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Interferons and graft-versus-host disease

Edited by
Jaebok Choi, Markus Y. Mapara and Byungsuk Kwon

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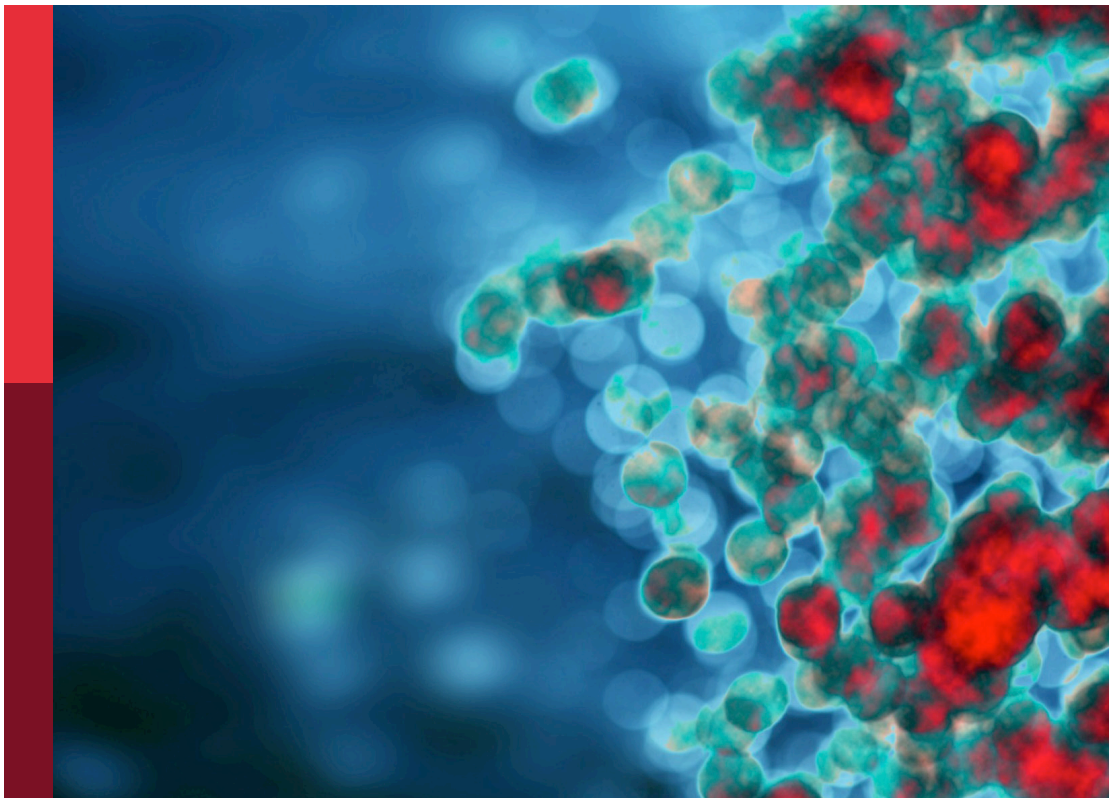

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Interferons and graft-versus-host disease

Topic editors

Jaebok Choi — Washington University in St. Louis, United States
Markus Y. Mapara — Columbia University, United States
Byungsuk Kwon — University of Ulsan, Republic of Korea

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EDITORIAL
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Editorial: Interferons and GvHD

*Boram Kim and Jaebok Choi**

Division of Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, MO, United States

Keywords: interferons, GvHD, allogeneic hematopoietic cell transplant (alloHCT), JAK - STAT signaling pathway, JAK inhibitors, LYG1, CD27, CD70

Editorial on the Research Topic

Interferons and Graft-versus-Host Disease

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the most effective treatment and curative approach for patients with hematological malignancies. The life-saving benefits of all-HSCT include graft-versus-leukemia (GvL) effects and healthy immune reconstitution. However, the majority of patients develops graft-vs-host-disease (GvHD), leading to organ/tissue damages in the skin, liver, and GI tract. For that reason, extensive researches on GvHD have been made and identified IFNs as therapeutic targets. Nonetheless, the pleiotropic nature of IFNs has hindered our goals to optimally control GvHD.

The goals of the articles collected in the Research Topic are to define the roles and mechanisms of IFNs in GvHD and to provide insights into novel therapeutic approaches to restrain GvHD while enhancing the benefits of allo-HSCT.

To expand the investigation on mechanisms of primary human T cell responses in GvHD, xenogeneic cell transplant models have been utilized. In depth, Hess et al. highlighted the development of humanized mouse models and xenogeneic transplant model system, leading to investigation on human T cell biology. The models essentially benefit from the human T cell receptors (TCRs) having cross-reactivity to murine MHC in addition to cytokines and co-stimulatory proteins. Hess et al. further explained the importance of TCR, the ability to recognize non-self-antigens on MHC, and cellular therapy by suppressing or enhancing the secretion of cytokines and co-stimulatory/inhibitory signaling pathways.

In the review by Zhao et al. the authors discussed the effects of IFNs on T cell and their function and epigenetic regulations during GvHD. They elaborated the paradoxical roles of IFNs in alloreactive T cell differentiation and function and the development of GvHD. The process of GvHD is also critically controlled by epigenetic regulators, such as DNA methylation, histone modification, and chromatin remodeling. In turn, these regulators modulate T cell differentiation and function by influencing IFN signaling and therapeutic potentials. Conclusively, the authors suggest future studies on epigenetic mechanisms of IFNs to better understand how IFNs regulate GvHD.

Not only essential for differentiation and expansion of immune cells, IFNs play an important role in the GI tract. Haring et al. explored the pathophysiology of GvHD caused by the imbalance of cytokine network in the GI tract. The authors summarize that similar to IBD, type I and type III IFNs are important for maintaining the intestinal epithelial barrier integrity and controlling immune responses in GvHD. However, the conditioning regimen prior to allo-HSCT damages the epithelial cells and results in the release of DAMPs and PAMPs, leading to local inflammation together with inflammatory cytokines and activation of APCs. This in turn results in the activation and expansion of the alloreactive T cells causing further tissue destruction and inflammation together with cytokines including type II IFN and chemokines in the GI tract. Overall, the review

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

***Correspondence:**
Jaebok Choi
jchoi25@wustl.edu

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shows the highly complex and interconnected cytokines network and the effect of its imbalance on intestinal mucosal inflammation, suggesting that identifying targets downstream of IFNs and receptors might provide novel therapeutic strategies for GvHD and IBD.

The use of JAK inhibitors is a promising therapeutic strategy for GvHD and cytokine release syndrome. Assal and Mapara comprehensively reviewed the role of the JAK/STAT pathway in T cell activation and expansion, APC function, and Tregs expansion. The knowledge led to the idea of disrupting the JAK/STAT pathway by using ruxolitinib or baricitinib to ameliorate GvHD. Clinical evidence has also shown that ruxolitinib administration to GvHD patients has been successful. The study by Wang et al. also showed similar results; in the retrospective study, 70 Chinese patients with steroid-refractory chronic GvHD (SR-cGvHD) received ruxolitinib, then the overall response, complete remission, and partial remission rates were examined. Twenty-four weeks after ruxolitinib treatment, those rates were significantly improved, compared to those of the patient group without ruxolitinib treatment. The authors also showed that $CD4^+$ cells, total B cells, and IL-10 were significantly increased after the treatment, whereas NK cells, Tregs, and ST2 were significantly decreased. Collectively, these results suggest that ruxolitinib is a safe and effective treatment for SR-cGvHD.

Targeting TCR and co-stimulatory pathways also serves as a therapeutic strategy to control GvHD. In the review by Lutfi et al. authors illustrated the CD27-CD70 co-stimulatory pathway and its therapeutic potential in combination with immune checkpoint inhibitors and in GvHD. The CD27-CD70 pathway leads to survival and activation of T, B, and NK cells, and the activity is shown to be increased under pro-inflammatory conditions and IFN-γ secretion. With the understanding of its nature, studies to targeting the CD27-CD70 pathway to improve outcomes have been made. For instance, a CD27 agonizing monoclonal antibody, varlilumab, showed to improve antineoplastic response in combination with immune checkpoint inhibitors by enhancing $CD8^+$ T cell expansion and proliferation. On the other hand, an administration of anti-CD70 monoclonal antibody and CD70 KO mice resulted in increased GvHD. Further studies explained that while APC-expressed CD70 provides a co-stimulatory signal, T-cell-expressed CD70 served an inhibitory role in T-cell response. These findings proposed that this complex mechanism may provide a potential therapeutic intervention as an oncologic therapy and to attenuate GvHD by modulating the pathway.

