death-inducing signaling complex (DISC). Subsequently, Fas interacts with the adaptor protein Fas-associated death domain protein (FADD) through homologous domain (40). This triggers the autocatalytic cleavage of pro-caspase 8 into caspase 8 and activation of downstream molecules, such as caspase 3, caspase 6, and caspase 7, which eventually induce apoptosis. Caspase 8 can activate the mitochondrial cell death pathway as well, resulting in activation of cytochrome c and caspase 9 (40).

Fas or FasL deficiency in mice (Fas receptor mutation *lpr* mice and FasL deficiency *gld* mice) causes accumulation of $TCR\alpha\beta^+CD3^+B220^+CD4^-CD8^-$ double negative (DN) T cells and systemic lupus erythematosus like autoimmune disease, which indicated Fas/FasL pathway plays an important role in T cell negative selection in thymus (41, 42). Fas mutation in human can also cause autoimmune lymphoproliferative syndrome (ALPS) (43). Activation-induced cell death (AICD), defined as activated T cells undergoing apoptosis after ligation of TCR by antigen or mitogen, has critical regulatory function of T cell response. Fas/FasL pathway is essential for AICD of T cells, T cell selection during development, as well as mature T cell re-stimulation by antigens (44, 45).

Fas/FasL in GVHD

Increased expression of Fas and FasL is observed in both $CD8^+$ and $CD4^+$ T cells during GVHD (46–48) and is associated with the severity of GVHD (48, 49). Blockade of Fas/FasL pathway led to decreased overall mortality in GVHD (50, 51) and reduced tissue specific organ damage (52). Meanwhile, single-nucleotide polymorphism (SNP) analysis showed that SNP of Fas in recipients can be used to improve prognostic stratification of GVHD (53, 54). Furthermore, selective depletion of host-sensitized donor lymphocytes by pre-treatment of soluble FasL can prevent GVHD (54–56). These results indicate that Fas/FasL is a key molecule in the pathogenesis of GVHD. Mizrahi et al. (57) found that short-term mobilization of peripheral blood by FasL reduced GVHD and improved survival following lipopolysaccharide stimulation, while retaining GVT activity. Likewise, engineered T cells displaying novel form of FasL (streptavidin-FasL) eliminated alloreactive T cells without significantly affecting GVT effect (58). However, the expression level of Fas failed to serve as a sensitive and specific marker for GVHD (59).

Variable mechanisms have been proposed for the function of Fas/FasL pathway in GVHD. Using murine parent to F1 models, it was reported that FasL pathway was important for

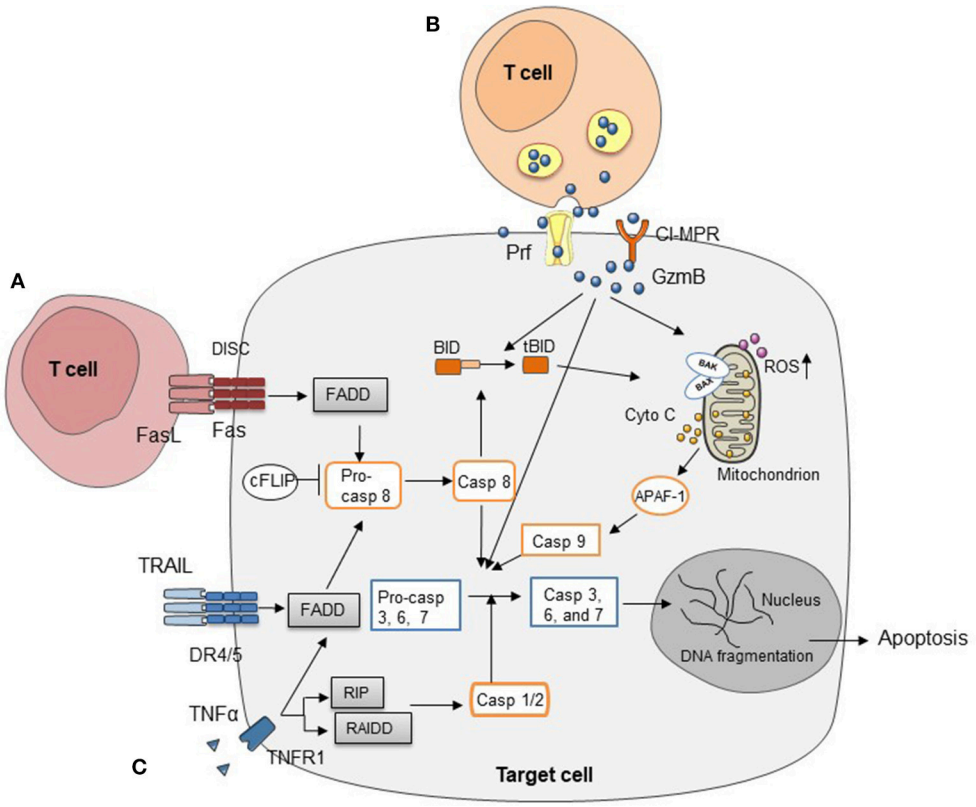


FIGURE 1 | Three major cytotoxic pathways in HCT **(A)** FasL on T cells induces target cell apoptosis by engaging Fas on cell surface. **(B)** Cell apoptosis mediated by perforin/granzymes stored in the cytotoxic granules of T cells. **(C)** Cytokines secreted by T cells, such as TNFα, IFNγ, and TRAIL, mediate target cell apoptosis through various signaling pathways.

both $CD4^+$ and $CD8^+$ T cell-mediated GVHD. Host mice receiving FasL-deficient donor T cells developed significantly less GVHD compared with WT donor T cells (60). FasL-deficiency in donor T cell did not affect T cell proliferation, homing, activation, cytokine production, and anti-tumor activity, but decreased mature T cell expansion after allo-HCT (50, 60). However, allo-HCT of FasL-deficient T cells led to decreased donor cell engraftment and subsequent chimerism (61). On the recipient side, both Fas-deficient and FasL-deficient mice had higher GVHD mortality compared with WT mice (62, 63). Together, these findings show that Fas/FasL pathway in the host is vital to resist donor cell engraftment and subsequent GVHD, while important for donor cell engraftment in allogeneic host to form stable chimerism after non-myeloablative conditioning. Therefore, how to attenuate Fas-mediated GVHD, while not affecting donor cell engraftment is a great challenge. Further study showed brief exposure of unstimulated naïve donor lymphocytes to FasL *in vitro* preferentially depleted FasL-sensitive cells, and attenuated GVHD without impairing engraftment or GVT activity (64). In addition, FasL had been found to enhance the killing activity of $CD25^+$ regulatory T cells (killer Treg) and abrogate autoimmunity. Infusion of killer Treg cells increased apoptosis of effector lymphocytes and ameliorated GVHD severity (65).

Previously, it was believed that $CD4^+$ T cells cause cytotoxicity mainly through Fas/FasL pathway while $CD8^+$ T cells prefer the perforin/granzyme pathway (66). However, reports afterwards demonstrated that the perforn/granzyme pathway was involved in cytotoxic function of $CD4^+$ T cells and Fas/FasL is important for that of $CD8^+$ T cells as well, though the potency was variable (60, 67). Maeda et al. (68) reported that deficiency in either perforin or FasL in $CD8^+$ T cells decreased the development of GVHD, indicating that both were required for the function of alloreactive $CD8^+$ T cells. However, another study showed that donor T cell cytotoxicity via Fas/FasL or perforin was not prerequisite for induction of GVHD (69). T cells lacking perforin and FasL function can still cause lethal GVHD after bone marrow transplantation (69). Furthermore, it was reported that memory $CD8^+$ T cells in the host mediated resistance to donor cell engraftment through a mechanism that was independent of FasL and perforin pathways (70). Sleater et al. (71) demonstrated that the absence of either perforin or Fas had little impact on rejection of pancreatic islet. However, simultaneous disruption of both pathways prevented allograft rejection despite T cell infiltration. These findings painted a complicated picture about how Fas/FasL in the host and donor cells affect GVHD. We postulate that the perforin/granzyme and Fas/FasL pathways comprise alternative

and required mechanisms for T cell-mediated cytotoxic function in the context of allo-HCT. In addition, FasL is the critical for NK cell-mediated cytotoxicity. Donor NK cells have been found to suppress GVHD while inducing GVT effect after allo-HCT (72, 73). Olson et al. also showed that co-injection of donor NK cells with alloreactive T cells decreased host GVHD severity by reducing cytokine production, T cell activation, and proliferation, via a mechanism that involved T cell apoptosis induced by NK cells through the FasL and perforin pathways (74).

FasL/Fas in Target Organ Damage

Skin, liver, and intestines are typical target organs in acute GVHD, while primary and second lymphoid organs are also susceptible. In a human skin explant model, higher GVHD score was associated with Fas expression in epithelium and blockade of Fas-mediated apoptosis decreased severity of cutaneous GVHD damage (75). Likewise, in oral mucosa lesions, allogeneic lymphocytes from FasL-defective mice did not induce vascular damage, or epithelial cell death in recipients, suggesting a major role of FasL by allogeneic lymphocyte-mediated mucosal GVHD (76). It was found that radiation conditioning prior to allo-HCT upregulated Fas expression on thymic stromal cells and donor alloreactive T cells used FasL to medicate thymic GVHD (77). In addition, bone marrow atrophy is mediated by p53-dependent up-regulation of Fas (78). Ceramide-rich macrodomains are sites where Fas is concentrated on cells. Sphingomyelinase-deficient mice, which cannot generate ceramide, revealed reduced GVHD-related organ damage, attenuated cytokine storm, and $CD8^+$ T cell proliferation. These results indicate that GVHD-mediated cutaneous damage is associated with Fas expression in recipients (79). However, studies of GVHD in liver and intestines are controversial. Hepatotoxicity is more likely through FasL-Fas pathway (80), while intestinal GVHD is associated with FasL-dependent TNFα level (81). Specifically, hepatic lesions were improved by administration of anti-FasL antibody whereas intestinal lesions were protected by anti-TNFα antibody but not by anti-FasL antibody (82). This result indicates that FasL and TNFα differentially contribute to GVHD pathogenesis. Contradictory results were also reported that administration of anti-FasL and anti-TNFα antibodies or using FasL-deficient donor T cells was not able to prevent intestinal GVHD (52, 82, 83). Furthermore, hematopoietic stem cells are also susceptible to FasL-induced cell apoptosis. A recent murine model study indicated that bone marrow cells pretreated with IFNγ increased expression of Fas and related caspases and proapoptotic genes which cause engraftment failure after allo-HCT (84). Therefore, it becomes evident that multiple pathways are involved in this sophisticated network and further investigations need to evaluate the role of Fas/FasL pathway in crosstalk with other molecules during GVHD target organ damage.

FasL/Fas System in GVT Effect

Initially, the FasL/Fas system was believed to engage in GVHD only (32). Depletion of FasL led to decreased lethal GVHD while GVT activity remained intact, suggesting that other molecular pathways are responsible for GVT effect (85). However, another report showed that $CD8^+$ T cell-mediated GVT activity depended on IFNγ and FasL but did not require TNFα, perforin or TRAIL (35). Other studies showed that FasL and perforin were both required for $CD8^+$ T cell-mediated GVT effect (86), while the perforin/granzyme pathway may be more dominant in GVT activity mediated by $CD8^+$ T cells (60). It is also possible that $CD4^+$ T cell-mediated GVT effect relies on both the Fas/FasL and the perforin/granzyme pathways (60). These studies used different donor-host combinations for allo-HCT and various tumor models, which may explain the discrepancy on the contributions of these pathways to GVT effect. While there is not a comprehensive all-in-one model to quantify the contributions of various pathways to GVT activity in different cancers that are enormously heterogenous, it is reasonable to conclude that the FasL/Fas system is important for GVT effect against certain tumors.

PERFORIN/GRANZYME PATHWAY IN ALLO-HCT, GVHD, AND GVT EFFECT

More than 30 years ago, a pore-forming molecule was observed on cells that were targeted by NK cells (87), which was later isolated, purified, and named perforin (88). A few years later, Jürg Tschopp's group purified a family of serine protease stored in cytoplasmic granules in cytotoxic T lymphocytes (CTLs) called granzymes (89). To date, five different granzymes have been identified in humans, named A, B, H, K, and M; while for mice, there are 10 functional granzymes, A, B, C, D, E, F, G, K, M, and N (90). Granzymes and perforin are packaged in CTLs and natural killer (NK) cells. When the killer cells engage their target cells, these proteins are released into the target cell membrane through synaptic cleft, where perforin mediates the influx of granzymes through forming pores on target cell membrane. Subsequently, granzymes cleave substrate proteins carrying out multiple functions. GzmA and B are expressed in CTLs and NK cells, whereas GzmK is expressed mainly in NK cells (91, 92). Different granzymes have various substrates specificity. GzmA and K exhibit tryptase-like activity and cleave substrates after arginine or lysine, whereas GzmB cleaves its target proteins after aspartic acid or glutamic acid. Among all these granzymes, GzmB, which is responsible for apoptosis, is the most extensively studied (93). Clipped by GzmB, pro-apoptotic BH3-only protein BID translocated to mitochondria causing cytochrome C release. Besides activation of mitochondria-mediated apoptosis, GzmB can directly process caspase-dependent pathways, including the effector caspase 3 and initiator caspase 8. A recent study also reported that GzmB directly attacked mitochondria and triggered increased production of reactive oxygen species (ROS) in target cells that was involved in causing apoptosis (94). To date, GzmB has been implicated in autoimmune disease, infection, cancer, and GVHD (95).

It was reported in 1996 that perforin was involved in the kinetics of GVHD induced by allogeneic T cells (52). Graubert et al. (33, 96) further showed that the cytotoxic effect of GzmB was pivotal for GVHD mediated by $CD8^+$ T cells, but not by $CD4^+$ T cells, and restricted in MHC I-mismatched GVHD.

Then the contribution of this pathway to the GVT response was examined a few years later. Tsukada et al. (38) used mouse leukemia models to show that perforin-deficient donor cells lost GVT activity, leading to early death of the hosts due to leukemia outgrowth. On the other hand, a recent report showed that perforin-dependent $CD8^+$ T cell apoptosis after donor lymphocyte infusion (DLI) impaired T cell proliferation and limited vaccine-based GVT effect (97). In addition, a recent published study from Galleu et al. (98) stated that mesenchymal stromal cells (MSCs) could be induced to undergo apoptosis in a perforin-dependent manner, which was essential to initiate MSC-induced immunosuppression after infusion to GVHD patients. Moreover, the cytotoxic activity delivered by either host $CD8^+$ T cells or host $CD56^+$ NK cells was correlated with less severe GVHD for patients who get MSC therapy. Therefore, the contribution of the perforin/granzyme pathway to GVHD and GVT effect is more complicated than initially believed and is involved in multiple aspects of GVHD.

Perforin/Granzyme Pathway in T Regulatory Cells

Grossman et al. first showed that human adaptive Treg cells (converted from $CD4^+$ conventional T cells) preferentially express GzmB and can kill allogeneic target cells in a perforin-dependent manner (99). On the other hand, human $CD4^+CD25^+$ natural Treg cells express GzmA but very little GzmB. Both Treg subtypes display perforin-dependent cytotoxicity against autologous target cells, including activated $CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes, and both immature and mature dendritic cells (100). Based on *in vitro* activation of human T cells, these findings suggest that the perforin/granzyme pathway is one of the mechanisms that human Treg cells use to control immune responses. A recent study from Choi et al. (101) reported that the hypomethylating agent azacytidine could drive Foxp3 expression in non-Treg cells and convert them into Tregs that could suppress GVHD without decreasing GVT effect in a murine model. And the suppressive function in those converted Tregs was partially dependent on perforin, but not GzmB. However, our studies with *in vivo* mouse tumor models showed that GzmB is important for natural Treg cell-mediated suppression of anti-tumor response (102). For natural Treg cell-mediated allogeneic T cell response, it was learned that GzmB was not required for donor natural Treg cell-mediated suppression of murine GVHD (103). Furthermore, our recent work has proven that GzmB is not required for natural Treg cell-mediated suppression of GVT effect either (104). Therefore, it seems that inhibiting GzmB will cause minimal influence on natural Treg-mediated suppression of murine GVHD and GVT effect. However, GzmA has recently been reported to be required for Treg-mediated suppression of murine GVHD, providing protection against GI tract damage (105). In a recent clinical study, Ukena S et al. analyzed $CD4^+CD25^{hi}CD127^{lo}$ Treg population from patients with and without GVHD after allo-HCT and found that higher GzmA expression in Treg cells had better tolerance to allo-graft (106).

Perforin/Granzyme Pathway in $CD8^+$ and $CD4^+CD25^-$ Conventional T Cells

Using MHC I-mismatched and MHC-fully mismatched murine models, Graubert et al. reported in 1996 that GzmB was important for $CD8^+$ T cells to cause lethal GVHD. GzmB deficiency in $CD8^+$ T cells significantly decreased the lethality and severity of GVHD after transplantation (96). Recent studies by our lab added to two new discoveries. First, while $GzmB^{-/-}$ $CD8^+$ T cells exhibit reduced ability to cause GVHD, which was expected, surprisingly $GzmB^{-/-}$ $CD8^+$ T cells showed significantly enhanced GVT activity with several tumor models (107). GzmB-mediated activation-induced T cell death may account for the different anti-tumor immune responses between WT and $GzmB^{-/-}$ $CD8^+$ T cells. Secondly, we have found that a TLR5 agonist, could not only enhance GVT activity via activating antigen presenting cells (APCs) (108), but also stimulate up-regulation of endogenous GzmB inhibitor, Spi6, in accessory immune cells including APCs. In addition, our new report showed that Spi6 protects alloreactive T cells from GzmB-mediated mitochondrial damage, preserving their ability to cause GVHD without affecting GVT effect (109). Yet our study also suggests a novel function for Spi6, which contributes to GzmB-independent protection of intestinal epithelial cells in murine GVHD (110).

Initially, it was thought that GzmB was not important for $CD4^+$ T cell-mediated GVHD. However, from our study published recently, we found that GzmB expression was upregulated in $CD4^+CD25^-$ conventional T cells after allo-HCT (111). $GzmB^{-/-}$ $CD4^+CD25^-$ T cells exhibited enhanced expansion which was due to decreased activation-induced cell death (AICD). More GI tract damage and more cytokine production were observed in the hosts mice receiving $GzmB^{-/-}$ $CD4^+CD25^-$ T cells. Using both MHC-mismatched (B6 to BALB/c) and minor antigen mismatched (129/SvJ to B6) models, we confirmed that $GzmB^{-/-}$ $CD4^+CD25^-$ T cells caused more severe GVHD compared to WT counterparts (111). On the flip side, $GzmB^{-/-}$ $CD4^+CD25^-$ conventional T cells partially lost GVT effect compared with WT T cells (104).

These new results reveal a more complicated paradigm for this pathway in allo-HCT as GzmB function in different T cell subsets ($CD4^+$ vs. $CD8^+$) unexpectedly leads to opposite outcomes in GVHD and GVT effect. Therefore, simply targeting GzmB in total T cell population is probably not beneficial for improving allo-HCT. Instead, disabling GzmB function in selected $CD8^+$ donor T cells but not in $CD4^+CD25^-$ donor T cells may lead to favorable outcomes desired for allo-HCT patients.

OTHER CYTOTOXIC PATHWAYS IN ALLO-HCT

Cytokines and their receptors are involved in different stages of GVHD, from T cell activation, differentiation, trafficking to direct tissue injury. In completed clinical trials, blockade of single cytokines alone, such as TNFα or interleukin-1 (IL-1), failed to improve clinical outcomes (24, 112) although there were evidence

showing significant correlation between IL-1, IL-1β, and TNF-α and GVHD occurrence (113). The reason for these results is elusive and related to insufficient insight of complexity of the cytokine network. Latest advances in immunology and novel therapeutic agents suggest that the strategy of targeting cytokines needs to be revisited and may provide salutary effects on GVHD and GVT management.

TNFα

TNFα is a type II transmembrane protein which can be cleaved to a soluble form. It has been well-studied and known as a pro-inflammatory cytokine (114, 115). Holler et al. first reported that increased blood level of TNFα was observed in acute GVHD (116). Soluble TNF receptors (TNFRs) were also associated with GVHD related complications (117). Choi et al. demonstrated the dynamical change of TNFR1 level before and after allo-HCT and its correlation with high grade II-IV GVHD (118). Clinical trial investigating the combination of TNFα monoclonal antibody (Etanercept) plus methylprednisolone as initial therapy for GVHD found substantial majority of remission, delayed onset of acute GVHD and reduced organ damage (112). Subsequent phase II trial revealed that lower TNFR1 level was linked with better prognosis. However, adding Etanercept to standard prophylaxis did not affect the overall rate of GVHD (119). In a multicenter prospective study, Etanercept arm had lower rate of GVHD initially, but achieved similar response in the end (120). Infliximab, a murine-human chimerized monoclonal antibody against TNFα, failed to lower the risk of GVHD in a small prospective study (121). In addition, TNFα has been a promising target in GVHD prevention particularly in gastrointestinal system (122). It should be noted that none of the studies stratify the patients based on their TNFα or TNFRs levels and these ambiguous findings should prompt us to revamp the conventional concept of TNFα. On the flip side, TNFα performs critical regulatory function in Treg cells after allo-HCT (123). In a murine allo-HCT study, donor Treg cells primed by TNFα can decrease GVHD, prolong animal survival and maintain GVT effect (124). Overall speaking, TNFα plays a fundamental role in allo-HCT, including GVHD initiation and progression, affecting clinical outcome and response to therapy, yet it functions much more like a pro-inflammatory cytokine than a cytotoxic molecule.

IFNγ

IFNγ plays a central role in host defense by regulating both innate and adaptive immunity, including specific effects on T cell differentiation and proliferation (125). IFNγ exerts paradoxical effect in GVHD. Exogenous IL-12 treatment stimulates IFNγ-mediated protection against GVHD after lethal irradiation conditioning on the day of allo-HCT (126). However, using IFNγ knockout mice, two groups independently reported that neither donor nor host derived IFNγ is required for the development of GVHD (127, 128). Further studies confirm that the protective effect of IFNγ may depend on IL-12, IL-18, or Fas (129–131). Although the exact mechanism of IFNγ in GVHD remains unclear, it may implicate that IFNγ signaling in recipient non-hematopoietic cells is more important in the process of GVHD development (125). In a recent study, Kim et al. (132) showed that human MSCs, primed with IFNγ before infusion, displayed stronger suppression of GVHD *in vivo* in an indoleamine 2,3-dioxygenase (IDO)-dependent manner. On the other aspect, IFNγ production is essential for tumor eradication as well (133). GVT effect was diminished in the hosts receiving IFNγ-deficient donor cells as IFNγ was also shown to promote FasL-dependent GVT activity of $CD8^+$ T cells (35). Furthermore, lack of IFNγ led to impaired Treg function and exacerbated GVHD (134). Among these studies, we note that IFNγ may function as a cytotoxic molecule as well as a proinflammatory cytokine. While

TABLE 1 | Contribution of different cytotoxic pathways in allo-HCT.

Cytotoxic pathway	Influence on GVHD	Influence on GVT	Target organs
Fas/FasL	Contributes to both $CD4^+$ and $CD8^+$ T cell-mediated GVHD (46–54, 60, 67, 68). FasL in NK cells inhibits GVHD (72–74).	Controversial; seems more important for $CD4^+$ T cell-mediated GVT (28, 35, 60, 85, 86).	Damage skin, liver, thymus, HSC, controversial for GI (75–84).
Perforin	Involved in $CD8^+$ T cell-mediated GVHD (52, 68) Perforin in NK cells inhibits GVHD (74).	Critical for $CD8^+$ T cell-mediated GVT (38, 97).	Not defined (31).
GzmB	GzmB is involved in $CD8^+$ T cell-mediated GVHD (107). GzmB decreases $CD4^+$ T cell-induced GVHD (111). GzmB does not affect natural Treg cell mediated suppression of GVHD (103).	GzmB damages $CD8^+$ T cell-mediated GVT (107). GzmB contributes to optimal GVT induced by $CD4^+$ T cells (104). GzmB does not affect natural Treg cell mediated suppression of GVT (103).	Not defined (31, 105, 107).
GzmA	GzmA is required for Treg-mediated suppression of GVHD (105, 106).	No report	Protects GI GVHD (105).
IFNγ	Controversial; Can be either protective against GVHD (126, 129–132), or dispensable for GVHD (127, 128). IFNγ increases Treg-mediated suppression GVHD (134).	IFNγ is critical for GVT effect (35, 36, 133).	No report
TNFα	TNFα is associated with GVHD development (112, 119).	No report	Damages skin, liver, GI (122).
TRAIL	TRAIL in T cells decreases GVHD (137). Soluble TRAIL prevents GVHD (138).	TRAIL is required for GVT effect (37, 136, 137).	No report

both functions are involved in GVHD and GVT effect, a better mechanistic understanding of the INFγ signaling is still required for dissociating the GVT effect from GVHD.

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) belongs to TNF superfamily. TRAIL can induce target cell apoptosis though binding to death receptor (DR) 4 or 5 (135). TRAIL is upregulated after allogeneic stimulation and does not affect donor T cell proliferation and cytokine production. It has been reported that TRAIL contributes to optimal GVT effect since $TRAIL^{-/-}$ donor T cells exhibit decreased anti-tumor activity (37). NK cell-derived TRAIL was also shown to kill acute lymphoblastic leukemia cells after HCT (136). A study using over-expression system revealed that $TRAIL^{+}$ T cells induced less GVHD but augmented GVT effect (137), while another study reported that the level of soluble TRAIL in peripheral blood after allo-HCT was corelated with better prognosis with less GVHD (138), suggesting that TRAIL may be a feasible target for GVHD and GVT management.

CONCLUSION AND PERSPECTIVE

The prevention and treatment of GVHD without impairing the GVT effect remains a major challenge for allo-HCT. Over the past decades, intriguing studies in the field of cytotoxic pathways open new avenues that can potentially diminish GVHD while largely preserving the GVT effect. It has been established that Fas/FasL, perforin/granzyme and cytokines are three major pathways contributing to T cell-mediated cytotoxicity in allo-HCT (**Table 1**). However, our understanding of these complicated pathways remains limited. There is still a barrier where current animal models cannot precisely mirror the clinical situation, leading to compounding discrepancies that hinder the translation into clinical practice. We anticipate that the improved insights of the cytotoxic pathways coupled with advanced technologies targeting these pathways will in the near future promote translation of preclinical discoveries into clinical implementation in GVHD management (139). New therapies, such as targeting GzmB, may emerge to overcome this devastating complication.

AUTHOR CONTRIBUTIONS

WD searched literature and wrote the manuscript. XC searched literature and wrote the manuscript.

FUNDING

This work was supported by NIH research Grant R01CA184728 (to XC).

ACKNOWLEDGMENTS

This work attempts to comprehend most studies on the cytotoxic pathways relevant to allogeneic hematopoietic cell transplantation. However, we apologize for not being able to be comprehensive in including all publications on this topic due to limitation of space.

REFERENCES

1. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol.* (2007) 7:340–52. doi: 10.1038/nri2000
2. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med.* (2006) 354:1813–26. doi: 10.1056/NEJMra052638
3. Zahid MF, Rizzieri DA. Haploidentical hematopoietic stem cell transplantation: expanding the horizon for hematologic disorders. *Adv Hematol.* (2016) 2016:1423493. doi: 10.1155/2016/1423493
4. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol.* (2007) 25:139–70. doi: 10.1146/annurev.immunol.25.022106.141606
5. Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. *Blood* (2004) 103:767–76. doi: 10.1182/blood-2003-02-0342
6. Vogelsang GB, Lee L, Bensen-Kennedy DM. Pathogenesis and treatment of graft-versus-host disease after bone marrow transplant. *Annu Rev Med.* (2003) 54:29–52. doi: 10.1146/annurev.med.54.101601.152339
7. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
8. Michael M, Shimoni A, Nagler A. Regulatory T cells in allogeneic stem cell transplantation. *Clin Dev Immunol.* (2013) 2013:608951. doi: 10.1155/2013/608951
9. Sarantopoulos S, Blazar BR, Cutler C, Ritz J. B cells in chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* (2015) 21:16–23. doi: 10.1016/j.bbmt.2014.10.029
10. Shimabukuro-Vornhagen A, Hallek MJ, Storb RF, von Bergwelt-Baildon MS. The role of B cells in the pathogenesis of graft-versus-host disease. *Blood* (2009) 114:4919–27. doi: 10.1182/blood-2008-10-161638
11. Suck G, Linn YC, Tonn T. Natural killer cells for therapy of leukemia. *Transfus Med Hemother.* (2016) 43:89–95. doi: 10.1159/000445325
12. Ullah MA, Hill GR, Tey SK. Functional reconstitution of natural killer cells in allogeneic hematopoietic stem cell transplantation. *Front Immunol.* (2016) 7:144. doi: 10.3389/fimmu.2016.00144
13. de Witte T, Hoogenhout J, de Pauw B, Holdrinet R, Janssen J, Wessels J, et al. Depletion of donor lymphocytes by counterflow centrifugation successfully prevents acute graft-versus-host disease in matched allogeneic marrow transplantation. *Blood* (1986) 67:1302–8.
14. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* (1990) 75:555–62.
15. Platzbecker U, Ehninger G, Bornhauser M. Allogeneic transplantation of CD34+ selected hematopoietic cells–clinical problems and current challenges. *Leuk lymphoma* (2004) 45:447–53. doi: 10.1080/10428190310001615684
16. Champlin RE, Passweg JR, Zhang MJ, Rowlings PA, Pelz CJ, Atkinson KA, et al. T-cell depletion of bone marrow transplants for leukemia from donors other than HLA-identical siblings: advantage of T-cell antibodies with narrow specificities. *Blood* (2000) 95:3996–4003.
17. Yu XZ, Bidwell SJ, Martin PJ, Anasetti C. CD28-specific antibody prevents graft-versus-host disease in mice. *J Immunol.* (2000) 164:4564–8. doi: 10.4049/jimmunol.164.9.4564
18. Blazar BR, Sharpe AH, Taylor PA, Panoskaltsis-Mortari A, Gray GS, Korngold R, et al. Infusion of anti-B7.1 (CD80) and anti-B7.2 (CD86) monoclonal antibodies inhibits murine graft-versus-host disease lethality in part via direct effects on CD4+ and CD8+ T cells. *J Immunol.* (1996) 157:3250–9.
19. Beyersdorf N, Ding X, Hunig T, Kerkau T. Superagonistic CD28 stimulation of allogeneic T cells protects from acute graft-versus-host disease. *Blood* (2009) 114:4575–82. doi: 10.1182/blood-2009-04-218248
20. Saha A, Aoyama K, Taylor PA, Koehn BH, Veenstra RG, Panoskaltsis-Mortari A, et al. Host programmed death ligand

1 is dominant over programmed death ligand 2 expression in regulating graft-versus-host disease lethality. *Blood* (2013) 122:3062–73. doi: 10.1182/blood-2013-05-500801
21. Fujiwara H, Maeda Y, Kobayashi K, Nishimori H, Matsuoka K, Fujii N, et al. Programmed death-1 pathway in host tissues ameliorates Th17/Th1-mediated experimental chronic graft-versus-host disease. *J Immunol.* (2014) 193:2565–73. doi: 10.4049/jimmunol.1400954
22. Cooke KR, Gerbitz A, Crawford JM, Teshima T, Hill GR, Tesolin A, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. *J Clin Invest.* (2001) 107:1581–9. doi: 10.1172/JCI12156
23. Couriel D, Saliba R, Hicks K, Ippoliti C, de Lima M, Hosing C, et al. Tumor necrosis factor-alpha blockade for the treatment of acute GVHD. *Blood* (2004) 104:649–54. doi: 10.1182/blood-2003-12-4241
24. Antin JH, Weisdorf D, Neuberg D, Nicklow R, Clouthier S, Lee SJ, et al. Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* (2002) 100:3479–82. doi: 10.1182/blood-2002-03-0985
25. Theiss-Suennemann J, Jorss K, Messmann JJ, Reichardt SD, Montes-Cobos E, Luhder F, et al. Glucocorticoids attenuate acute graft-versus-host disease by suppressing the cytotoxic capacity of CD8(+) T cells. *J Pathol.* (2015) 235:646–55. doi: 10.1002/path.4475
26. Markey KA, MacDonald KP, Hill GR. The biology of graft-versus-host disease: experimental systems instructing clinical practice. *Blood* (2014) 124:354–62. doi: 10.1182/blood-2014-02-514745
27. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity, and cancer immunoediting. *Nat Rev Immunol.* (2006) 6:836–48. doi: 10.1038/nri1961
28. Schmaltz C, Alpdogan O, Muriglan SJ, Kappel BJ, Rotolo JA, Ricchetti ET, et al. Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood* (2003) 101:2440–5. doi: 10.1182/blood-2002-07-2109
29. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol.* (2002) 20:323–70. doi: 10.1146/annurev.immunol.20.100201.131730
30. Borsotti C, Franklin AR, Lu SX, Kim TD, Smith OM, Suh D, et al. Absence of donor T-cell-derived soluble TNF decreases graft-versus-host disease without impairing graft-versus-tumor activity. *Blood* (2007) 110:783–6. doi: 10.1182/blood-2006-10-054510
31. van den Brink MR, Burakoff SJ. Cytolytic pathways in haematopoietic stem-cell transplantation. *Nat Rev Immunol.* (2002) 2:273–81. doi: 10.1038/nri775
32. Schmaltz C, Alpdogan O, Horndasch KJ, Muriglan SJ, Kappel BJ, Teshima T, et al. Differential use of Fas ligand and perforin cytotoxic pathways by donor T cells in graft-versus-host disease and graft-versus-leukemia effect. *Blood* (2001) 97:2886–95. doi: 10.1182/blood.V97.9.2886
33. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. *J Clin Invest.* (1997) 100:904–11. doi: 10.1172/JCI119606
34. Yu Y, Cho HI, Wang D, Kaosaard K, Anasetti C, Celis E, et al. Adoptive transfer of Tc1 or Tc17 cells elicits antitumor immunity against established melanoma through distinct mechanisms. *J Immunol.* (2013) 190:1873–81. doi: 10.4049/jimmunol.1201989
35. Ramirez-Montagut T, Chow A, Kochman AA, Smith OM, Suh D, Sindhi H, et al. IFN-gamma and Fas ligand are required for graft-versus-tumor activity against renal cell carcinoma in the absence of lethal graft-versus-host disease. *J Immunol.* (2007) 179:1669–80. doi: 10.4049/jimmunol.179.3.1669
36. Tateishi K, Ohta M, Guleng B, Kanai F, Tanaka Y, Asaoka Y, et al. TRAIL-induced cell death cooperates with IFN-gamma activation in the graft-versus-tumor effect against colon tumors. *Int J Cancer* (2006) 118:2237–46. doi: 10.1002/ijc.21658
37. Schmaltz C, Alpdogan O, Kappel BJ, Muriglan SJ, Rotolo JA, Ongchin J, et al. T cells require TRAIL for optimal graft-versus-tumor activity. *Nat Med.* (2002) 8:1433–7. doi: 10.1038/nm1202-797
38. Tsukada N, Kobata T, Aizawa Y, Yagita H, Okumura K. Graft-versus-leukemia effect and graft-versus-host disease can be differentiated by cytotoxic mechanisms in a murine model of allogeneic bone marrow transplantation. *Blood* (1999) 93:2738–47.
39. Pan L, Teshima T, Hill GR, Bungard D, Brinson YS, Reddy VS, et al. Granulocyte colony-stimulating factor-mobilized allogeneic stem cell transplantation maintains graft-versus-leukemia effects through a perforin-dependent pathway while preventing graft-versus-host disease. *Blood* (1999) 93:4071–8.
40. Bouillet P, O'Reilly LA. CD95, BIM and T cell homeostasis. *Nat Rev Immunol.* (2009) 9:514–9. doi: 10.1038/nri2570
41. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* (1992) 356:314–7. doi: 10.1038/356314a0
42. Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* (1994) 76:969–76. doi: 10.1016/0092-8674(94)90375-1
43. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* (1995) 268:1347–9. doi: 10.1126/science.7539157
44. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med.* (1995) 181:71–7. doi: 10.1084/jem.181.1.71
45. Mogil RJ, Radvanyi L, Gonzalez-Quintial R, Miller R, Mills G, Theofilopoulos AN, et al. Fas (CD95) participates in peripheral T cell deletion and associated apoptosis *in vivo*. *Int Immunol.* (1995) 7:1451–8. doi: 10.1093/intimm/7.9.1451
46. Wasem C, Frutschi C, Arnold D, Vallan C, Lin T, Green DR, et al. Accumulation and activation-induced release of preformed Fas (CD95) ligand during the pathogenesis of experimental graft-versus-host disease. *J Immunol.* (2001) 167:2936–41. doi: 10.4049/jimmunol.167.5.2936
47. Shustov A, Nguyen P, Finkelman F, Elkon KB, Via CS. Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-gamma production. *J Immunol.* (1998) 161:2848–55.
48. Wu Q, Chen H, Fang J, Xie W, Hong M, Xia L. Elevated Fas/FasL system and endothelial cell microparticles are involved in endothelial damage in acute graft-versus-host disease: a clinical analysis. *Leuk Res.* (2012) 36:275–80. doi: 10.1016/j.leukres.2011.08.005
49. Liem LM, van Lopik T, van Nieuwenhuijze AE, van Houwelingen HC, Aarden L, Goulmy E. Soluble Fas levels in sera of bone marrow transplantation recipients are increased during acute graft-versus-host disease but not during infections. *Blood* (1998) 91:1464–8.
50. Via CS, Nguyen P, Shustov A, Drappa J, Elkon KB. A major role for the Fas pathway in acute graft-versus-host disease. *J Immunol.* (1996) 157:5387–93.
51. Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. *J Exp Med.* (1996) 183:657–61. doi: 10.1084/jem.183.2.657
52. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. *J Exp Med.* (1996) 183:2645–56. doi: 10.1084/jem.183.6.2645
53. Kim DD, Yun J, Won HH, Cheng L, Su J, Xu W, et al. Multiple single-nucleotide polymorphism-based risk model for clinical outcomes after allogeneic stem-cell transplantation, especially for acute graft-versus-host disease. *Transplantation* (2012) 94:1250–7. doi: 10.1097/TP.0b013e3182708e7c
54. Bohana-Kashtan O, Morisot S, Hildreth R, Brayton C, Levitsky HI, Civin CI. Selective reduction of graft-versus-host disease-mediating human T cells by *ex vivo* treatment with soluble Fas ligand. *J Immunol.* (2009) 183:696–705. doi: 10.4049/jimmunol.0800561
55. Prigozhina TB, Elkin G, Khitrin S, Slavin S. Depletion of donor-reactive cells as a new concept for improvement of mismatched bone marrow engraftment using reduced-intensity conditioning. *Exp Hematol.* (2004) 32:1110–7. doi: 10.1016/j.exphem.2004.07.017
56. Georgantas RWIII, Bohana-Kashtan O, Civin CI. *Ex vivo* soluble fas ligand treatment of donor cells to selectively reduce murine acute graft versus host disease. *Transplantation* (2006) 82:471–8. doi: 10.1097/01.tp.0000229435.58898.c5
57. Mizrahi K, Yaniv I, Ash S, Stein J, Askenasy N. Apoptotic signaling through Fas and TNF receptors ameliorates GVHD in mobilized peripheral blood grafts. *Bone Marrow Transplant.* (2014) 49:640–8. doi: 10.1038/bmt.2014.12