Previous studies suggest that recombinant human LYG1 protein (rhLYG1) can promote the activation and IFN-γ production of $CD4^+$ T cells, thereby inhibiting tumor growth. Liu et al. in turn hypothesized that LYG1 participated in the development of GvHD and explored the role and mechanisms of LYG1 during GvHD. As hypothesized, the study discovered that LYG1 deficiency in donor T cells reduced the severity of GvHD. In depth, LYG1 deficiency in donor T cells inhibited the activation of $CD4^+$ T cells and expression of IFN-γ, while promoting the expression of FOXP3, thereby suppressing the expression of CXCL9 and CXCL10 and the infiltration of allogeneic $CD4^+$ T cells into target organs. Despite of a modest improvement in the overall survival by LYG1 blockade, these findings demonstrate that targeting LYG1 may be as a potential therapeutic strategy to reduce GvHD.

Huisman et al. provide significant insight on the risk of off-target toxicity *via* allo-HLA across-reactivity. Using third-party donor-derived virus-specific T cells, Huisman et al. investigated whether HLA-restriction, HLA background, and/or virus-specificity could predict the risk of allo-HLA cross-reactivity. The results demonstrated that HLA-B*08:01-restricted T cells, had the highest allo-HLA cross-reactivity regardless of virus-specificity, suggesting a potential strategy to reduce the risk of off-target toxicity by selecting T cells with a specific HLA restriction and background.

Overall, the articles in the Research Topic provided sufficient reviews on the recent advances in the roles of IFNs in GvHD. They also highlighted promising therapeutic strategies, such as CD27 agonizing monoclonal antibodies, JAK inhibitors, and targeting LYG1, to modulate IFN signaling to restrain GvHD while preserving the benefits of all-HSCT. Indeed, further researches should be followed to reveal the exact mechanisms of IFNs in the context of transplantation to provide promising transplantation therapies.

AUTHOR CONTRIBUTIONS

BK and JC wrote the manuscript. JC is a guest associate editor of the Research Topic. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Magnitude of Off-Target Allo-HLA Reactivity by Third-Party Donor-Derived Virus-Specific T Cells Is Dictated by HLA-Restriction

Wesley Huisman[1,2]*, *Didier A. T. Leboux*[1], *Lieve E. van der Maarel*[1], *Lois Hageman*[1], *Derk Amsen*[2], *J. H. Frederik Falkenburg*[1] *and Inge Jedema*[1]

[1] Department of Hematology, Leiden University Medical Center, Leiden, Netherlands, [2] Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory for Blood Cell Research, Amsterdam, Netherlands

T-cell products derived from third-party donors are clinically applied, but harbor the risk of off-target toxicity *via* induction of allo-HLA cross-reactivity directed against mismatched alleles. We used third-party donor-derived virus-specific T cells as model to investigate whether virus-specificity, HLA restriction and/or HLA background can predict the risk of allo-HLA cross-reactivity. Virus-specific $CD8^{pos}$ T cells were isolated from HLA-A*01:01/B*08:01 or HLA-A*02:01/B*07:02 positive donors. Allo-HLA cross-reactivity was tested using an EBV-LCL panel covering 116 allogeneic HLA molecules and confirmed using K562 cells retrovirally transduced with single HLA-class-I alleles of interest. HLA-B*08:01-restricted T cells showed the highest frequency and diversity of allo-HLA cross-reactivity, regardless of virus-specificity, which was skewed toward multiple recurrent allogeneic HLA-B molecules. Thymic selection for other HLA-B alleles significantly influenced the level of allo-HLA cross-reactivity mediated by HLA-B*08:01-restricted T cells. These results suggest that the degree and specificity of allo-HLA cross-reactivity by T cells follow rules. The risk of off-target toxicity after infusion of incompletely matched third-party donor-derived virus-specific T cells may be reduced by selection of T cells with a specific HLA restriction and background.

Keywords: virus-specific T cells, allo-HLA cross-reactivity, adoptive T cell immunotherapy, HLA-mismatched donor, cytotoxic T cells, Graft Versus Host Disease

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Antoine Toubert, Université Paris Diderot, France

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Federico Simonetta, Geneva University Hospitals (HUG), Switzerland
Benedetto Bruno, University of Turin, Italy

***Correspondence:**
Wesley Huisman
w.huisman@lumc.nl

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KEY POINTS

- HLA-restriction determines the scope of off-target reactivities.
- HLA background shapes the broadness of off-target reactivities.

INTRODUCTION

Adoptive transfer of autologous or human leukocyte antigen (HLA)-matched patient-specific T-cell products, including antigen-specific T cells, Chimeric Antigen Receptor (CAR) T cells and T-cell receptor (TCR) modified T cells are clinically applied and show feasibility and safety (1–5). Nevertheless, the complex logistics and delays associated with the generation of these products for adoptive immunotherapy strategies are hampering easy broad application. Off-the shelf T-cell

products, generated from cells of healthy third-party donors and suitable for treatment of multiple patients, may be an elegant solution, but such products are often only partially HLA-matched with the recipient.

In our study we focused on virus-specific T-cell products derived from healthy third-party donors that can be used for the treatment of uncontrolled viral reactivations and/or viral disease in patients without easy access to autologous or donor-derived virus-specific T cells. Reactivations of cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus (AdV) are frequently seen and associated with high morbidity and mortality in immune-compromised patients (6, 7), like patients after allogeneic stem cell transplantation (AlloSCT), but also patients after solid organ transplantation. For patients transplanted with stem cells from a virus-non-experienced donor or in general for solid-organ donors there is no easy access to (HLA-matched) memory virus-specific T cells. Adoptive transfer of partially HLA-matched virus-specific T cells from healthy third-party donors is a potential strategy to temporarily provide anti-viral immunity to these patients. However, these third party donor-derived virus-specific T cells have not been tolerized by thymic negative selection to the non-matched HLA molecules that are present within the patient (8, 9), thereby implying the risk of off-target toxicity due to allo-HLA cross-reactivity directed against the mismatched HLA alleles (10).

It was demonstrated that third-party derived virus-specific T cells can exert allo-HLA cross-reactivity directed against mismatched HLA alleles *in vitro* (11–14). The viral specificity as well as the allo-HLA cross-reactivity was shown to be mediated by the same T-cell receptor (TCR) complex (11, 14). Additionally, TCR cross-reactivity could be a major trigger of graft rejection, as shown by the association between viral reactivation and graft rejection in recipients of solid organs (15, 16). Despite the clearly documented allo-HLA cross-reactivity of virus-specific T-cell populations documented *in vitro*, only low rates (∼5%) of off-target toxicity/*de novo* Graft vs. Host Disease (GVHD) were observed in stem cell recipients that were treated with partially HLA-matched virus-specific T cells (17–21). There are several potential reasons for this discrepancy: (1) the specific allogeneic peptide/HLA complex recognized by the cross-reactive virus-specific T cells is not present in the patient, (2) removal of the *in vitro* off-target (>10% cytotoxic) virus-specific T cells from the product prior to administration to the patient and/or selection of T-cell products that do not show *in vitro* allo-HLA reactivity (18), (3) low T-cell numbers of cross-reactive virus-specific T cells administered and/or limited *in vivo* proliferation, (4) Rejection of the partly HLA-matched third party virus-specific T cells by the recipient (22). In the last example, such rejection prevents toxicity, but it also diminishes the short-term protection afforded by the third-party derived T cells. In a recent phase I/II clinical study by Neuenhahn et al., survival/persistence was only demonstrated for adoptively transferred virus-specific T cells of the original stem cell donor (8/8 HLA-matched), but not for virus-specific T cells derived from third-party donors with a higher degree of HLA-mismatch (22).

It would be useful if we could predict which non-matched HLA molecules are recognized by third-party derived T cells so that specific donors and/or specific T-cell populations can be selected with a low likelihood of exerting off-target reactivity. Thus far, recurrent off-target reactivity toward the same non-matched HLA molecule was only found for T-cell populations isolated from different individuals using the exact same TCR (public TCR) (14, 23, 24). A classic example of such public cross-reactivity is the HLA-B*08:01-restricted EBV-EBNA3A^{FLR}-specific T-cell population that contains a dominant public TCR showing cross-reactivity against non-self HLA-B*44:02 (23, 24). Importantly, this public TCR is not found in the T-cell repertoire of HLA-B*08:01/HLA-B*44:02 positive individuals, demonstrating the deletion of this otherwise potentially auto-reactive public TCR during *in vivo* thymic selection. Many antiviral T-cell responses are, however, not so clearly dominated by a single dominant public TCR, making predictions of cross-reactivity more difficult.

The aim of this study was to investigate whether we could identify and predict allo-HLA cross-reactivity patterns by third-party donor-derived T cells, using virus-specific T cells as a model. We investigated whether the allo-HLA cross-reactivity by third-party donor-derived virus-specific T cells was influenced by virus-specificity, HLA-restriction and/or HLA background of the donors. Our data show that the level of allo-HLA cross-reactivity is not affected by viral-specificity, but surprisingly strongly associated with HLA restriction and influenced by the HLA background of the donors.

MATERIALS AND METHODS

Collection of Donor Material

After informed consent according to the Declaration of Helsinki, healthy donors (homozygously) expressing HLA-A*01:01 and HLA-B*08:01 or HLA-A*02:01 and HLA-B*07:02 were selected from the Sanquin database and the biobank of the department of Hematology, Leiden University Medical Center (LUMC). Two donors expressing HLA-A*02:01/HLA-B*07:02 were not homozygous. Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Isopaque separation and used directly or thawed after cryopreservation in the vapor phase of liquid nitrogen. Donor characteristics (HLA typing, CMV and EBV serostatus) are provided in **Table 1** (Donors 1–24). Healthy donors expressing HLA-B*08:01 and HLA-B*13:02 or HLA-B*35:01 (**Table 1**; donors 25–30) were selected from the biobank of the department of Hematology (LUMC).

Isolation and Expansion of Virus-Specific T Cells

Phycoerythrin (PE), allophycocyanin (APC), BV421, BV510 and/or peridinin-chlorophyll-protein (PerCP)-labeled pMHC-tetramer complexes were used for fluorescence-activated cell sorting (FACSorting). The pMHC-tetramers used (for generation see **Supplementary Material and Methods**) are shown in **Supplementary Table 1**. PeptideMHC-tetramer positive, CD8^{pos}/CD4^{neg} T cells were sorted and seeded at 10,000 cells per well in U-bottom microtiter plates for the generation of bulk T-cell populations. After 2 weeks of culture,

TABLE 1 | HLA typing and CMV/EBV serostatus of healthy donors.

#	CMV	EBV	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
1	Pos	Pos	01:01		08:01		07:01		03:01	15:01	02:01	06:02	N.D	
2	Pos	Pos	01:01		08:01		07:01		03:01	01:02	02:01	05:01	01:01	04:01
3	Neg	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
4	Pos	Pos	01:01		08:01		07:01		03:01		02:01		04:01	
5	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	
6	Neg	Pos	01:01		08:01		07:01		03:01		02:01		01:01	09:01
7	Pos	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
8	Neg	Pos	01:01		08:01		07:01		03:XX		02:XX		N.D	
9	Pos	Pos	01:01		08:01		07:01		03:01		02:01		01:01	04:01
10	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	04:02/01
11	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	05:01
12	Neg	Pos	01:01		08:01		07:01		03:01		02:01		04:01	05:01
13	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
14	Pos	Pos	02:01		07:02	44:02	07:02	05:01	15:01	04:01	06:02	03:01	04:XX	02:01
15	Pos	Pos	02:01	03:01	07:02		07:02		15:01		06:02		04:01	03:01
16	Pos	Pos	02:01	03:01	07:02	44:02	07:02	05:01	15:01	01:01	06:02	05:01	04:01	14:01
17	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	05:01
18	Pos	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
19	Neg	Pos	02:01		07:02		07:02		15:01		06:02		04:01	
20	Pos	Pos	02:01		07:02		07:02		15:01		06:02		02:01	04:01
21	Neg	Pos	02:01		07:02		07:02		15:01		06:02		04:01	13:01
22	Pos	Pos	02:01		07:02		07:02		07:01	15:01	03:03	06:02	04:01	13:01
23	Neg	Pos	02:01		07:02		07:02		15:XX		06:XX		N.D	
24	Pos	Pos	02:01		07:02		07:02		15:XX		06:XX		N.D	
25	Pos	Pos	01:01	68:01	08:01	35:01	04:01	07:01	01:01	03:01	02	05:01	04:01	04:02
26	Neg	pos	01:01	24:02	08:01	35:01	04:01	07:01	03:01	08:01	02:01	04:02	04:01	
27	Pos	pos	02:01	24:02	08:01	35:01	07:01	11:01	02:02	03:01	02:02	03:01	02:01	13:01
28	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	03:01	04:01	02:01	03:01	04:01	
29	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	04:07	15:01	03:01	06:02	04:01	
30	Pos	pos	01:01	30:01	08:01	13:02	07:01	06:02	03:01	07:01	02:01	02:02	04:01	09:01

*Virus-specific T cells restricted to HLA-A*01:01/HLA-B*08:01 or HLA-A*02:01/HLA-B*07:02 were isolated from donors # 1–12 and donors # 13–24, respectively. CMV and EBV serostatus are indicated for each donor. HLA typing was determined either by serology, where the second digits could not be determined (XX) or with high resolution HLA typing, unless indicated by N.D. HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T cells were additionally isolated from donors # 25–30 for specific experiments to investigate the role of HLA-backgrounds. Blanks indicate homozygosity for the given allele.*

pMHC-tetramer^{pos} T-cell populations were considered pure if they contained ≥97% pMHC-tetramer^{pos} cells. Polyclonality of the sorted virus-specific T cells was assessed by T-cell receptor-variable β (TCR-Vβ) family analysis using the TCR-Vβ kit (Beckman Coulter, Fullerton, USA). Sub-populations expressing a single TCR-Vβ family were sorted from the bulk using monoclonal antibodies from the TCR-Vβ kit. Sub-populations were then non-specifically expanded. Sub-populations using one specific TCR-Vβ family were considered pure if ≥95% of the population was positive for that TCR-Vβ family. Sorting was performed on a FACS ARIA (BD) and analyzed using Diva software (BD). All analyses were performed on a FACS Calibur (BD), and analyzed using Flowjo Software (TreeStar, Ashland, USA). Procedures to isolate and expand virus-specific T cells are described in the **Supplementary Material and Methods**.

Selection and Generation of Stimulator Cells for Functional Analyses

EBV-transformed lymphoblastoid cell-lines (EBV-LCLs) were generated according to standard protocols (25). EBV-LCLs were selected to cover a total of 116 frequently occurring HLA molecules, as listed in **Table 2**. HLA-deficient K562 cells were transduced with 40 different single HLA-class-I molecules (**Supplementary Table 2**) including common and rare HLA-class-I molecules. EBV-LCLs and HLA-deficient K562 cell-lines were cultured in stimulator medium consisting of Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, USA), 100 U/mL penicillin (Lonza), 100 μg/mL streptavidin (Lonza) and 2.7 mM L-glutamine (Lonza). Generation of various cell-lines is described in the **Supplementary Material and Methods**.

TABLE 2 | HLA typing of the HLA-mismatched EBV-LCL panel.

EBV-LCL	HLA-A		HLA-B		HLA-C		HLA-DR		HLA-DQ		HLA-DP	
UBX	01:01	03:01	08:01	18:01	01:02	07:01	03:01	10:XX	02:01	05:01	01:01	02:01
ACD	01:01	24:02	39:06	51:01	07:02	14:02	01:02	11:01	03:01	05:01	04:01	04:02
GME	26:01	02:06	38:01	35:01	12:03	04:01	04:04	01:01	05:01	03:02	04:01	04:02
ABF	30:04	68:02	38:01	55:01	03:03	12:03	03:01	13:01	02:01	06:03	02:01	04:01
LSR	32:01	68:01	35:03	52:01	12:02	12:03	15:02	16:02	05:02	06:01	04:01	14:01
GML	23:01		41:01	51:01	15:02	17:01	07:01	15:01	02:01	06:02	02:01	04:01
UCE	03:01	11:01	07:02	27:05	02:02	07:02	11:01	14:54	03:01	05:03	02:01	16:01
GMS	01:01	11:01	51:01	50:01	15:02	06:02	07:01	04:07	02:01	03:01	03:01	02:01
WKD	11:01	24:02	15:02	40:01	07:02	08:01	08:03	09:XX	03:03	06:01	05:01	
UVN	03:01	11:01	14:02	35:01	04:01	08:02	01:01	13:02	05:01	06:09	05:01	10:01
MWX	01:01	34:01	15:21	35:03	04:03	12:03	01:01	15:02	05:01	06:01	06:01	13:01
GMK	01:01		07:02	57:01	06:02	07:02	04:04	13:01	03:02	06:03	04:02	15:01
MSV	03:01	33:01	07:02	14:02	07:02	08:02	01:02	04:05	03:03	05:01	02:01	04:01
CBF	02:01	11:01	35:01	44:02	04:01	05:01	03:01	09:01	02:01	03:03	01:01	04:01
AVZ	02:20	24:02	08:01	14:01	07:01	08:02	03:01	07:01	02:01	02:02	02:01	04:01
BSR	02:01	68:01	35:03	37:01	04:01	06:02	04:03	10:01	03:01	05:01	02;01	04:01
RHP	03:01	31:01	07:02	40:01	03:04	07:02	13:02	15:01	06:02	06:04	04:01	13:01
SOM	02:60	23:01	15:10	57:03	03:04	18:02	11:01	13:01	03:01	05:01	04:02	40:01
UBM	03:01	24:02	15:01	44:03	03:04	16:01	04:01	07:01	02:02	03:02	03:01	
UBG	02:01	30:02	15:01	39:01	03:03	12:03	01:01	13:01	05:04	06:03	02:01	04:01
LMB	29:02		44:03	51:01	14:02	16:01	07:01	08:01	02:02	04:02	04:01	11:01
FAQ	23:01	68:02	14:02	38:01	08:02	12:03	13:01	13:03	03:01	06:03	02:01	
OBB	01:01	02:01	07:02	08:01	07:01	07:02	03:01	15:01	02:01	06:02	01:01	05:01

The panel of HLA-mismatched EBV-LCLs was composed of high resolution HLA-typed EBV-LCLs that together covered all HLA-class-I and almost all frequently HLA-class-II molecules that are frequently (2%) occurring in the Caucasian population. The HLA typing was determined molecularly. XX indicates that only the allele group could be determined (2 digit resolution). Blanks indicate homozygosity for the given allele.