58. Yolcu ES, Singh NP. Mouse T cells engineered to display on their surface a novel form of FasL protein undergo apoptosis when stimulated with alloantigens: implications for graft-versus-host disease. *Transplant Proc.* (2013) 45:1850–2. doi: 10.1016/j.transproceed.2013.01.029
59. Socie G, Mary JY, Lemann M, Daneshpouy M, Guardiola P, Meignin V, et al. Prognostic value of apoptotic cells and infiltrating neutrophils in graft-versus-host disease of the gastrointestinal tract in humans: TNF and Fas expression. *Blood* (2004) 103:50–7. doi: 10.1182/blood-2003-03-0909
60. Hsieh MH, Korngold R. Differential use of FasL- and perforin-mediated cytolytic mechanisms by T-cell subsets involved in graft-versus-myeloid leukemia responses. *Blood* (2000) 96:1047–55.
61. Nusair S, Gincberg G, Almogi-Hazan O, Breuer R, Or R, Wallach-Dayan SB. Failure of chimerism formation and tolerance induction from Fas ligand mutant bone marrow donors after nonmyeloablative conditioning. *Transpl Immunol.* (2012) 27:184–8. doi: 10.1016/j.trim.2012.07.001
62. van Den Brink MR, Moore E, Horndasch KJ, Crawford JM, Hoffman J, Murphy GF, et al. Fas-deficient lpr mice are more susceptible to graft-versus-host disease. *J Immunol.* (2000) 164:469–80. doi: 10.4049/jimmunol.164.1.469
63. van den Brink MR, Moore E, Horndasch KJ, Crawford JM, Murphy GF, Burakoff SJ. Fas ligand-deficient gld mice are more susceptible to graft-versus-host-disease. *Transplantation* (2000) 70, 184–191.
64. Askenasy N, Mizrahi K, Ash S, Askenasy EM, Yaniv I, Stein J. Depletion of naive lymphocytes with fas ligand *ex vivo* prevents graft-versus-host disease without impairing T cell support of engraftment or graft-versus-tumor activity. *Biol Blood Marrow Transplant.* (2013) 19:185–95. doi: 10.1016/j.bbmt.2012.10.004
65. Yolcu ES, Kaminitz A, Mizrahi K, Ash S, Yaniv I, Stein J, et al. Immunomodulation with donor regulatory T cells armed with Fas-ligand alleviates graft-versus-host disease. *Exp Hematol.* (2013) 41:903–11. doi: 10.1016/j.exphem.2013.04.016
66. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, et al. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* (1994) 265:528–30. doi: 10.1126/science.7518614
67. Blazar BR, Taylor PA, Vallera DA. CD4+ and CD8+ T cells each can utilize a perforin-dependent pathway to mediate lethal graft-versus-host disease in major histocompatibility complex-disparate recipients. *Transplantation* (1997) 64:571–6. doi: 10.1097/00007890-199708270-00004
68. Maeda Y, Levy RB, Reddy P, Liu C, Clouthier SG, Teshima T, et al. Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. *Blood* (2005) 105:2023–7. doi: 10.1182/blood-2004-08-3036
69. Marks L, Altman NH, Podack ER, Levy RB. Donor T cells lacking Fas ligand and perforin retain the capacity to induce severe GvHD in minor histocompatibility antigen mismatched bone-marrow transplantation recipients. *Transplantation* (2004) 77:804–12. doi: 10.1097/01.TP.0000110416.96307.D5
70. Zimmerman Z, Shatry A, Deyev V, Podack E, Mammolenti M, Blazar BR, et al. Effector cells derived from host CD8 memory T cells mediate rapid resistance against minor histocompatibility antigen-mismatched allogeneic marrow grafts without participation of perforin, Fas ligand, and the simultaneous inhibition of 3 tumor necrosis factor family effector pathways. *Biol Blood Marrow Transplant.* (2005) 11:576–86. doi: 10.1016/j.bbmt.2005.05.006
71. Sleater M, Diamond AS, Gill RG. Islet allograft rejection by contact-dependent CD8+ T cells: perforin and FasL play alternate but obligatory roles. *Am J Transplant.* (2007) 7:1927–33. doi: 10.1111/j.1600-6143.2007.01889.x
72. Asai O, Longo DL, Tian ZG, Hornung RL, Taub DD, Ruscetti FW, et al. Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation. *J Clin Invest.* (1998) 101:1835–42. doi: 10.1172/JCI1268
73. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* (2002) 295:2097–100. doi: 10.1126/science.1068440
74. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* (2010) 115:4293–301. doi: 10.1182/blood-2009-05-222190
75. Ruffin N, Ahmed SS, Osorio LM, Wang XN, Jackson GH, Collin MP, et al. The involvement of epithelial Fas in a human model of graft versus host disease. *Transplantation* (2011) 91:946–51. doi: 10.1097/TP.0b013e318212c833
76. Deschaumes C, Verneuil L, Ertault-Daneshpouy M, Adle-Biassette H, Galateau F, Ainoun F, et al. CD95 ligand-dependant endothelial cell death initiates oral mucosa damage in a murine model of acute graft versus host disease. *Lab Invest.* (2007) 87:417–29. doi: 10.1038/labinvest.3700541
77. Na IK, Lu SX, Yim NL, Goldberg GL, Tsai J, Rao U, et al. The cytolytic molecules Fas ligand and TRAIL are required for murine thymic graft-versus-host disease. *J Clin Invest.* (2010) 120:343–56. doi: 10.1172/JCI 39395
78. Yada S, Takamura N, Inagaki-Ohara K, O'Leary MK, Wasem C, Brunner T, et al. The role of p53 and Fas in a model of acute murine graft-versus-host disease. *J Immunol.* (2005) 174:1291–7. doi: 10.4049/jimmunol.174.3.1291
79. Rotolo JA, Stancevic B, Lu SX, Zhang J, Suh D, King CG, et al. Cytolytic T cells induce ceramide-rich platforms in target cell membranes to initiate graft-versus-host disease. *Blood* (2009) 114:3693–706. doi: 10.1182/blood-2008-11-191148
80. Kondo T, Suda T, Fukuyama H, Adachi M, Nagata S. Essential roles of the Fas ligand in the development of hepatitis. *Nat Med.* (1997) 3:409–13. doi: 10.1038/nm0497-409
81. Lin T, Brunner T, Tietz B, Madsen J, Bonfoco E, Reaves M, et al. Fas ligand-mediated killing by intestinal intraepithelial lymphocytes. Participation in intestinal graft-versus-host disease. *J Clin Invest.* (1998) 101:570–7. doi: 10.1172/JCI896
82. Hattori K, Hirano T, Miyajima H, Yamakawa N, Tateno M, Oshimi K, et al. Differential effects of anti-Fas ligand and anti-tumor necrosis factor alpha antibodies on acute graft-versus-host disease pathologies. *Blood* (1998) 91:4051–5.
83. Stuber E, Buschenfeld A, von Freier A, Arendt T, Folsch UR. Intestinal crypt cell apoptosis in murine acute graft versus host disease is mediated by tumour necrosis factor alpha and not by the FasL-Fas interaction: effect of pentoxifylline on the development of mucosal atrophy. *Gut* (1999) 45:229–35. doi: 10.1136/gut.45.2.229
84. Chen J, Feng X, Desierto MJ, Keyvanfar K, Young NS. IFN-gamma-mediated hematopoietic cell destruction in murine models of immune-mediated bone marrow failure. *Blood* (2015) 126:2621–31. doi: 10.1182/blood-2015-06-652453
85. Hattori K, Hirano T, Ushiyama C, Miyajima H, Yamakawa N, Ebata T, et al. A metalloproteinase inhibitor prevents lethal acute graft-versus-host disease in mice. *Blood* (1997) 90:542–8.
86. Ito M, Shizuru JA. Graft-vs.-lymphoma effect in an allogeneic hematopoietic stem cell transplantation model. *Biol Blood Marrow Transplant.* (1999) 5:357–68. doi: 10.1016/S1083-8791(99)70012-1
87. Dourmashkin RR, Deteix P, Simone CB, Henkart P. Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin Exp Immunol.* (1980) 42:554–60.
88. Millard PJ, Henkart MP, Reynolds CW, Henkart PA. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J Immunol.* (1984) 132:3197–204.
89. Masson D, Tschopp J. A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell* (1987) 49:679–85. doi: 10.1016/0092-8674(87)90544-7
90. Ewen CL, Kane KP, Bleackley RC. A quarter century of granzymes. *Cell Death Differ.* (2012) 19:28–35. doi: 10.1038/cdd.2011.153
91. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Ann Rev Immunol.* (2008) 26:389–420. doi: 10.1146/annurev.immunol.26.021607.090404
92. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate proinflammatory cytokine responses. *J Immunol.* (2015) 194:491–7. doi: 10.4049/jimmunol.1401214
93. Bots M, Medema JP. Granzymes at a glance. *J Cell Sci.* (2006) 119:5011–4. doi: 10.1242/jcs.03239
94. Jacquemin G, Margiotta D, Kasahara A, Bassoy EY, Walch M, Thiery J, et al. Granzyme B-induced mitochondrial ROS are required for apoptosis. (2014) *Cell Death Differ.* 22:862–74. doi: 10.1038/cdd.2014.180

95. Boivin WA, Cooper DM, Hiebert PR, Granville DJ. Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma. *Lab Invest.* (2009) 89:1195–220. doi: 10.1038/labinvest.2009.91
96. Graubert TA, Russell JH, Ley TJ. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. *Blood* (1996) 87:1232–7.
97. Capitini CM, Nasholm NM, Duncan BB, Guimond M, Fry TJ. Graft-versus-host disease impairs vaccine responses through decreased CD4+ and CD8+ T cell proliferation and increased perforin-mediated CD8+ T cell apoptosis. *J Immunol.* (2013) 190:1351–9. doi: 10.4049/jimmunol.1200391
98. Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, et al. Apoptosis in mesenchymal stromal cells induces *in vivo* recipient-mediated immunomodulation. *Sci Transl Med.* (2017) 9:eaam7828. doi: 10.1126/scitranslmed.aam7828
99. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* (2004) 104:2840–8. doi: 10.1182/blood-2004-03-0859
100. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* (2004) 21:589–601. doi: 10.1016/j.immuni.2004.09.002
101. Choi J, Ritchey J, Prior JL, Holt M, Shannon WD, Deych E, et al. *In vivo* administration of hypomethylating agents mitigate graft-versus-host disease without sacrificing graft-versus-leukemia. *Blood* (2010) 116:129–39. doi: 10.1182/blood-2009-12-257253
102. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* (2007) 27:635–46. doi: 10.1016/j.immuni.2007.08.014
103. Cai SF, Cao X, Hassan A, Fehniger TA, Ley TJ. Granzyme B is not required for regulatory T cell-mediated suppression of graft-versus-host disease. *Blood* (2010) 115:1669–77. doi: 10.1182/blood-2009-07-233676
104. Du W, Leigh ND, Bian G, Alqassim E, O'Neill RE, Mei L, et al. Granzyme B contributes to the optimal graft-versus-tumor effect mediated by conventional CD4+ T Cells. *J Immunol Res Ther.* (2016) 1:22–8.
105. Velaga S, Ukena SN, Dringenberg U, Alter C, Pardo J, Kershaw O, et al. Granzyme A is required for regulatory T-Cell mediated prevention of gastrointestinal graft-versus-host disease. *PLoS ONE* (2015) 10:e0124927. doi: 10.1371/journal.pone.0124927
106. Ukena SN, Velaga S, Geffers R, Grosse J, Baron U, Buchholz S, et al. Human regulatory T cells in allogeneic stem cell transplantation. *Blood* (2011) 118:e82–92. doi: 10.1182/blood-2011-05-352708
107. Bian G, Ding X, Leigh ND, Tang Y, Capitano ML, Qiu J, et al. Granzyme B-mediated damage of CD8+ T cells impairs graft-versus-tumor effect. *J Immunol.* (2013) 190:1341–50. doi: 10.4049/jimmunol.1201554
108. Ding X, Bian G, Leigh ND, Qiu J, McCarthy PL, Liu H, et al. A TLR5 agonist enhances CD8(+) T cell-mediated graft-versus-tumor effect without exacerbating graft-versus-host disease. *J Immunol.* (2012) 189:4719–27. doi: 10.4049/jimmunol.1201206
109. Du W, Mohammadpour H, O'Neill RE, Kumar S, Chen C, Qiu M, et al. Serine protease inhibitor 6 protects alloreactive T cells from Granzyme B-mediated mitochondrial damage without affecting graft-versus-tumor effect. *Oncoimmunology* (2018) 7:e1397247. doi: 10.1080/2162402X.2017.1397247
110. Mohammadpour H, Du W, O'Neill R, Khalili S, Qiu J, Repasky EA, et al. Host-derived serine protease inhibitor 6 provides granzyme B-independent protection of intestinal epithelial cells in murine graft-versus-host disease. *Biol Blood Marrow Transplant.* (2018) 24:2397–408. doi: 10.1016/j.bbmt.2018.07.003
111. Du W, Leigh ND, Bian G, O'Neill RE, Mei L, Qiu J, et al. Granzyme B-mediated activation-induced death of CD4+ T cells inhibits murine acute graft-versus-host disease. *J Immunol.* (2015) 195:4514–23. doi: 10.4049/jimmunol.1500668
112. Levine JE, Paczesny S, Mineishi S, Braun T, Choi SW, Hutchinson RJ, et al. Etanercept plus methylprednisolone as initial therapy for acute graft-versus-host disease. *Blood* (2008) 111:2470–5. doi: 10.1182/blood-2007-09-112987
113. Rowbottom AW, Riches PG, Downie C, Hobbs JR. Monitoring cytokine production in peripheral blood during acute graft-versus-host disease following allogeneic bone marrow transplantation. *Bone Marrow Transplant.* (1993) 12:635–41.
114. Bazzoni F, Beutler B. The tumor necrosis factor ligand and receptor families. *N Engl J Med.* (1996) 334:1717–25. doi: 10.1056/NEJM199606273342607
115. Brenner D, Blaser H, Mak TW. Regulation of tumour necrosis factor signalling: live or let die. *Nat Rev Immunol.* (2015) 15:362–74. doi: 10.1038/nri3834
116. Holler E, Kolb HJ, Moller A, Kempeni J, Liesenfeld S, Pechumer H, et al. Increased serum levels of tumor necrosis factor alpha precede major complications of bone marrow transplantation. *Blood* (1990) 75:1011–6.
117. Or R, Kalinkovich A, Nagler A, Weisman Z, Naparstek E, Weiss L, et al. Soluble tumor necrosis factor (sTNF) receptors: a possible prognostic marker for bone marrow transplantation-related complications. *Cytokines Mol Ther.* (1996) 2:243–50.
118. Choi SW, Kitko CL, Braun T, Paczesny S, Yanik G, Mineishi S, et al. Change in plasma tumor necrosis factor receptor 1 levels in the first week after myeloablative allogeneic transplantation correlates with severity and incidence of GVHD and survival. *Blood* (2008) 112:1539–42. doi: 10.1182/blood-2008-02-138867
119. Choi SW, Stiff P, Cooke K, Ferrara JL, Braun T, Kitko C, et al. TNF-inhibition with etanercept for graft-versus-host disease prevention in high-risk HCT: lower TNFR1 levels correlate with better outcomes. *Biol Blood Marrow Transplant.* (2012) 18:1525–32. doi: 10.1016/j.bbmt.2012.03.013
120. Alousi AM, Weisdorf DJ, Logan BR, Bolanos-Meade J, Carter S, Difronzo N, et al. Etanercept, mycophenolate, denileukin, or pentostatin plus corticosteroids for acute graft-versus-host disease: a randomized phase 2 trial from the blood and marrow transplant clinical trials network. *Blood* (2009) 114:511–7. doi: 10.1182/blood-2009-03-212290
121. Hamadani M, Hofmeister CC, Jansak B, Phillips G, Elder P, Blum W, et al. Addition of infliximab to standard acute graft-versus-host disease prophylaxis following allogeneic peripheral blood cell transplantation. *Biol Blood Marrow Transplant.* (2008) 14:783–9. doi: 10.1016/j.bbmt.2008.04.006
122. Levine JE. Implications of TNF-alpha in the pathogenesis and management of GVHD. *Int J Hematol.* (2011) 93:571–7. doi: 10.1007/s12185-011-0803-1
123. Mancusi A, Piccinelli S, Velardi A, Pierini A. The effect of TNF-alpha on regulatory T cell function in graft-versus-host disease. *Front Immunol.* (2018) 9:356. doi: 10.3389/fimmu.2018.00356
124. Leclerc M, Naserian S, Pilon C, Thiolat A, Martin GH, Pouchy C, et al. Control of GVHD by regulatory T cells depends on TNF produced by T cells and TNFR2 expressed by regulatory T cells. *Blood* (2016) 128:1651–9. doi: 10.1182/blood-2016-02-700849
125. Wang H, Yang YG. The complex and central role of interferon-gamma in graft-versus-host disease and graft-versus-tumor activity. *Immunol Rev.* (2014) 258:30–44. doi: 10.1111/imr.12151
126. Yang YG, Dey BR, Sergio JJ, Pearson DA, Sykes M. Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. *J Clin Invest.* (1998) 102:2126–35. doi: 10.1172/JCI4992
127. Murphy WJ, Welniak LA, Taub DD, Wiltrout RH, Taylor PA, Vallera DA, et al. Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. *J Clin Invest.* (1998) 102:1742–8. doi: 10.1172/JCI3906
128. Wang H, Asavaroengchai W, Yeap BY, Wang MG, Wang S, Sykes M, et al. Paradoxical effects of IFN-gamma in graft-versus-host disease reflect promotion of lymphohematopoietic graft-versus-host reactions and inhibition of epithelial tissue injury. *Blood* (2009) 113:3612–9. doi: 10.1182/blood-2008-07-168419
129. Reddy P, Teshima T, Hildebrandt G, Duffner U, Maeda Y, Cooke KR, et al. Interleukin 18 preserves a perforin-dependent graft-versus-leukemia effect after allogeneic bone marrow transplantation. *Blood* (2002) 100:3429–31. doi: 10.1182/blood-2002-04-1252
130. Reddy P, Teshima T, Kukuruga M, Ordemann R, Liu C, Lowler K, et al. Interleukin-18 regulates acute graft-versus-host disease by enhancing Fas-mediated donor T cell apoptosis. *J Exp Med.* (2001) 194:1433–40. doi: 10.1084/jem.194.10.1433
131. Dey BR, Yang YG, Szot GL, Pearson DA, Sykes M. Interleukin-12 inhibits graft-versus-host disease through an Fas-mediated mechanism associated with alterations in donor T-cell activation and expansion. *Blood* (1998) 91:3315–22.

132. Kim DS, Jang IK, Lee MW, Ko YJ, Lee DH, Lee JW, et al. Enhanced immunosuppressive properties of human mesenchymal stem cells primed by interferon-gamma. *EBioMedicine* (2018) 28:261–73. doi: 10.1016/j.ebiom.2018.01.002
133. Yang YG, Sergio JJ, Pearson DA, Szot GL, Shimizu A, Sykes M. Interleukin-12 preserves the graft-versus-leukemia effect of allogeneic CD8 T cells while inhibiting CD4-dependent graft-versus-host disease in mice. *Blood* (1997) 90:4651–60.
134. Koenecke C, Lee CW, Thamm K, Fohse L, Schafferus M, Mittrucker HW, et al. IFN-gamma production by allogeneic Foxp3+ regulatory T cells is essential for preventing experimental graft-versus-host disease. *J Immunol.* (2012) 189:2890–6. doi: 10.4049/jimmunol.1200413
135. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* (1997) 277:818–21. doi: 10.1126/science.277.5327.818
136. Lelaidier M, Diaz-Rodriguez Y, Cordeau M, Cordeiro P, Haddad E, Herblot S, et al. TRAIL-mediated killing of acute lymphoblastic leukemia by plasmacytoid dendritic cell-activated natural killer cells. *Oncotarget* (2015) 6:29440–55. doi: 10.18632/oncotarget.4984
137. Ghosh A, Dogan Y, Moroz M, Holland AM, Yim NL, Rao UK, et al. Adoptively transferred TRAIL+ T cells suppress GVHD and augment antitumor activity. *J Clin Invest.* (2013) 123:2654–62.doi: 10.1172/JCI 66301
138. Nomura S, Ishii K, Inami N, Uoshima N, Ishida H, Yoshihara T, et al. Role of soluble tumor necrosis factor-related apoptosis-inducing ligand concentrations after stem cell transplantation. *Transpl Immunol.* (2007) 18:115–21. doi: 10.1016/j.trim.2007.05.012
139. Choi SW, Reddy P. Current and emerging strategies for the prevention of graft-versus-host disease. *Nat Rev Clin Oncol.* (2014) 11:536–47. doi: 10.1038/nrclinonc.2014.102

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 06 December 2018
doi: 10.3389/fimmu.2018.02887

Inhibition of Human Dendritic Cell ER Stress Response Reduces T Cell Alloreactivity Yet Spares Donor Anti-tumor Immunity

Brian C. Betts[1,2,3], Frederick L. Locke[1,2], Elizabeth M. Sagatys[4], Joseph Pidala[1,2], Kelly Walton[2,3], Meghan Menges[2], Jordan Reff[2], Asim Saha[5,6], Julie Y. Djeu[2], John V. Kiluk[7], Marie C. Lee[7], Jongphil Kim[8], Chang Won Kang[9], Chih-Hang Anthony Tang[10], Jeremy Frieling[11], Conor C. Lynch[11], Alan List[12], Paulo C. Rodriguez[2], Bruce R. Blazar[5,6], Jose R. Conejo-Garcia[2], Juan R. Del Valle[9], Chih-Chi Andrew Hu[10] and Claudio Anasetti[1,2]*

[1] Department of Blood and Marrow Transplantation and Cellular Immunotherapy, Tampa, FL, United States, [2] Department of Immunology, Moffitt Cancer Center, Tampa, FL, United States, [3] Division of Hematology, Oncology, and Transplantation, University of Minnesota, Minneapolis, MN, United States, [4] Department of Hematopathology and Laboratory Medicine, Moffitt Cancer Center, Tampa, FL, United States, [5] Division of Blood and Marrow Transplantation, Department of Pediatrics, Masonic Cancer Center, University of Minnesota, Minneapolis, MN, United States, [6] The Center for Immunology, University of Minnesota Medical School, Minneapolis, MN, United States, [7] Comprehensive Breast Program, Moffitt Cancer Center, Tampa, FL, United States, [8] Department of Biostatistics and Bioinformatics, Moffitt Cancer Center, Tampa, FL, United States, [9] Department of Chemistry, University of South Florida, Tampa, FL, United States, [10] Department of Translational Tumor Immunology, The Wistar Institute, Philadelphia, PA, United States, [11] Department of Tumor Biology, Moffitt Cancer Center, Tampa, FL, United States, [12] Department of Malignant Hematology, Moffitt Cancer Center, Tampa, FL, United States*

OPEN ACCESS

Edited by:
Aurore Saudemont, GlaxoSmithKline, United Kingdom

Reviewed by:
Alain Haziot, Institut National de la Santé et de la Recherche Médicale (INSERM), France
Ralf Dressel, Universitätsmedizin Göttingen, Germany

***Correspondence:**
Brian C. Betts
bett0121@umn.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *08 October 2018*
Accepted: *26 November 2018*
Published: *06 December 2018*

Citation:
Betts BC, Locke FL, Sagatys EM, Pidala J, Walton K, Menges M, Reff J, Saha A, Djeu JY, Kiluk JV, Lee MC, Kim J, Kang CW, Tang C-HA, Frieling J, Lynch CC, List A, Rodriguez PC, Blazar BR, Conejo-Garcia JR, Del Valle JR, Hu C-CA and Anasetti C (2018) Inhibition of Human Dendritic Cell ER Stress Response Reduces T Cell Alloreactivity Yet Spares Donor Anti-tumor Immunity. Front. Immunol. 9:2887. doi: 10.3389/fimmu.2018.02887

Acute graft- vs. -host disease (GVHD) is an important cause of morbidity and death after allogeneic hematopoietic cell transplantation (HCT). We identify a new approach to prevent GVHD that impairs monocyte-derived dendritic cell (moDC) alloactivation of T cells, yet preserves graft- vs.-leukemia (GVL). Exceeding endoplasmic reticulum (ER) capacity results in a spliced form of X-box binding protein-1 (XBP-1s). XBP-1s mediates ER stress and inflammatory responses. We demonstrate that siRNA targeting XBP-1 in moDCs abrogates their stimulation of allogeneic T cells. B-I09, an inositol-requiring enzyme-1α (IRE1α) inhibitor that prevents XBP-1 splicing, reduces human moDC migration, allo-stimulatory potency, and curtails moDC IL-1β, TGFβ, and p40 cytokines, suppressing Th1 and Th17 cell priming. B-I09-treated moDCs reduce responder T cell activation via calcium flux without interfering with regulatory T cell (Treg) function or GVL effects by cytotoxic T lymphocytes (CTL) and NK cells. In a human T cell mediated xenogeneic GVHD model, B-I09 inhibition of XBP-1s reduced target-organ damage and pathogenic Th1 and Th17 cells without impacting donor Tregs or anti-tumor CTL. DC XBP-1s inhibition provides an innovative strategy to prevent GVHD and retain GVL.

Keywords: GvHD, GvL, er stress, XBP-1S, dendritic cell (DC)

INTRODUCTION

DCs are a relevant biologic target for GVHD prevention (1), though current immune suppressive GVHD treatment strategies primarily act upon donor T cells (2–4). Beyond antigen presentation and costimulation, DCs produce proinflammatory cytokines that fuel alloreactive T cells (5). Activated DCs secrete IL-1β via the inflammasome (6), which promotes the differentiation of pathogenic Th17 cells (7, 8). In response to ER stress, the inflammasome is regulated by XBP-1, a transcription factor that is spliced and activated by the RNAse subunit of IRE1α (9, 10). XBP-1s allows the ER to synthesize lipids, expand its size, and produce chaperones to fold ER client proteins that can protect stressed cells from death (9, 11, 12). Prolonged ER stress leads to hyperactivation of IRE1α and secondary inflammasome activation via interactions between XBP-1s and NLRP3 (9, 13). Additionally, XBP-1s regulates DC responses to inflammatory stimuli, such as lipopolysaccharide (LPS) (14).

Inflammasome activity within recipient tissue directly impacts murine GVHD severity (15). NLRP3-deficient mice are partially protected from acute GVHD, while transfer of allogeneic NLRP3-deficient donor T cells to normal hosts has no protective effect (15). This suggests that inflammasome activity in antigen present cells, and/or DCs, contribute to acute GVHD. Furthermore, microRNA-155 is known to regulate DC inflammasome activity (15). MicroRNA-155 deficient murine DCs exhibit impaired IL-1β production from the inflammasome, and such recipient mice also develop significantly less severe acute GVHD (16). In other studies, GVHD but not no GVHD transplant control mice were shown to induce inflammasome activation in myeloid-derived suppressor cells within the first 5 days post-transplant resulting in a loss of their suppressor function (17). Inflammasome activation has been linked to the expression of the purinergic receptors P2x7R and P2Y2, that can sense ATP from damage-associated molecular patterns produced during GVHD, while neutralizing ATP or purinergic receptor triggering reduces GVHD (18, 19). Similarly in patients, high ATP levels have been documented in the peritoneal fluid of patients with severe GVHD (18). Altogether, this evidence strongly supports the pathogenic role of the inflammasome in acute GVHD biology (20).

However, translating this concept to clinical transplantation remains a challenge. Administering IL-1 receptor antagonist failed to prevent acute GVHD in some (21), but not all (22) murine models, and in patients treated in the early post- or peri-HCT period, respectively (23). While the inflammasome and IL-1β are biologically relevant to GVHD pathogenesis, the best strategy to target IL-1β and control donor T cells remains uncertain (15, 23).

Given the fundamental contributions of the inflammasome to acute GVHD and XBP-1s as a regulator of inflammasome activation, targeting ER stress via XBP-1s inactivation could represent a novel strategy to prevent acute GVHD. In support of this possibility is work in autoimmune syndromes showing that blocking ER stress with tauroursodeoxycholic acid ameliorates experimental autoimmune encephalomyelitis and reduces Th17 differentiation (24). In a donor *B cell* dependent *chronic* GVHD model, suppressing XBP-1s in donor B cells reduces murine chronic GVHD (25). While these findings in murine chronic GVHD are important, translational questions regarding how the ER stress response influences human acute GVHD pathogenesis were not addressed.

Our present work is distinct from observations in murine chronic GVHD, as we demonstrate that siRNA knock down or a small molecule inhibitor of XBP-1s can ameliorate DC-allostimulation of human T cells, and using a human skin xenograft model we show that pharmacologic inhibition of XBP-1s can reduce donor alloreactivity *in vivo*. Mechanistically, we also demonstrate how blocking the ER stress response of DCs impacts responding donor T cell activation and differentiation. Herein, we provide human data that support XBP-1s^{+} DCs are relevant biologic targets to prevent acute GVHD, without loss of Treg function or anti-tumor activity by CTLs and NK cells.

Abbreviations: GVHD, graft-vs.-host disease; HCT, hematopoietic cell transplantation; moDC, monocyte-derived dendritic cell; ER, endoplasmic reticulum; CTL, stress, cytotoxic T lymphocytes ; LPS, lipopolysaccharide; GVL, graft- vs.-leukemia.

MATERIALS AND METHODS

Medium

Unless otherwise stated, cells were cultured in complete RPMI supplemented with 10% heat-inactivated, pooled human serum (26).

mAbs and Flow Cytometry

Fluorochrome-conjugated anti-mouse or -human monoclonal antibodies included anti-CD3, CD4, CD8, CD25, CD25RO, CD83, CD86, HLA-DR, CD127, CCR6, CCR7, Ki-67, Foxp3, IFNγ, IL-4, IL-17A, XBP-1s, and phosphorylated STAT3 Y705 (**Supplemental Table 1**). Live/Dead Fixable Yellow Dead Cell Stain (Life Technologies) was used to determine viability. Live events were acquired on a FACSCanto (FlowJo software, ver. 7.6.4).

moDC Phenotyping

LPS-mature moDCs were surfaced stained for CD83, followed by fixation, permeabilization (eBioscience) and intracellular staining for XBP-1s. All other moDC maturation phenotyping was performed as described (19, 21).

siRNA Knock Down of XBP-1

Immature, human moDCs were loaded with XBP-1 or control siRNAs (Dharmacon) using polyethyleneimine (Polyplus), and then stimulated with LPS (1 μg/ml) for 24 h in the presence of B-I09 (20 μM) or DMSO (0.1%). XBP-1 knock down was confirmed by flow cytometry. The moDCs were then used as stimulators in 5-day alloMLRs.

moDC Experiments and alloMLRs

Immature human moDCs were cytokine-generated and differentiated with LPS as described (27). B-I09, XBP-1s inhibitor, was synthesized and purified as reported (28). For XBP-1s expression experiments, moDCs were stimulated with LPS for 24 h while treated with B-I09 (20 μM) or DMSO (0.1%). Supernatant cytokines were quantified using commercial ELISA kits (Thermo Fisher Scientific Inc) after 24 h of LPS-stimulation

in the presence of B-I09 or DMSO. RT-PCR to analyze the levels of regulated IRE1-dependent decay (RIDD) substrates, and total and spliced XBP-1 mRNA was performed after LPS stimulation.

moDC chemotaxis was quantified by migration through a 5 μm pore filter in the chamber of a 24-well transwell plate (29). The lower chamber was filled with 500 μl RPMI enriched with 10% heat-inactivated, human pooled serum and B-I09 (20 μM) or DMSO (0.1%). moDCs pre-treated with B-I09 or DMSO during LPS-maturation were added to the upper chamber at 1×10^5/50 μl. CCL19 or CCL21 (300 ng/ml, R&D systems) was added to the lower well and moDC migration was analyzed after 3 h.

moDC stimulatory capacity was measured in 5-day allogeneic mixed leukocyte reactions (alloMLR). Purified T cells were obtained from healthy human donors (OneBlood) as described (26). AlloMLRs were plated at a moDC:T cell ratio of 1:30. The MLRs consisted of DMSO (0.1%) or B-I09 (20 μM) pre-treated moDCs, DMSO or B-I09 treated MLR medium only, or both. T cell proliferation was determined by a colorimetric assay (Promega) (26, 30).