Cytokine Production Assays to Determine T-Cell Reactivity

Interferon-γ (IFN-γ) production by virus-specific T cells was quantified using standard enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (Sanquin Reagents, The Netherlands). Responder T cells were co-cultured with stimulator cells at a ratio of 1:10 (responder: stimulator) for 16 h at 37°C in T-cell medium used for expansion of T-cell populations as described in **Supplementary Material** using 25 IU/ml Interleukin-2 (IL-2) instead of 100 IU/ml IL-2. Recognition of HLA-mismatched EBV-LCLs, HLA-matched peptide-pulsed EBV-LCLs and K562 cells transduced with specific HLA molecules was defined as production of ≥200 pg/ml of IFN-γ.

Statistical Analysis

Statistical analyses were only performed on quantitative data and were performed using non-parametric tests. The Fisher's-Exact-test was used to assess the differences in cross-reactivity (present or absent) of HLA-mismatched EBV-LCLs between groups (i.e., HLA-A*01:01- and HLA-B*08:01-restricted virus-specific T cells). Differences in the numbers of recognized HLA-mismatched EBV-LCLs was first assessed by the Kruskal-Wallis test. Differences between two groups were then further assessed with the non-parametric Mann-Whitney U test. *p*-values were adjusted by the Bonferroni correction for multiple testing. Statistical analyses were conducted using GraphPad Prism (GraphPad Software, version 8).

RESULTS

Virus-Specific T-Cell Populations Show Profound and Diverse Cross-Reactivity Against a Panel of HLA-Mismatched EBV-LCLs

To study the influence of HLA restriction and antigen specificity on the level of allo-HLA cross-reactivity mediated by virus-specific T cells, bulk virus-specific T-cell populations targeting single epitopes were isolated from total PBMCs of (homozygous) HLA-A*01:01/HLA-B*08:01^{pos} or HLA-A*02:01/HLA-B*07:02^{pos} healthy donors. Two donors did not homozygously express HLA-A*02:01/HLA-B*07:02. All donors were EBV seropositive and 5 out of 12 HLA-A*01:01/HLA-B*08:01^{pos} donors and 9 out of 12 HLA-A*02:01/HLA-B*07:02^{pos} donors were CMV seropositive (**Table 1**). The serostatus for AdV was unknown for all donors. Virus-specific T cells were isolated by FACS using pMHC-tetramers for various peptides ($n = 21$) from CMV, EBV, and AdV (**Supplementary Table 1**). In total,

TABLE 3 | Isolated virus-specific T-cell populations.

				Number of isolated T-cell populations/maximum number of isolations (%)		
Virus	**Antigen**	**HLA**	**Peptide**	**Per specificity**	**Per virus**	**Per HLA**
CMV	pp50	HLA-A*01:01	VTEHDTLLY	4/5 (80%)	**CMV:** 45/56 (80.3%)	**HLA-A*01:01:** 22/28 (78.6%)
	pp65	HLA-A*01:01	YSEHPTFTSQY	4/5 (80%)		
	pp65	HLA-A*02:01	NLVPMVATV	7/9 (78%)		
	IE-1	HLA-A*02:01	VLEETSVML	5/9 (56%)		
	pp65	HLA-B*07:02	TPRVTGGGAM	8/9 (89%)		
	pp65	HLA-B*07:02	RPHERNGFTVL	8/9 (89%)		**HLA-A*02:01:** 69/90 (76.7%)
	IE-1	HLA-B*08:01	ELRRKMMYM	5/5 (100%)		
	IE-1	HLA-B*08:01	QIKVRVDMV	4/5 (80%)		
EBV	LMP2	HLA-A*01:01	ESEERPPTPY	5/6 (83%)	**EBV:** 95/114 (83.3%)	
	LMP2	HLA-A*02:01	FLYALALLL	11/12 (92%)		
	LMP2	HLA-A*02:01	CLGGLLTMV	9/12 (75%)		
	EBNA3C	HLA-A*02:01	LLDFVRFMGV	7/12 (58%)		**HLA-B*07:02**: 34/42 (81%)
	BMLF1	HLA-A*02:01	GLCTLVAML	10/12 (83%)		
	BRLF1	HLA-A*02:01	YVLDHLIVV	12/12 (100%)		
	EBNA3A	HLA-B*07:02	RPPIFIRRL	11/12 (92%)		
	BZLF1	HLA-B*08:01	RAKFKQLL	9/12 (75%)		
	EBNA3A	HLA-B*08:01	FLRGRAYGL	10/12 (83%)		**HLA-B*08:01:** 39/46 (84.8%)
	EBNA3A	HLA-B*08:01	QAKWRLQTL	11/12 (92%)		
AdV	HEXON	HLA-A*01:01	TDLGQNLLY	9/12 (75%)	**AdV:** 24/36 (66.7%)	
	E1A	HLA-A*02:01	LLDQLIEEV	8/12 (67%)		
	HEXON	HLA-B*07:02	KPYSGTAYNAL	7/12 (58%)		

*Twelve donors were used to isolate HLA-A*01:01/B*08:01-restricted virus-specific T-cell populations and 12 donors were used to isolate HLA-A*02:01/B*07:02-restricted virus-specific T-cell populations. Five out of 12 HLA-A*01:01/B*08:01^{pos} donors were seropositive for CMV and 9 out of 12 HLA-A*02:01/B*07:02^{pos} donors were seropositive for CMV. CMV, Cytomegalovirus; EBV, Epstein-Barr virus; AdV, Adenovirus.*

45 CMV, 95 EBV and 24 AdV-specific T-cell populations were isolated (**Table 3**). CMV and EBV-specific T-cell populations could be isolated from all CMV^{pos} and EBV^{pos} donors, respectively. Although no AdV serostatus was known, AdV-specific T-cell populations could be isolated from 18 out of 24 donors. We isolated 22 different HLA-A*01:01-restricted virus-specific T-cell populations, 69 HLA-A*02:01-restricted virus-specific T-cell populations, 34 HLA-B*07:02-restricted virus-specific T-cell populations and 39 HLA-B*08:01-restricted virus-specific T-cell populations (**Table 3**). These T-cell populations were analyzed for allo-HLA cross-reactivity using a panel of HLA-mismatched EBV-LCLs, expressing the most frequent (>2%) HLA-class-I and class-II antigens in the Caucasian population (**Table 2**). EBV-specific T-cell populations were only tested against HLA-mismatched EBV-LCLs that did not express the specific restriction molecules to avoid recognition of EBV-derived peptides in self-HLA. In total, 65 out of 164 (39%) virus-specific T-cell populations produced interferon-γ in response to stimulation with at least one HLA-mismatched EBV-LCL (**Supplementary Figure 1A**).

Next, we investigated whether the T-cell populations that did not recognize any HLA-mismatched EBV-LCL contained smaller sub-population(s) of T cells that could recognize HLA-mismatched EBV-LCLs, but were missed in the initial bulk analysis. Sub-populations were sorted based on expression of single TCR-Vβ families. Twenty-four different TCR-Vβ families can be identified with the provided monoclonal antibodies in the kit that was used for flow cytometry, covering around 70% of the human TCR-Vβ repertoire (26). Sub-populations that could not be stained with the antibody kit could not be separated from the bulk populations using this strategy and were not analyzed for recognition of HLA-mismatched EBV-LCLs. In total, 165 sub-populations expressing a single TCR-Vβ family were isolated from the 99 bulk T-cell populations that initially did not show reactivity against the EBV-LCL panel. These sub-populations were subsequently analyzed for their capacity to recognize HLA-mismatched EBV-LCLs. We observed that 31 of these isolated sub-populations contained T cells that were capable of exerting allo-HLA cross-reactivity (**Supplementary Figure 1B**). Additionally, 193 sub-populations were sorted from bulk T-cell populations that did already demonstrate HLA-mismatched EBV-LCL recognition in the initial analysis (derived from 65 initial bulk populations). Eighty-six of these isolated sub-populations contained T cells that recognized HLA-mismatched EBV-LCLs. Recognition of additional HLA-mismatched EBV-LCLs could be observed that were not detected in the initial analysis of 25 different bulk T-cell populations (**Supplementary Figure 2**). In summary, a total of 83

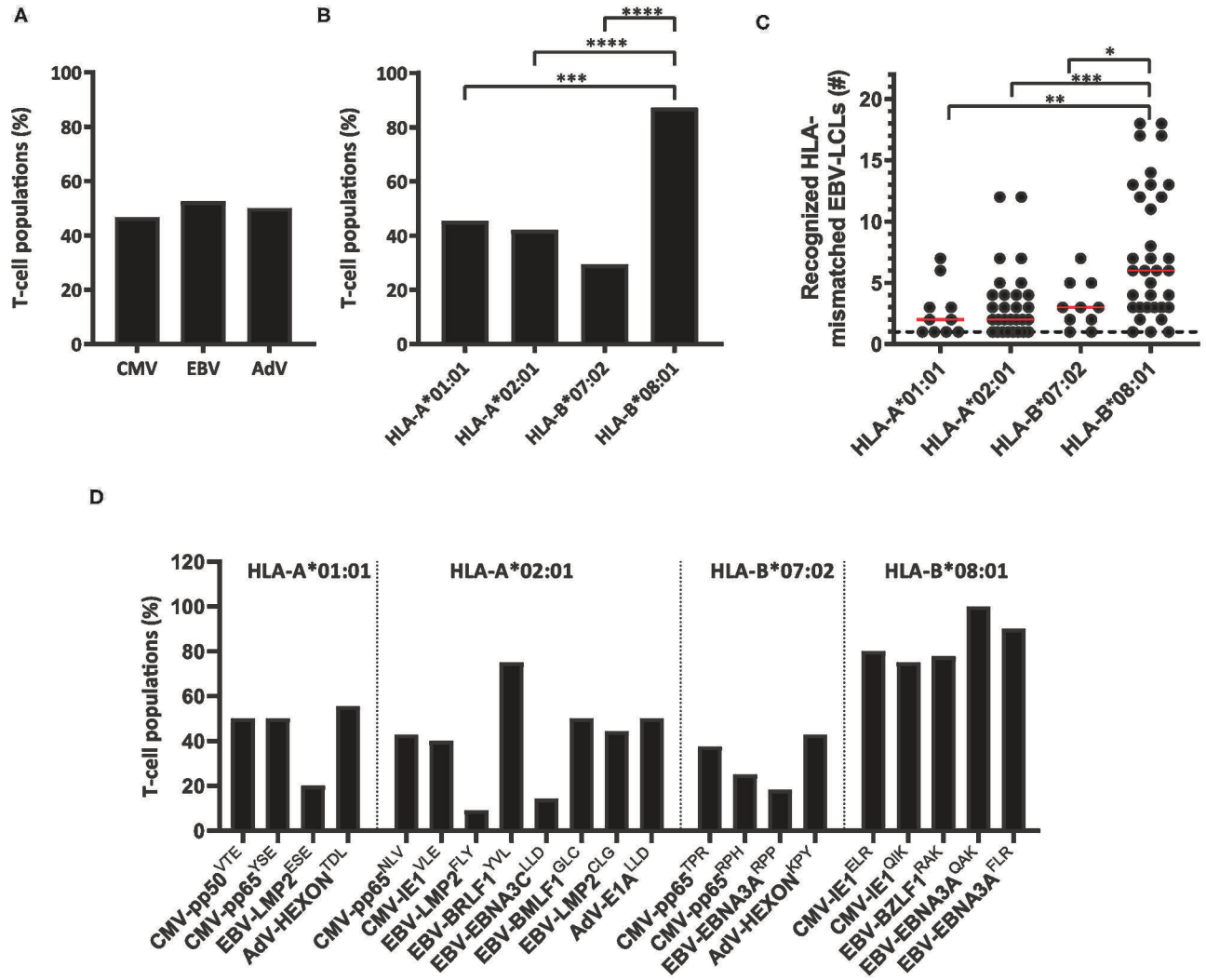

FIGURE 1 | The scope of HLA-mismatched EBV-LCL recognition by virus-specific T-cell populations. Virus-specific T-cell populations ($n = 164$) were stimulated with a panel of HLA-mismatched EBV-LCLs for 16 h and IFNγ production was measured by ELISA. EBV-specific T-cell populations were tested only against those HLA-mismatched EBV-LCLs that did not express the specific restriction molecule of the viral specificity of those T cells. In total, 83 virus-specific T-cell populations contained T cells that showed recognition of HLA-mismatched EBV-LCLs, defined as production of >200 pg IFNγ/ml. **(A,B)** Shown are the frequencies of virus-specific T-cell populations that recognized HLA-mismatched EBV-LCLs per virus-specificity **(A)** and per HLA-restriction of the viral specificity **(B)**. **(C)** The number of recognized HLA-mismatched EBV-LCLs is shown for the 83 virus-specific T-cell populations that contained T cells that showed cross-reactivity against one or more HLA-mismatched EBV-LCLs (117 sub-populations were included). Recognition of the same HLA-mismatched EBV-LCL by the bulk T-cell populations and sub-population(s) derived from those initial populations was counted once. **(D)** Shown are frequencies of virus-specific T-cell populations recognizing HLA-mismatched EBV-LCLs for each viral specificity. Statistical differences were assessed with the Chi-Squared Fishers Exact-Test (A/B) or the Mann-Whitney *t*-test **(C)**. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Red lines represent medians. AdV, Adenovirus; EBV, Epstein-Barr virus; CMV, Cytomegalovirus.

bulk T-cell populations contained T cells that showed detectable cross-reactivity against one or more HLA-mismatched EBV-LCL(s).

These 83 T-cell populations were used to investigate whether the virus specificity (CMV, EBV or AdV) or HLA restriction (HLA-A*01:01, HLA-A*02:01, HLA-B*07:02 or HLA-B*08:01) of the virus-specific reactivity influences the occurrence and frequency of HLA-mismatched EBV-LCL recognition. A similar proportion of the virus-specific T-cell populations targeting CMV, EBV or AdV exerted reactivity against at least one HLA-mismatched EBV-LCL (**Figure 1A**). In contrast, a significantly larger fraction of the HLA-B*08:01-restricted virus-specific T-cell populations showed recognition of HLA-mismatched EBV-LCLs, as compared to the HLA-A*01:01, HLA-A*02:01, and HLA-B*07:02-restricted virus-specific T-cell populations (**Figure 1B**). To assess the broadness of the cross-reactivity patterns, we counted how many different HLA-mismatched EBV-LCLs were recognized by the individual T-cell populations (65 bulk T-cell populations including 86 additional sub-populations derived from these T-cell populations, and 31

sub-populations derived from the 18 bulk T-cell populations that did not recognize any HLA-mismatched EBV-LCLs in the initial analysis). In these analyses, HLA-B*08:01-restricted virus-specific T cells exhibited a significantly broader cross-reactivity pattern, illustrated by reactivity against a median of 6 different HLA-mismatched EBV-LCLs, whereas HLA-A*01:01, HLA-A*02:01 and HLA-B*07:02-restricted virus-specific T-cell populations showed reactivity against a median of only 2, 2 and 3 HLA-mismatched EBV-LCLs, respectively (**Figure 1C**). Within the different HLA-B*08:01-restricted T-cell populations, similar high frequencies of T cells capable of exerting cross-reactivity against HLA-mismatched EBV-LCLs were observed, regardless of viral antigen-specificity (**Figure 1D**). These results show that the occurrence and frequency of cross-reactivity against HLA-mismatched EBV-LCLs is highly affected by HLA-restriction and not by virus-specificity (CMV, EBV or AdV).