Calcium Flux Assay

Human moDCs were stimulated with LPS (1 μg/ml) for 24 h in the presence of B-I09 (20 μM) or DMSO (0.1%), and then used to stimulate allogeneic T cells in 5-day alloMLRs. The T cells were then rested for 24 h at 37°C after primary stimulation, transferred to FluoroDish (WPI, 35 mm, 5×10^5 T cells/200 μl) plates coated with Cell-Tak (Corning), loaded with Fluo-4 dye (Thermofisher) for 30 min and washed, and finally restimulated with fresh B-I09- or DMSO-treated moDCs (3×10^4) during live cell imaging to monitor calcium flux in real time (Moffitt Cancer Center, Analytic Microscopy Core).

Live T cells were observed with a Leica TCS SP8 AOBS laser scanning confocal microscope through a 20X/0.8NA or 40X/1.3NA Plan Apochromat objective lens (Leica Microsystems CMS GmbH, Germany). A 488 nm laser line was applied to excite the sample and tunable emission was set to capture the Fluo-4 spectrum. Images were captured at 400 Hz scan speed with photomultiplier detectors and LAS X software version 3.1.5 (Leica Microsystems).

Time lapse images were analyzed using the Definiens Tissue Studio v4.7 (Definiens AG, Munich, Germany) software suite. The green fluorescent channel images were segmented by green intensity and cell size. The image was analyzed as an 8 bit image and intensity was measured from 0 to 255 grayscale fluorescent units. The cells were then quantified for green intensity per field for each time point imaged and then plotted for intensity over time.

REVERSE TRANSCRIPTION AND QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) TO DETECT THE EXPRESSION LEVELS OF RIDD SUBSTRATES

Human moDCs were stimulated with LPS (1 μg/ml) for 24 h in the presence of B-I09 (20 μM) or DMSO (0.1%). moDCs were then harvested and total RNA was isolated using TRIzol reagent (Invitrogen). Complementary DNA was synthesized from RNA using Maxima H Minus reverse transcriptase (Thermo Scientific). The following sets of primers were used together with iTaq Universal SYBR Green Supermix (Roche) in qPCR to detect the expression levels of human GAPDH (GGA TGA TGT TCT GGA GAG CC and CAT CAC CAT CTT CCA GGA GC); human XBP1s (CTG AGT CCG AAT CAG GTG CAG and ATC CAT GGG GAG ATG TTC TGG); human XBP1t (TGG CCG GGT CTG CTG AGT CCG and ATC CAT GGG GAG ATG TTC TGG); human Bloc1S1 (CCC AAT TTG CCA AGC AGA CA and CAT CCC CAA TTT CCT TGA GTG C); human CD59 (TGA TGC GTG TCT CAT TAC CAA AGC and ACA CAG GTC CTT CTT GCA GCA G); and human Scara3 (AAC TTC CTG CAC ACA CTG GC and CAA ACC AGT TGC ACA TCC AG).

Treg Experiments

Tregs were defined as $CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$ cells (31, 32). Treg potency was determined using suppression assays (26). DMSO (0.1%) or B-I09 (20 μM) was only added to the initial culture to expand the Tregs. No drug was added to the suppression assay medium. Conventional, alloreactive T cell (Tconv) proliferation was measured by Ki-67 expression using flow cytometry. To test the effect of XBP-1s blockade on natural (nTreg) or *in vitro* induced Tregs (iTreg), circulating Tregs were isolated from healthy donor blood by magnetic bead purification ($CD4^+$, $CD25^+$). Tconv ($CD4^+$, $CD25^-$) were also purified from the donor sample and stimulated with allogeneic moDCs and IL-2 for iTreg differentiation. The enriched nTregs were also cultured with IL-2 (20IU/ml) and allogeneic moDCs (pretreated with DMSO or B-I09) at a ratio of 1:30. DMSO (0.1%) or B-I09 (20 μM) was added to the co-culture once on day 0 as indicated. After 5 days, the cells were harvested and analyzed by flow cytometry. Tregs were enumerated using CountBright beads (Thermo Fisher Scientific Inc). In select experiments, TGFβ1 (4 ng/ml) (R&D Systems) was added to the medium on alternating days.

Th1, Th2, and Th17 Phenotype Experiments

T cells were cultured with DMSO- or B-I09-pretreated, allogeneic moDCs, DMSO (0.1%) or B-I09 (20 μM) was added once on day 0. For Th17 experiments only, the T cells were first CD4-purified by magnetic bead isolation and supplemented with IL-1β or TGFβ as indicated, and anti-IFNγ antibody (26). On day +5, the T cells were harvested and stained to identify the following T helper subsets: Th17 - $CD4^+$, $IL\text{-}17A^+$; Th1 - $CD4^+$, $IFN\gamma^+$; and Th2 - $CD4^+$, $IL\text{-}4^+$.

Tumor Lysis Experiments and T Cell Recall Response

Human peripheral blood mononuclear cells (PBMCs, $5x10^5$) were stimulated with irradiated (30Gy) U937 cells (American Type Culture Collection) at a 1:1 ratio on day 0 and +7. DMSO (0.1%) or B-I09 (20 μM) was added on day 0. $CD8^+$ T cells were isolated on days +12-14 (to prevent non-specific killing by NK cells), and then cultured with fresh U937 cells at the stated effector-to-target ratios for 4 h at 37°C (26). Unprimed $CD8^+$ T cells served as a negative control. No drug was added.

Tumor lysis was determined by a colorimetric LDH release assay (Thermo Fisher Scientific Inc) (26, 33). Percent lysis was calculated as follows: [(test optical density (OD) – spontaneous OD)/(maximum OD – spontaneous OD)] × 100 (26, 33).

To determine T cell recall response to nominal antigen, T cells were cultured with autologous moDCs loaded with a mixed CMV, EBV, influenza, and tetanus peptide pool (JPT). DMSO (0.1%) or B-I09 (20 μM) was added once on day 0 of the culture. T cell proliferation was determined after 3 days of culture (34).

NK Cell Experiments

Human natural killer cells (NK cells) were isolated from healthy donor PBMCs by magnetic bead purification (Miltenyi Biotec Inc). NK cells were cultured with K562 cells at the stated effector-to-target ratios for 5 h at 37°C in the presence of DMSO (0.1%) or B-I09 (20μM) (35). Tumor lysis was determined by a colorimetric LDH release assay (33, 35).

NK cell proliferation was assessed by allogeneic moDC (moDC: NK cell ratio 1:10) or cytokine stimulation (IL-2 200 IU/ml and IL-15 10 ng/ml) (35). DMSO (0.1%) or B-I09 (20 μM) was added once on day 0 of the culture. NK cell proliferation was determined after 5 days using a colorimetric assay.

Xenograft Model and *in vivo* CTL Generation

NSG mice were transplanted with a 1 cm^2 human skin graft using a well-established model (33, 36, 37). Skin was procured from consented mastectomy patients (MCC 17634, an IRB-approved protocol). After 30 days of rest, mice received 5 × 10^6 fresh, human PBMCs (OneBlood) i.p. using a random donor allogeneic to the skin graft (26, 36, 37). Each transplant experiment used a unique donor pair of skin and PBMCs. B-I09 30 mg/kg or a polyethylene glycol-based vehicle (26) was given by i.p. injection 5 days a week for 3 weeks. On day +21, mice were humanely euthanized; skin grafts, host lung, host liver, and host spleen were harvested for analysis. Skin rejection and xenogeneic GVHD scoring was performed blinded according to standard criteria (26, 33, 38). Processed spleens cells were phenotyped by flow cytometry. To generate $CD8^+$ CTL *in vivo*, mice were transplanted with 30 × 10^6 human PBMCs and also received irradiated U937 cells (10x10^6) on day 0 and +7 (26, 33). Control mice received PBMCs alone without tumor. Mice did not receive skin grafts for these experiments. Mice received B-I09 or vehicle exactly as stated. On days +10-12, the mice were humanely euthanized and the spleens were harvested. Human $CD8^+$ T cells within the spleens were purified by magnetic beads. Tumor lysis assays were performed *in vitro*.

NSG Mice for Xenograft Model and *in vivo* CTL Generation

NSG mice (male or female, 6–24 weeks old) were used in the described *in vivo* experiments. NSG mice were purchased from The Jackson Laboratory and raised at the Moffitt Cancer Center vivarium. Experiments were performed according to an Institutional Animal Care and Use Committee (IACUC)–approved protocol in adherence to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Statistical Analysis

Data are reported as mean values ±SEM. Normality was tested by the Anderson-Darling test. For comparisons of dependent data, the paired *t*-test was used. The Mann-Whitney test was used for comparisons of *in vivo* mouse and patient skin sample data. ANOVA was used for group comparisons, including a Dunnett's or Tukey post-test with correction for multiple-comparisons. The statistical analysis was conducted using Prism software version 5.04 (GraphPad). Statistical significance was defined by $P < 0.05$ (two-tailed).

RESULTS

XBP-1s Inhibition Reduces the Stimulatory Potency of moDCs Toward Allogeneic T Cells

XBP-1 mRNA is constitutively spliced by IRE1α in moDCs at steady-state (14, 39). The TLR4 agonist, LPS, triggers ER stress and augments XBP-1 splicing (40). We first tested the effect of XBP-1s blockade on human moDC stimulatory capacity in alloMLRs. To genetically suppress XBP-1s, human moDCs were treated with XBP-1 or control siRNA and stimulated with LPS for 24 h (**Figure 1A**). Allogeneic T cells responding to the XBP-1 siRNA-treated moDCs showed a significant reduction in proliferation compared to controls (**Figure 1B**).

B-I09 blocks the RNAse activity of IRE1α, suppressing XBP-1 splicing (28). B-I09 (20 μM) has shown on-target XBP-1s inhibition in murine B cells and chronic lymphocytic leukemia (28). Similar to its effects on murine B cells, B-I09 significantly reduces XBP-1s in human, LPS-stimulated moDCs (**Supplemental Figures 1A,B**). We confirmed on-target inhibition of XBP-1s in moDCs by B-I09 via RNA expression, and verified that key regulated IRE1 dependent decay (RIDD) substrates, BlocS1 (41) and CD59 (42), were unaffected by the inhibitor compared to DMSO (**Figure 1C**). IRE1α also exhibits kinase activity and phosphorylates c-Jun N-terminal kinase (JNK) in response to ER stress (43). Disruption of JNK signaling could interfere with moDC function (44). However, B-I09 did not suppress IRE1α-mediated JNK phosphorylation in human moDCs (**Supplemental Figures 2A,B**).

Despite the inhibitory effect of B-I09 on moDC XBP-1 splicing, B-I09 did not impair LPS-mediated moDC maturation or viability (**Supplemental Figures 3A–D**). Pre-treating the moDCs with B-I09 (20 μM) during LPS-maturation had no significant effect on the stimulation of allogeneic T cell proliferation (**Figure 1D**, condition 1 vs. condition 4). Adding B-I09 to the allogeneic co-culture alone produced a modest reduction in T cell proliferation (**Figure 1D**, condition 2 vs. condition 5). moDC-allostimulated T cell proliferation was significantly impaired when the moDCs were first pre-treated with B-I09 during LPS-maturation and added once again to the alloMLR medium (**Figure 1D**, condition 3 vs. condition 6). Therefore, moDCs require XBP-1s suppression during maturation and also during their interactions with T cells to fully inhibit the alloresponse. Based on these data, allogeneic co-cultures described hereafter used either DMSO- (0.1%) or

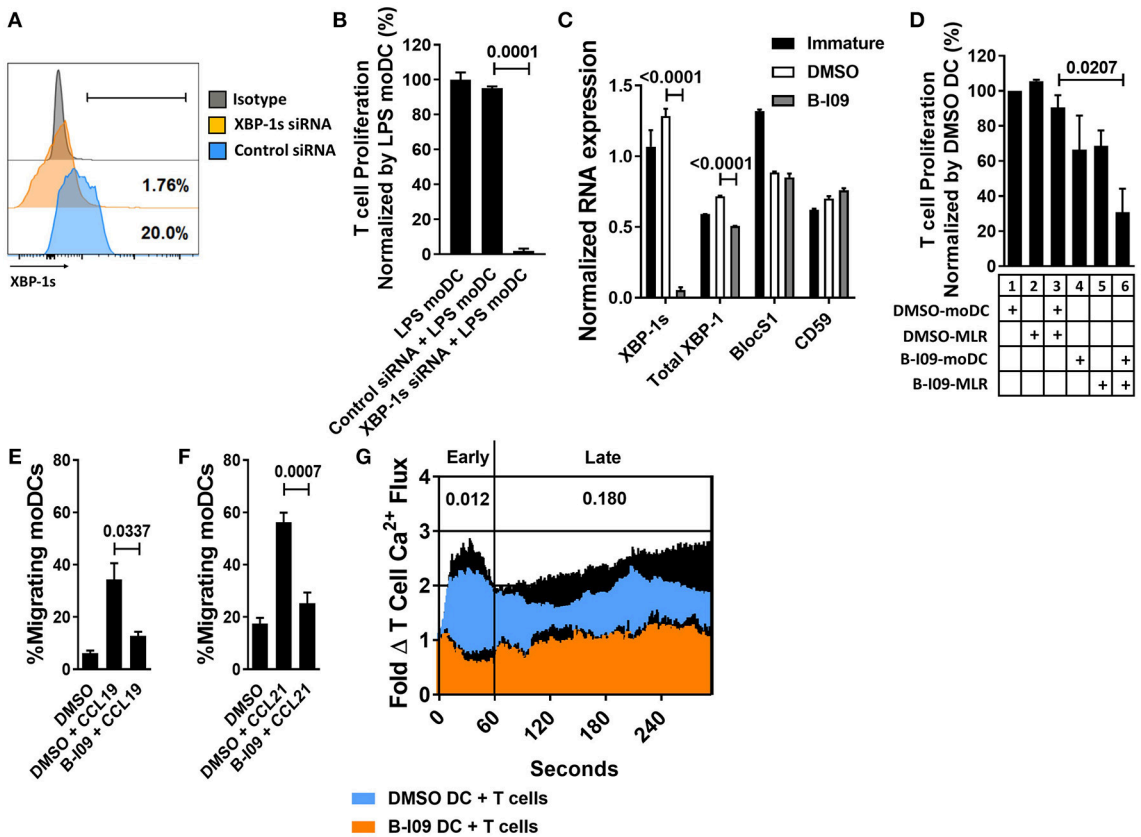


FIGURE 1 | XBP-1s inhibition reduces the stimulatory potency of moDCs toward allogeneic T cells**.** Human moDCs were stimulated with LPS (1 μg/ml) for 24 h in the presence of XBP-1 or control siRNA. **(A)** Representative histograms show XBP-1s expression in siRNA-treated moDCs. **(B)** T cells were stimulated by XBP-1- or control-siRNA-treated moDCs in alloMLRs. T cell proliferation (MTS colormetric assay) after 5 days is shown. AlloMLRs were plated in replicates of 5 at a moDC: T cell ratio of 1:30. 1 representative experiment of 2 independent studies is shown, Dunnett's test. **(C)** Human moDCs were stimulated with LPS (1 μg/ml) for 24 h in the presence of B-I09 (20 μM) or DMSO (0.1%). Bar graph shows triplicate mean XBP-1s or total XBP-1 mRNA, vs. the RIDD components BlocS1 and CD59 in B-I09- or DMSO-treated moDCs after 24 h of LPS stimulation as measured by RT-PCR. 1 representative experiment of 4 independent studies is shown, Tukey's test. **(D)** T cell proliferation (MTS colormetric assay) measured in 5-day MLRs using B-I09- or DMSO-treated allogeneic, moDCs. Table depicts whether moDCs were pre-treated with DMSO (0.1%) or B-I09 (20 μM), or if DMSO (0.1%) or B-I09 (20 μM) was added to the MLR medium. Replicate means from 4 independent experiments are shown, Dunnett's test. **(E,F)** Human moDCs were LPS-stimulated for 24 h with B-I09 or DMSO. Bar graphs show proportion of migrating moDCs in transwell assays testing CCL19 or CCL21 (300 ng/ml, 3 h) chemotaxis. Replicate means from 4 independent experiments are shown for each, Dunnett's test. **(G)** Human moDCs were stimulated with LPS (1 μg/ml) for 24 h in the presence of B-I09 (20 μM) or DMSO (0.1%), and then used to activate allogeneic T cells for 5 days. T cells were rested for 24 h at 37°C, loaded with Fluo-4 dye for 30 min, then restimulated with fresh B-I09- or DMSO-treated moDCs during live cell imaging to monitor calcium flux in real time. Replicate means from 3 independent studies are shown. *P* values are shown for early (<60 s) and late (>60 s) T cell calcium flux after initial moDC:T cell interaction by comparing the mean AUCs after stimulation with B-I09- or DMSO-treated moDCs, paired *t*-test.

B-I09-pretreated (20 μM), LPS-stimulated moDCs. B-I09 or vehicle was also added to the MLR medium once on day 0 to maintain XBP-1s suppression during culture unless otherwise indicated.

B-I09 was not toxic toward human T cells in treated allogeneic co-cultures (**Supplemental Figure 4A**) and we were unable to detect significant amounts of XBP-1s in DC-allostimulated T cells (**Supplemental Figure 4B**). Although CD3/CD28 bead-stimulation produced significant amounts of XBP-1s in responder T cells (**Supplemental Figure 4C**), B-I09 had no suppressive effect on these responder T cells (**Supplemental Figure 4D**). Taken together, these findings indicate that the immune suppressive effect of XBP-1s inhibition acts primarily on human moDCs, impairing their allostimulatory capacity, and secondarily limits allogeneic T cells.

XBP-1s Blockade Impairs Human moDC Migration and Induction of T Cell Calcium Flux

TLR4 facilitates the migration of moDCs by inducing CCR7 surface expression (45). In trans-well assays, B-I09 abrogates moDCs motility toward the CCR7 ligands, CCL19, and CCL21, compared to vehicle-treated cells (**Figures 1E,F**). T cell activation is a chief function of DCs. Despite intact potential by the allogeneic moDCs to mediate costimulation by CD86 (**Supplemental Figure 3A**), we identified that T cell activation

via early (<60 s) phase calcium flux was reduced in response to B-I09-treated moDCs (**Figure 1G**).

XBP-1s Inhibited moDCs Reduce Th1 Differentiation

Since Th1 cells are implicated in GVHD pathogenesis (46), we investigated the effect of B-I09 on Th1 and Th2 responses *in vitro*. B-I09 reduced LPS-stimulated moDC production of p40 cytokines, implicated in Th1 differentiation (**Figure 2A**), without impairing moDC TNFα production (**Figure 2B**). T cells stimulated by allogeneic moDCs pretreated with B-I09, along with B-I09 added to the co-culture once on day 0 had a significantly reduced Th1 response compared to controls (**Figures 2C,D,G**). Conversely, XBP-1s blockade significantly increased the amount of Th2 cells after 5 days of culture in a proportional manner (**Figures 2E-G**). The shifts in Th1 were consistent with reduced p40 cytokine production by moDCs treated with B-I09.

Targeting XBP-1s Abrogates moDC Production of IL-1β, TGFβ, and Diminishes Alloresponder Th17 Differentiation

We next investigated whether B-I09 could reduce Th17-inducing cytokines by LPS-stimulated moDCs. XBP-1s blockade significantly suppressed IL-1β production by human moDCs

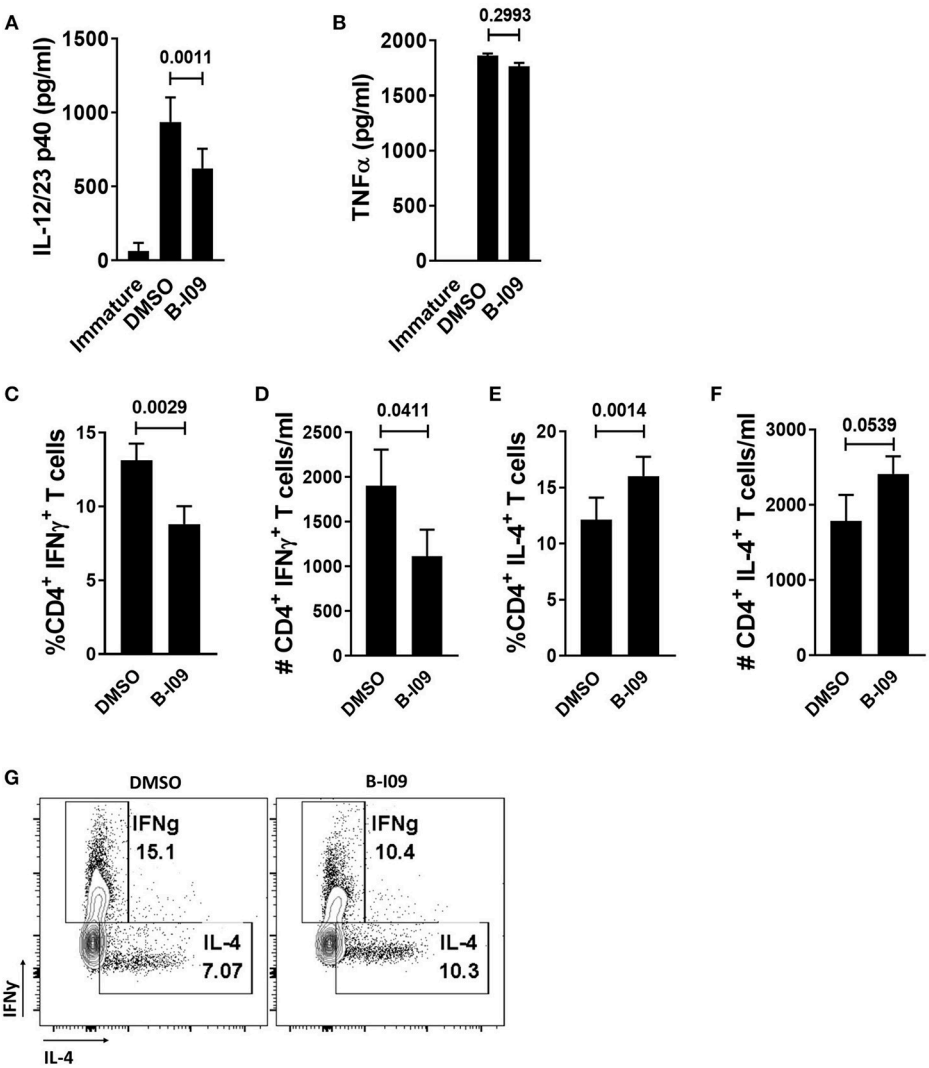


FIGURE 2 | XBP-1s inhibited moDCs reduce Th1 differentiation**. (A,B)** ELISAs were used to determine the concentration of IL-12/23 p40 cytokines or TNFα in the supernatants of B-I09- or DMSO-treated moDCs after 24 h of LPS stimulation. Replicate means from 8 (IL-12/23 p40) and 2 (TNFα) independent experiments are shown, Dunnett's test. **(C–G)** T cells were cultured with B-I09 or DMSO pre-treated moDCs (moDC:T cell ratio of 1:30), and additional B-I09 (20 μM) or DMSO (0.1%) was added once on day 0. Harvested T cells were evaluated on day +5 for Th1 ($CD4^+$, $IFN\gamma^+$) and Th2 ($CD4^+$, $IL\text{-}4^+$) phenotype. Percentage or absolute numbers of Th1 **(C,D)** and Th2 **(E,F)** are shown. **(G)** Representative contour plots show $CD4^+$ Th1 vs. Th2 cells on day +5 of the allogeneic co-culture. Means of 8 independent experiments are shown, paired t-test.

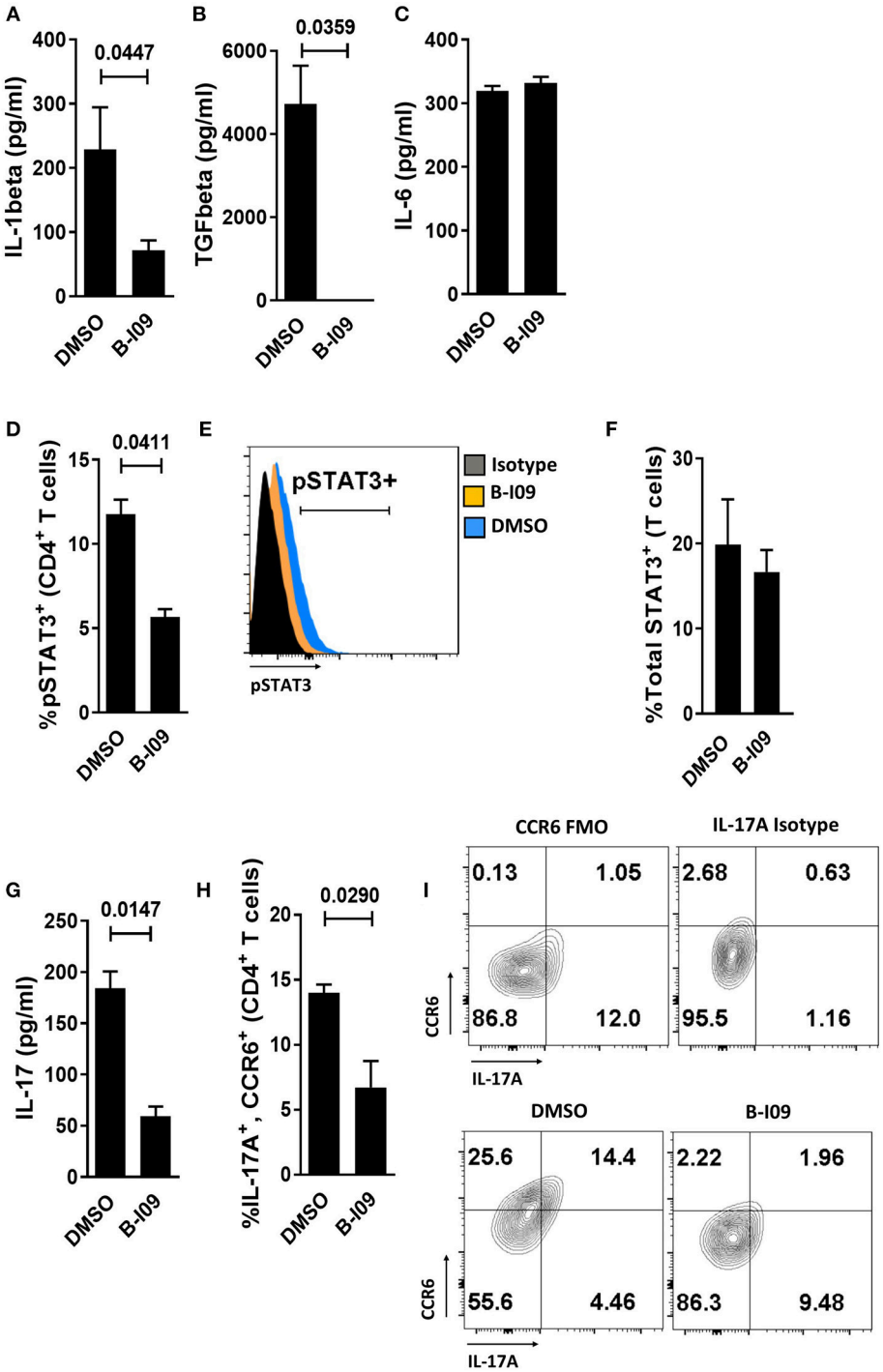


FIGURE 3 | Targeting XBP-1s abrogates human moDC production of IL-1β, TGFβ, and diminishes alloresponder Th17 differentiation**. (A–C)** Supernatant concentrations of IL-1β, TGFβ, or IL-6 from LPS-stimulated moDCs exposed to B-I09 or DMSO after 24 h of culture are shown. Replicate means from 5 (IL-1 β), 3 (TGFβ), and 4 (IL-6) independent experiments are shown, paired *t*-test. **(D–H)** T cells were cultured with B-I09 or DMSO pre-treated moDCs (moDC:T cell ratio of 1:30), and additional B-I09 (20 μM) or DMSO (0.1%) was added once on day 0. pSTAT3$^+$ CD4$^+$ T cells were analyzed at day +5 by flow cytometry **(D)** and representative histograms are shown **(E)**. Means from 3 independent experiments are shown, paired *t*-test. **(F)** Total STAT3 was measured in T cells from co-cultures of DCs and T cells treated with B-I09 or DMSO. Means from 3 experiments are shown. In similarly treated co-cultures, the supernatant concentration of IL-17 was quantified **(G)** and Th17 (CD4$^+$, CCR6$^+$, IL-17A$^+$) differentiation was evaluated by flow cytometry **(H,I)**. Replicate means from 3 (IL-17 ELISA) and 4 (Th17) independent experiments, paired *t*-test. **(I)** Representative contour plots are shown.

(**Figure 3A**) and eliminated TGFβ from the supernatant of LPS-stimulated moDCs (**Figure 3B**). IL-1β is an essential co-factor for IL-6 signal transduction (47). Despite ample IL-6 production by B-I09- or DMSO-treated moDCs (**Figure 3C**), $CD4^+$ T cells co-cultured with these moDCs in the presence of B-I09 displayed significantly less STAT3 phosphorylation (**Figures 3D,E**). Total STAT3 expression within T cells stimulated by B-I09- or DMSO-treated moDCs was similar (**Figure 3F**). Moreover, Th17 differentiation was significantly decreased by XBP-1s inhibition (**Figures 3G–I**) and partially rescued by adding IL-1β, but not TGFβ, to B-I09 treated co-cultures (**Supplemental Figure 5**).

moDC XBP-1s Directs iTreg Differentiation via TGFβ

As XBP-1s-inhibited moDCs impaired Th17 differentiation, we were surprised to observe that such moDCs also significantly reduced responder Treg frequency in treated alloMLRs (**Figures 4A,B**), though the suppressive function of antigen-specific Tregs remained intact (**Figure 4C**). We then investigated whether the reduction in Tregs stimulated by B-I09-treated moDCs was due to impaired differentiation of iTregs or suppression of nTregs. Treg-depleted Tconv or purified nTregs were cultured with DMSO-

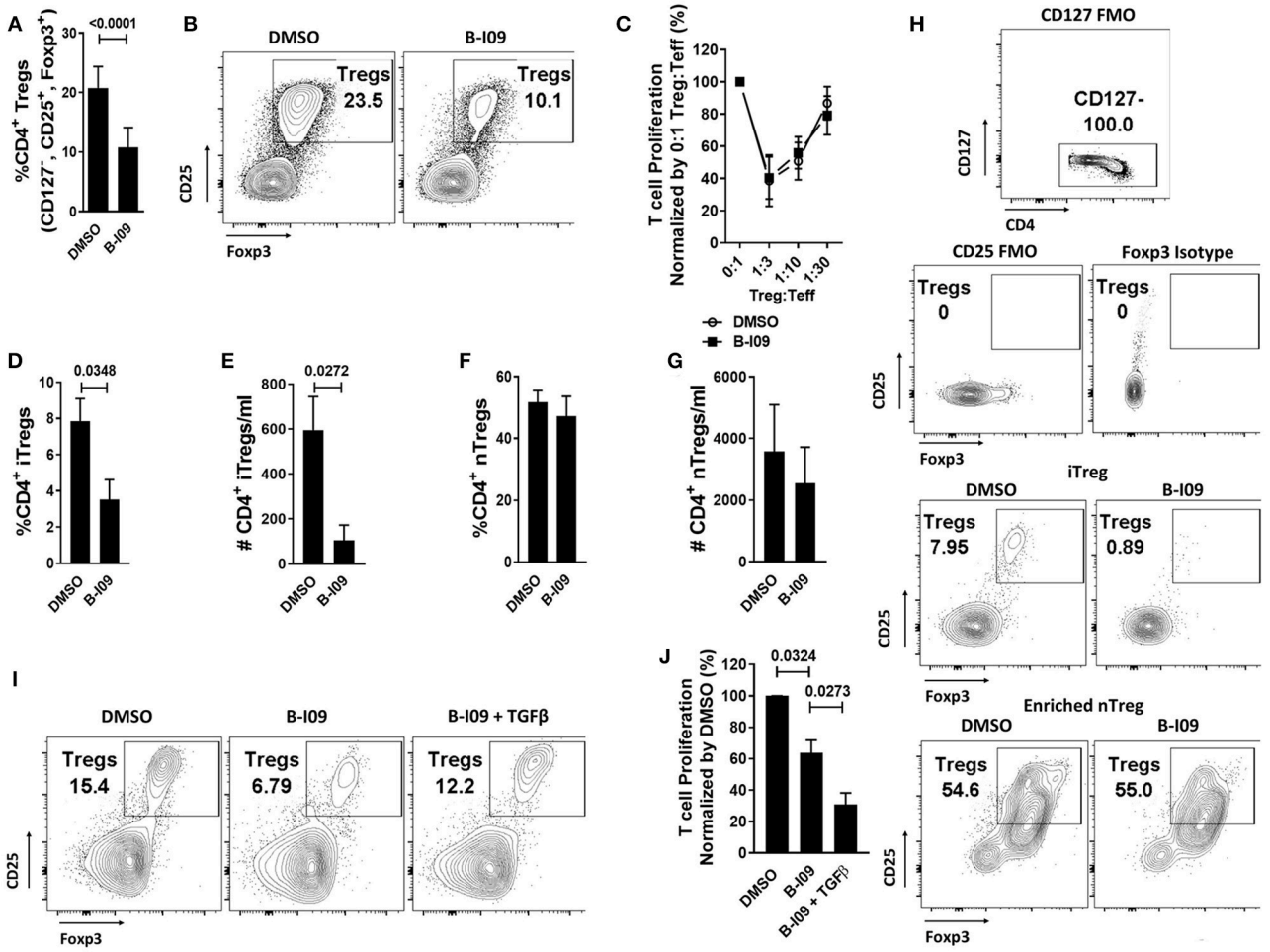


FIGURE 4 | moDC XBP-1s direct human iTreg differentiation via TGFβ. T cells were cultured with B-I09 or DMSO pre-treated moDCs (moDC:T cell ratio of 1:30), and additional B-I09 (20μM) or DMSO (0.1%) was added once on day 0. **(A)** Percentage of Tregs ($CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$) in the 5-day co-cultures, with representative contour plots shown **(B)**. Means from 6 independent experiments are shown. **(C)** The suppressive capacity of harvested moDC-allostimulated Tregs was tested at different ratios of Treg to T cell responders stimulated by fresh allogeneic moDCs (moDC:responder T cell ratio 1:30) in alloMLRs. No additional B-I09 or DMSO was added. Graph shows mean percent T effector (Teff) proliferation measured by Ki-67. Means from 3 independent experiments are shown. To generate inducible Tregs, Treg-depleted $CD4^+$ Tconv were stimulated by B-I09 or DMSO-pretreated allogeneic moDCs at a moDC:T cell ratio of 1:30. B-I09 or DMSO was added once on day 0. Magnetic bead enriched natural Tregs ($CD4^+$, $CD25^+$) were similarly treated in allogeneic Treg:moDC co-cultures. Treg populations were evaluated by flow cytometry on day +5. Means from 4 iTreg and 4 nTreg independent experiments are shown, paired *t*-test. Percentage and absolute number of iTreg **(D,E)** and nTreg **(F,G)** are shown. **(H)** Representative contour plots are shown for iTreg and nTreg. **(I)** Representative contour plots show that adding recombinant human TGFβ rescues iTreg generation in Treg-depleted alloMLRs treated with B-I09 vs. DMSO. 1 representative experiment of 2 independent studies is shown. **(J)** T cell proliferation (MTS colormetric assay) at day +5 among Treg-depleted alloMLRs treated with B-I09 or DMSO, with recombinant human TGFβ added as indicated. Replicate means from 4 independent experiments are shown, Dunnett's test.

or B-I09-pretreated, allogeneic moDCs, and DMSO or B-I09 was also added to the media, respectively. XBP-1s inhibition significantly reduced both the frequency and absolute number of iTregs stimulated by allogeneic moDCs (**Figures 4D,E,H**) in contrast to nTregs (**Figures 4F,G,H**). Based on these data, we surveyed known moDC-mediated mechanisms for iTreg generation. The expression of indolamine 2,3-deoxygenase (48) was similar among B-I09- or DMSO-treated moDCs (**Supplemental Figures 6A,B**), as was STAT5 phosphorylation among the DC-allostimulated T cells (**Supplemental Figures 6C,D**). Instead, we discovered that exogenous TGFβ rescued the differentiation of iTregs in the co-cultures containing XBP-1s-inhibited moDCs (**Figure 4I**). Moreover, adding TGFβ to Treg-depleted, MLRs stimulated by B-I09-treated moDCs provided even greater suppression of allogeneic T cells (**Figure 4J**). These data suggest that XBP-1s-mediated TGFβ production by human moDCs contributes to allogeneic iTreg differentiation, and that moDC XBP-1s activity is not required for Treg suppressive potency or nTreg responses.

XBP-1s Is Dispensable for Anti-tumor Activity by CD8$^+$ Cytotoxic T Lymphocytes and NK Cells

While inhibiting moDC-XBP-1s in alloMLRs impaired the proliferation of responder T cells, CTLs generated with B-I09-treated stimulators exhibited intact tumor specific lytic function (**Figure 5A**). Compared to unloaded moDCs, T cell responses to clinically relevant pathogens using peptide-loaded, B-I09 exposed autologous moDCs permitted a measureable response to CMV, EBV, influenza, and tetanus albeit significantly less robust

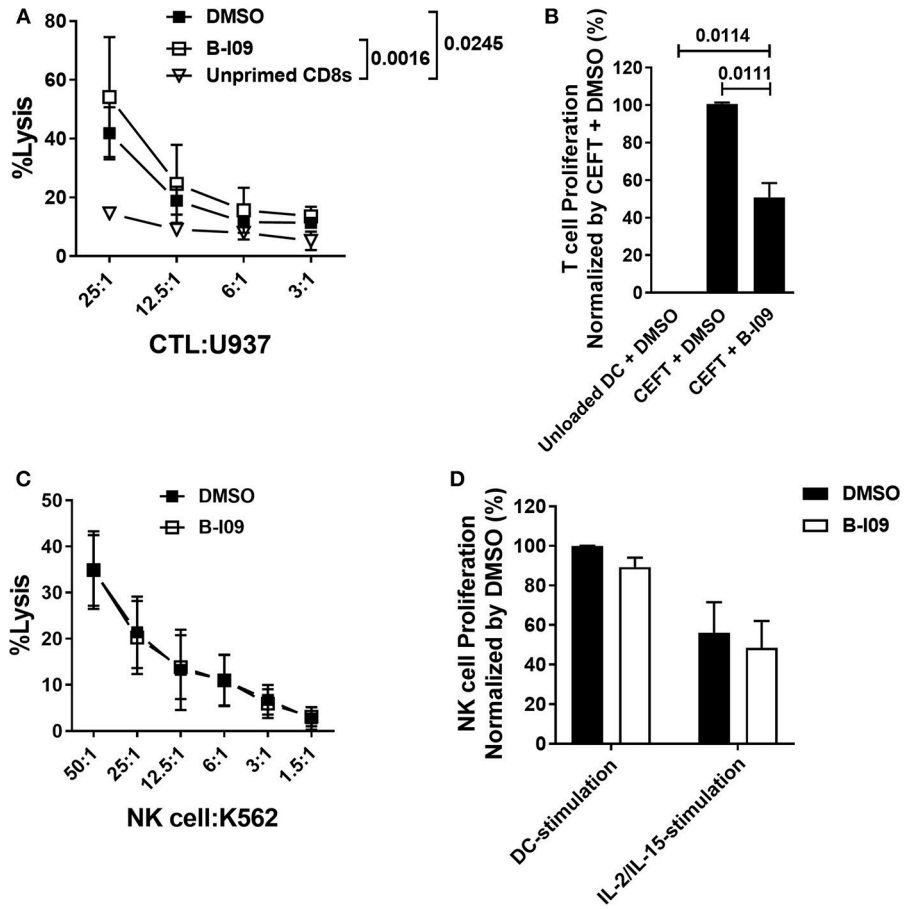


FIGURE 5 | XBP-1s is dispensable for anti-tumor activity by CD8$^+$ cytotoxic T lymphocytes and NK cells. **(A)** Replicate mean specific lysis by human CD8$^+$ CTL generated *in vitro* using PBMCs stimulated by irradiated U937cells (1:1) on days 0 and +7 of a 10–12 day culture. B-I09 or DMSO was added once on days 0 and +7. Tumor-specific killing by purified CD8$^+$ T cells was determined using fresh U937 cells. U937 lysis was measured by a colorimetric assay after 4 h. Triplicate means from 3 independent experiments are shown. **(B)** T cell proliferation stimulated by autologous moDCs loaded with CMV, EBV, influenza, or tetanus peptides is shown. B-I09 or DMSO was added once on day 0. T cell proliferation (MTS colormetric assay) was measured on day +3. Replicate means from 4 independent experiments are shown, Dunnett's test. **(C)** Mean specific lysis by human NK cells against K562 targets is shown. B-I09 or DMSO was added at the outset of the culture. K562 lysis was measured by a colorimetric assay after 4 h. Replicate means from 3 independent experiments are shown. **(D)** NK cell proliferation stimulated by allogeneic moDCs (moDC:NK cell ratio 1:10) or IL-2 plus IL-15. NK cell proliferation (MTS colormetric assay) was measured on day +5. Replicate means from 3 independent experiments are shown.

than DMSO-treatment of peptide-loaded DCs (**Figure 5B**). In evaluating the effect of XBP-1s-inhibited moDCs on responder T cell memory phenotypes after 5 days of stimulation, we identified that blocking the moDC ER stress response increased the proportion of non-alloreactive, naïve $CD8^+$ T cells vs. alloreactive central and effector memory $CD8^+$ T cells compared to vehicle controls (**Supplemental Figures 7A,B**). The proportion of responder $CD4^+$ T cell memory subsets was similar regardless of B-I09- or DMSO-treated moDC-stimulation (**Supplemental Figures 7C,D**).

NK cells and moDCs both constitutively express XBP-1s (49). The functional significance of XBP-1s in NK cells is not known. Because NK cells can mediate important anti-tumor effects after allo-HCT, we investigated the effect of B-I09 on NK cell lytic capacity and proliferative responses. Human NK cells readily destroyed K562 targets in the presence of B-I09 or DMSO (**Figure 5C**). Despite their exposure to B-I09 NK, cells proliferated when stimulated by allogeneic, immature moDCs or a cocktail of IL-2 and IL-15 (**Figure 5D**).

XBP-1s Blockade Reduces Human Skin Graft Rejection and Xenogeneic GVHD, Yet Preserves *in vivo* Generation of Anti-tumor CTL

Skin is a highly immunogenic tissue and a critical target-organ in GVHD (1). To test the efficacy of B-I09 *in vivo* using human immune cells, NSG mice were transplanted with a human skin xenograft using a well-established model (33, 36). After 30 days to heal, mice were later injected with 5×10^6 human donor PBMCs allogeneic to the skin to induce graft rejection and xenogeneic GVHD (33, 36). B-I09 or vehicle was administered at 30mg/kg by intraperitoneal injection 5 days a week for 3 weeks. XBP-1s blockade significantly reduced skin graft rejection by the allogeneic PBMCs, as measured on day +21 after the adoptive transfer of allogeneic PBMCs (**Figures 6A,B**). Importantly, B-I09 also significantly reduced xenogeneic GVHD by the human T cells against murine liver (**Figures 6A,C**) and modestly against mouse lungs that did not quite reach statistical significance (**Figures 6A,D**). *In vivo* XBP-1s inhibition by B-I09 could be seen in CD3 negative cells isolated from the recipient spleens (**Figure 6E**). Consistent with *in vitro* observations, B-I09 significantly reduced the amount of human Th17 cells ($CD4^+$, IL-$17A^+$) in the mouse spleens (**Figures 7A,B**). Human Tregs ($CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$) recovered from the host spleens were similar among B-I09- or vehicle-treated mice (**Figures 7C,D**). While B-I09-treated mice demonstrated a significant decrease in Th1 cells ($CD4^+$, IFNγ^+), Th2 cells ($CD4^+$, IL-4^+) were not increased as observed *in vitro* (**Figures 7E-G**).

We used an established method to generate human anti-tumor CTL *in vivo* and then test their specific killing (26, 33, 50). NSG mice received human PBMCs (30×10^6) and were inoculated with irradiated U937 cells (10×10^6) on days 0 and +7. Mice were treated with B-I09 or vehicle as described above, and human $CD8^+$ T cells were isolated from the spleens of euthanized recipients during days +10-12. $CD8^+$ CTLs from B-I09-treated mice demonstrated tumor killing equal to CTLs from vehicle-treated recipients, and both were significantly more potent than $CD8^+$ CTLs from unvaccinated controls (**Figure 7H**). Preliminary data from a pilot cohort of patients undergoing allogeneic HCT (5 with acute GVHD and 5 without), suggests that the amount of intracellular XBP-1s is significantly increased in $CD1b^+$, epidermal DCs among skin biopsies from patients with acute GVHD compared to no GVHD controls (**Supplemental Figures 8A–C**). Though not statistically significant, we also observed a trend toward increased $CD1b^+$, XBP-$1s^+$epidermal DCs in the GVHD cohort (**Supplemental Figure 8B**). This observation among a limited number of patients is provocative and merits the prospective study of XBP-$1s^+$ DCs in acute GVHD pathogenesis.

DISCUSSION

We demonstrated that blocking XBP-1s in human moDCs significantly reduced IL-1β and IL12/23p40 production during their interaction with donor T cells, suppressing Th1 and Th17 differentiation. While XBP-1s inhibition restrained T cell alloreactivity, anti-tumor responses by CTLs and NK cells remained intact. While rodent models have shown targeting XBP-1s in B cells reduces chronic GVHD without impairing graft- vs.-leukemia (GVL) effects (25), we now demonstrate that suppressing XBP-1s in human DCs limits acute GVHD without impairing donor immunity toward cancer. We also detected a partial response toward clinically relevant infectious antigens by T cells stimulated by XBP-1s-inhibited, peptide loaded moDCs.

We showed XBP-1s inhibition protected human skin grafts from alloreactive T cell rejection *in vivo*. Xenogeneic GVHD mediated by human T cells was also decreased by targeting XBP-1s. Altogether, our data show that XBP-1s is a relevant therapeutic target to suppress key aspects of DC function and prevent GVHD after alloHCT.

Our results are consistent with prior studies which demonstrate that eliminating ASC or the NLRP3 inflammasome in bone marrow transplant recipients significantly reduces GVHD in mice (15). Our approach substantially extends these data by interrogating XBP-1s in human cells and demonstrating regulation of NLRP3 activation in the context of ER stress (9, 51). While targeting either the inflammasome or XBP-1s reduces Th17 differentiation (15), XBP-1s inhibition differs from NLRP3 blockade by also significantly reducing Th1 and iTreg responses. This defect in moDC supported human iTreg generation is correctable by adding exogenous TGFβ, which is otherwise absent from B-I09-treated moDCs. We show XBP-1s blockade does not diminish the number or frequency of allo-stimulated nTregs or total Treg suppressive potency. Interestingly, XBP-1s inhibition was not detrimental to human Tregs *in vivo*. In the xenogeneic transplant experiments, recipient mice were transplanted with whole human PBMCs. Though alloMLRs are restricted to T cells and moDCs, we speculate the more diverse non-moDC constituents of the PBMC inoculum may potentially rescue Tregs *in vivo* by providing TGFβ (52). Additionally,

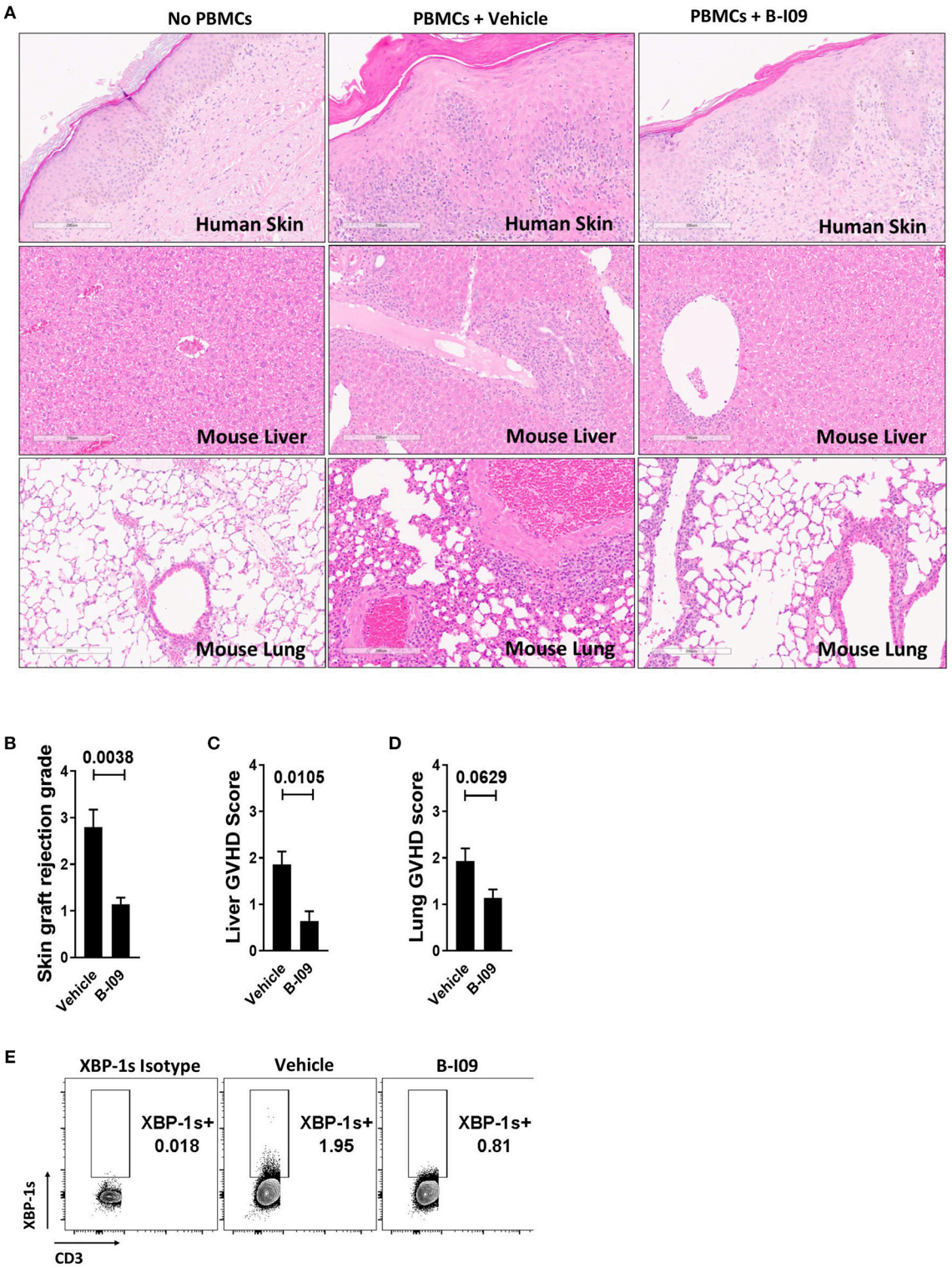


FIGURE 6 | XBP-1s blockade reduces human skin graft rejection and xenogeneic GVHD. NSG mice received a 1 cm^2 split thickness human skin graft. After 30 days of rest to permit engraftment, 5×10^6 human PBMCs (allogeneic to the skin) were injected into the mice. Unique pairs of donor skin and allogeneic PBMCs were used for each set of experiments. B-I09 30 mg/kg or vehicle was given by i.p. injection 5 days a week for 3 weeks. Mice were humanely euthanized and the skin graft, spleen, lung, and liver were harvested from the recipient on day +21 from time of PBMC injection. **(A)** Representative H&E sections compare skin graft rejection and xenogeneic GVHD in the liver and lung among no PBMC controls, mice that received PBMCs plus vehicle, and mice that received PBMCs plus B-I09 (100X). **(B–D)** Bar graphs show skin graft rejection and xenogeneic GVHD scores (blinded assessment) at day +21. **(E)** Representative contour plots show the amount of detectable XBP-1s in CD3 negative cells residing in the murine spleen at day +21. Pooled data from two independent experiments, up to 7 mice per group, Mann–Whitney test.

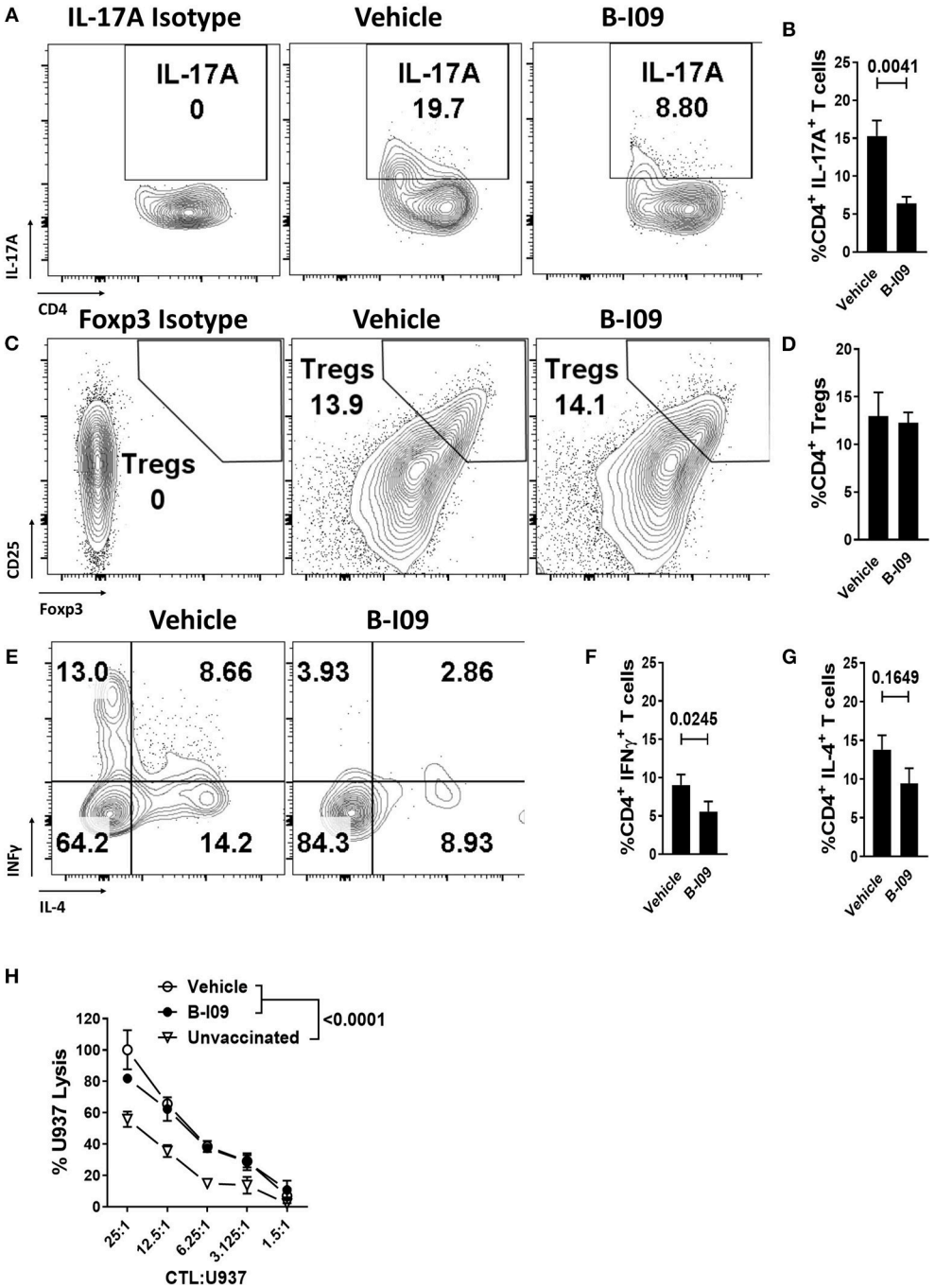


FIGURE 7 | XBP-1s inhibition reduces pathogenic Th17 and Th1 cells, yet preserves generation of anti-tumor CTL and Tregs *in vivo*. NSG mice were transplanted with human skin grafts and allogeneic PBMCs and treated with vehicle or B-I09 exactly as described. On day +21, the mouse spleens were harvested and human T cell phenotypes were determined by flow cytometry. The amount of human **(A,B)** Th17s ($CD4^+$, IL-$17A^+$), **(C,D)** Tregs ($CD4^+$, $CD127^-$, $CD25^+$, $Foxp3^+$), Th1s ($CD4^+$ IFNγ^+), and Th2s ($CD4^+$, IL-4^+) **(E–G)** isolated from the recipient spleen at day +21 are shown. Pooled data from two independent experiments, up to 7 mice per group, Mann-Whitney test. **(H)** Replicate mean specific lysis by human $CD8^+$ CTL generated *in vivo* using NSG mice transplanted with human PBMCs (30×10^6) and vaccinated with irradiated U937cells (10×10^6) on days 0 and +7. Mice received B-I09 or vehicle as described. For these experiments, recipients did not receive human skin. U937 lysis was measured by a colorimetric assay after 4 h using purified human $CD8^+$ T cells from recipient spleens at days +10–12. Replicate mean tumor lysis values shown are from 1 of 2 independent experiments, Tukey's test.

the human and murine TGFβ1 genes share 66% nucleotide homology and it is possible that cross-reactive murine TGFβ could also rescue the human iTregs *in vivo* (53).

Targeting XBP-1s with B-I09 or siRNA supports translation of our proposed strategy in GVHD prevention. While we show that anti-tumor responses by CTLs and NK cells remain intact *in vitro*, we acknowledge that these experiments do not fully replicate the biology of GVL *in vivo*. Despite this limitation, our study design demonstrates the relevance of DC XBP-1s in human GVHD and that targeting XBP-1s does not impair donor anti-tumor immunity. We surmise that the ability of the DC to express critical T cell costimulatory molecules, such as CD86, despite XBP-1s inhibition is important for the preserved GVL effect. The essential role for CD86 in CD8 CTL-mediated tumor clearance is well-demonstrated (54–56). Furthermore, the blunting of GVHD is likely driven by impaired DC production of IL-1β and reduced differentiation of pathogenic Th1 and Th17 cells by XBP-1s blockade.

Silencing XBP-1 in intratumoral suppressive DCs enhances T cell responses to cancer antigens, a result that is distinct from our observations in GVHD (57). It is reasonable that ER stress mediators support antigen-presentation by DCs during acute GVHD. In contrast, sustained, unremitting ER stress in tumor bed DCs, along with associated metabolic alterations (57), abrogates their immunostimulatory activity. For example, in ovarian cancer, ER stress leads to lipid peroxidation, impaired antigen presentation, and blunted stimulatory capacity toward responder T cells (57). Alternatively, context-dependent ER stress effects could depend on tumor or GVHD target-organ location, and/or be influenced by a different cytokine milieu altogether. Understanding the context-dependent effects of ER stress in cancer vs. inflammation is an area of active interest.

The ER stress response of DCs represents a novel biologic target to prevent GVHD in humans. In summary, targeting DC XBP-1s is an innovative approach to selectively impair alloreactive T cells and pathogenic Th1/Th17 differentiation, while maintaining donor immune mediated anti-leukemia responses, that deserves consideration for clinical trials of acute GVHD prophylaxis. Additionally, a prospective investigation of epidermal XBP-1s$^+$, CD1b$^+$ DCs and their potential involvement in the pathogenesis of acute GVHD warrants future study.

AUTHOR CONTRIBUTIONS

BB designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. KW, MM, JR, and AS performed experiments and edited the manuscript. FL, JP, JD, and BRB. discussed experimental design, analyzed and interpreted data, and edited the manuscript. ES scored skin graft rejection, analyzed, and interpreted tissue data, and edited the manuscript. ML and JVK harvested skin grafts and edited the manuscript. AL, JF, JK, CL, PR, and JC-G discussed experimental design and edited the manuscript. JRD, CK, C-HT, and C-CH discussed experimental design, performed experiments, synthesized B-I09, interpreted chemical analysis, and edited the manuscript. CA designed experiments, interpreted data, and edited the manuscript.

FUNDING

This work was supported by K08 HL11654701 (BB), R01 HL133823 (BB), R01 HL56067, and R01 HL11879 (BRB), R01 CA190860 (JRD and C-CH), R01 CA163910 (C-CH), and R21 CA199553 (JRD) from the U.S. National Institutes of Health. The Flow Cytometry, USF Comparative Medicine and Vivarium, Analytic Microscopy, and Tissue Cores at Moffitt/USF were also utilized in completing this work. The core facilities are supported partially by the Moffitt Cancer Center Support Grant, P30-CA076292.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02887/full#supplementary-material

REFERENCES

1. Young JW, Merad M, Hart DN. Dendritic cells in transplantation and immune-based therapies. *Biol Blood Marrow Transl.* (2007) 13:23–32. doi: 10.1016/j.bbmt.2006.10.023
2. Vaeth M, Bauerlein CA, Pusch T, Findeis J, Chopra M, Mottok A, et al. Selective NFAT targeting in T cells ameliorates GvHD while maintaining antitumor activity. *Proc Natl Acad Sci USA.* (2015) 112:1125–30. doi: 10.1073/pnas.1409290112
3. Singh K, Stempora L, Harvey RD, Kirk AD, Larsen CP, Blazar BR, et al. Superiority of rapamycin over tacrolimus in preserving nonhuman primate Treg half-life and phenotype after adoptive transfer. *Am J Transl.* (2014) 14:2691–703. doi: 10.1111/ajt.12934
4. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* (2006) 108:390–9. doi: 10.1182/blood-2006-01-0329
5. Antin JH, Ferrara JL. Cytokine dysregulation and acute graft-versus-host disease. *Blood* (1992) 80:2964–8.
6. Gardella S, Andrei C, Costigliolo S, Olcese L, Zocchi MR, Rubartelli A. Secretion of bioactive interleukin-1beta by dendritic cells is modulated by interaction with antigen specific T cells. *Blood* (2000) 95:3809–15.
7. Ikeda S, Saijo S, Murayama MA, Shimizu K, Akitsu A, Iwakura Y. Excess IL-1 signaling enhances the development of Th17 cells by downregulating TGF-beta-induced Foxp3 expression. *J Immunol.* (2014) 192:1449–58. doi: 10.4049/jimmunol.1300387
8. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* (2009) 30:576–87. doi: 10.1016/j.immuni.2009.02.007
9. Maly DJ, Papa FR. Druggable sensors of the unfolded protein response. *Nat Chem Biol.* (2014) 10:892–901. doi: 10.1038/nchembio.1664
10. Tavernier SJ, Osorio F, Vandersarren L, Vetters J, Vanlangenakker N, Van Isterdael G, et al. Regulated IRE1-dependent mRNA decay sets the threshold for dendritic cell survival. *Nat Cell Biol.* (2017) 19:698–710. doi: 10.1038/ncb3518
11. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* (2011) 334:1081–6. doi: 10.1126/science.1209038

12. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol.* (2007) 8:519–29. doi: 10.1038/nrm2199
13. Bronner DN, Abuaita BH, Chen X, Fitzgerald KA, Nunez G, He Y, et al. Endoplasmic reticulum stress activates the inflammasome via nlrp3- and caspase-2-driven mitochondrial damage. *Immunity* (2015) 43:451–62. doi: 10.1016/j.immuni.2015.08.008
14. Iwakoshi NN, Pypaert M, Glimcher LH. The transcription factor XBP-1 is essential for the development and survival of dendritic cells. *J Exp Med.* (2007) 204:2267–75. doi: 10.1084/jem.20070525
15. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber FC, Guarda G, et al. The Nlrp3 inflammasome regulates acute graft-versus-host disease. *J Exp Med.* (2013) 210:1899–910. doi: 10.1084/jem.20130084
16. Chen S, Smith BA, Iype J, Prestipino A, Pfeifer D, Grundmann S, et al. MicroRNA-155-deficient dendritic cells cause less severe GVHD through reduced migration and defective inflammasome activation. *Blood* (2015) 126:103–12. doi: 10.1182/blood-2014-12-617258
17. Koehn BH, Apostolova P, Haverkamp JM, Miller JS, McCullar V, Tolar J, et al. GVHD-associated, inflammasome-mediated loss of function in adoptively transferred myeloid-derived suppressor cells. *Blood* (2015) 126:1621–8. doi: 10.1182/blood-2015-03-634691
18. Wilhelm K, Ganesan J, Muller T, Durr C, Grimm M, Beilhack A, et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7. *Nat Med R.* (2010) 16:1434–8. doi: 10.1038/nm.2242
19. Klambt V, Wohlfeil SA, Schwab L, Hulsdunker J, Ayata K, Apostolova P, et al. A novel function for P2Y2 in myeloid recipient-derived cells during graft-versus-host disease. *J Immunol.* (2015) 195:5795–804. doi: 10.4049/jimmunol.1501357
20. Apostolova P, Zeiser R. The role of purine metabolites as DAMPs in acute graft-versus-host disease. *Front Immunol.* (2016) 7:439. doi: 10.3389/fimmu.2016.00439
21. Vallera DA, Taylor PA, Vannice JL, Panoskaltsis-Mortari A, Blazar BR. Interleukin-1 or tumor necrosis factor-alpha antagonists do not inhibit graft-versus-host disease induced across the major histocompatibility barrier in mice. *Transplantation* (1995) 60:1371–4.
22. Abhyankar S, Gilliland DG, Ferrara JL. Interleukin-1 is a critical effector molecule during cytokine dysregulation in graft versus host disease to minor histocompatibility antigens. *Transplantation* (1993) 56:1518–23. doi: 10.1097/00007890-199312000-00045
23. Antin JH, Weisdorf D, Neuberg D, Nicklow R, Clouthier S, Lee SJ, et al. Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* (2002) 100:3479–82. doi: 10.1182/blood-2002-03-0985
24. Brucklacher-Waldert V, Ferreira C, Stebegg M, Fesneau O, Innocentin S, Marie JC, et al. Cellular Stress in the context of an inflammatory environment supports TGF-beta-Independent T Helper-17 Differentiation. *Cell Rep.* (2017) 19:2357–70. doi: 10.1016/j.celrep.2017.05.052
25. Schutt SD, Wu Y, Tang CH, Bastian D, Nguyen H, Sofi MH, et al. Inhibition of the IRE-1alpha/XBP-1 pathway prevents chronic GVHD and preserves the GVL effect in mice. *Blood Adv.* (2018) 2:414–427. doi: 10.1182/bloodadvances.2017009068
26. Betts BC, Veerapathran A, Pidala J, Yang H, Horna P, Walton K, Cubitt CL, et al. Targeting aurora kinase A and JAK2 prevents GVHD while maintaining Treg and antitumor CTL function. *Sci Transl Med.* (2017) 9:eaai8269. doi: 10.1126/scitranslmed.aai8269
27. Betts BC, St Angelo ET, Kennedy M, Young JW. Anti-IL6-receptor-alpha (tocilizumab) does not inhibit human monocyte-derived dendritic cell maturation or alloreactive T-cell responses. *Blood* (2011) 118:5340–3. doi: 10.1182/blood-2011-06-363390
28. Tang CH, Ranatunga S, Kriss CL, Cubitt CL, Tao J, Pinilla-Ibarz JA, et al. Inhibition of ER stress-associated IRE-1/XBP-1 pathway reduces leukemic cell survival. *J Clin Invest.* (2014) 124:2585–98. doi: 10.1172/JCI73448
29. Scandella E, Men Y, Legler DF, Gillessen S, Prikler L, Ludewig B, et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Blood* (2004) 103:1595–601. doi: 10.1182/blood-2003-05-1643
30. Betts BC, Abdel-Wahab O, Curran SA, St Angelo ET, Koppikar P, Heller G, Levine et al. Janus kinase-2 inhibition induces durable tolerance to alloantigen by human dendritic cell-stimulated T cells yet preserves immunity to recall antigen. *Blood* (2011) 118:5330–9. doi: 10.1182/blood-2011-06-363408
31. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med.* (2006) 203:1701–11. doi: 10.1084/jem.20060772
32. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med.* (2006) 203:1693–700. doi: 10.1084/jem.20060468
33. Betts BC, Bastian D, Iamsawat S, Nguyen H, Heinrichs JL, Wu Y, et al. Targeting JAK2 reduces GVHD and xenograft rejection through regulation of T cell differentiation. *Proc Natl Acad Sci USA.* (2018) 115:1582–7. doi: 10.1073/pnas.1712452115
34. Locke FL, Menges M, Veerapathran A, Coppola D, Gabrilovich D, Anasetti C. Survivin-specific CD4+ T cells are decreased in patients with survivin-positive myeloma. *J Immunother Cancer* (2015) 3:20. doi: 10.1186/s40425-015-0065-1
35. Curran SA, Shyer JA, St Angelo ET, Talbot LR, Sharma S, Chung DJ, et al. Human dendritic cells mitigate NK-Cell dysfunction mediated by nonselective JAK1/2 blockade. *Cancer Immunol Res.* (2017) 5:52–60. doi: 10.1158/2326-6066.CIR-16-0233
36. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. *Ex vivo*-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. *Transplantation* (2010) 90:1321–7. doi: 10.1097/TP.0b013e3181ff8772
37. Zaitsu M, Issa F, Hester J, Vanhove B, Wood KJ. Selective blockade of CD28 on human T cells facilitates regulation of alloimmune responses. *JCI Insight* (2017) 2:89381. doi: 10.1172/jci.insight.89381
38. Bejarano PA, Levi D, Nassiri M, Vincek V, Garcia M, Weppler D, et al. The Pathology of full-thickness cadaver skin transplant for large abdominal defects: a proposed grading system for skin allograft acute rejection. *Am J Surg Pathol.* (2004) 28:670–5. doi: 10.1097/00000478-200405000-00016
39. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, et al. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* (2013) 39:311–23. doi: 10.1016/j.immuni.2013.08.001
40. Martinon F, Chen X, Lee AH, Glimcher LH. TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nat Immunol.* (2010) 11:411–8. doi: 10.1038/ni.1857
41. Bright MD, Itzhak DN, Wardell CP, Morgan GJ, Davies FE. Cleavage of BLOC1S1 mRNA by IRE1 is sequence specific, temporally separate from xbp1 splicing, and dispensable for cell viability under acute endoplasmic reticulum stress. *Mol Cell Biol.* (2015) 35:2186–202. doi: 10.1128/MCB.00013-15
42. Satpute-Krishnan P, Ajinkya M, Bhat S, Itakura E, Hegde RS, Lippincott-Schwartz J. ER stress-induced clearance of misfolded GPI-anchored proteins via the secretory pathway. *Cell* (2014) 158:522–33. doi: 10.1016/j.cell.2014.06.026
43. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* (2006) 7:880–5. doi: 10.1038/sj.embor.7400779
44. Wang Y, Lavender P, Watson J, Arno M, Lehner T. Stress-activated dendritic cells (DC) Induce Dual Interleukin (IL)-15- and IL1beta-mediated pathways, which may elicit CD4+ memory t cells and interferon (IFN)-stimulated Genes. *J Biol Chem.* (2015) 290:15595–609. doi: 10.1074/jbc.M115.645754
45. Anderson AE, Swan DJ, Sayers BL, Harry RA, Patterson AM, von Delwig A, et al. LPS activation is required for migratory activity and antigen presentation by tolerogenic dendritic cells. *J Leukoc Biol.* (2009) 85:243–50. doi: 10.1189/jlb.0608374
46. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. *Blood* (2011) 118:5011–20. doi: 10.1182/blood-2011-03-340315
47. Basu R, Whitley SK, Bhaumik S, Zindl CL, Schoeb TR, Benveniste EN, et al. IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the TH17 cell-iTreg cell balance. *Nat Immunol.* (2015) 16:286–95. doi: 10.1038/ni.3099

48. Chung DJ, Rossi M, Romano E, Ghith J, Yuan J, Munn DH, et al. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. *Blood.* (2009) 114:555–63. doi: 10.1182/blood-2008-11-191197
49. Osorio F, Tavernier SJ, Hoffmann E, Saeys Y, Martens L, Vetters J, et al. The unfolded-protein-response sensor IRE-1alpha regulates the function of CD8alpha+ dendritic cells. *Nat Immunol.* (2014) 15:248–57. doi: 10.1038/ni.2808
50. Wilson J, Cullup H, Lourie R, Sheng Y, Palkova A, Radford KJ, Dickinson AM, et al. Antibody to the dendritic cell surface activation antigen CD83 prevents acute graft-versus-host disease. *J Exp Med.* (2009) 206:387–98. doi: 10.1084/jem.20070723
51. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol.* (2013) 13:397–411. doi: 10.1038/nri3452
52. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity* (2015) 42:607–12. doi: 10.1016/j.immuni.2015.04.005
53. Geiser AG, Kim SJ, Roberts AB, Sporn MB. Characterization of the mouse transforming growth factor-beta 1 promoter and activation by the Ha-ras oncogene. *Mol Cell Biol.* (1991) 11:84–92. doi: 10.1128/MCB.11.1.84
54. Mokyr MB, Kalinichenko TV, Gorelik L, Bluestone JA. Importance of the B7–2 molecule for low dose melphalan-induced acquisition of tumor-eradicating immunity by mice bearing a large MOPC-315 tumor. *J Immunol.* (1998) 160:1866–74.
55. Mokyr MB, Kalinichenko TV, Gorelik L. Potentiation of antitumor CTL response by GM-CSF involves a B7-dependent mechanism. *Cell Immunol.* (1997) 178:152–61. doi: 10.1006/cimm.1997.1130
56. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med.* (1998) 188:1075–82. doi: 10.1084/jem.188.6.1075
57. Cubillos-Ruiz JR, Silberman PC, Rutkowski MR, Chopra S, Perales-Puchalt A, Song M, Zhang S, et al. ER Stress Sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell* (2015) 161:1527–38. doi: 10.1016/j.cell.2015.05.025

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 06 December 2018
doi: 10.3389/fimmu.2018.02853

The Role of Micronutrients in Graft-VS.-Host Disease: Immunomodulatory Effects of Vitamins A and D

Xiao Chen[1]* *and Christopher G. Mayne*[2]

[1] Division of Hematology & Oncology, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, United States, [2] Biology Department, Viterbo University, La Crosse, WI, United States

Graft-vs.-host disease (GVHD) remains a major obstacle to the success of allogeneic hematopoietic stem cell transplantation (HSCT). GVHD occurs because donor T cells in the allograft recognize the genetically disparate host as foreign and attack the transplant recipient's tissues. While genetic incompatibility between donor and recipient is the primary determinant for the extent of alloimmune response, GVHD incidence and severity are also influenced by non-genetic factors. Recent advances in immunology establish that environmental factors, including dietary micronutrients, contribute significantly to modulating various immune responses and may influence the susceptibility to autoimmune and inflammatory diseases of experimental animals and humans. Emerging clinical and preclinical evidence indicates that certain micronutrients may participate in regulating GVHD risk after allogeneic HSCT. In this review, we summarize recent advances in our understanding with respect to the potential role of micronutrients in the pathogenesis of acute and chronic GVHD, focusing on vitamins A and D.