Cross-Reactivity Against HLA-Mismatched EBV-LCLs Is Mediated by Recognition of Allogeneic HLA Molecules

To investigate if the recognition of HLA-mismatched EBV-LCLs was indeed caused by recognition of allogeneic HLA molecules, HLA-deficient EBV^{neg} K562 cell-lines transduced with single HLA-class-I molecules were used as stimulator cells (12, 27). T-cell populations exhibiting a clear pattern of EBV-LCL recognition, corresponding with the expression of a single HLA allele, were tested against K562 cells transduced with the respective HLA-molecule. For example, a population of EBV-EBNA3A^{QAK}-specific T cells recognized HLA-mismatched EBV-LCL ABF, which uniquely expressed HLA-A*30:04 and HLA-B*55:01 (**Figure 2A**). Recognition of K562 cells that were transduced with HLA-B*55:01 confirmed part of this respective cross-reactivity pattern (**Figure 2B**). In another example, a population of EBV-BRLF1^{YVL}-specific T cells recognized HLA-mismatched EBV-LCLs ACD, WKD, AVZ and UBM that all expressed HLA-A*24:02 (**Figure 2A**) and this was confirmed by recognition of K562 cells transduced with HLA-A*24:02 (**Figure 2B**). Some virus-specific T-cell populations (especially HLA-B*08:01-restricted T cells) showed more complex reactivity patterns when tested against the EBV-LCL panel, that could not be (fully) attributed to recognition of a single allogeneic HLA-molecule. The first representative example shows CMV-pp65^{RPH}-specific T cells that recognized multiple different EBV-LCLs, not allowing direct complete elucidation of the HLA allele(s) being recognized (**Figure 2C**). Only part of the reactivity could be explained by the unique shared expression of HLA-B*40:01 in EBV-LCLs WKD and RHP, that were both recognized. EBV-LCL UCE was the only EBV-LCL expressing HLA-B*27:05. However, the HLA alleles underlying the recognition of EBV-LCLs GML, GMS, and MWX could not be deduced. Similarly, EBV-LMP2^{CLG}-specific T cells recognized 4 EBV-LCLs with unique shared expression of HLA-B*35:01 or HLA-B*35:03, while the recognition of EBV-LCL GMK could not be traced back to a specific HLA allele (**Figure 2C** and **Supplementary Figure 3**). Recognition of the HLA molecules that were anticipated to partly underlie the cross-reactivity patterns was confirmed using K562 cells transduced with the respective HLA molecules (**Figure 2D**). No recognition was observed of K562 cells transduced with irrelevant HLA molecules, whereas recognition of K562 cells transduced with the HLA restriction molecule of the respective virus-specific T-cell population only occurred upon exogenous peptide loading (**Figures 2B,D**). Allo-HLA cross-reactive virus-specific T cells also showed to be able to lyse HLA-mismatched target cells (**Supplementary Figure 4**), in line with previous studies (11, 14). These results show that recognition of HLA-mismatched EBV-LCLs can be mediated by recognition of single or multiple allogeneic HLA-molecules.

HLA-B*08:01-Restricted Virus-Specific T Cells Recognize Multiple Allogeneic HLA Molecules, Skewed Toward Recognition of HLA-B Alleles

The cross-reactivity patterns against the EBV-LCL panel of more than half of the HLA-B*08:01-restricted T-cell populations were rather complex and extensive (observed in 11 out of the 12 HLA-B*08:01^{pos} donors), even when the complexity of the T-cell populations was reduced by selecting for cells expressing a single TCR-Vβ family (Representative examples; **Figure 3A**). No correlation could be observed for recognition of EBV-LCLs that show shared expression of specific HLA-class-II molecules. To investigate whether the reactivity patterns of these HLA-B*08:01-restricted T-cell populations could be (fully) attributed to recognition of a limited number of allogeneic HLA-class-I alleles, a panel of 40 different single HLA-class-I-transduced K562 cell-lines was used as stimulator cells (**Supplementary Table 2**). With this panel we covered 63% of the HLA-A, 73% of the HLA-B and 37% of the HLA-C alleles present in our EBV-LCL panel. Testing the HLA-B*08:01-restricted T-cell populations against this K562 panel revealed recognition of multiple specific groups of allogeneic HLA alleles by single T-cell populations, which could in part explain the cross-reactivity patterns observed when tested against the EBV-LCL panel (**Figures 3B,C**).

Next, we determined if the cross-reactivity of HLA-B*08:01-restricted T-cell populations was skewed toward HLA-A, B, or C molecules. In total, 22 HLA-B*08:01-restricted bulk or sub-populations (derived from the 11 HLA-B*08:01^{pos} donors that contained complex and extensive cross-reactive virus-specific T-cell populations) were tested against the K562 panel expressing a selection of HLA-A, B, and C alleles. Twenty-one out of 22 HLA-B*08:01-restricted T-cell populations recognized at least one allogeneic HLA-B molecule and 1 HLA-B*08:01-restricted T-cell population (CMV-IE1^{QIK} from donor 7) only recognized multiple HLA-A molecules in this panel (**Figure 4A** and **Supplementary Figure 5**). Twelve out of 21 allo-HLA-B-reactive HLA-B*08:01-restricted T-cell populations recognized only allogeneic HLA-B molecules and 9 T-cell populations additionally recognized allogeneic HLA-A and/or HLA-C molecules (**Supplementary Figure 5**). The number of allogeneic HLA-class-I molecules in the K562 panel recognized by the 22 HLA-B*08:01-restricted virus-specific T-cell populations ranged from 1 to 10 per T-cell population (median

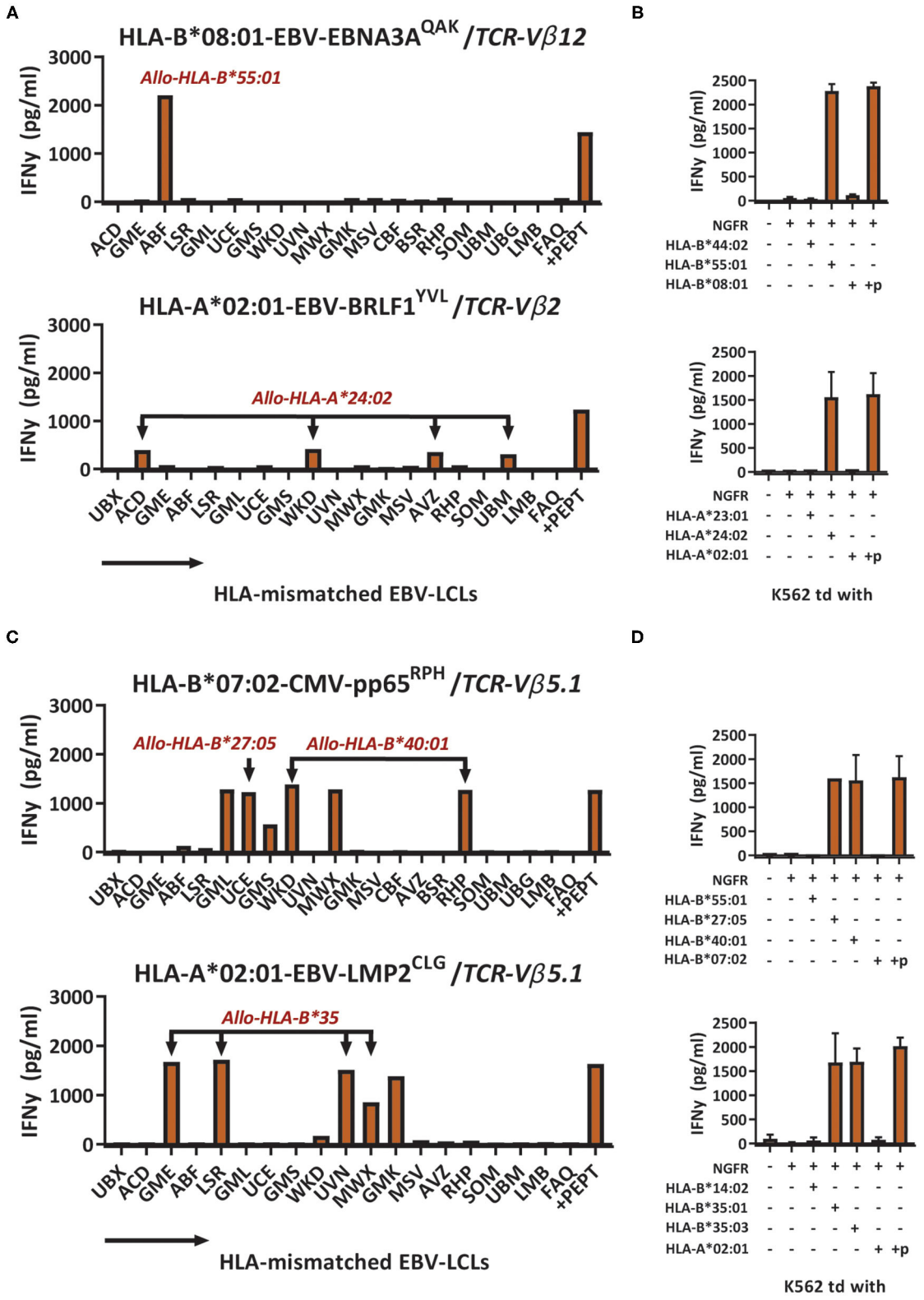

FIGURE 2 | Cross-reactivity against HLA-mismatched EBV-LCLs is mediated by recognition of allogeneic HLA molecules. Sub-populations expressing a single TCR-Vβ family were sorted from the bulk virus-specific T-cell populations targeting a single epitope and were stimulated with a panel of HLA-mismatched EBV-LCLs

(Continued)

FIGURE 2 | for 16 h and IFNγ production was measured by ELISA. EBV-specific T-cell populations were tested only against those HLA-mismatched EBV-LCLs that did not express the specific restriction molecule of the viral reactivity of those T cells. Reactivity was defined as production of >200 pg/ml IFNγ. **(A)** Shown are two representative examples of virus-specific T-cell sub-populations that showed production of IFNγ (y-axis) in response to stimulation with HLA-mismatched EBV-LCLs (x-axis). **(B)** EBV-EBNA3A^{QAK} and EBV-BRLF1^{YVL}-specific T cells were stimulated with K562 cells transduced with the HLA molecules that were expected to be recognized based on the patterns of reactivity against the EBV-LCL panel. K562 cells were also transduced with HLA molecules that were not expected to be recognized as negative control. K562 cells transduced with HLA-A*02:01 or HLA-B*08:01 exogenously loaded with 10^{-6}M of the respective viral peptide were used as positive control. **(C)** Shown are two representative examples of virus-specific T-cell sub-populations that showed production of IFNγ (y-axis) in response to stimulation with HLA-mismatched EBV-LCLs (x-axis) that shared multiple different allogeneic HLA molecules. **(D)** CMV-pp65^{RPH} and EBVLMP2^{CLG}-specific T-cell populations were stimulated with K562 cells transduced with HLA molecules that were expected to be recognized based on the reactivities seen against the EBV-LCL panel. K562 cells transduced with HLA-B*07:02 or HLA-B*08:01 exogenously loaded with 10^{-6}M of the respective viral peptide were used as positive control. TCR, T-cell Receptor. Vβ, Variable Beta Chain. *+p*, peptide pulsed; NGFR, Nerve Growth Factor Receptor; td, transduced.

of 3; **Figure 4B**). HLA-B*35:01, B*44:02 and B*44:03 were most frequently recognized, whereas HLA-B*13:02, HLA-B*14:02 and HLA-B*41:01 were never recognized by HLA-B*08:01-restricted T cells (**Figure 4C**).

To investigate whether the complex and extensive recognition of allogeneic HLA molecules could be mediated by one T-cell clone, we generated T-cell clones from three cross-reactive EBV-EBNA3A^{QAK}-specific T-cell populations (donor #4; donor #8 and #12; **Supplementary Figure 5**). Indeed, all T-cell clones recognized multiple HLA-B alleles in the K562 panel, in the same pattern as the initial EBV-EBNA3A^{QAK}-specific T-cell populations (1 representative example per donor; **Figure 5**), demonstrating that single T-cell clones can exert complex and extensive cross-reactivity against allogeneic HLA molecules. Although these T-cell clones expressed different TCR-Vβ families, all T-cell clones showed a recurrent pattern of recognition of both HLA-B*15:01, HLA-B*35:01, HLA-B*35:03, HLA-B*40:01, and HLA-B*44:03.

The HLA Background of Donors Shapes the Allo-HLA Cross-Reactivity of HLA-B*08:01-Restricted T Cells

Virus-specific T cells from HLA-B*08:01 homozygous donors frequently recognized HLA-B*35:01. We therefore reasoned that heterozygosity for HLA-B*35:01 would purge much of the cross-reactivity from the HLA-B*08:01-restricted TCR-repertoire through thymic negative selection. For these reasons, HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations ($n = 35$) were isolated from 3 HLA-B*08:01/HLA-B*35:01^{pos} heterozygous donors (**Table 1**). Contrary, HLA-B*13:02 was never recognized by T cells from HLA-B*08:01^{pos} donors. Therefore, we also isolated T-cell populations ($n = 10$) with the same specificities from 3 HLA-B*08:01^{pos} donors, heterozygous for HLA-B*13:02 (**Table 1**). Strikingly, only 33% of the T-cell populations isolated from HLA-B*08:01/B*35:01^{pos} heterozygous donors recognized one or more HLA-mismatched EBV-LCLs, while 90% of the corresponding T-cell populations from HLA-B*08:01^{pos} homozygous donors demonstrated recognition of HLA-mismatched EBV-LCLs (**Figure 6A**). In contrast, 80% of the T-cell populations isolated from HLA-B*08:01/HLA-B*13:02 heterozygous donors recognized one or more HLA-mismatched EBV-LCLs (**Figure 6A**). HLA-B*08:01-restricted T cells isolated from HLA-B*08:01/HLA-B*35:01 donors also recognized significantly fewer HLA-mismatched EBV-LCLs than the corresponding T-cell populations isolated from HLA-B*08:01/HLA-B*13:02 heterozygous or HLA-B*08:02 homozygous donors (**Figure 6B**). These results show that the occurrence and broadness of allo-HLA cross-reactivity by virus-specific-specific T cells is influenced by the HLA background of the donors.

DISCUSSION

In this study, we demonstrated that 50% (83/164) of virus-specific T-cell populations contained T cells that cross-reacted against HLA-mismatched EBV-LCLs, in line with previous findings (11). We showed that the level of allo-HLA cross-reactivity is highly influenced by HLA restriction and not by the viral specificity of the virus-specific T-cell populations. HLA-B*08:01-restricted virus-specific T cells showed the highest frequencies and diversities of allo-HLA cross-reactivity compared to the HLA-A*01:01, HLA-A*02:01 or HLA-B*07:02-restricted virus-specific T-cell populations. Cross-reactivity against HLA-mismatched EBV-LCLs was shown to be mediated by recognition of allogeneic HLA molecules, which was confirmed by recognition of EBV^{neg} K562 cells transduced with specific HLA-class-I molecules, illustrating that the peptides presented by these allogeneic HLA molecules were not EBV or B-cell-derived. HLA-B*08:01-restricted virus-specific T cells showed a skewed pattern of recognition of a group of allogeneic HLA-B alleles, with HLA-B*35:01 being recognized most often. We demonstrated that cross-reactivities against multiple allogeneic HLA-class-I molecules by HLA-B*08:01-restricted EBV-EBNA3^{QAK}-specific T cells could be mediated by single T-cell clones. Finally, heterozygosity for HLA-B*35:01, but not HLA-B*13:02 significantly reduced the degree of HLA cross-reactivity by HLA-B*08:01-restricted T cells, demonstrating that the HLA background of donors influences the off-target reactivity of virus-specific T cells.

Several groups have investigated whether the allo-HLA cross-reactive risk of virus-specific T cells could be predicted. In most of these studies, allo-HLA cross-reactive patterns could only be predicted when a T-cell population used a public TCR (14, 23, 24). Public T-cell populations could often be found by analysis of sub-populations of T cells expressing a single TCR-Vβ family. However, no pattern of allo-HLA cross-reactivity could be