Keywords: vitamin A, vitamin D, retinoic acid, vitamin D receptor, graft-vs.-host disease, allogeneic hematopoietic stem cell transplantation

OPEN ACCESS

Edited by:
Xue-Zhong Yu,
Medical University of South Carolina, United States

Reviewed by:
Michael Uhlin,
Karolinska Institutet (KI), Sweden
Luiza Guilherme,
University of São Paulo, Brazil

***Correspondence:**
Xiao Chen
xchen@mcw.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *01 October 2018*
Accepted: *20 November 2018*
Published: *06 December 2018*

Citation:
Chen X and Mayne CG (2018) The Role of Micronutrients in Graft-VS.-Host Disease: Immunomodulatory Effects of Vitamins A and D. Front. Immunol. 9:2853. doi: 10.3389/fimmu.2018.02853

MICRONUTRIENTS AND IMMUNITY

Micronutrients are compounds that are only needed in small amounts, yet are essential for the proper growth and development of the human body. These vitamins and minerals are indispensible for the production and function of various enzymes and hormones that are critical for maintaining optimal physical and mental function. An aberrant micronutrient status contributes to the increased susceptibility to various infectious, inflammatory, and metabolic conditions such as colitis, diabetes, cancer, obesity, and cardiovascular disease.

The importance of the micronutrients vitamins A and D in health has been recognized since the early twentieth century. More recent advances have led to the discovery of the critical role of these molecules in the immune system (1). Current highlights within this field include the finding that maternal vitamin A levels significantly influence the proper development of secondary lymphoid organs in offspring and determine the fitness of their immune system in later life (2). Lack of vitamin A-mediated signaling in utero substantially reduced the anti-pathogen immune response of newborn mice (2). Similarly, vitamin D also plays a role at the maternal-fetal interface, preventing inflammatory responses such as pre-eclampsia (3). These immunomodulatory effects may be long

lasting as maternal vitamin D deficiency has been shown to contribute to a greater likelihood of atopic responses in the neonatal lung (4, 5). These findings reveal how nutritional status during fetal life can profoundly affect immune responses in adulthood, highlighting the importance of vitamins A and D in the development and maintenance of a competent, yet tightly regulated immune system.

GRAFT-VS.-HOST DISEASE (GVHD) AND NUTRITIONAL FACTORS

GVHD remains a major obstacle limiting the broader application of allogeneic hematopoietic stem cell transplantation (HSCT), an effective treatment for a number of malignant and non-malignant hematological disorders (6–8). GVHD is the consequence of a normal, yet exaggerated, immune reaction elicited by donor T cells when they encounter alloantigens expressed by the transplant recipient. Acute GVHD (aGVHD) pathophysiology is characterized by strong inflammatory components while chronic GVHD (cGVHD) displays more autoimmune manifestations (9–13). The pathogenesis of GVHD is a complex process involving a variety of host and donor immune cells (**Figures 1**, **2**). The major determinant for the development and the severity of GVHD is the genetic disparity between the donor and recipient. However, some non-genetic factors such as the level of exposure to damage associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) are also important components of GVHD pathophysiology, due to their ability to amplify inflammatory responses (14). In addition, other host factors may also influence the function of various immune cells and modulate the alloimmune response.

Nutritional status is a significant variable among patients undergoing allogeneic HSCT. In fact, nutritional support appears to affect the development of GVHD, with adequate enteral nutrition being associated with reduced GVHD risk as compared to parenteral nutrition (15–18). These studies indicate that the interaction between certain oral nutrition and the gastrointestinal tract can modulate GVHD risk. Thus, patient nutritional status may be an independent and modifiable factor influencing GVHD severity (19). It is conceivable that an improved nutritional status may provide patients with an increased ability to tolerate treatment-associated toxicity and recover from GVHD-associated tissue damage. More importantly, certain micronutrients may also be actively involved in regulating the initiation, development, and resolution of inflammatory

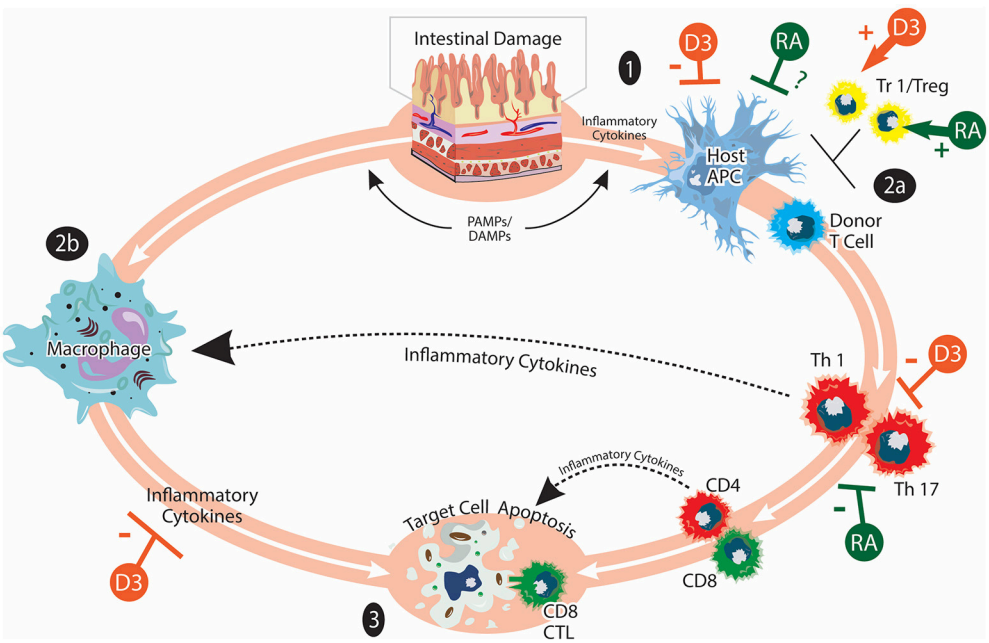


FIGURE 1 | Acute GVHD and vitamins A and D. aGVHD pathogenesis involves: (1) Activation of host APCs due to release of inflammatory cytokines and PAMPs/DAMPs from tissue damaged by HSCT conditioning. Intestinal damage by conditioning serves to amplify inflammatory responses. (2) Activation of donor T cells when they encounter host APC. Donor T cells undergo differentiation, expansion, and acquisition of tissue homing specificity during this stage (2a). Inflammatory cytokines produced by donor T cells and bacterial LPS can further activate innate immune cells such as macrophages (2b). (3) Inflammatory mediators from donor T cells and innate immune cells lead to target cell apoptosis. Cytotoxic CD8 T cells can mediate direct cell killing. Tr1/Tregs play immunomodulatory roles in aGVHD pathogenesis. The effects of vitamin A/RA (shown in green) on aGVHD are complex and not completely understood. Vitamin A/RA promotes donor T-cell intestinal homing. Inhibiting donor T-cell RAR signaling suppresses the induction of gut-homing molecules and favors Treg cell differentiation. It has also been reported that RA inhibits donor T cell expansion and cytokine production. The potential effects of vitamin A/RA on host APCs are currently under investigation. The effects of vitamin D (shown in orange) on aGVHD may include suppressing the activation of host APCs, inhibiting the activation and cytokine production of donor T cells as well as promoting the induction of Tr1/Treg. The figure is adapted from Ferrara et al. (9).

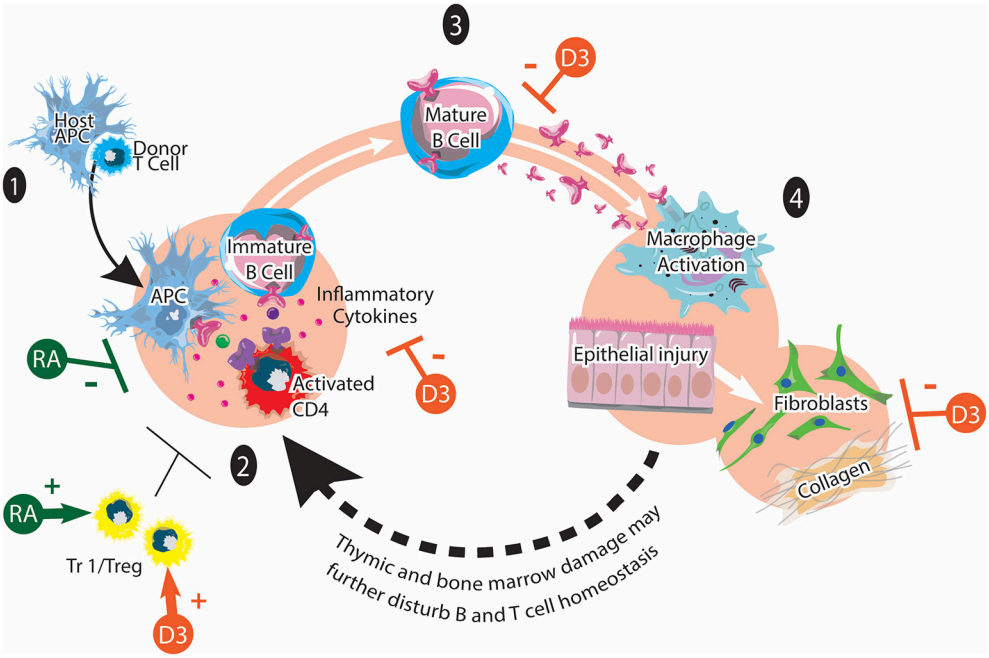


FIGURE 2 | Chronic GVHD and vitamins A and D. cGVHD pathogenesis involves: (1) Early inflammation and tissue injury. An existing inflammation and danger signals activate innate immune cells and recruits donor T cells to the tissue. (2) Dysregulated immunity with loss of tolerance. Activated CD4 T cells stimulate the maturation of auto-reactive B cells. (3) Mature B cells produce various autoantibodies against host antigens. (4) Aberrant tissue repair and fibrosis via macrophages and fibroblasts. Tr1/Treg play immunomodulatory roles in chronic GVHD pathogenesis. It has been reported that synthetic retinoid (shown in green) reduces cGVHD by inhibiting Th1 and Th17 cells. It may also facilitate the generation of Tr1/Tregs. The potential effects of vitamin D on cGVHD (shown in orange) may include positive effects on Tr1/Treg function and polarization as well as inhibitory effects on proinflammatory T cell polarization, inflammatory cytokines, autoantibody secretion and collagen production. This figure is adapted from Cooke et al. (12).

responses after HSCT. In this review, we briefly summarize the potential roles of vitamins A and D in GVHD pathogenesis.

EFFECTS OF VITAMIN A ON GVHD

Vitamin A is a multifunctional vitamin involved in a wide range of biological processes. Most biological effects of vitamin A are exerted by its major metabolite, retinoic acid (RA) (20). The conversion from vitamin A to RA requires two hydrolysis steps catalyzed first by alcohol dehydrogenases (ADHs), followed by aldehyde dehydrogenases (RALDHs). RALDHs are the rate-limiting enzymes for RA synthesis and are expressed in limited tissues (21). Heterodimers of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) mediate RA signaling. These heterodimers bind to retinoic acid responsive elements (RARE) of target genes and regulate gene transcription. One of the most important physiological functions of vitamin A and RA is to regulate immune responses, and dysregulated retinoid signaling can lead to a weakened immunity against pathogens and/or the loss of immune homeostasis (20, 22).

RA has pleiotropic effects on cells of the innate and adaptive immune system (23–25). It can target T cells, B cells, antigen presenting cells (APCs), and innate lymphoid cells (ILCs) to regulate immune responses. RA induces the expression of gut-homing molecules CCR9 and α4β7 on various immune cells, augmenting cell migration to the intestines (26–28). At steady state, RA promotes the induction of tolerogenic dendritic cells (DCs). However, in the presence of inflammatory cytokines such as IL-15, RA promotes the induction of inflammatory DCs and intensifies pathogenic mucosal immune responses (29). RA has also been shown to influence the development of DC subsets in the spleen and intestines (30–33). RA also plays a central role in modulating intestinal $CD4^+$ T cell responses and enhances the stability of natural regulatory T cells (Tregs) (34). Together with TGF-β, RA promotes the conversion of naïve T cells into induced-Tregs at the expense of Th17 cells (35, 36). Vitamin A deficiency is associated with impaired oral tolerance, suggesting an important role of RA in maintaining intestinal homeostasis (37, 38). On the other hand, the RA-RAR-α axis is important for $CD4^+$ T cell activation and effector function under inflammatory conditions (39, 40). Finally, RA promotes the induction of ILC3 but suppresses the generation and cytokine production of ILC2 (41). These observations demonstrate the complex and sometimes paradoxical functions of RA within the immune system, as it can possess either pro-inflammatory or anti-inflammatory properties depending on the context.

In the context of GVHD, Koenecke and colleagues used a dietary approach to first examine how recipient vitamin A levels affect donor T cell trafficking after experimental HSCT

(42). Vitamin A-deficient (VAD) recipient mice had a reduced percentage and absolute number of donor T cells in the intestine, which was attributable to diminished expression of gut-homing molecules α4β7 and CCR9. VAD recipients survived longer than control vitamin A-normal (VAN) mice due to gastrointestinal protection, though they developed more severe hepatic GVHD. These results indicated that vitamin A affects GVHD target organ tropism of donor T cells, with a particularly important role in controlling the migration of donor T cells to the intestine, a critical GVHD target organ (43). We and others then used a genetic approach to examine the role of RA signaling in GVHD pathogenesis. These studies consistently showed that genetic ablation of RAR-α on donor T cells significantly decreased the ability of these cells to cause lethal GVHD (44, 45). This was largely due to reduced expression of gut-homing molecules CCR9 and α4β7 on donor T cells with diminished intestinal migration. In contrast, administrating RA exogenously to recipient mice increased expression of gut-homing molecules on donor T cells and increased their gut-tropism, leading to a significantly increased overall mortality (44–46). In addition, inhibiting RAR-α reduced donor T cell differentiation toward a Th1 phenotype and favored the induction of Tregs (45), which also contribute to the decreased ability of these cells to cause GVHD. Importantly, genetic inhibition of RAR-α signaling on donor T cells does not compromise their ability to mediate the graft-vs.-leukemia effect.

In an effort to improve the translational potential of this research, we treated donor mice with BMS493, a pan-RAR antagonist. Recipients of BMS493-treated donor T cells showed improved overall survival after HSCT compared to recipients of vehicle-treated donor T cells, indicating that pharmacological inhibition of the retinoic acid pathway on donor T cells can reduce their alloreactivity and ability to cause GVHD (47). Interestingly, chronic vitamin A deficiency changed the composition of the donor T cell compartment with a reduction in the percentage of $CD4^+$ T cells, resulting in reduced ability of transferred T cells from VAD mice to cause lethal GVHD (47). Thus, both host and donor vitamin A levels appear to affect the development of experimental GVHD (42, 47). While most preclinical studies suggest a detrimental effect of RA on GVHD, it has also been reported that RA treatment reduces aGVHD (48) and a synthetic retinoid ameliorates cGVHD (49). Differences in mouse GVHD models used, RA levels *in situ*, and local cytokine milieu could all potentially contribute to these differing observations.

Apart from above preclinical studies, emerging clinical data also demonstrate the involvement of vitamin A/RA in GVHD pathogenesis. A recent study found that lower levels of vitamin A are associated with increased intestinal GVHD in children receiving allogeneic HSCT (50). The incidence of grades 2–4 GVHD was also significantly higher in patients with lower vitamin A levels. These observations appear in contrast to a murine study in which vitamin A deficiency is associated with a reduced intestinal GVHD and improved overall survival (42). This discrepancy could be due to inherent differences between mouse model and human disease or increased severity of experimentally induced vitamin A deficiency compared to clinical deficiency/insufficiency. In addition, the study by Lounder et al. actually used serum vitamin A levels above or below a median value, instead of vitamin A deficiency or sufficiency, to separate patient groups. Finally, there was also evidence that low serum vitamin A levels are associated with more severe ocular GVHD in allogeneic HSCT patients (51). Thus, both preclinical and clinical data indicate a significant involvement of vitamin A and RA pathway in GVHD pathogenesis.

EFFECTS OF VITAMIN D ON GVHD

Vitamins D and A are similar in that they are the only two vitamins whose active metabolites have hormone-like properties. Indeed, the active metabolite of vitamin D, calcitriol, is a well-established secosteroid hormone with multiple roles throughout the human body (52). Though vitamin D may be acquired nutritionally, a large proportion of vitamin D is synthesized in the human body. This synthesis is initiated in the skin as UV-B rays cause the photolysis of 7-dehydrocholesterol, forming vitamin D_3. In the liver, vitamin D_3 is hydroxylated to 25-hydroxyvitamin D_3 ($25(OH)D_3$) by enzymes such as CYP2R1 and CYP27A1 (53, 54). 25-hydroxyvitamin D_3 is the principal circulating metabolite of vitamin D and $25(OH)D_3$ concentration is typically used as an indicator of vitamin D status. This inactive $25(OH)D_3$ is hydroxylated once more in the kidney via the enzyme CYP27B1 to become the biologically active hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), also known as calcitriol.

Vitamin D utilizes similar signaling mechanisms to vitamin A. Calcitriol binds to the vitamin D receptor (VDR), which heterodimerizes with RXR (55, 56). VDR-RXR heterodimers that are bound to calcitriol act as transcriptional regulators by binding vitamin D response elements (VDREs) of target genes (57). The classical physiological roles of vitamin D (via calcitriol) are in calcium and phosphate homeostasis and bone metabolism, with other roles being referred to as "non-classical" functions. The discovery of vitamin D binding within immune cells in the early 1980s and eventual description of VDR expression in immune cells were key steps in the study of the non-classical effects of vitamin D on the immune system (58–60).

It has been shown in numerous studies that calcitriol inhibits maturation and inflammatory cytokine production of DCs (61–64). These changes in DC differentiation and function also result in a skew toward a more tolerogenic DC profile with the ability to drive Treg, T-regulatory cell type 1 (Tr1), and Th2 cell development (65). Importantly, calcitriol also appears to exhibit direct effects on CD4 T cell populations to modify immune function. *In vitro* treatment of T cells with calcitriol inhibits proliferation under several activating conditions (66, 67). Calcitriol has been shown to be effective as a treatment in numerous mouse models of diseases that are driven by Th1 and Th17 cells, suggesting a global immunomodulatory effect on these cell types (68–71). Calcitriol also appears to inhibit proliferation and pathogenicity of CD8 T cells since VDR-deficient CD8 T cells are hyperproliferative

and proinflammatory (72, 73). Calcitriol inhibits production of IFN-γ and stimulates IL-4 secretion in invariant natural killer (iNKT) cells (70). Finally, calcitriol leads to decreased B cell proliferation and differentiation to plasma cells. However, it is unclear if these effects are due to direct effects on the B cell, or due to reduced interactions with CD4 T cells (74).

There is a significant history of studies on the effects of vitamin D and its analogs on allograft survival in several tissues (75, 76). However, preclinical studies in animal models of allogeneic HSCT are rather limited. To our knowledge, only one study of VDR agonism has been reported in animal models of GVHD. In this study, a vitamin D analog reduced aGVHD severity and immune cell infiltration in liver, skin, and spleen of rats (77). *In vitro* studies utilizing human monocyte-derived DCs recapitulated previously results showing that vitamin D led to the development of more immature tolerogenic DCs. Vitamin D-treated DCs activated allogeneic CD4 and CD8 T cells with a greater IL-10 to IFN-γ ratio, and these T cells were less proliferative in mixed lymphocyte reactions (MLRs) (78). In another study, alloreactive T cells were shown to express greater levels of VDR (79). Addition of calcitriol to the MLR led to a decrease in the percentage of proliferating T cells. This appeared to be due to direct action of calcitriol on the alloreactive T cells since the allogeneic DC were matured in the absence of calcitriol and irradiated prior to be used in the MLR (79).

The first reports for a potential role of vitamin D in human GVHD came through candidate gene studies analyzing known *VDR* polymorphisms. Interestingly, the results of these studies are quite variable. Some studies suggest roles for various polymorphisms in GVHD when present in the recipient only (80–82), some studies suggest a role for VDR genotype in both the donor and recipient (83), while another more recent study found no significant association between GVHD and VDR polymorphisms in the donor nor recipient (84). Taken together, these results suggest that in some instances the *a* allele of *VDR* may play a role in aGVHD risk when present in recipients of HSCT. However, not all studies have found such an association and the results may vary between different populations (84). One complicating factor of these studies was that the vitamin D status of the individuals studied was often unknown. Thus, any differences in VDR activity associated with disease could be obfuscated depending on whether an individual's vitamin D stores were sufficient to provide for VDR function.

Though genetic studies of *VDR* suggest a potential role for vitamin D signaling in GVHD, patient serum levels of vitamin D may provide a more direct method of investigation. Indeed, it appears that individuals undergoing HSCT are at particular risk for vitamin D deficiency/insufficiency (85–89). Several retrospective studies have thus investigated whether vitamin D status prior to HSCT corresponds with subsequent development of GVHD (90–92). These studies seem to suggest a relatively consistent association of cGVHD with lower vitamin D status, whereas the results for aGVHD are more variable. In a more recent study, however, levels of vitamin D pre-HSCT did not correlate to development of aGVHD nor cGVHD in a group of pediatric patients (93). Interestingly, the one-year survival rate did differ significantly, with 35% mortality in the deficient group vs. 0% in the insufficient and 7% in the sufficient groups, suggesting a beneficial effect of higher vitamin D levels on overall survival after allogeneic HSCT (93).

Given the potential association of vitamin D status and GVHD, the effect of supplementation was further investigated. Even though HSCT patients may be particularly at risk for vitamin D insufficiency/deficiency, supplementation can increase their vitamin D status (86, 90). Rosenblatt et al. reported observation of two patients with steroid refractory cGVHD who were treated with supplemental vitamin D for bone mineral abnormalities. Impressively, both had their symptoms wane to the point that they were removed from immunosuppression after vitamin D treatment (78). A marked improvement in cGVHD was also observed in a subsequent analysis of 12 adult HSCT patients who were given 1,000 IU/day vitamin D to treat osteopenia or osteoporosis (94).

To date, we are aware of only one published prospective study of vitamin D supplementation in HSCT patients (95). This investigation demonstrates that vitamin D may play a role in the prevention of cGVHD, as suggested previously (78, 90–92, 94). Intriguingly, there was no difference in aGVHD among the patient groups in this study. It is worth noting that in this study vitamin D supplementation began only 3 days prior to transplantation. Indeed, the authors show that significantly higher levels of $25(OH)D_3$ were not observed in the serum until day 7 in high-dose and day 21 in low-dose patients (95). This signifies that aGVHD may have been initiated in the absence of sufficiently elevated levels of vitamin D. Further investigation into the effect of earlier supplementation to raise serum vitamin D levels prior to HSCT to prevent aGVHD is of interest. Overall, the data surrounding vitamin D and the immune system as well as the initial studies on vitamin D and GVHD suggest that it is highly likely that vitamin D could exhibit positive effects in the prevention and/or treatment of GVHD (96).

Conclusions and Perspectives

In conclusion, we believe that small molecules like vitamins A and D could have the potential to influence the development of GVHD after allogeneic HSCT. These micronutrients may modulate crosstalk between the various immune cells involved in the pathogenesis of GVHD, thus influencing disease initiation, progression, and resolution (**Figures 1**, **2**). Their levels may also have prognostic value, serving as independent risk factors for predicting the severity of organ-specific or systemic GVHD. Most importantly, nutritional intervention before and after allogeneic HSCT may be used as an adjuvant therapy to reduce GVHD risk and improve the outcome of allogeneic HSCT (95, 97, 98). We propose that GVHD research using animal models should consider dietary composition. More preclinical studies in this understudied research area will provide new insights into how nutritional factors contribute to GVHD pathogenesis. Finally, more prospective randomized controlled clinical trials

are needed to fully reveal the potential of using micronutrients such as vitamins A and D as simple and inexpensive approaches with minimal side effects to mitigate clinical GVHD.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by a grant from the National Institutes of Health, National Institute of Allergy and Infectious Diseases RO1 AI125334 (XC).

ACKNOWLEDGMENTS

The authors thank Derek Fuchsberger for artistic design on the figures.

REFERENCES

1. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol.* (2008) 8:685–98. doi: 10.1038/nri2378
2. van de Pavert SA, Ferreira M, Domingues RG, Ribeiro H, Molenaar R, Moreira-Santos L, et al. Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. *Nature* (2014) 508:123–7. doi: 10.1038/nature13158
3. Tamblyn JA, Hewison M, Wagner CL, Bulmer JN, Kilby MD. Immunological role of vitamin D at the maternal-fetal interface. *J Endocrinol.* (2015) 224:R107–21. doi: 10.1530/JOE-14-0642
4. Clark A, Mach N. Role of vitamin D in the hygiene hypothesis: the interplay between vitamin D, vitamin D receptors, gut microbiota, and immune response. *Front. Immunol.* (2016) 7:627. doi: 10.3389/fimmu.2016.00627
5. Macpherson AJ, de Aguero MG, Ganal-Vonarburg SC. How nutrition and the maternal microbiota shape the neonatal immune system. *Nat Rev Immunol.* (2017) 17:508–17. doi: 10.1038/nri.2017.58
6. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol.* (2007) 7:340–52. doi: 10.1038/nri2000
7. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol.* (2007) 25:139–70. doi: 10.1146/annurev.immunol.25.022106.141606
8. Choi SW, Reddy P. Current and emerging strategies for the prevention of graft-versus-host disease. *Nat Rev Clin Oncol.* (2014) 11:536–47. doi: 10.1038/nrclinonc.2014.102
9. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
10. Zeiser R, Blazar BR. Acute graft versus host disease – biologic process, prevention, and therapy. *N Engl J Med.* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
11. Zeiser R, Blazar BR. Pathophysiology of chronic graft versus host disease and therapeutic targets. *N Engl J Med.* (2017) 377:2565–79. doi: 10.1056/NEJMra1703472
12. Cooke KR, Luznik L, Sarantopoulos S, Hakim FT, Jagasia M, Fowler DH, et al. The biology of chronic graft-versus-host disease: a task force report from the national institutes of health consensus development project on criteria for clinical trials in chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* (2017) 23:211–34. doi: 10.1016/j.bbmt.2016.09.023
13. MacDonald KP, Hill GR, Blazar BR. Chronic graft-versus-host disease: biological insights from preclinical and clinical studies. *Blood* (2017) 129:13–21. doi: 10.1182/blood-2016-06-686618
14. Toubai T, Mathewson ND, Magenau J, Reddy P. danger signals and graft-versus-host disease: current understanding and future perspectives. *Front. Immunol.* (2016) 7:539. doi: 10.3389/fimmu.2016.00539
15. Mattsson J, Westin S, Edlund S, Remberger M. Poor oral nutrition after allogeneic stem cell transplantation correlates significantly with severe graft-versus-host disease. *Bone Marrow Transplant.* (2006) 38:629–33. doi: 10.1038/sj.bmt.1705493
16. Svahn BM, Remberger M, Heijbel M, Martell E, Wikstrom M, Eriksson B, et al. Case-control comparison of at-home and hospital care for allogeneic hematopoietic stem-cell transplantation: the role of oral nutrition. *Transplantation* (2008) 85:1000–7. doi: 10.1097/TP.0b013e31816a3267
17. Seguy D, Duhamel A, Rejeb MB, Gomez E, Buhl ND, Bruno B, et al. Better outcome of patients undergoing enteral tube feeding after myeloablative conditioning for allogeneic stem cell transplantation. *Transplantation* (2012) 94:287–94. doi: 10.1097/TP.0b013e3182558f60
18. Beckerson J, Szydlo RM, Hickson M, Mactier CE, Innes AJ, Gabriel IH. Impact of route and adequacy of nutritional intake on outcomes of allogeneic haematopoietic cell transplantation for haematologic malignancies. *Clin Nutr.* (2018). doi: 10.1016/j.clnu.2018.03.008. [Epub ahead of print].
19. Kerby EH, Li Y, Getz KD, Smith EC, Smith LT, Bunin NJ, et al. Nutritional risk factors predict severe acute graft-versus-host disease and early mortality in pediatric allogeneic hematopoietic stem cell transplantation. *Pediatr Blood Cancer* (2018) 65:e26853. doi: 10.1002/pbc.26853
20. Stephensen CB. Vitamin A, infection, and immune function. *Annu. Rev. Nutr.* (2001) 21:167–92. doi: 10.1146/annurev.nutr.21.1.167
21. Kumar S, Sandell LL, Trainor PA, Koentgen F, Duester G. Alcohol and aldehyde dehydrogenases: retinoid metabolic effects in mouse knockout models. *Biochim. Biophys. Acta* (2012) 1821:198–205. doi: 10.1016/j.bbalip.2011.04.004
22. Larange A, Cheroutre H. Retinoic acid and retinoic acid receptors as pleiotropic modulators of the immune system. *Annu Rev Immunol.* (2016) 34:369–94. doi: 10.1146/annurev-immunol-041015-055427
23. Hall JA, Grainger JR, Spencer SP, Belkaid Y. The role of retinoic acid in tolerance and immunity. *Immunity* (2011) 35:13–22. doi: 10.1016/j.immuni.2011.07.002
24. Cassani B, Villablanca EJ, De Calisto J, Wang S, Mora JR. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. *Mol Aspects Med.* (2012) 33:63–76. doi: 10.1016/j.mam.2011.11.001
25. Raverdeau M, Mills KH. Modulation of T cell and innate immune responses by retinoic acid. *J. Immunol.* (2014) 192:2953–8. doi: 10.4049/jimmunol.1303245
26. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* (2004) 21:527–38. doi: 10.1016/j.immuni.2004.08.011
27. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B., et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* (2006) 314:1157–60. doi: 10.1126/science.1132742
28. Kim MH, Taparowsky EJ, Kim CH. Retinoic acid differentially regulates the migration of innate lymphoid cell subsets to the gut. *Immunity* (2015) 43:107. doi: 10.1016/j.immuni.2015.06.009
29. DePaolo RW, Abadie V, Tang F, Fehlner-Peach H, Hall JA, Wang W, et al. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature* (2011) 471:220–4. doi: 10.1038/nature09849
30. Beijer MR, Molenaar R, Goverse G, Mebius RE, Kraal G, den Haan JM. A crucial role for retinoic acid in the development of Notch-dependent murine splenic CD8- CD4- and CD4+ dendritic cells. *Eur J Immunol.* (2013) 43:1608–16. doi: 10.1002/eji.201343325
31. Klebanoff CA, Spencer SP, Torabi-Parizi P, Grainger JR, Roychoudhuri R, Ji Y, et al. Retinoic acid controls the homeostasis of pre-cDC-derived splenic and intestinal dendritic cells. *J. Exp. Med.* (2013) 210:1961–76. doi: 10.1084/jem.20122508

32. Saurer L, McCullough KC, Summerfield A. *In vitro* induction of mucosa-type dendritic cells by all-trans retinoic acid. *J Immunol.* (2007) 179:3504–14. doi: 10.4049/jimmunol.179.6.3504
33. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, et al. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. *Mucosal Immunol.* (2013) 6:847–56. doi: 10.1038/mi.2012.123
34. Zhou X, Kong N, Wang J, Fan H, Zou H, Horwitz D, et al. Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. *J Immunol.* (2010) 185:2675–79. doi: 10.4049/jimmunol.1000598
35. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* (2007) 317:256–60. doi: 10.1126/science.1145697
36. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced Treg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med*. (2007) 204:1765–74. doi: 10.1084/jem.20070719
37. Nakamoto A, Shuto E, Tsutsumi R, Nakamoto M, Nii Y, Sakai T. Vitamin A deficiency impairs induction of oral tolerance in mice. *J Nutr Sci Vitaminol.* (2015) 61:147–53. doi: 10.3177/jnsv.61.147
38. Turfkruyer M, Rekima A, Macchiaverni P, Le Bourhis L, Muncan V, van den Brink GR, et al. Oral tolerance is inefficient in neonatal mice due to a physiological vitamin A deficiency. *Mucosal Immunol.* (2016) 9:479–91. doi: 10.1038/mi.2015.114
39. Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, et al. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity* (2011) 34:435–47. doi: 10.1016/j.immuni.2011.03.003
40. Pino-Lagos K, Guo Y, Brown C, Alexander MP, Elgueta R, Bennett KA, et al. A retinoic acid-dependent checkpoint in the development of CD4+ T cell-mediated immunity. *J. Exp. Med.* (2011) 208:1767–75. doi: 10.1084/jem.20102358
41. Spencer SP, Wilhelm C, Yang Q, Hall JA, Bouladoux N, Boyd A, et al. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science* (2014) 343:432–7. doi: 10.1126/science.1247606
42. Koenecke C, Prinz I, Bubke A, Schreder A, Lee CW, Pabst O, et al. Shift of graft versus host disease organ tropism by dietary vitamin A. *PLoS ONE* (2012) 7:e38252. doi: 10.1371/journal.pone.0038252
43. Hill GR, Ferrara JLM. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood* (2000) 95:2754–9.
44. Chen X, Dodge J, Komorowski R, Drobyski WR. A critical role for the retinoic acid signaling pathway in the pathophysiology of gastrointestinal graft-versus-host disease. *Blood* (2013) 121:3970–80. doi: 10.1182/blood-2012-08-445130
45. Aoyama K, Saha A, Tolar J, Riddle MJ, Veenstra RG, Taylor PA, et al. Inhibiting retinoic acid signaling ameliorates graft-versus-host disease by modifying T-cell differentiation and intestinal migration. *Blood* (2013) 122:2125–34. doi: 10.1182/blood-2012-11-470252
46. Wang D, Yu Y, Haarberg K, Fu J, Kaosaard K, Nagaraj S, et al. Dynamic change and impact of myeloid-derived suppressor cells in allogeneic bone marrow transplantation in mice. *Biol Blood Marrow Transplant.* (2013) 19:692–702. doi: 10.1016/j.bbmt.2013.01.008
47. Dodge J, Stephans A, Lai J, Drobyski WR, Chen X. Effects of donor vitamin A deficiency and pharmacologic modulation of donor T cell retinoic acid pathway on the severity of experimental graft-versus-host disease. *Biol Blood Marrow Transplant*. (2016) 22:2141–28. doi: 10.1016/j.bbmt.2016.09.001
48. Yang H, Gu J, Zhu Q, Lu H, Wang K, Ni X, et al. Protection of acute GVHD by all-trans retinoic acid through suppression of T cell expansion and induction of regulatory T cells through IL-2 signaling. *Int Immunopharmacol.* (2015) 28:911–6. doi: 10.1016/j.intimp.2015.03.042
49. Nishimori H, Maeda Y, Teshima T, Sugiyama H, Kobayashi K, Yamasuji Y, et al. Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17. *Blood* (2012) 119:285–95. doi: 10.1182/blood-2011-01-332478
50. Lounder DT, Khandelwal P, Dandoy CE, Jodele S, Grimley MS, Wallace G, et al. Lower levels of vitamin A are associated with increased gastrointestinal graft-versus-host disease in children. *Blood* (2017) 129:2801–07. doi: 10.1182/blood-2017-02-765826
51. Tong J, Hu R, Zhao Y, Xu Y, Zhao X, Jin X. Serum vitamin A levels may affect the severity of ocular graft-versus-host disease. *Front Med.* (2017) 4:67. doi: 10.3389/fmed.2017.00067
52. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr.* (2008) 87:1080S—6S. doi: 10.1093/ajcn/87.4.1080S
53. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci USA.* (2004) 101:7711–5. doi: 10.1073/pnas.0402490101
54. Sawada N, Sakaki T, Ohta M, Inouye K. Metabolism of vitamin D by human CYP27A1. *Biochem Biophys Res Commun.* (2000) 273:977–84. doi: 10.1006/bbrc.2000.3050
55. Haussler MR, Norman AW. Chromosomal receptor for a vitamin D metabolite. *Proc Natl Acad Sci USA.* (1969) 62:155–62. doi: 10.1073/pnas.62.1.155
56. Jin CH, Kerner SA, Hong MH, Pike JW. Transcriptional activation and dimerization functions in the human vitamin D receptor. *Mol Endocrinol.* (1996) 10:945–57.
57. Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh J-C, et al. Molecular mechanisms of vitamin D action. *Calcif Tissue Int.* (2013) 92:77–98. doi: 10.1007/s00223-012-9619-0
58. Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, et al. Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. *Proc Natl Acad Sci USA.* (1981) 78:4990–4. doi: 10.1073/pnas.78.8.4990
59. Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab.* (1983) 57:1308–10. doi: 10.1210/jcem-57-6-1308
60. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science* (1983) 221:1181–3. doi: 10.1126/science.6310748
61. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol.* (2000) 164:2405–11. doi: 10.4049/jimmunol.164.5.2405
62. Piemonti L, Monti P, Sironi M, Fraticelli P, Leone BE, Dal Cin E, et al. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol.* (2000) 164:4443–51. doi: 10.4049/jimmunol.164.9.4443
63. Adorini L, Penna G, Giarratana N, Roncari A, Amuchastegui S, Daniel KC, et al. Dendritic cells as key targets for immunomodulation by Vitamin D receptor ligands. *J Steroid Biochem Mol Biol.* (2004) 89–90:437–41. doi: 10.1016/j.jsbmb.2004.03.013
64. Adorini L, Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. *Hum Immunol.* (2009) 70:345–52. doi: 10.1016/j.humimm.2009.01.016
65. Penna G, Amuchastegui S, Giarratana N, Daniel KC, Vulcano M, Sozzani S, et al. 1,25-Dihydroxyvitamin D3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J Immunol.* (2007) 178:145–53. doi: 10.4049/jimmunol.178.1.145
66. Mahon BD, Wittke A, Weaver V, Cantorna MT. The targets of vitamin D depend on the differentiation and activation status of CD4 positive T cells. *J Cell Biochem.* (2003) 89:922–32. doi: 10.1002/jcb.10580
67. Correale J, Ysrraelit MC, Gaitán MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain* (2009) 132:1146–60. doi: 10.1093/brain/awp033
68. Tang J, Zhou R, Luger D, Zhu W, Silver PB, Grajewski RS, et al. Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response. *J Immunol.* (2009) 182:4624–32. doi: 10.4049/jimmunol.0801543
69. Mayne CG, Spanier JA, Relland LM, Williams CB, Hayes CE. 1,25-Dihydroxyvitamin D3 acts directly on the T lymphocyte vitamin D receptor to inhibit experimental autoimmune encephalomyelitis. *Eur J Immunol.* (2011) 41:822–32. doi: 10.1002/eji.201040632

70. Cantorna MT, Snyder L, Lin Y-D, Yang L. Vitamin D and 1,25(OH)2D Regulation of T cells. *Nutrients* (2015) 7:3011–21. doi: 10.3390/nu7043011
71. Hayes CE, Hubler SL, Moore JR, Barta LE, Praska CE, Nashold FE. Vitamin D Actions on CD4+ T Cells in Autoimmune Disease. *Front Immunol.* (2015) 6:100. doi: 10.3389/fimmu.2015.00100
72. Rigby WF, Stacy T, Fanger MW. Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D3 (calcitriol). *J Clin Invest.* (1984) 74:1451–5. doi: 10.1172/JCI111557
73. Chen J, Bruce D, Cantorna MT. Vitamin D receptor expression controls proliferation of naïve CD8+ T cells and development of CD8 mediated gastrointestinal inflammation. *BMC Immunol.* (2014) 15:6. doi: 10.1186/1471-2172-15-6
74. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol.* (2007) 179:1634–47. doi: 10.4049/jimmunol.179.3.1634
75. Kallio E, Häyry P, Pakkala S. MC1288, a vitamin D analogue, reduces short- and long-term renal allograft rejection in the rat. *Transplant Proc.* (1996) 28:311.
76. Hullett DA, Cantorna MT, Redaelli C, Humpal-Winter J, Hayes CE, Sollinger HW, et al. Prolongation of allograft survival by 1,25-dihydroxyvitamin D3. *Transplantation* (1998) 66:824–8. doi: 10.1097/00007890-199810150-00002
77. Pakkala I, Taskinen E, Pakkala S, Räisänen-Sokolowski A. MC1288, a vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation. *Bone Marrow Transplant.* (2001) 27:863–7. doi: 10.1038/sj.bmt.1702873
78. Rosenblatt J, Bissonnette A, Ahmad R, Wu Z, Vasir B, Stevenson K, et al. Immunomodulatory effects of vitamin D: implications for GVHD. *Bone Marrow Transplant.* (2010) 45:1463–8. doi: 10.1038/bmt.2009.366
79. Joseph RW, Bayraktar UD, Kim TK, St John LS, Popat U, Khalili J, et al. Vitamin D receptor upregulation in alloreactive human T cells. *Hum Immunol.* (2012) 73:693–8. doi: 10.1016/j.humimm.2012.04.019
80. Middleton PG, Cullup H, Dickinson AM, Norden J, Jackson GH, Taylor PRA, et al. Vitamin D receptor gene polymorphism associates with graft-versus-host disease and survival in HLA-matched sibling allogeneic bone marrow transplantation. *Bone Marrow Transplant.* (2002) 30:223–8. doi: 10.1038/sj.bmt.1703629
81. Rocha V, Porcher R, Fernandes JF, Filion A, Bittencourt HWS Jr, Vilela G, et al. Association of drug metabolism gene polymorphisms with toxicities, graft-versus-host disease and survival after HLA-identical sibling hematopoietic stem cell transplantation for patients with leukemia. *Leukemia* (2009) 23:545–56. doi: 10.1038/leu.2008.323
82. Cho HJ, Shin D-Y, Kim JH, Bae J-Y, Lee K-H, See CJ, et al. Impact of vitamin D receptor gene polymorphisms on clinical outcomes of HLA-matched sibling hematopoietic stem cell transplantation. *Clin Transplant.* (2012) 26:476–83. doi: 10.1111/j.1399-0012.2011.01523.x
83. Bogunia-Kubik K, Middleton P, Norden J, Dickinson A, Lange A. Association of vitamin D receptor polymorphisms with the outcome of allogeneic haematopoietic stem cell transplantation. *Int J Immunogenet.* (2008) 35:207–13. doi: 10.1111/j.1744-313X.2008.00758.x
84. Kamel AM, El-Fishawi S, Rasekh EO, Radwan ER, Zeidan A, El-Said A, et al. Variability of contribution of vitamin D receptor gene polymorphisms to outcome of HLA-matched sibling allogeneic bone marrow transplantation. *Leuk Lymphoma* (2018) 4:1–10. doi: 10.1080/10428194.2018.1459608
85. Kreutz M, Eissner G, Hahn J, Andreesen R, Drobnik W, Holler E. Variations in 1 alpha,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3 serum levels during allogeneic bone marrow transplantation. *Bone Marrow Transplant.* (2004) 33:871–3. doi: 10.1038/sj.bmt.1704448
86. Robien K, Strayer LG, Majhail N, Lazovich D, Baker KS, Smith AR, et al. Vitamin D status among long-term survivors of hematopoietic cell transplantation. *Bone Marrow Transplant.* (2011) 46:1472–79. doi: 10.1038/bmt.2010.326
87. Urbain P, Ihorst G, Biesalski H-K, Bertz H. Course of serum 25-hydroxyvitamin D status and its influencing factors in adults undergoing allogeneic hematopoietic cell transplantation. *Ann Hematol.* (2012) 91:759–66. doi: 10.1007/s00277-011-1365-2
88. Myers KC, Howell JC, Wallace G, Dandoy C, El-Bietar J, Lane A, et al. Poor growth, thyroid dysfunction and vitamin D deficiency remain prevalent despite reduced intensity chemotherapy for hematopoietic stem cell transplantation in children and young adults. *Bone Marrow Transplant.* (2016) 51:980–4. doi: 10.1038/bmt.2016.39
89. Wallace G, Jodele S, Myers KC, Dandoy CE, El-Bietar J, Nelson A, et al. Vitamin D deficiency in pediatric hematopoietic stem cell transplantation patients despite both standard and aggressive supplementation. *Biol Blood Marrow Transplant.* (2016) 22:1271–4. doi: 10.1016/j.bbmt.2016.03.026
90. Glotzbecker B, Ho VT, Aldridge J, Kim HT, Horowitz G, Ritz J, et al. Low levels of 25-hydroxyvitamin D before allogeneic hematopoietic SCT correlate with the development of chronic GVHD. *Bone Marrow Transplant.* (2013) 48:593–7. doi: 10.1038/bmt.2012.177
91. von Bahr L, Blennow O, Alm J, Björklund A, Malmberg K-J, Mougiakakos D, et al. Increased incidence of chronic GvHD and CMV disease in patients with vitamin D deficiency before allogeneic stem cell transplantation. *Bone Marrow Transplant.* (2015) 50:1217–23. doi: 10.1038/bmt.2015.123
92. Hansson MEA, Norlin A-C, Omazic B, Wikström A-C, Bergman P, Winiarski J, et al. Vitamin D levels affect outcome in pediatric hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2014) 20:1537–43. doi: 10.1016/j.bbmt.2014.05.030
93. Beebe K, Magee K, McNulty A, Stahlecker J, Salzberg D, Miller H, et al. Vitamin D deficiency and outcomes in pediatric hematopoietic stem cell transplantation. *Pediatr Blood Cancer* (2018) 65:1–6. doi: 10.1002/pbc.26817
94. Silva F, Pérez-Simón JA, Caballero-Velazquez T, Sánchez-Guijo FM, Villanueva-Gomez F, Vazquez L, et al. Effect of vitamin D treatment in chronic GVHD. *Bone Marrow Transplant*. (2011) 46:1395–7. doi: 10.1038/bmt.2010.317
95. Caballero-Velázquez T, Montero I, Sánchez-Guijo F, Parody R, Saldaña R, Valcarcel D, et al. Immunomodulatory effect of vitamin D after allogeneic stem cell transplantation: results of a prospective multicenter clinical trial. *Clin Cancer Res.* (2016) 22:5673–81. doi: 10.1158/1078-0432.CCR-16-0238
96. Benrashid M, Moyers K, Mohty M, Savani BN. Vitamin D deficiency, autoimmunity, and graft-versus-host-disease risk: implication for preventive therapy. *Exp Hematol.* (2012) 40:263–7. doi: 10.1016/j.exphem.2012.01.006
97. Carpenter PA. Vitamin A to reduce gut leak and GVHD? *Blood* (2017) 129:2715–7. doi: 10.1182/blood-2017-03-773226
98. Zheng J, Taylor B, Chen X. Role of vitamin A in modulating graft-versus-host disease. *J Immunol Res Ther*. (2018) 3:124–8.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 June 2018
doi: 10.3389/fimmu.2018.01461

Inducible T-Cell Co-Stimulator Impacts Chronic Graft-Versus-Host Disease by Regulating Both Pathogenic and Regulatory T Cells

Mengmeng Zhang[1], Yongxia Wu[2], David Bastian[2], Supinya Iamsawat[2], Jinsam Chang[3], Anusara Daenthanasanmak[2], Hung D. Nguyen[2], Steven Schutt[2], Min Dai[4], Fangping Chen[1], Woong-Kyung Suh[3] and Xue-Zhong Yu[2,5]**

[1] Department of Hematology, Xiangya Hospital, Central South University, Changsha, China, [2] Department of Microbiology and Immunology, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, United States, [3] Institut de Recherches Cliniques de Montréal (IRCM), Montreal, QC, Canada, [4] Department of Hematology, Nanfang Hospital, Southern Medical University, Guangzhou, China, [5] Department of Medicine, Medical University of South Carolina, Charleston, SC, United States

OPEN ACCESS

Edited by:
Aurore Saudemont, GlaxoSmithKline, United Kingdom

Reviewed by:
Benedetto Bruno, Università degli Studi di Torino, Italy
Evelyn Ullrich, Goethe-Universität Frankfurt am Main, Germany

***Correspondence:**
Woong-Kyung Suh
woong-kyung.suh@ircm.qc.ca;
Xue-Zhong Yu
yux@musc.edu

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: 23 March 2018
Accepted: 12 June 2018
Published: 22 June 2018

Citation:
Zhang M, Wu Y, Bastian D, Iamsawat S, Chang J, Daenthanasanmak A, Nguyen HD, Schutt S, Dai M, Chen F, Suh W-K and Yu X-Z (2018) Inducible T-Cell Co-Stimulator Impacts Chronic Graft-Versus-Host Disease by Regulating Both Pathogenic and Regulatory T Cells. Front. Immunol. 9:1461. doi: 10.3389/fimmu.2018.01461

The incidence of chronic graft-versus-host disease (cGVHD) is on the rise and still the major cause of morbidity and mortality among patients after allogeneic hematopoietic stem cell transplantation (HCT). Both donor T and B cells contribute to the pathogenesis of cGVHD. Inducible T-cell co-stimulator (ICOS), a potent co-stimulatory receptor, plays a key role in T-cell activation and differentiation. Yet, how ICOS regulates the development of cGVHD is not well understood. Here, we investigated the role of ICOS in cGVHD pathogenesis using mice with germline or regulatory T cell (Treg)-specific ICOS deficiency. The recipients of $ICOS^{-/-}$ donor grafts had reduced cGVHD compared with wild-type controls. In recipients of $ICOS^{-/-}$ donor grafts, we observed significant reductions in donor T follicular helper (Tfh), Th17, germinal center B-cell, and plasma cell differentiation, coupled with lower antibody production. Interestingly, Tregs, including follicular regulatory T (Tfr) cells, were also impaired in the absence of ICOS. Using ICOS conditional knockout specific for $Foxp3^{+}$ cells, we found that ICOS was indispensable for optimal survival and homeostasis of induced Tregs during cGVHD. Furthermore, administration of anti-ICOS alleviated cGVHD severity *via* suppressing T effector cells without affecting Treg generation. Taken together, ICOS promotes T- and B-cell activation and differentiation, which can promote cGVHD development; however, ICOS is critical for the survival and homeostasis of iTregs, which can suppress cGVHD. Hence, ICOS balances the development of cGVHD and could offer a potential target after allo-HCT in the clinic.

Keywords: inducible T-cell co-stimulator, chronic graft-versus-host disease, regulatory T cells, T follicular helper, follicular regulatory T

INTRODUCTION

The morbidity and mortality associated with chronic graft-versus-host disease (cGVHD) has raised in the past two decades, due to improvements in patient care during the acute phase after allogeneic hematopoietic stem cell transplantation (allo-HCT), the use of peripheral blood stem cells instead of the bone marrow as grafts, and increasing age of donors or recipients (1–3). Patients with cGVHD

show various clinical symptoms that can resemble those observed in various autoimmune disorders, such as fibrosis that can result in organ failure (4). The development of cGVHD involves aberrant effector T (Teff) and B cell activation, differentiation and costimulation, coupled with decreased regulatory T cell (Treg) generation and development (5–7).

Naïve CD4 T cells can differentiate into Th1, Th2, and Th17 subsets, among others. Tregs, *via* the transcription factor-Foxp3, limit the Teff and B cell response. IFN-γ, a Th1-signature cytokine, increases in patients in early stages post allo-HCT (3–8 months), but is notably decreased in later stages (≥9 months), suggesting that Th1 is required for the initiation of cGVHD (8–10). Th2 cells were originally reported as the dominate subset mediating cGVHD, yet conflicting data have obscured this finding (10–12). Th17 cells secrete IL-17 and IL-21 and can induce fibrosis (11–13). Thymic damage after conditioning leads to decreased Treg development, and subsequently an inability to suppress autoreactive and alloreactive immune cells (9, 14). T follicular helper (Tfh) cells provide support to B cells in germinal center (GC) formation, which facilitate B cell differentiation into plasma cells, leading to auto- and/or allo-antibody deposition in target organs (15). Follicular regulatory T (Tfr) cells, derived from natural Treg precursors, can control GC responses by suppressing B and Tfh cell responses (16). Thus, the aforementioned mechanisms contribute to both the complexity and development of cGVHD.

Inducible T-cell co-stimulator (ICOS), a member of the CD28 family, is expressed on activated murine T cells, NKT cells, and type 2 innate lymphoid cells. ICOS is implicated in almost all T-cell differentiation and cytokine production patterns (17). Depending on the context, ICOS has been documented to promote Th1 or Th2 skewing (18), maintain Th17 under inflammatory conditions (19–21), and promote Tfh cell differentiation (22, 23). ICOS also contributes to Treg development and suppressive function in both mice and humans; $ICOS^{-/-}$ mice have reduced Treg percentage and number versus healthy controls (24–26). In addition, ICOS is important for GC formation and T-cell-dependent antibody responses, reflected by a profound defect in B-cell maturation and immunoglobulin isotype switching in both $ICOS^{-/-}$ mice and humans associated with reduced help from Tfh cells (27–29).

Previous studies have shown that $ICOS^{-/-}$ T cells have reduced IFN-γ yet elevated IL-4, which resulted in alleviated acute GVHD (aGVHD) (30); blocking ICOS confirmed this reduced GVHD severity (31). Antibody blockade of ICOS in mice with cGVHD using a bronchiolitis obliterans cGVHD mode can also improve pulmonary function by decreasing Tfh and GC responses (32). However, the role of ICOS in T-cell differentiation and Treg generation, development, and function is unknown in cGVHD. Utilizing a murine model of allogeneic bone marrow transplantation (BMT), we demonstrate a vital role for ICOS in promoting pathogenic T/B-cell differentiation, and further identified that ICOS was indispensable for Treg development and survival during cGVHD development. Importantly, we observe that ICOS blockade prior to cGVHD onset preserved Tregs and was efficacious in reducing cGVHD severity.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 (B6, H-$2K^b$, CD45.2), B6Ly5.2 (CD45.1), and BALB/c (H-$2K^d$) mice were purchased from National Cancer Institute (Frederick, MD, USA). $Rag1^{-/-}$ B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). ICOS germline knockout (KO) (29) and $ICOS^{fl/fl}$ (33) mice were generated in 129 background and backcrossed 12 generations into B6. $ICOS^{fl/fl}$ mice were bred with $Foxp3^{YFP\text{-}Cre}$ (JAX016959) mice to generate Treg-specific ICOS KO mice ($Foxp3^{YFP\text{-}Cre}ICOS^{fl/fl}$). Mice between 8 and 10 weeks old were used as recipients, and 6 and 8 weeks old mice were used as donors in this study. All mice were bred under specific pathogen-free conditions in the animal facility of the Medical University of South Carolina (Charleston, SC, USA). All animal experiments were approved by the Institutional Animal Care and Use of Committee.

cGVHD Model

A major histocompatibility complex-mismatched (B6 to BALB/c) mouse model was used as previously described (34). Briefly, BALB/c recipients were lethally irradiated with total body irradiation (TBI) at 650 cGy using a RAD 320 X-ray Irradiator (Precision X-ray Inc., North Branford, CT, USA) and received 5×10^6 T-cell-depleted bone marrow (TCD-BM) cells, with or without 0.5×10^6 whole splenocytes (SPLs) or 0.25×10^6 CD25-depleted splenocytes ($CD25^-$SPLs) from WT, ICOS KO, $Cre^-ICOS^{fl/fl}$, or $Foxp3^{YFP\text{-}Cre}ICOS^{fl/fl}$ B6 donor mice *via* tail vein. Recipients were monitored for survival, body weight, and clinical syndromes of cGVHD described previously (35). As published previously, anti-ICOS (7E.17G9. G1, rIgG2b; produced at National Cell Culture, Minneapolis, MN, USA) or irrelevant rat-IgG were injected i.p. at 200 μg/mouse from day 0 to day 28, 3 times/week after BMT (31).

aGVHD Model

BALB/c recipients were lethally irradiated with TBI at 700 cGy and injected with 5×10^6 BM from $Rag1^{-/-}$ B6 mice and enriched 0.5×10^6 $CD25^{hi}Ly5.1^-CD4$ T cells on day 0, and then recipients were transferred with 0.5×10^6 $CD25^-Ly5.1^+$ T cells on day 3. Recipients were monitored with survival, body weight loss, and clinical twice per week for 80 days.

Flow Cytometry

Recipient's splenocytes and thymocytes were isolated and stained for surface markers and intracellular markers and cytokines using standard flow cytometric protocols as previously described (35). Stained cells were analyzed by LSR II (BD Biosciences, San Jose, CA, USA) and Flow Jo (Tree Star, Ashland, OR, USA).

Serum Autoantibody Detection

Serum autoantibodies were detected as previously described (35). Succinctly, double-stranded DNA (dsDNA) made from calf thymus were pre-coated on ELISA plate (Corning Inc.) and then incubated with diluted serum. Biotin-IgG, IgG1, and IgG2c (Southern Biotech) followed by HRP-streptavidin antibodies and TMB substrate (eBioscience) were utilized. Plates were read out

by a Multiscan FC (Thermo Scientific, MA, USA) ELISA plate reader.

Trichrome Staining

Six-micrometer cryosections were stained with a Masson trichrome staining kit (Sigma-Aldrich) for detection of collagen deposition. Collagen deposition was quantified on trichrome-stained sections as a ratio of area of blue staining to area of total staining by use of ImageJ 1.51s (National Institutes of Health, USA) analysis tool.

iTregs Generation and Enrichment

$CD4^+CD25^-$ T cells were purified from WT or $ICOS^{-/-}$ B6 spleens and lymph nodes by MACS. $CD11c^+$ dendritic cells were purified from BALB/c mice using CD11c microbeads (Miltenyi). $CD4^+CD25^-$ T cells were co-cultured with $CD11c^+$ DCs at 1:10 (DC:T cell) ratio with IL-2 (5 ng/ml), TGF-β (5 μg/ml), and retinoic acid (40 nM) for 5 days. iTregs were enriched from bulk culture using positive selection with CD25 microbeads and LS columns (Miltenyi). Purity of iTregs was usually 90–95% as in these experiments.

Statistical Analysis

Results were presented as mean ± 1 SEM, a two-tailed Student's *t*-test is utilized for accessing statistical significance among groups, and the log-rank test is utilized for evaluating recipient survival among groups by GraphPad Prism 6.

RESULTS

ICOS Contributes to the Progression of cGVHD

Inducible T-cell co-stimulator is expressed on activated CD4 and CD8 T cells and promotes T cell alloresponses to mediate GVHD (36). We therefore evaluated the ICOS expression on allogeneic T cells using a cGVHD transition model, B6 to BALB/c. We found that ICOS expression was significantly increased on donor CD4 T cells in the spleen of the recipients with cGVHD 60 days post-BMT compared with those without cGVHD; although ICOS expression was comparable on donor CD8 T cells and Tregs (**Figure 1A**). These data implicate ICOS expression on T cells in cGVHD, especially on the CD4 subset.

To test how ICOS affects the development of cGVHD, we initially used ICOS germline KO strain on B6 background as donors and BALB/c as recipients, in which the recipients develop aGVHD and transit to cGVHD (34). Consistent with previous reports (25, 28, 29, 37, 38), WT and ICOS KO displayed a comparable frequency of B220, CD4, and CD8, with a moderate decrease in regulatory and effector-memory CD4 T (Tem) cells (data not shown). Given that donor Tem have not been shown to impact GVHD development (39), and Tregs alleviate GVHD (40), we used CD25-depleted splenocytes plus TCD-BM as donor grafts to induce cGVHD. Recipients transplanted with $ICOS^{-/-}$ grafts had reduced body weight loss (**Figure 1B**) and lower cGVHD clinical scores at later time points, but not at early time points compared with those receiving WT grafts (**Figure 1C**). As fibrosis is a key feature of cGVHD (6), we measured fibrosis in the recipient skin and lung 60 days post-BMT and found that $ICOS^{-/-}$ donor cells induced less sclerodermatous-like pathology, reflected by retention of subcutaneous fat in the skin and less fibrosis in both skin and lung tissues (**Figures 1D,E**). These results indicate that ICOS expression on donor grafts promoted the progression of cGVHD.

ICOS Promotes Treg and Tfh Development in cGVHD

We then examined Treg and Tfh differentiation 60 days post-BMT, as they play critical roles in the development of cGVHD (7). We observed that recipients of $ICOS^{-/-}$ donor grafts had significantly reduced Treg frequency compared with WT controls in the spleen, but not the thymus (**Figures 2A,B**, and data not shown), suggesting that ICOS influences the generation of iTregs but not the development of nTregs. The recipients of $ICOS^{-/-}$ grafts also had significantly reduced Tfh among $CD4^+Foxp3^-$ and Tfr among $CD4^+Foxp3^+$ cells compared with those of WT grafts (**Figures 2A,B**). A marked elevation in ICOS expression on Tfr cells was observed compared with that on Tfh cells among WT donor T cells (**Figure 2C**), suggesting that ICOS may play a greater role in Tfr differentiation than in Tfh cells. Notably, we observed that follicular-like CD8 T cells, which resemble Tfh cells and expressed ICOS, were decreased in the recipients of $ICOS^{-/-}$ donor grafts compared with those of WT grafts (Figure S1A in Supplementary Material), suggesting that ICOS played a role in follicular-like CD8 T cell development during cGVHD pathogenesis.

To further understand how ICOS expression impacts cGVHD, we evaluated the kinetics of Treg, Tfr, and Tfh differentiation in the spleen of recipients at day 15, 30, 45, and 60 after BMT. In the recipients of WT or $ICOS^{-/-}$ donor grafts, Tregs began to gradually increase after day 15, peaked on day 45, and stabilized through day 60 post-BMT. However, Tregs were significantly reduced from day 30 to day 60 in the recipients of $ICOS^{-/-}$ grafts when compared with those of WT (**Figure 2D**), indicating that ICOS promoted Treg development during cGVHD development. In both groups, Tfh cells among $CD4^+Foxp3^-$ peaked at day 15 and slowly declined through day 60. However, Tfh cells were significantly lower from day 30 to day 60 in the recipients of $ICOS^{-/-}$ grafts (**Figure 2D**), indicating that ICOS similarly affected Tfh cells development. Tfr cells among $CD4^+Foxp3^+$ retained relatively high levels at day 15, yet gradually decreased over time, and finally sustained a steady state from day 45 to day 60; albeit, significantly fewer Tfr cells were generated from $ICOS^{-/-}$ T cells compared with WT T cells in later stages of cGVHD development (**Figure 2D**). Taken together, these data indicate that ICOS affects Treg and Tfh differentiation by day 30 after BMT, correlated with disease onset.

ICOS Promotes Th17 Differentiation

Given that ICOS controls the memory T-cell pool (25) and is essential for CD4 T cell activation (37), we measured CD44 and CD69 expression on T cells derived from WT and ICOS KO donor grafts. We observed comparable percentages of $CD44^+$ cells among CD4 and CD8 T cells (**Figure 3A**), but a lower

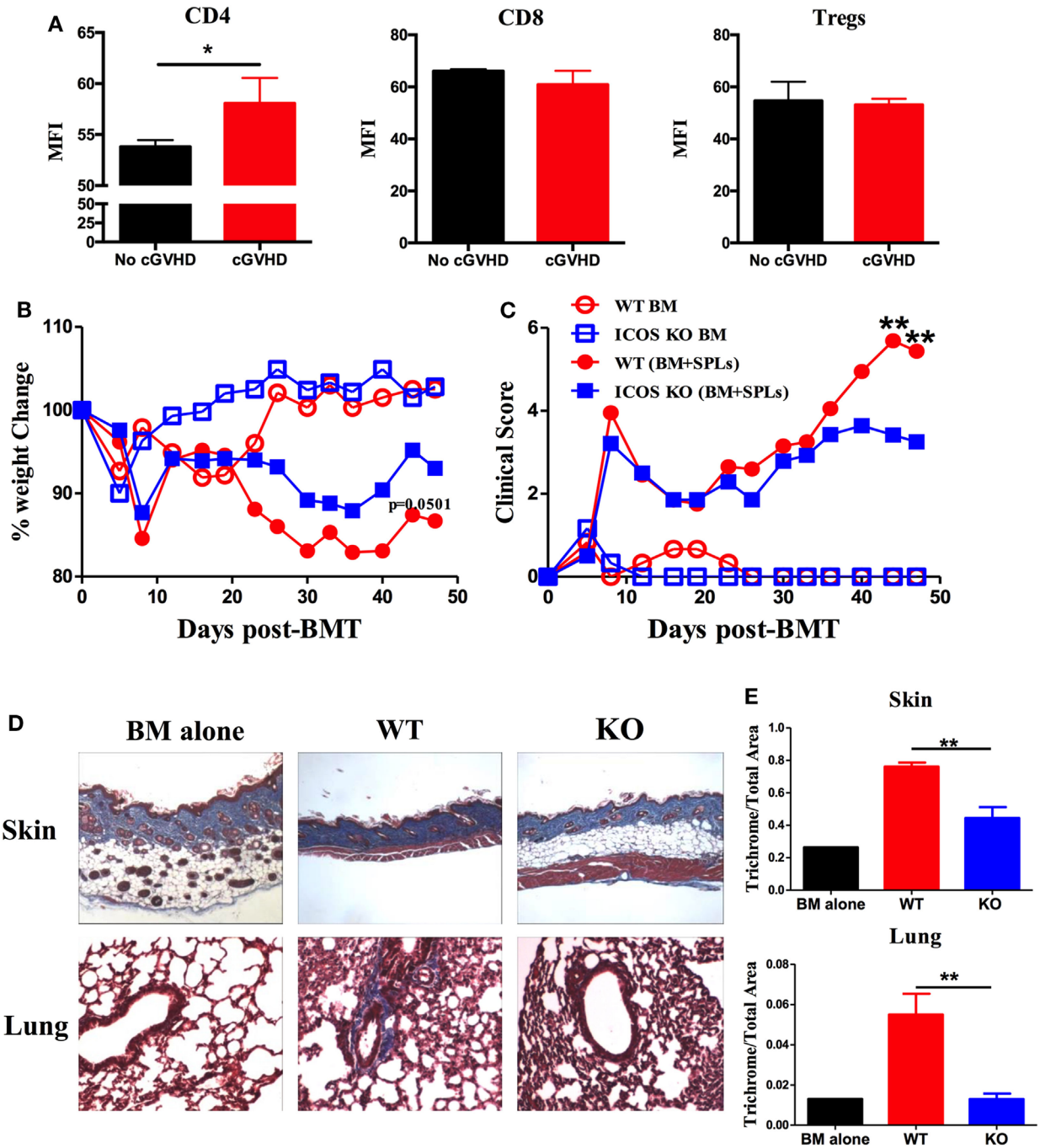


FIGURE 1 | Inducible T-cell co-stimulator (ICOS) contributes to the progression of chronic graft-versus-host disease (cGVHD). **(A)** Lethally irradiated BALB/c mice were transplanted with 5×10^6 T-cell-depleted bone marrow (TCD-BM) or plus 0.5×10^6 whole SPLs from wild-type (WT) B6 mice. Spleens were processed and analyzed by flow cytometry. Mean fluorescence intensity (MFI) of ICOS on gated donor CD4, CD8, and regulatory T cells (Tregs) are shown, $n = 3$–5 mice/group. BALB/c mice were lethally irradiated and transferred with 0.25×10^6 $CD25^-$SPLs and 5×10^6 TCD-BM from WT or $ICOS^{-/-}$ mice on B6 background. Body weight **(B)** and clinical scores **(C)** of cGVHD were monitored bi-weekly for 50 days, $n = 8$ mice/group. Recipient skin and lung were harvested at day 60 after bone marrow transplantation (BMT) and processed for Masson's trichrome staining. Representative images from one experiment are shown **(D)**. **(E)** Collagen deposition of skin and lung was qualified by ImageJ as the ratio of collagen area to the whole area of tissue, $n = 4$ mice/group. $^*p < 0.05$ and $^{**}p < 0.01$.

percentage of CD69 on CD4 (data not shown), suggesting that ICOS is required for allogeneic T cell activation in cGVHD development.