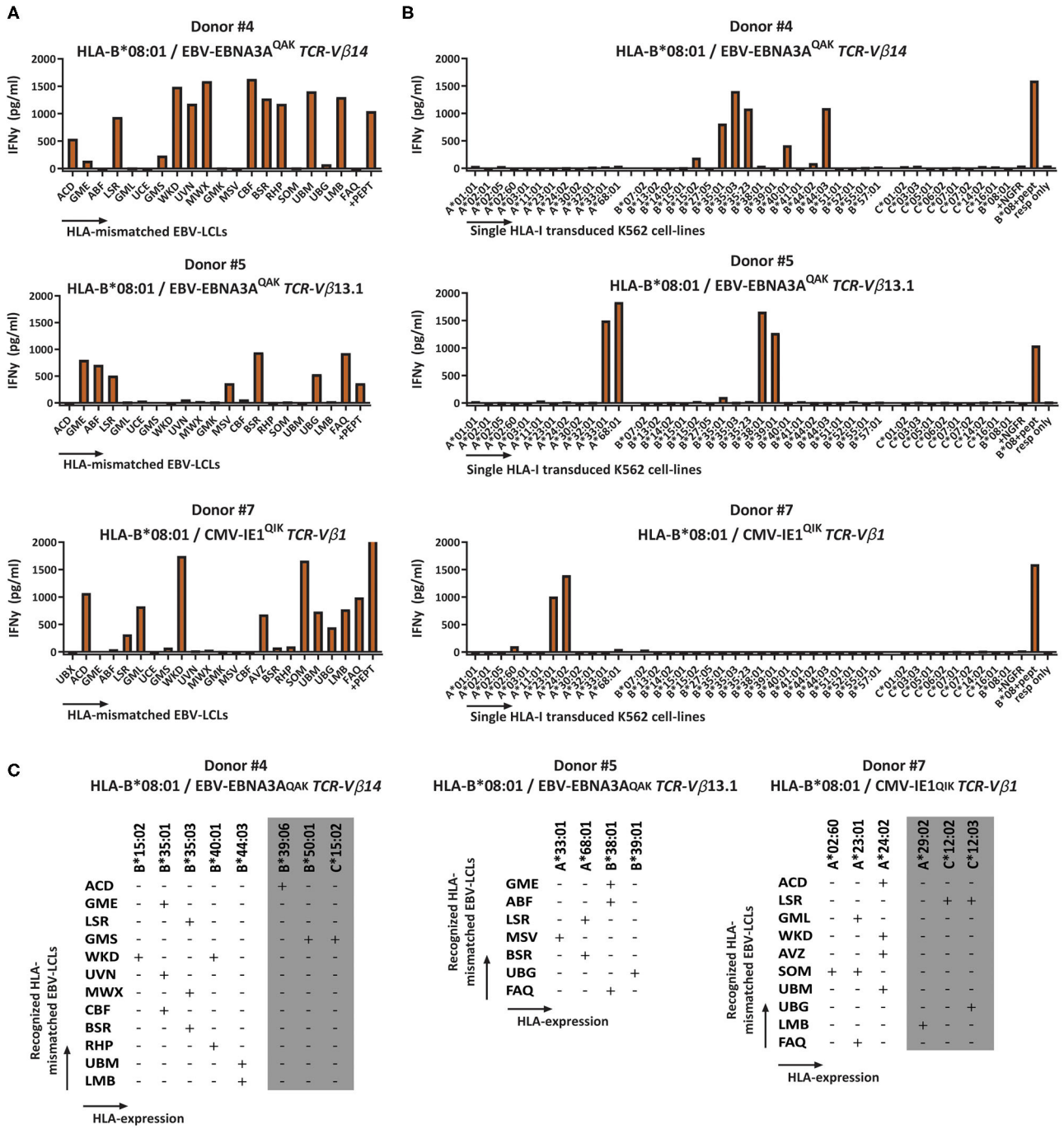

FIGURE 3 | Recognition of multiple HLA-mismatched EBV-LCLs and multiple allogeneic HLA molecules by virus-specific T-cell populations. HLA-B*08:01-restricted T-cell populations that showed reactivity against multiple HLA-mismatched EBV-LCLs were subsequently tested against a panel of single HLA-class-I-transduced K562 cell-lines ($n = 40$) and IFNγ production was measured by ELISA after 16 h to analyze which specific HLA molecules were being recognized. Reactivity was defined as production of >200 pg/ml IFNγ. The K562 panel covered 63% of HLA-A, 73% of HLA-B and 37% of the HLA-C molecules that were present in the EBV-LCL panel. **(A)** Shown are three representative examples of HLA-B*08:01-restricted virus-specific T-cell populations, sorted for expression of a single TCR-Vβ family tested for production of IFNγ (y-axis) in response to stimulation with a panel of HLA mismatched EBV-LCLs (x-axis) **(B)** A panel of single HLA-class-I transduced K562 cell-lines allowed partial deduction of the recognized allogeneic HLA molecules. **(C)** Listed are the EBV-LCLs that were recognized by the respective virus-specific T-cell populations. The reactivity pattern observed with the EBV-LCL panel could partly be explained by recognition of specific HLA-class-I alleles confirmed with the K562 panel. However, some EBV-LCLs did not express any of the HLA molecules present in the K562 panel. Their recognition might be explained by recognition of HLA molecules that were not present in our K562 panel (gray). TCR, T-cell Receptor; Vβ, Variable Beta Chain; NGFR, Nerve growth factor receptor; Resp, responder.

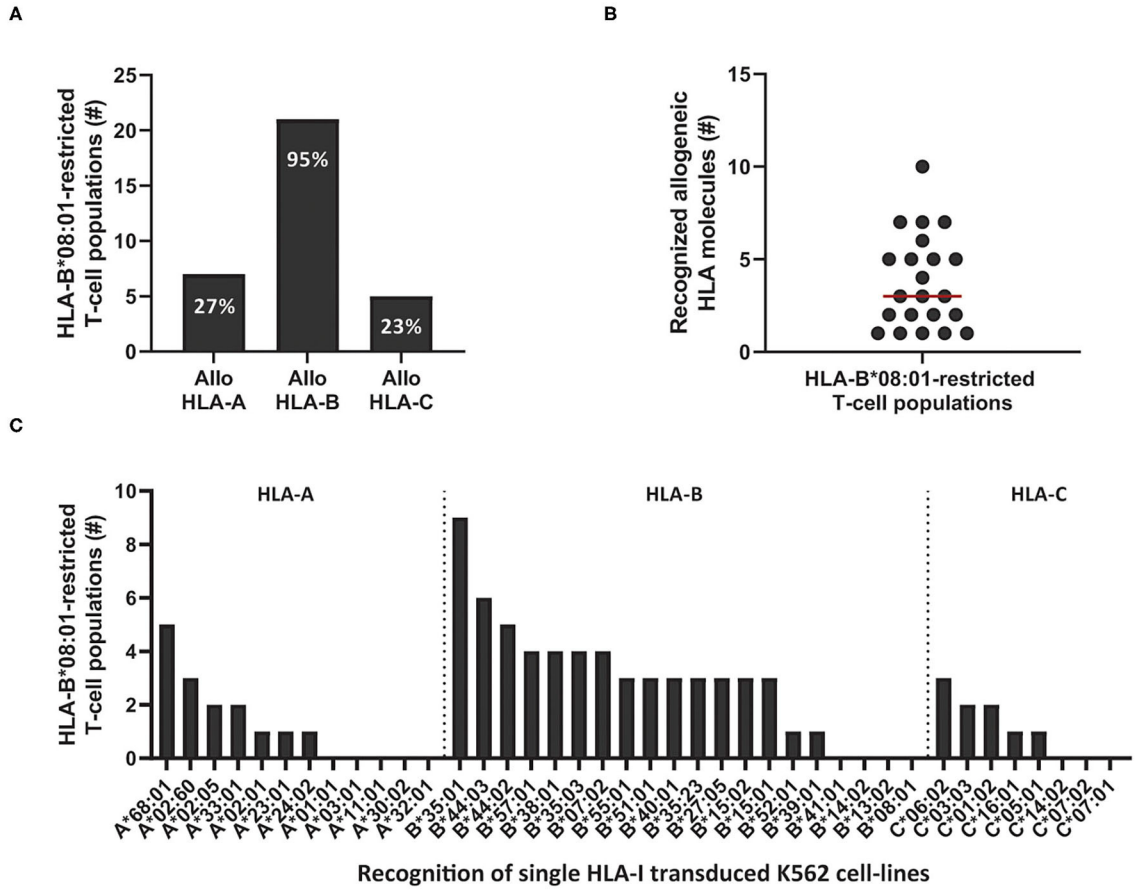

FIGURE 4 | Cross-reactivity by HLA-B*08:01-restricted virus-specific T cells is skewed toward recognition of certain allogeneic HLA-B alleles. Eleven out of 12 HLA-B*08:01^{pos} donors contained HLA-B*08:01-restricted T-cell populations ($n = 22$) with no clear recognition pattern when tested against the HLA-mismatched EBV-LCL panel. To analyze which HLA molecules were being recognized, virus-specific T-cell populations, were stimulated with a panel of single HLA-class-I-transduced K562 cell-lines ($n = 40$) for 16 h and IFNγ production was measured by ELISA. Reactivity was defined as production of >200 pg/ml IFNγ. **(A)** Shown are the number of HLA-B*08:01-restricted T-cell populations that contained T cells that recognized allogeneic HLA-A, B or C alleles. Some populations were allocated to multiple groups. **(B)** Shown are the number of recognized allogeneic HLA molecules for each HLA-B*08:01-restricted T-cell population. Red line represents median **(C)** Shown are the numbers of HLA-B*08:01-restricted T-cell populations (y-axis) that show recognition of specific allogeneic HLA-A, B or C alleles (x-axis).

observed in our study, except for HLA-A*02:01-restricted EBV-LMP2^{CLG}-specific T cells sorted for expression of TCR-Vβ5.1 (**Figure 2C**). Although virus-specific T cells often expressed the same TCR-Vβ family, differences in the Complementary Determining Region 3 (CDR3) or a different TCR-alpha chain could result in variation in the allo-HLA cross-reactivity patterns. Allo-HLA cross-reactivity can therefore not be predicted based on TCR-Vβ-family usage alone and may only result in clear patterns if the TCR-Vβ family consist of a public TCR (27).

Similar to other studies we observed that part of the allo-HLA cross-reactive T-cell populations showed recognition of HLA-mismatched EBV-LCLs, but no recognition of our panel of single HLA-class-I transduced K562 cells expressing 58% ($n = 37/64$) of the HLA-class-I molecules present in the EBV-LCL panel (11). The scope our current study was not to fully unravel the recognized allogeneic peptide in allo-HLA molecules. However, this may demonstrate that allo-HLA cross-reactive T cells do not solely recognize an household peptide in the context of allogeneic HLA, but potentially also lineage-specific peptide-allo-HLA cross-reactivity exists (28). Also recognition of HLA-class-II molecules by HLA-class-I-restricted CD8^{pos} virus-specific T cells has previously been described (11). However, in our study we did not see a correlation with the pattern of recognition against the EBV-LCL panel and the expression of specific HLA-class-II molecules. Therefore, HLA-class-II-restricted cross-reactivity was not further analyzed in depth in our current study.

Finding third-party donors with anti-viral T cells that are fully (