Effector CD4 T cells drive the pathogenesis of cGVHD (7). ICOS has a distinct role in Th1 and Th2 differentiation depending on disease context (18), but is known to consistently promote

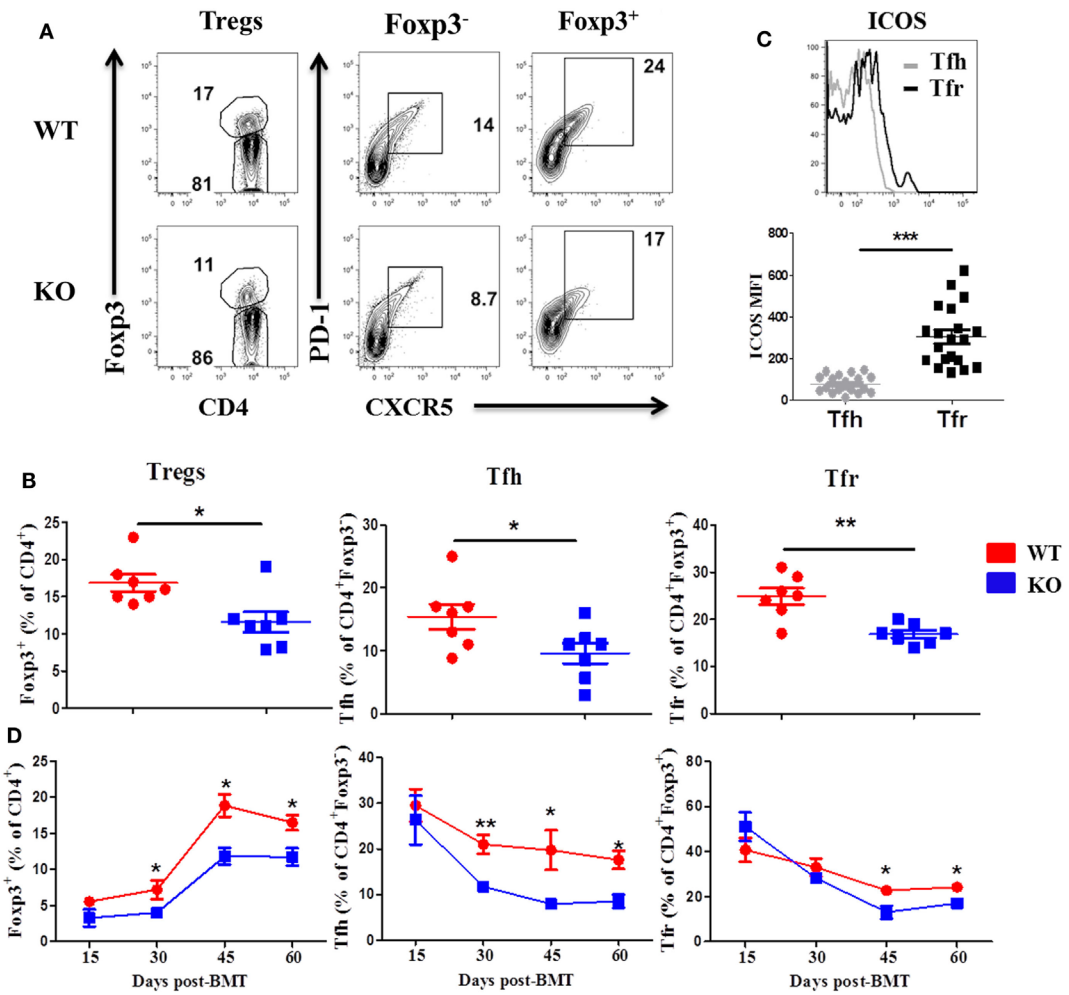


FIGURE 2 | Inducible T-cell co-stimulator (ICOS) promotes development of regulatory T cell (Treg) and T follicular helper (Tfh) cells. Bone marrow transplantation (BMT) was performed as described in **Figures 1B–E**. 60 days after BMT, recipient spleens were harvested and processed for flow cytometry. Representative contour plots from individual mice **(A)** and dot plots of mean percentage **(B)** of Tregs (Foxp3$^+$) on gated donor H-2K^{b+}CD4 T cells, Tfh cells (PD-1$^+$CXCR5$^+$) on gated H-2K^{b+}CD4$^+$Foxp3$^-$, and follicular regulatory T (Tfr) cells (PD-1$^+$CXCR5$^+$) on gated H-2K^{b+}CD4$^+$Foxp3$^+$ T cells are shown, $n = 7$ mice/group. Representative histogram plots of individual mice from wild-type (WT) group [**(C)** top] and mean fluorescence intensity (MFI) of ICOS on Tfh and Tfr cells are shown [**(C)**, bottom]. BMT parameters were the same as described in **Figures 1B–E**, at day 15, 30, 45, and 60 post-BMT, spleens were collected and analyzed by flow cytometry. Summary of percentage of Tregs, Tfh, and Tfr are shown over time **(D)**, $n = 3$–4 mice/group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Th17 development (19, 41). We therefore tested the impact of ICOS expression on CD4 T-cell differentiation in cGVHD. Upon comparison of cytokine secretion by T cells, we observed similar IFN-γ and IL-4/5 secretion by WT or ICOS$^{-/-}$ CD4 T cells, yet IL-17A production was dramatically decreased by ICOS$^{-/-}$ CD4 T cells (**Figures 3B,C**). We then assessed kinetics of Th1, Th2, and Th17 cells during cGVHD pathogenesis and found that IFN-γ and IL-4/5 production were again comparable in WT and ICOS$^{-/-}$ T cells (**Figure 3D**). However, ICOS$^{-/-}$ donor T cells produced lower levels of IL-17A from 45 to 60 days post-BMT when compared with WT T cells (**Figure 3D**). These results suggested that ICOS was necessary for Th17, but dispensable for Th1 or Th2, differentiation during cGVHD development.

Given that ICOS also promotes CD8 T-cell activation and expansion (42) that can contribute to cGVHD (43), we quantified the effect of ICOS expression on CD8 T cells after BMT. We observed reduced CD69 expression and IFN-γ secretion on donor ICOS$^{-/-}$ CD8 T cells, but not IL-17 (data not shown), which was correlated with reduced cGVHD severity (11). Taken together, ICOS controls both CD4 and CD8 T-cell activation and differentiation in cGVHD pathogenesis.

ICOS Induces GC B-Cell Development and Plasma Cell Differentiation

Inducible T-cell co-stimulator is required for the differentiation of Tfh cells (23), which promote GC B-cell formation, plasma cell differentiation, and antibody production (18). These activated donor B cells then act as antigen-presenting cells (APCs) to stimulate T cells (44). We next examined the effect of ICOS

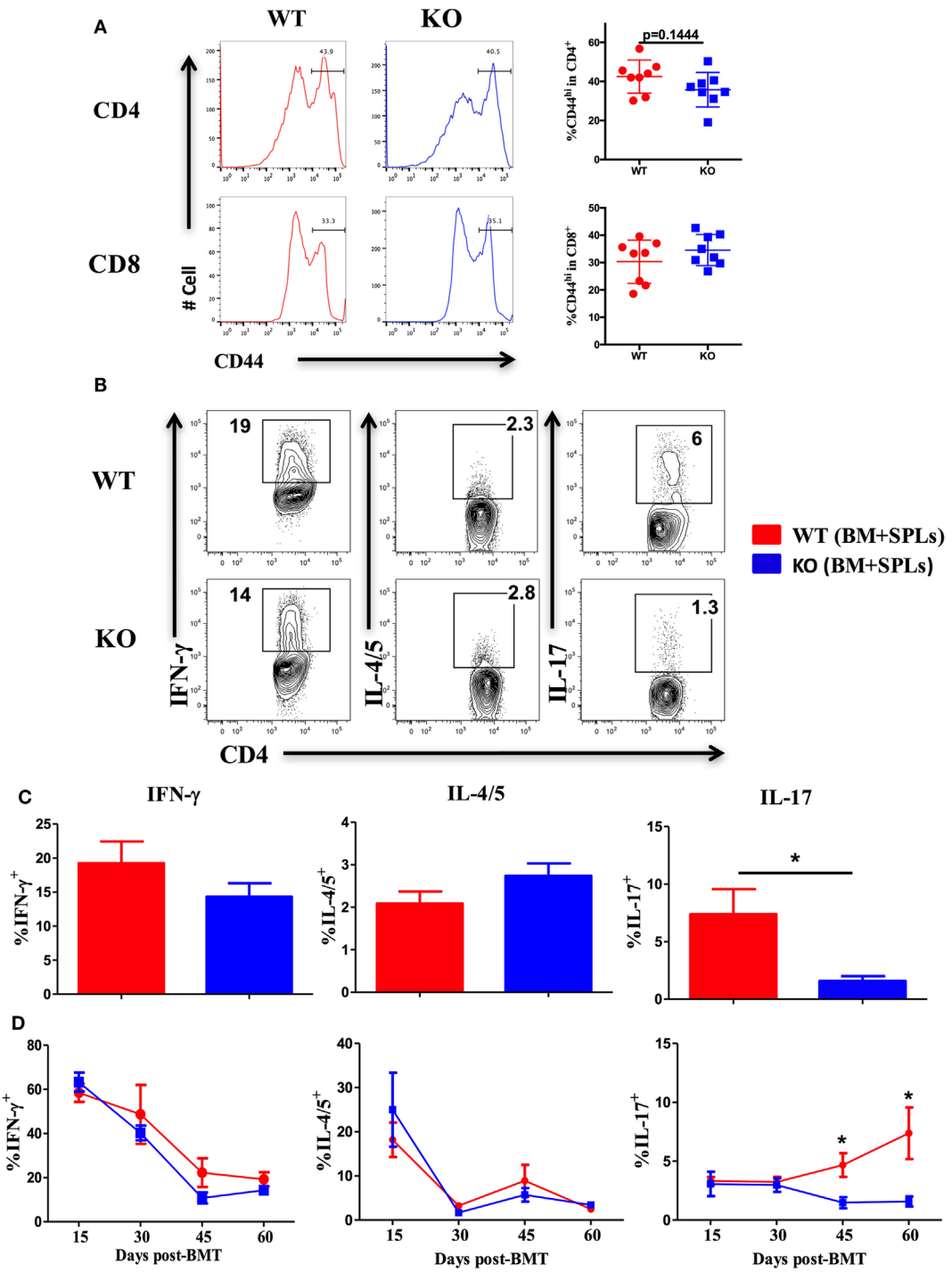


FIGURE 3 | Inducible T-cell co-stimulator promotes Th17 cell differentiation. Bone marrow transplantation (BMT) was setup as described in **Figures 1B–E**. 60 days after BMT, recipient spleens were harvested and processed for flow cytometry. **(A)** Representative histograms plots and mean percentage of CD44 gated on H-2K^{b+}CD4 and CD8 T cells are shown, $n = 8$ mice/group. Representative contour plots of individual mice **(B)** and bar graphs of mean percentage **(C)** of IFN-γ$^+$, IL-4/5$^+$, and IL-17$^+$ on gated H-2K^{b+}CD4$^+$ cells are shown, $n = 6$–8 mice/group. Summary of mean percentage of IFN-γ$^+$, IL-4/5$^+$, and IL-17$^+$ on gated H-2K^{b+}CD4$^+$ cells are shown over time **(D)**, $n = 3$–4 mice per group. *$p < 0.05$.

on donor B-cell activation and differentiation. The recipients of ICOS$^{-/-}$ donor grafts had a lower frequency of Fas$^+$GL-7$^+$ GC B cells and B220^{low}CD138$^+$ plasma cells when compared with those of WT grafts (Figure S1B in Supplementary Material). Albeit, B-cell reconstitution and expression of co-stimulatory molecules were comparable (Figure S1B in Supplementary Material

and data not shown). These data suggest that ICOS expression on donor T cells promotes GC formation and plasma cell differentiation. In kinetic experiments, we observed fewer GC and plasma cells in the recipients of ICOS$^{-/-}$ donor grafts starting at day 30 post-BMT (data not shown), which correlated with a reduction in Tfh cells during cGVHD development (**Figure 2D**). We tested serum autoantibody specific for dsDNA and we found significantly lower levels of total IgG, IgG1, and IgG2c in the sera taken from recipients of ICOS$^{-/-}$ grafts (Figure S1C in Supplementary Material). Taken together, ICOS on donor T cells affects B-cell and plasma cell differentiation and antibody production.

ICOS Promotes Treg Survival and Homeostasis *In Vivo*

To further elucidate the role of ICOS in Tregs, we generated mice with a Foxp3-specific ICOS deletion and performed BMT using Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ or Cre^{-}ICOS$^{fl/fl}$ mice as donors. Given that Foxp3 gene is located on the X chromosome, this allele can be randomly silenced in female mice; we therefore chose male mice as donors to confirm ICOS deletion. Due to decreased CD25^{+}Foxp3^{+} cells in Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ mice (data not shown), we used CD25-depleted donor splenocytes (CD25^{-}SPL) to induce cGVHD. The recipients of CD25^{-}SPL from Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ donors had more severe cGVHD than those with Cre^{-}ICOS$^{fl/fl}$ donors, evidenced by lower body weight maintenance and higher clinical scores (**Figure 4A**). These data suggest that ICOS is required for optimal Treg development and/or function during cGVHD development. To determine at what stage ICOS affects Treg development, we evaluated Foxp3 expression in the thymus of recipients. We observed that frequencies of Foxp3^{+} among CD4^{+}CD8^{-} cells were comparable among cohorts (data not shown), suggesting that ICOS is dispensable for the development of nTregs in recipient thymus. While frequencies of CD4^{+}Foxp3^{+} cells were similar in the spleen (**Figure 4B**), we observed significant reductions in absolute number and survival of Foxp3^{+} cells in the recipients of Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ donor grafts when compared with those of Cre^{-}ICOS$^{fl/fl}$ grafts (**Figure 4B**). These data suggest that ICOS promotes Treg survival but not generation in lymphoid organs. In addition, Tregs derived from Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ donor cells exhibited an activated phenotype, with significantly higher frequencies of CD44^{hi}CD62L^{lo} (effector) cells and lower CD44^{lo}CD62L^{hi} (naïve) cells compared with those derived from Cre^{-}ICOS$^{fl/fl}$ donor cells (**Figure 4C**), suggesting that ICOS is critical for maintaining homeostasis of Tregs.

To corroborate a role of ICOS in effector cell generation, we examined the phenotype of donor T cells and observed that donor CD4 T cells exhibited higher CD44^{hi}CD62L^{lo} frequencies (**Figure 5A**) coupled with higher ICOS expression (**Figure 5B**) but had lower CD44^{lo}CD62L^{hi} frequencies in the recipients of Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ (**Figure 5A**). Moreover, donor CD4 T cells isolated from recipients of Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ produced more pro-inflammatory cytokines, including IFN-γ, IL-17, and IL-21 (**Figure 5C**). Similar results were observed in donor CD8 T cells, although less dramatic (data not shown). These results suggest that ICOS may be required for optimal suppressive function of Tregs.

ICOS Promotes Tfr Development *In Vivo*

Given the requirement for ICOS in Tfr cells (22), we compared the presence of Tfh and Tfr cells in the recipients of Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ or Cre^{-}ICOS$^{fl/fl}$ donor grafts. We observed a higher percentage of Tfh among CD4^{+}Foxp3^{-} cells, but lower percentage of Tfr among CD4^{+}Foxp3^{+} cells, in recipients of Foxp3$^{YFP\text{-}Cre}$ICOS$^{fl/fl}$ grafts (**Figure 6A**). Consistently, B-cell reconstitution (B220^{+}) was significantly decreased (**Figures 6B,C**), whereas B-cell activation (CD40 and CD86 expression) (**Figure 6D**) and GC B and plasma cell differentiation were significantly increased (**Figures 6B,C**), suggesting that ICOS is required for Tfr cells to inhibit B-cell activation and differentiation. In addition, we observed that the percentages of follicular-like CD8 T cells increased and secreted more IL-21 in ICOS-deficient donor Tregs (Figures S2A,B in Supplementary Material). This suggests that Tfr cells can inhibit follicular-like CD8 T cells that promote B-cell differentiation. Overall, these results indicate that ICOS is indispensable for Tfr development and suppressive function.

ICOS Is Required for the Optimal Function and Stability of iTregs *In Vivo*

To further test how ICOS impacts stability and function of alloantigen-reactive iTregs, we stimulated CD25-depleted CD4 T cells from WT or ICOS KO mice with allogeneic APCs under Treg-polarization conditions as previously described (45). We then compared the suppressive function of iTregs between groups pertaining to their ability to suppress the induction of GVHD when co-transplanted with CD25^{-} WT T cells. While WT iTregs were able to significantly alleviate GVHD, ICOS KO iTregs were compromised (**Figures 7A,B**). These results suggest that ICOS is required for the optimal function of iTregs to suppress GVHD development.

To understand the underlying mechanism, we measured molecular markers related to Treg function. When compared with WT counterparts, ICOS$^{-/-}$ iTregs had lower expression of PD-1 and CD39, both known to be positively correlated with Treg suppressive function (46, 47), and a higher expression of CD127, conversely known to negatively impact Treg suppressive function (48) and stability (49) (**Figure 7C**). However, ICOS had a little effect on the expression of CD73, CTLA-4, GITR, and Nrp1 (data not shown). Chemokine receptors are important for Treg migration into areas of inflammation (50). We observed that ICOS$^{-/-}$ iTregs expressed lower levels of chemokine receptor CXCR5 and gut-homing adhesion molecule α4β7 (**Figure 7C**), but did not affect expression of CCR4, CCR5, or CCR9 (data not shown). These data suggest that ICOS promotes iTreg migration to target organs. ICOS$^{-/-}$ iTregs also displayed higher frequencies of 7-AAD^{+} cells compared with WT iTregs in recipient spleens (**Figure 7D**), suggesting that ICOS is crucial for the survival of iTregs *in vivo*. Furthermore, ICOS$^{-/-}$ iTregs exhibited significantly lower percentages of Foxp3, yet higher IFN-γ or IL-4/5 in recipient spleen and liver (**Figure 7E**), suggesting that iTregs are more prone to lose Foxp3 and subsequently differentiate into Th1 or 2 cells.

We then directly compared the suppressive capacity of WT or ICOS$^{-/-}$ iTregs on day 14 post-BMT. We found that WT iTregs had a greater capacity to suppress donor CD4 Teffs (Ly5.1^{+}) (Figure S3A in Supplementary Material), but not Ly5.1^{+}CD8^{+} T cells (data

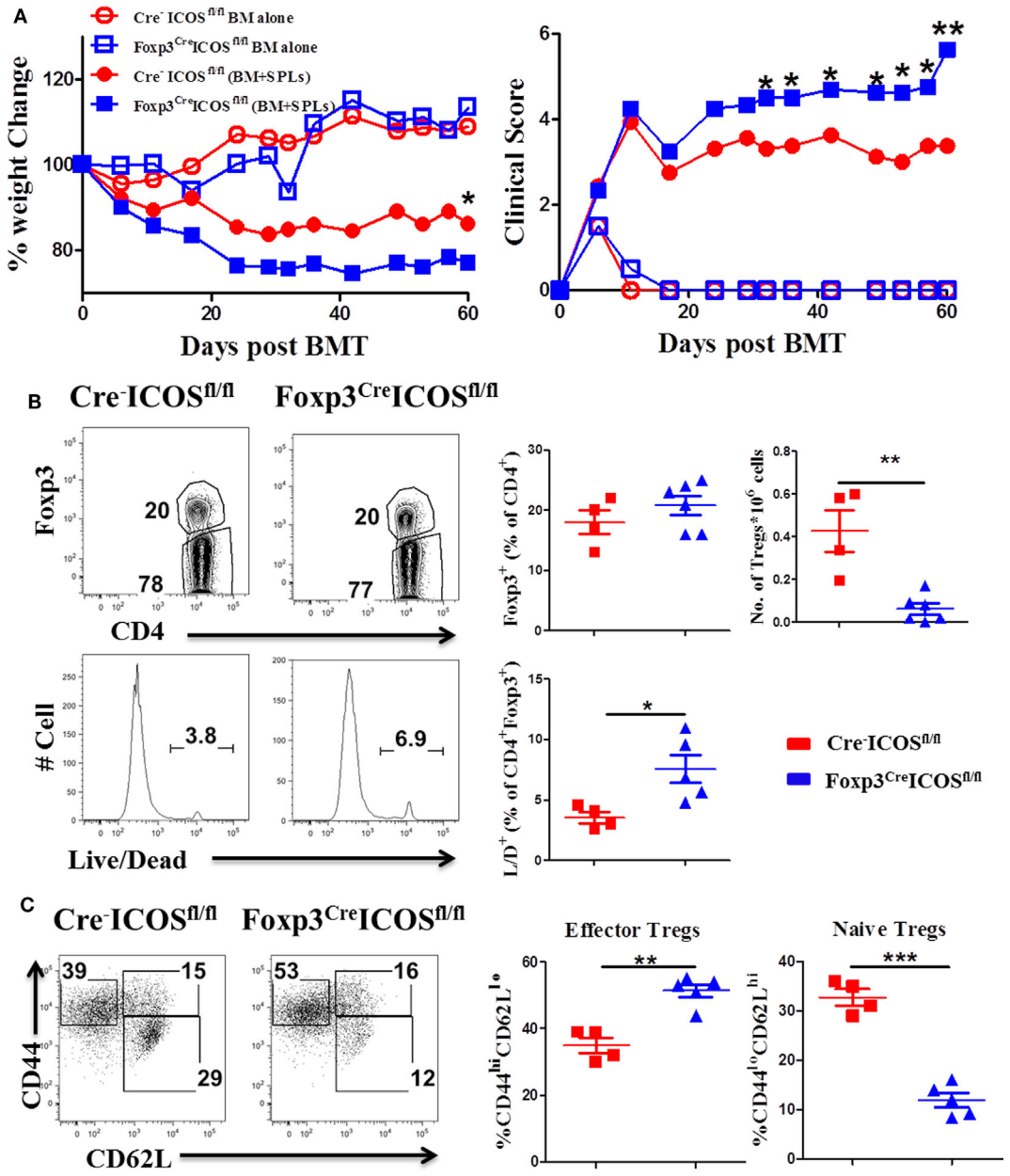


FIGURE 4 | Inducible T-cell co-stimulator (ICOS) is indispensable for regulatory T cells (Tregs) survival and homeostasis in chronic graft-versus-host disease. Lethally irradiated BALB/c mice were transplanted with 0.25 × 10^6 CD25$^-$SPLs and 5 × 10^6 T-cell-depleted bone marrow from Cre$^-$ICOS$^{fl/fl}$ or Foxp3^{Cre}ICOS$^{fl/fl}$ mice on B6 background. Body weight and clinical score **(A)** were monitored weekly, n = 6–8 mice/group. Spleens were harvested at day 60 after bone marrow transplantation (BMT) and subjected to FACS staining. Representative flow images from individual mice and mean percentage of Foxp3 gated on H-2K^{b+}CD4$^+$ T cells and live/dead gated on H-2K^{b+}CD4$^+$ Foxp3$^+$ are shown **(B)**. Representative dot plots from individual mice and dot graphs of mean percentage of CD44^{hi}CD62L^{lo} and CD44^{lo}CD62L^{hi} gated on H2K^{b+}CD4$^+$Foxp3$^+$ T cells are shown **(C)**. n = 4–5 mice/group. *p < 0.05, **p < 0.01, and ***p < 0.001.

not shown). In addition, WT iTregs induced significantly more death on Teffs than ICOS$^{-/-}$ iTregs (Figure S3B in Supplementary Material), suggesting that ICOS facilitates iTreg-mediated death of Teffs. We further evaluated the pro-inflammatory cytokines produced by Teffs and found that ICOS was required for iTregs to inhibit IFN-γ, but not IL-17, production by donor CD4 Teffs (Figure S3C in Supplementary Material). Taken together, these results suggest that ICOS is required for the survival, stability, function, and migration of Tregs *in vivo*.

Treatment of Anti-ICOS Antibody Alleviates cGVHD

To determine the feasibility of clinical translation, we next evaluated whether pharmacologically blocking ICOS could attenuate

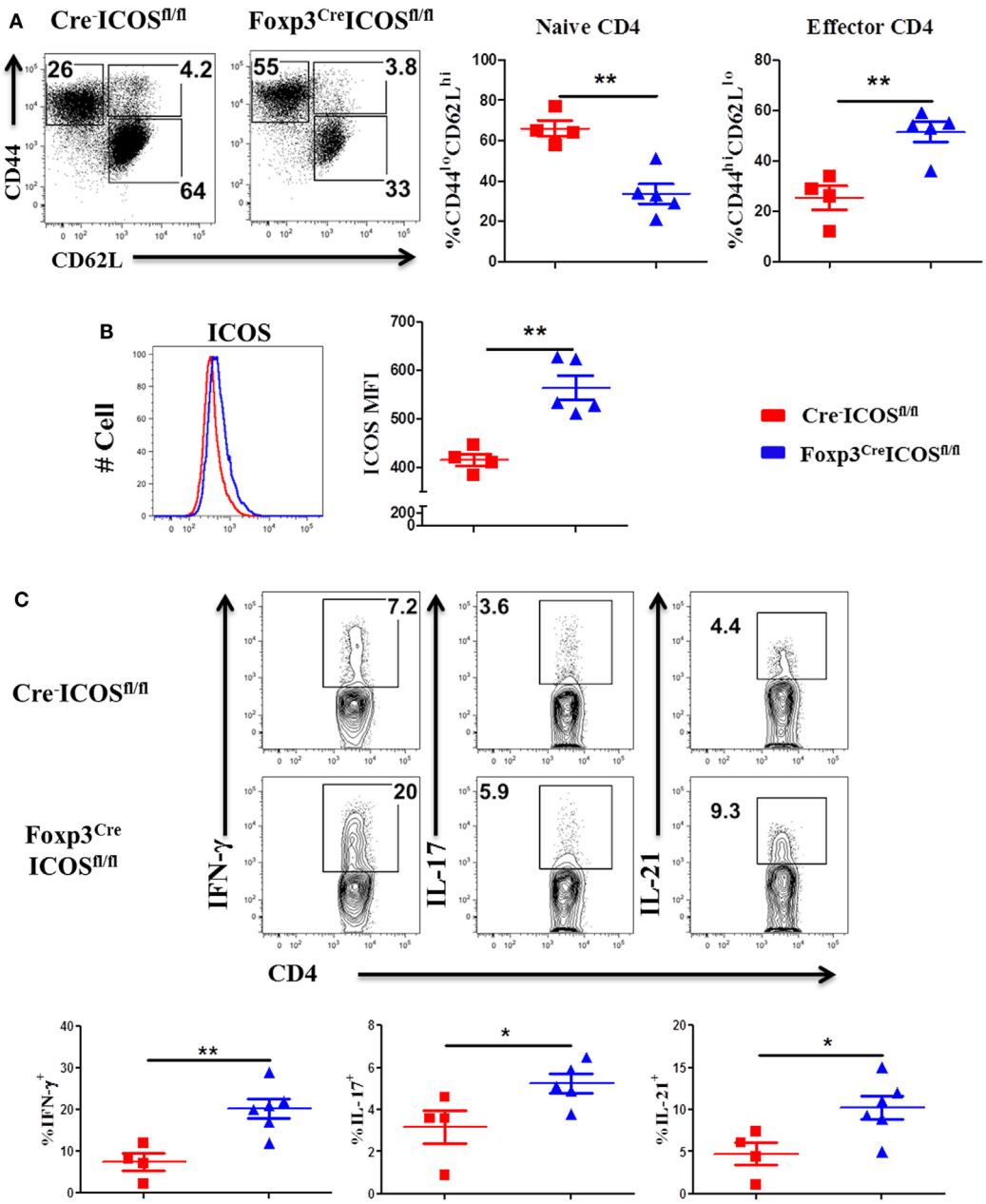


FIGURE 5 | Inducible T-cell co-stimulator (ICOS) on regulatory T cells affects activation of effector T cells. Bone marrow transplantation (BMT) was performed as described in **Figure 4**. Splenocytes were harvested and processed for flow cytometry 60 days after BMT. Representative dot plots from individual mice and mean percentage of $CD44^{hi}CD62L^{lo}$ and $CD44^{lo}CD62L^{hi}$ on gated H-$2K^{b+}CD4^+$ $Foxp3^-$ T cells are shown **(A)**. Representative histogram and mean fluorescence intensity (MFI) of ICOS on gated H-$2K^{b+}CD4^+Foxp3^-$ T cells are shown **(B)**. n = 4–5 mice/group. Single-cell suspension of splenocytes was processed for intracellular cytokine staining. The representative contour plots from individual mice and mean percentage of IFN-γ^+, IL-4/5$^+$, and IL-17$^+$ gated on H-$2K^{b+}CD4^+$ cells are shown **(C)**, n = 4–6 mice/group. *$p < 0.05$ and **$p < 0.01$.

cGVHD severity. As Treg development peaked at day 45 after BMT (**Figure 2D**) and ICOS was required for Treg fitness (**Figure 4**), we chose to administer α-ICOS mAb from day 0 to day 28. When compared with rat-IgG, α-ICOS treatment significantly reduced cGVHD severity, reflected by better body weight maintenance and lower clinical scores (**Figures 8A,B**). Accordingly, α-ICOS treatment reduced fibrosis in recipient skin and lung (**Figures 8C,D**).

To investigate the underlying mechanisms, we evaluated the effects of anti-ICOS on donor T-cell activation and differentiation on day 60 post-BMT. Indeed, treatment with α-ICOS reduced Tfh differentiation, but had no effect on Treg and Tfr (**Figures 9A,B**). This was consistent with preserved thymic function as reflected by percentages of $CD4^+CD8^+$ cells (Figure S4A in Supplementary Material). The recipients treated with α-ICOS

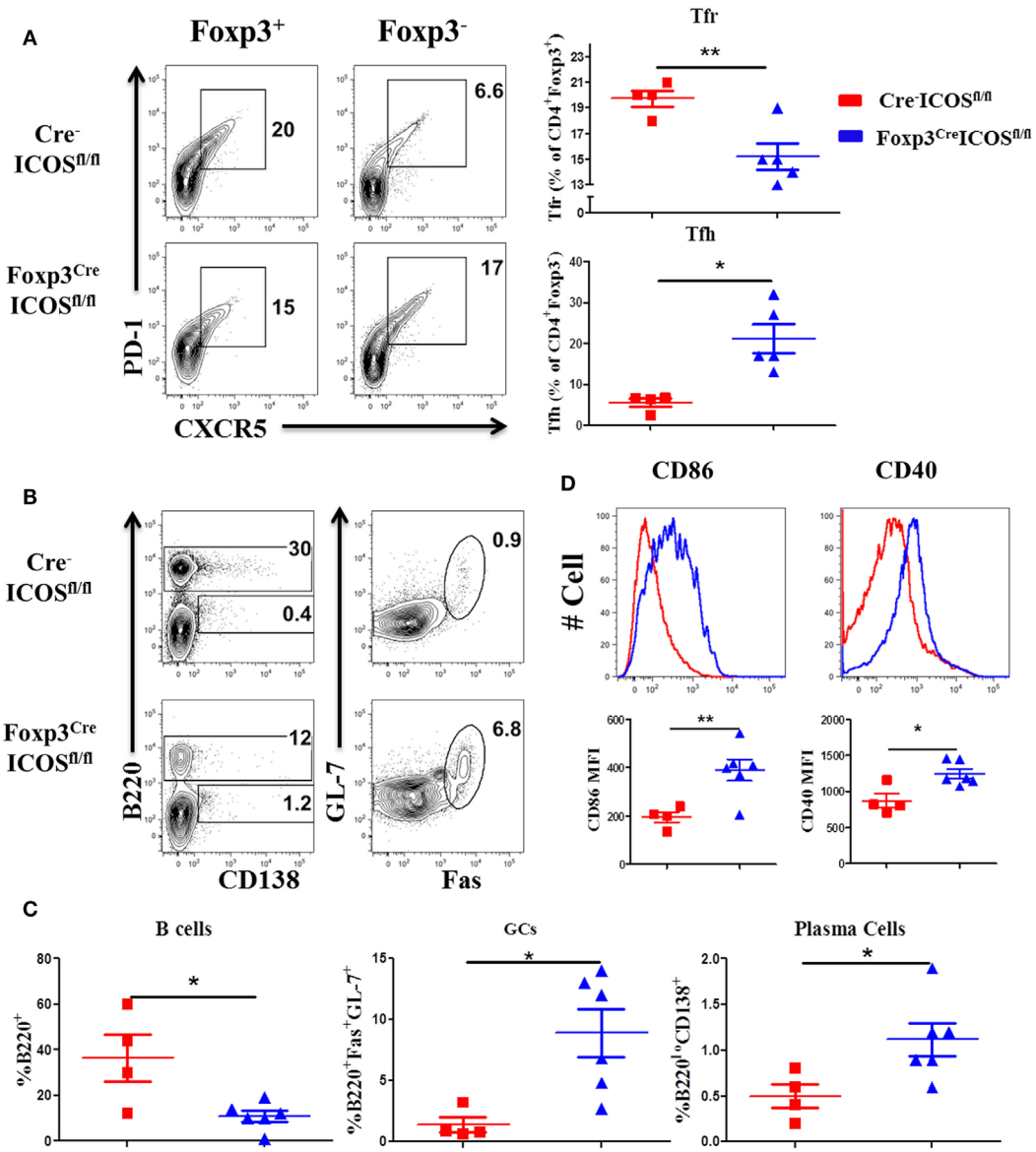


FIGURE 6 | Inducible T-cell co-stimulator (ICOS) promotes follicular regulatory T (Tfr) development. Bone marrow transplantation (BMT) was setup as described in **Figure 4**. Splenocytes were analyzed by flow cytometry at 60 days after BMT. Representative contour plots of individual mice and mean percentage of T follicular helper (Tfh) cells (PD-1$^+$CXCR5$^+$) on gated H-2K^{b+}CD4$^+$Foxp3$^-$ and Tfr cells (PD-1$^+$CXCR5$^+$) on gated H-2K^{b+}CD4$^+$Foxp3$^+$T cells are shown **(A)**, n = 4–5 mice/group. Representative contours plots of each group and mean percentage of B220$^+$ and B220$^-$CD138$^+$ plasma cells on gated H-2K^{b+} cells, GL-7$^+$Fas$^+$ germinal center B cells (GCs) gated on H-2K^{b+}B220$^+$ cells are shown **(B,C)**, n = 4–6 mice/group. Representative histograms and mean fluorescence intensity (MFI) of CD86 and CD40 gated on H-2K^{b+}B220$^+$ cells are shown **(D)**. *p < 0.05 and **p < 0.01.

also had decreased memory T (CD44^{hi}CD62L^{lo}) and increased naïve T-cell (CD44^{lo}CD62L^{hi}) frequencies (**Figures 9C,D**), which are known to be negatively and positively related with cGVHD severity, respectively (43, 51). Furthermore, we observed that α-ICOS treatment improved B-cell reconstitution as reflected by increased frequencies of donor B220$^+$ cells (Figures S4B,C in Supplementary Material), and reduced B-cell activation reflected by lower expression of CD86 (Figure S4D in Supplementary Material). However, α-ICOS treatment did not affect B-cell differentiation into GC and plasma cells. Taken together, α-ICOS treatment after BMT improved cGVHD outcomes by decreasing Teff-cell differentiation while restoring normal B-cell homeostasis and, importantly, by preserving thymic function and Treg development.

DISCUSSION

In this study, we demonstrate a critical role for ICOS in Teffs and Tregs, as well as B cells, in the pathogenesis of cGVHD. ICOS promoted cGVHD by boosting pathogenic T cells, pro-inflammatory

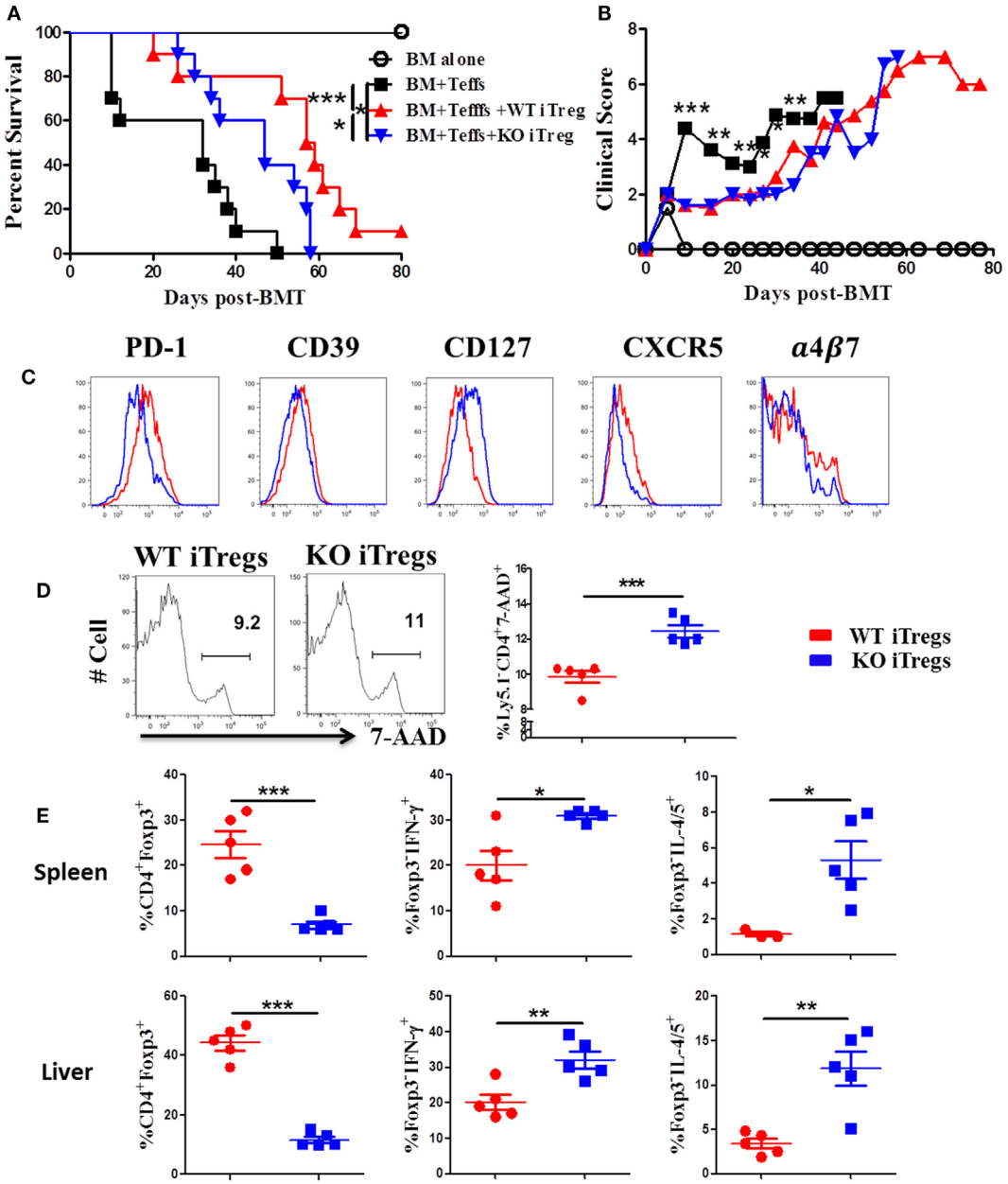


FIGURE 7 | Inducible T-cell co-stimulator (ICOS) is required for optimal function and stability of iTregs *in vivo*. Lethally irradiated BALB/c mice were transplanted with 5×10^6 BM from Rag1$^{-/-}$ B6 plus 0.5×10^6 CD25^{hi}CD4$^+$ cells isolated from wild-type (WT) or ICOS$^{-/-}$ mice (Ly5.1$^-$). Three days later, 0.5×10^6 CD25$^-$Ly5.1$^+$ B6 Teffs were injected into each recipient. Recipients were monitored for survival **(A)** and body weight loss **(B)** for 80 days, $n = 10$ mice/group. In separate experiments, spleens and livers were excised and processed for FACS staining on day 14 after allo-bone marrow transplantation (BMT). Histograms of PD-1, CD39, CD127, CXCR5, and α4β7 expressed on gated H-2K^{b+}Ly5.1$^-$CD4$^+$Foxp3$^+$ in spleen are shown **(C)**. Representative histograms from individual mice and mean percentage of 7-AAD on gated H-2K^{b+}Ly5.1$^-$CD4$^+$Foxp3$^+$ are shown **(D)**. Mean percentage of CD4$^+$Foxp3$^+$ gated on H-2K^{b+}Ly5.1$^-$, and Foxp3$^-$IFN-γ$^+$ and Foxp3$^-$IL-4/5$^+$ gated on H-2K^{b+}Ly5.1$^-$CD4$^+$ cells from spleens and livers are shown **(E)**. $n = 5$ mice/group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

cytokine production, and B-cell differentiation. Conversely, ICOS also facilitated Treg and Tfr development to restrict aberrant T and B cell responses and thus alleviate cGVHD. ICOS was required for Treg development, survival, and homeostasis in cGVHD development. Furthermore, ICOS blockade attenuated the severity of cGVHD by impeding the T-cell response and consequently B-cell differentiation; yet not affecting Treg development. In addition, we implicate follicular-like CD8 T cells, which are inhibited by Tfr, as a contributor to the pathogenic T cell pool in mediating cGVHD, and demonstrated that these cells required ICOS for differentiation. In summary, we found ICOS played a vital role in mediating cGVHD by regulating T and B cell differentiation and response, and that inhibiting ICOS could decrease cGVHD severity and spare Treg development.

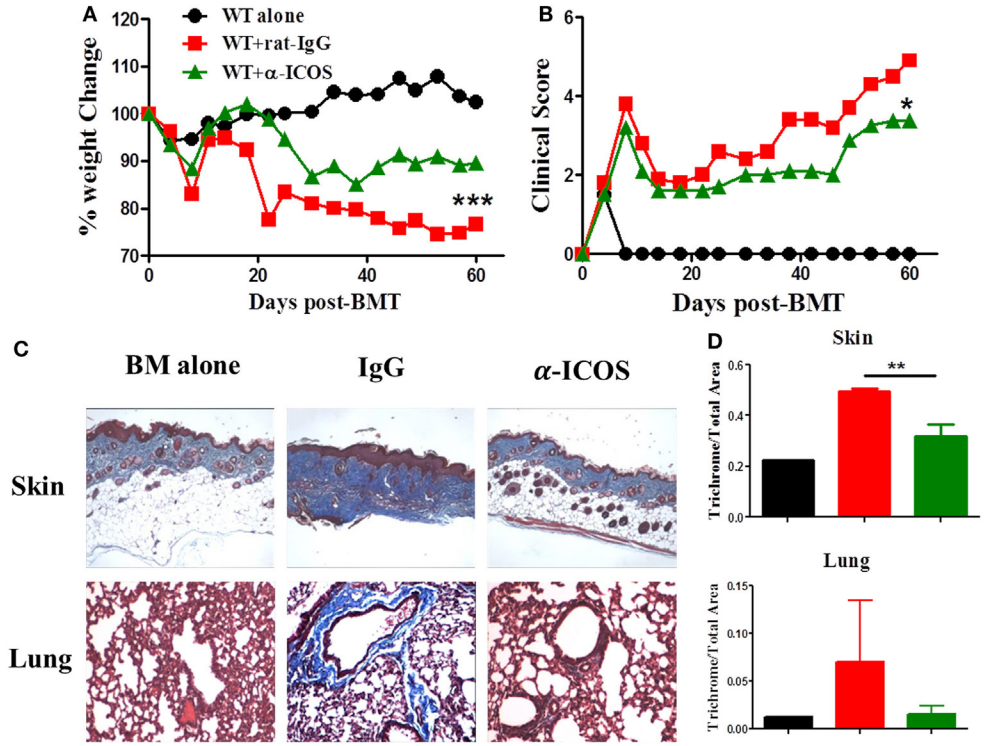


FIGURE 8 | Treatment with anti-inducible T-cell co-stimulator (ICOS) antibodies alleviates chronic graft-versus-host disease. Lethally irradiated BALB/c mice were transferred with 5×10^6 T-cell-depleted bone marrow and 0.5×10^6 SPLs on B6 background. Anti-ICOS Abs were administrated at 200 μg/mouse 3 times/week from day 0 to day 28 after bone marrow transplantation (BMT). Body weight maintenance **(A)** and clinical score **(B)** were monitored for 60 days, $n = 5$ mice/group. Representative pictures of skin and lung stained for collagen 60 days after transplantation are shown **(C)**. Bar graphs of ratios of collagen area to total areas of skin and lung qualified by ImageJ are shown **(D)**. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

It has been reported that ICOS promotes IFN-γ but reduce IL-4 production during the development of aGVHD (30). However, ICOS did not play a significant role in Th1 and Th2 differentiation during cGVHD development. These different results were likely due to the distinct pathophysiology of acute versus chronic GVHD (7). We observed that ICOS$^{-/-}$ donor T cells produced significantly lower IL-17 at day 45 but not in earlier stages (**Figure 3**), which was consistent with reports showing that ICOS is necessary for Th17 development and maintenance (41). Several mechanisms may account for this: (i) ICOS sustained IL-23R expression on Th17 cells through c-Maf (41, 52), (ii) IL-17 secretion was maintained through ICOS-mediated activation of PI3K pathway (21), and (iii) ICOS promoted IL-21 production by Th17 and Tfh cells (41) that maintain Stat3 activation to sustain the Th17 lineage (53, 54). ICOS$^{-/-}$ T cells also have reduced frequencies of Tfh cells and are unable to upregulate B-cell lymphoma 6 (Bcl-6) and c-Maf expression (22, 23). Consistently, we observed that ICOS controlled Tfh cell differentiation during cGVHD development (**Figure 2**). In kinetic experiments, ICOS had a dominant effect on Tfh differentiation 30 days after BMT. We interpret that CD28 regulated early Tfh differentiation, whereas ICOS maintained Tfh phenotype and homing to follicle areas by downregulating Kruppel-like factor2 (55).

Inducible T-cell co-stimulator is required for CD8 T-cell activation and IFN-γ secretion, in part because ICOS triggers IL-2 production (37, 42). Surprisingly, we observed that follicular-like CD8 T cells may also contribute to cGVHD pathogenesis. This population has been reported in models of chronic LCMV infection, which demonstrate secretion of pro-inflammatory cytokines and co-expression of CD28 and ICOS (56). Similarly, we observed that ICOS affects follicular-like CD8 T differentiation (Figure S1A in Supplementary Material). Quigley et al. reported that follicular CD8 T cells were localized in tonsil B cell follicles and supported B cell survival (57). Our data suggest that follicular-like CD8 T cells may function in a way akin to Tfh, specifically through IL-21 (Figure S2 in Supplementary Material)-mediated B-cell differentiation. Nonetheless, more studies are required to confirm the contribution of follicular-like CD8 T cells in cGVHD pathogenesis.

Aberrant donor B-cell differentiation is responsible for cGVHD development (58). Similar impairments in B-cell responses have been reported in both ICOS-deficient mice and humans (27, 28). We also found that the recipients of ICOS$^{-/-}$ donor grafts showed decreased GC development, plasma cell differentiation, and Ig production, which correlated with reduced Tfh cells during cGVHD development. These results confirm that ICOS is necessary to drive Tfh function and subsequently support B-cell differentiation likely *via* cell–cell contact (ICOS:ICOSL) (59) and IL-21 secretion (41, 60–62). ICOS was also shown to regulate extrafollicular Tfh cells that can induce B-cell differentiation into short-lived plasma

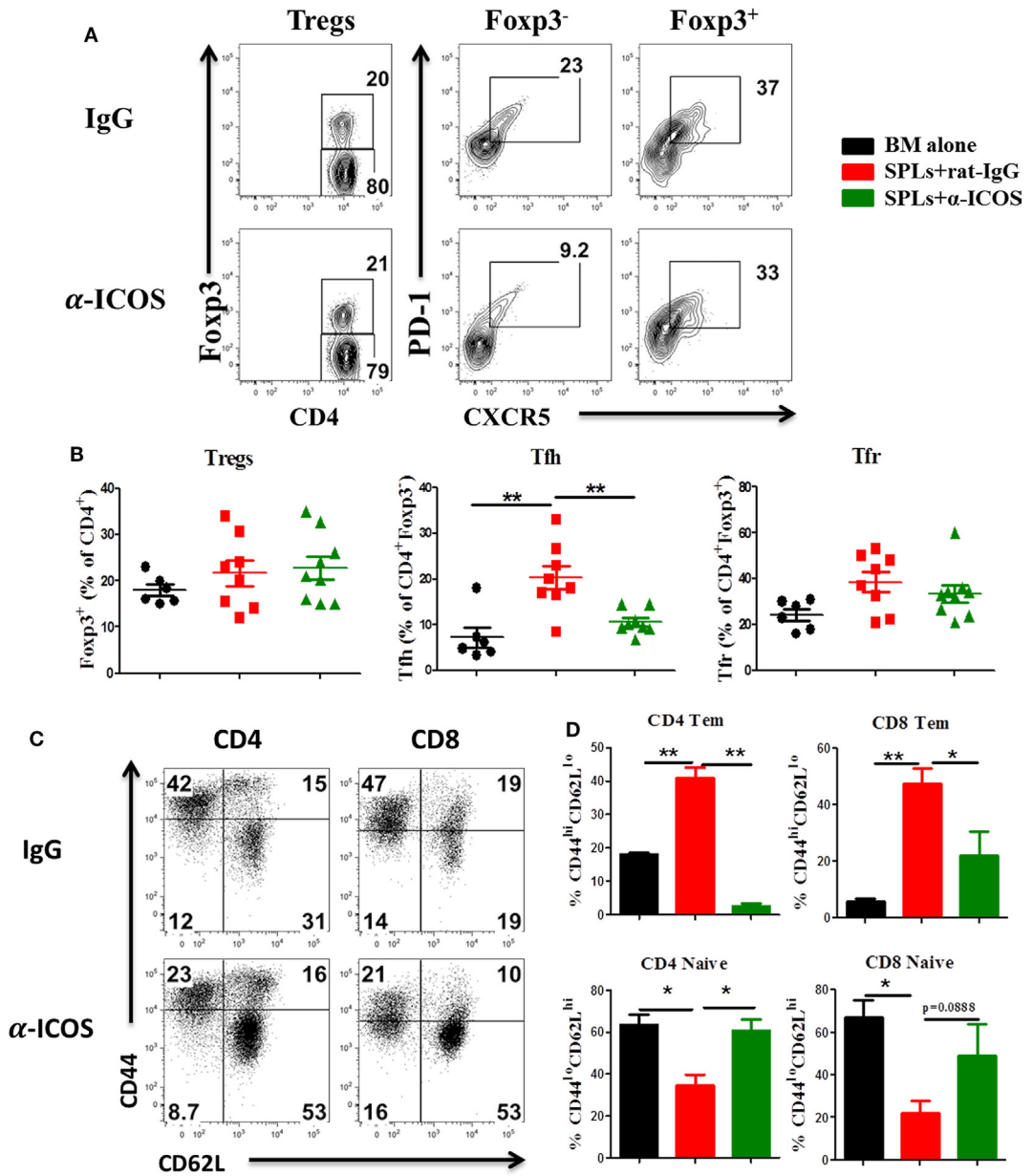


FIGURE 9 | Anti-inducible T-cell co-stimulator (ICOS) antibody treatment reduces effector T cells differentiation. Bone marrow transplantation (BMT) was performed as described in **Figure 8**. Splenocytes were stained and analyzed by flow cytometry at day 60 after BMT. Representative contour plots **(A)** and mean percentage **(B)** of regulatory T cells (Tregs) ($CD4^+Foxp3^+$) on gated donor H-$2K^{b+}$ T cells, T follicular helper (Tfh) cells ($PD\text{-}1^+CXCR5^+$) on gated H-$2K^{b+}CD4^+Foxp3^-$, and follicular regulatory T (Tfr) cells ($PD\text{-}1^+CXCR5^+$) on gated H-$2K^{b+}CD4^+Foxp3^+$ T cells are shown, two experiments were pooled together, $n = 8$–9 mice/group. Representative dot plots **(C)** and mean percentage **(D)** of $CD44^{hi}CD62L^{lo}$ and $CD44^{lo}CD62L^{hi}$ on gated H-$2K^{b+}CD4^+$ and H-$2K^{b+}CD8^+$ T cells are shown, $n = 3$–4 mice/group. $^*p < 0.05$ and $^{**}p < 0.01$.

cells, which then produce autoantibodies in a murine lupus model (63). Thus, we cannot exclude the possibility that a defect in extrafollicular Tfh cell function in the absence of ICOS was responsible for the observed impairment in B-cell response.

Previous studies indicate that ICOS controls Treg development and homeostasis (25). Consistently, we observed lower percentages of Tregs upon transplant with $ICOS^{-/-}$ donor T cells (**Figure 2**). Furthermore, ICOS was not only required for Treg generation but also for Treg homeostasis and survival (**Figure 4**), which could because ICOS promotes Treg sensitivity to IL-2 and hence resulting in better survival and Foxp3 stability (64, 65). $ICOS^{-/-}$ Tregs could not control the increased activation and differentiation of Teffs (**Figure 5**), indicating that ICOS was important for Treg suppressive function, possibly though impaired secretion of the suppressive cytokine IL-10 (24, 66).

Consistent with the improvement in aGVHD observed using anti-ICOS treatment (31), prophylactic anti-ICOS treatment also reduced cGVHD severity by decreasing effector T cells without affecting Treg development (**Figures 8** and **9**). Given ICOS had an impact on Treg generation within 30 days post-BMT, we interpret that prophylactic treatment still allowed Treg generation in the later stages of cGVHD development. Flynn et al. reported that anti-ICOS treatment was sufficient to reverse established cGVHD when administered from day 28 to day 56 post-BMT in a B6 to B10.BR murine model (32). Although the mechanisms accounting for this discrepancy are not clear, we postulate that different BMT models likely contributed to this, rather a *de novo* bronchiolitis cGVHD versus aGVHD to cGVHD transition model.

This study demonstrates that ICOS plays pleiotropic roles in the pathogenesis of cGVHD (Table S1 in Supplementary Material). Post allo-HCT, donor T cells activated *via* interaction with host and/or donor APCs upregulate ICOS in lymphoid tissues. (1) Activated CD4 T cells begin to differentiate into extrafollicular Tfh cells and promote B-cell maturation to short-lived plasma cells that produce auto- or allo-antibodies in the extrafollicular areas. (2) ICOS signaling promotes activated CD4 T cells to express CXCR5 and Bcl-6 and become pre-Tfh cells, which migrate into the follicular area and support B-cell differentiation into long-lived plasma cells by secreting IL-21 and IL-17. (3) Activated T cells also induce Th17 differentiation which produce IL-17 and IL-21 to promote Tfh function and B-cell differentiation into long-lived plasma cells. (4) In response to ICOS signaling, CD8 T cells begin to differentiate and expand; activated CD8 T cells secrete cytokines and/or cytotoxic molecules to induce cGVHD and express CXCR5 and PD-1 which then migrate to follicular areas to promote B cell differentiation into long-lived plasma cells. (5) These short-lived and/or long-lived plasma cells produce auto- and allo-antibodies that deposit into cGVHD target organs to induce cGVHD. (6) Th17 cells secrete cytokines such as IL-17 and IL-21 to facilitate fibroblast maturation and collagen production that subsequently deposits in target organs during cGVHD. (7) On the other hand, ICOS also promotes CD4 T cell differentiation into Tregs, which suppress pre-Tfh, Th17, and CD8 T cells, as well as fibroblasts through cell–cell contact (ICOS:ICOSL) supplemented by inhibitory cytokine secretion (TGF-β and IL-10); ICOS can also induce CXCR5 and Bcl-6 expression on Tregs and promote Tregs to migrate into follicular areas, dubbed Tfr cells, which inhibit Tfh, B cells responses, as well as plasma cells and antibody production (**Figure 10**). Although Treg and Tfr cells suppress pathogenic T and B cells, they cannot completely contain this response, thus the resultant effect of ICOS expression is exacerbated cGVHD. Our data provide rational to target ICOS for cGVHD prophylaxis in clinic, despite its pluralistic role in T-cell activation and differentiation.

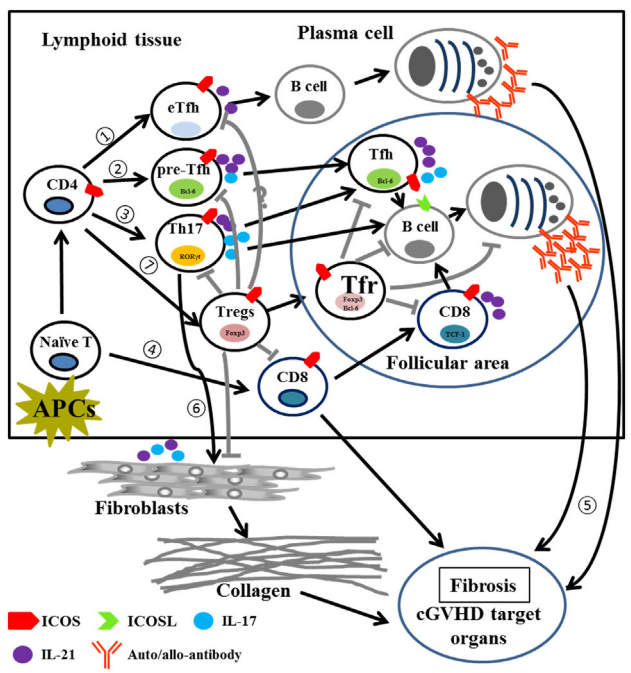


FIGURE 10 | Proposed model for the role of ICOS in pathogenic T cells and Tregs in cGVHD. Abbreviations: APCs, antigen-presenting cells; eTfh, extrafollicular T helper cells; Bcl-6, B-cell lymphoma 6; Foxp3, forkhead box P3; RORγt, retinoic acid receptor-related (RAR) orphan; TCF-1, T cell factor 1; cGVHD, chronic graft-versus-host disease; ICOS, inducible T-cell co-stimulator; Tregs, regulatory T cells.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "NIH Guide for Care and Use of Laboratory Animal, Institutional Animal Care and Use of Committee." The protocol was approved by the "Institutional Animal Care and Use of Committee."

AUTHOR CONTRIBUTIONS

MZ participated in experimental design, performed research, collected, analyzed and interpreted data, performed statistical analysis, and drafted and revised the manuscript. YW, DB, SI, JC, AD, HN, MD, SS, and MS performed research, collected and analyzed data, and edited the manuscript. FC interpreted data and edited manuscript. W-KS participated in experiment design, interpreted data, and edited the manuscript. X-ZY designed research, interpreted data, and revised the manuscript. All the authors read and approved the submitted version.

ACKNOWLEDGMENTS

The authors would like to thank to the Flow Cytometry Core, Small Animal Imaging Core at Medical University of South Carolina for their assistance. This work is partially supported by CIHR-84544 (W-KS), NIH grants NIH R01s CA169116, AI118305 and HL1937373, and SmartState Endowment in Cancer Stem Cell Biology & Therapy Program (X-ZY).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01461/full#supplementary-material.

REFERENCES

1. Arai S, Arora M, Wang T, Spellman SR, He W, Couriel DR, et al. Increasing incidence of chronic graft-versus-host disease in allogeneic transplantation: a report from the Center for International Blood and Marrow Transplant Research. *Biol Blood Marrow Transplant* (2015) 21(2):266–74. doi:10.1016/j.bbmt.2014.10.021
2. Jagasia M, Giglia J, Chinratanalab W, Dixon S, Chen H, Frangoul H, et al. Incidence and outcome of chronic graft-versus-host disease using National Institutes of Health consensus criteria. *Biol Blood Marrow Transplant* (2007) 13(10):1207–15. doi:10.1016/j.bbmt.2007.07.001
3. Flowers ME, Martin PJ. How we treat chronic graft-versus-host disease. *Blood* (2015) 125(4):606–15. doi:10.1182/blood-2014-08-551994
4. Lee SJ. Classification systems for chronic graft-versus-host disease. *Blood* (2017) 129(1):30–7. doi:10.1182/blood-2016-07-686642
5. Sarantopoulos S, Blazar BR, Cutler C, Ritz J. B cells in chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2015) 21(1):16–23. doi:10.1016/j.bbmt.2014.10.029
6. Zeiser R, Blazar BR. Pathophysiology of chronic graft-versus-host disease and therapeutic targets. *N Engl J Med* (2017) 377(26):2565–79. doi:10.1056/NEJMra1703472
7. MacDonald KP, Hill GR, Blazar BR. Chronic graft-versus-host disease: biological insights from preclinical and clinical studies. *Blood* (2017) 129(1):13–21. doi:10.1182/blood-2016-06-686618
8. Rozmus J, Schultz KR, Wynne K, Kariminia A, Satyanarayana P, Krailo M, et al. Early and late extensive chronic graft-versus-host disease in children is characterized by different Th1/Th2 cytokine profiles: findings of the Children's Oncology Group Study ASCT0031. *Biol Blood Marrow Transplant* (2011) 17(12):1804–13. doi:10.1016/j.bbmt.2011.05.011
9. Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR. Absence of regulatory T-cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease. *Blood* (2007) 110(10):3804–13. doi:10.1182/blood-2007-05-091074
10. Presland RB. Biology of chronic graft-vs-host disease: immune mechanisms and progress in biomarker discovery. *World J Transplant* (2016) 6(4):608–19. doi:10.5500/wjt.v6.i4.608
11. Bruggen MC, Klein I, Greinix H, Bauer W, Kuzmina Z, Rabitsch W, et al. Diverse T-cell responses characterize the different manifestations of cutaneous graft-versus-host disease. *Blood* (2014) 123(2):290–9. doi:10.1182/blood-2013-07-514372
12. Lim JY, Cho BS, Min CK, Park G, Kim YJ, Chung NG, et al. Fluctuations in pathogenic CD4+ T-cell subsets in a murine sclerodermatous model of chronic graft-versus-host disease. *Immunol Invest* (2014) 43(1):41–53. doi:10.3109/08820139.2013.843191
13. Nguyen V, Luzina I, Rus H, Tegla C, Chen C, Rus V. IL-21 promotes lupus-like disease in chronic graft-versus-host disease through both CD4 T cell- and B cell-intrinsic mechanisms. *J Immunol* (2012) 189(2):1081–93. doi:10.4049/jimmunol.1200318
14. Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood* (2005) 106(8):2903–11. doi:10.1182/blood-2005-03-1257
15. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* (2011) 29:621–63. doi:10.1146/annurev-immunol-031210-101400
16. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol* (2015) 36(7):410–8. doi:10.1016/j.it.2015.05.005
17. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* (2005) 23:515–48. doi:10.1146/annurev.immunol.23.021704.115611
18. Wikenheiser DJ, Stumhofer JS. ICOS co-stimulation: friend or foe? *Front Immunol* (2016) 7:304. doi:10.3389/fimmu.2016.00304
19. Paulos CM, Carpenito C, Plesa G, Suhoski MM, Varela-Rohena A, Golovina TN, et al. The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Sci Transl Med* (2010) 2(55):55ra78. doi:10.1126/scitranslmed.3000448
20. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* (2005) 6(11):1133–41. doi:10.1038/ni1261
21. Gao X, Gigoux M, Yang J, Leconte J, Yang X, Suh WK. Anti-chlamydial Th17 responses are controlled by the inducible costimulator partially through phosphoinositide 3-kinase signaling. *PLoS One* (2012) 7(12):e52657. doi:10.1371/journal.pone.0052657
22. Leavenworth JW, Verbinnen B, Yin J, Huang H, Cantor H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* (2015) 16(1):96–106. doi:10.1038/ni.3050
23. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* (2011) 34(6):932–46. doi:10.1016/j.immuni.2011.03.023
24. Redpath SA, van der Werf N, Cervera AM, MacDonald AS, Gray D, Maizels RM, et al. ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production during helminth infection. *Eur J Immunol* (2013) 43(3):705–15. doi:10.1002/eji.201242794
25. Burmeister Y, Lischke T, Dahler AC, Mages HW, Lam KP, Coyle AJ, et al. ICOS controls the pool size of effector-memory and regulatory T cells. *J Immunol* (2008) 180(2):774–82. doi:10.4049/jimmunol.180.5.3613
26. Takahashi N, Matsumoto K, Saito H, Nanki T, Miyasaka N, Kobata T, et al. Impaired CD4 and CD8 effector function and decreased memory T cell populations in ICOS-deficient patients. *J Immunol* (2009) 182(9):5515–27. doi:10.4049/jimmunol.0803256
27. Warnatz K, Bossaller L, Salzer U, Skrabl-Baumgartner A, Schwinger W, van der Burg M, et al. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood* (2006) 107(8):3045–52. doi:10.1182/blood-2005-07-2955
28. McAdam AJ, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, et al. ICOS is critical for CD40-mediated antibody class switching. *Nature* (2001) 409(6816):102–5. doi:10.1038/35051107
29. Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, et al. ICOS is essential for effective T-helper-cell responses. *Nature* (2001) 409(6816):105–9. doi:10.1038/35051113
30. Hubbard VM, Eng JM, Ramirez-Montagut T, Tjoe KH, Muriglan SJ, Kochman AA, et al. Absence of inducible costimulator on alloreactive T cells reduces graft versus host disease and induces Th2 deviation. *Blood* (2005) 106(9):3285–92. doi:10.1182/blood-2005-01-0410
31. Li J, Semple K, Suh WK, Liu C, Chen F, Blazar BR, et al. Roles of CD28, CTLA4, and inducible costimulator in acute graft-versus-host disease in mice. *Biol Blood Marrow Transplant* (2011) 17(7):962–9. doi:10.1016/j.bbmt.2011.01.018
32. Flynn R, Du J, Veenstra RG, Reichenbach DK, Panoskaltsis-Mortari A, Taylor PA, et al. Increased T follicular helper cells and germinal center B cells are required for cGVHD and bronchiolitis obliterans. *Blood* (2014) 123(25):3988–98. doi:10.1182/blood-2014-03-562231
33. Panneton V, Bagherzadeh Yazdchi S, Witalis M, Chang J, Suh WK. ICOS signaling controls induction and maintenance of collagen-induced arthritis. *J Immunol* (2018) 200(9):3067–76. doi:10.4049/jimmunol.1701305
34. Wu T, Young JS, Johnston H, Ni X, Deng R, Racine J, et al. Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. *J Immunol* (2013) 191(1):488–99. doi:10.4049/jimmunol.1300657
35. Schutt SD, Fu J, Nguyen H, Bastian D, Heinrichs J, Wu Y, et al. Inhibition of BTK and ITK with Ibrutinib is effective in the prevention of chronic graft-versus-host disease in mice. *PLoS One* (2015) 10(9):e0137641. doi:10.1371/journal.pone.0137641
36. Taylor PA, Panoskaltsis-Mortari A, Freeman GJ, Sharpe AH, Noelle RJ, Rudensky AY, et al. Targeting of inducible costimulator (ICOS) expressed on alloreactive T cells down-regulates graft-versus-host disease (GVHD) and facilitates engraftment of allogeneic bone marrow (BM). *Blood* (2005) 105(8):3372–80. doi:10.1182/blood-2004-10-3869
37. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* (2001) 409(6816):97–101. doi:10.1038/35051100
38. Guo F, Iclozan C, Suh WK, Anasetti C, Yu XZ. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. *J Immunol* (2008) 181(4):2285–91. doi:10.4049/jimmunol.181.4.2285
39. Juchem KW, Anderson BE, Zhang C, McNiff JM, Demetris AJ, Farber DL, et al. A repertoire-independent and cell-intrinsic defect in murine GVHD

induction by effector memory T cells. *Blood* (2011) 118(23):6209–19. doi:10.1182/blood-2011-01-330035
40. Rezvani K, Mielke S, Ahmadzadeh M, Kilical Y, Savani BN, Zeilah J, et al. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood* (2006) 108(4):1291–7. doi:10.1182/blood-2006-02-003996
41. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol* (2009) 10(2):167–75. doi:10.1038/ni.1690
42. Watanabe M, Hara Y, Tanabe K, Toma H, Abe R. A distinct role for ICOS-mediated co-stimulatory signaling in CD4+ and CD8+ T cell subsets. *Int Immunol* (2005) 17(3):269–78. doi:10.1093/intimm/dxh206
43. Grogan BM, Tabellini L, Storer B, Bumgarner TE, Astigarraga CC, Flowers ME, et al. Activation and expansion of CD8(+) T effector cells in patients with chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2011) 17(8):1121–32. doi:10.1016/j.bbmt.2011.03.007
44. Young JS, Wu T, Chen Y, Zhao D, Liu H, Yi T, et al. Donor B cells in transplants augment clonal expansion and survival of pathogenic CD4+ T cells that mediate autoimmune-like chronic graft-versus-host disease. *J Immunol* (2012) 189(1):222–33. doi:10.4049/jimmunol.1200677
45. Heinrichs J, Li J, Nguyen H, Wu Y, Bastian D, Daethanasanmak A, et al. CD8(+) Tregs promote GVHD prevention and overcome the impaired GVL effect mediated by CD4(+) Tregs in mice. *Oncoimmunology* (2016) 5(6):e1146842. doi:10.1080/2162402X.2016.1146842
46. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* (2007) 110(4):1225–32. doi:10.1182/blood-2006-12-064527
47. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* (2010) 236:219–42. doi:10.1111/j.1600-065X.2010.00923.x
48. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* (2006) 203(7):1701–11. doi:10.1084/jem.20060772
49. Simonetta F, Chiali A, Cordier C, Urrutia A, Girault I, Bloquet S, et al. Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells. *Eur J Immunol* (2010) 40(9):2528–38. doi:10.1002/eji.201040531
50. Sawant DV, Vignali DA. Once a Treg, always a Treg? *Immunol Rev* (2014) 259(1):173–91. doi:10.1111/imr.12173
51. Yamashita K, Choi U, Woltz PC, Foster SF, Sneller MC, Hakim FT, et al. Severe chronic graft-versus-host disease is characterized by a preponderance of CD4(+) effector memory cells relative to central memory cells. *Blood* (2004) 103(10):3986–8. doi:10.1182/blood-2003-09-3286
52. Schaefer JS, Montufar-Solis D, Vigneswaran N, Klein JR. ICOS promotes IL-17 synthesis in colonic intraepithelial lymphocytes in IL-10-/- mice. *J Leukoc Biol* (2010) 87(2):301–8. doi:10.1189/jlb.0409238
53. Deenick EK, Tangye SG. Autoimmunity: IL-21: a new player in Th17-cell differentiation. *Immunol Cell Biol* (2007) 85(7):503–5. doi:10.1038/sj.icb.7100114
54. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* (2007) 282(48):34605–10. doi:10.1074/jbc.M705100200
55. Weber JP, Fuhrmann F, Feist RK, Lahmann A, Al Baz MS, Gentz LJ, et al. ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med* (2015) 212(2):217–33. doi:10.1084/jem.20141432
56. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* (2016) 537(7620):417–21. doi:10.1038/nature19330
57. Quigley MF, Gonzalez VD, Granath A, Andersson J, Sandberg JK. CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. *Eur J Immunol* (2007) 37(12):3352–62. doi:10.1002/eji.200636746
58. Allen JL, Fore MS, Wooten J, Roehrs PA, Bhuiya NS, Hoffert T, et al. B cells from patients with chronic GVHD are activated and primed for survival via BAFF-mediated pathways. *Blood* (2012) 120(12):2529–36. doi:10.1182/blood-2012-06-438911
59. Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, et al. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* (2015) 517(7533):214–8. doi:10.1038/nature13803
60. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* (2008) 29(1):127–37. doi:10.1016/j.immuni.2008.06.001
61. Rasheed MA, Latner DR, Aubert RD, Gourley T, Spolski R, Davis CW, et al. Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. *J Virol* (2013) 87(13):7737–46. doi:10.1128/JVI.00063-13
62. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* (2010) 207(2):353–63. doi:10.1084/jem.20091738
63. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* (2008) 205(12):2873–86. doi:10.1084/jem.20080840
64. Kornete M, Sgouroudis E, Piccirillo CA. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. *J Immunol* (2012) 188(3):1064–74. doi:10.4049/jimmunol.1101303
65. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in Treg cell function. *Nat Immunol* (2016) 17(11):1322–33. doi:10.1038/ni.3540
66. Chen Y, Shen S, Gorentla BK, Gao J, Zhong XP. Murine regulatory T cells contain hyperproliferative and death-prone subsets with differential ICOS expression. *J Immunol* (2012) 188(4):1698–707. doi:10.4049/jimmunol.1102448

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership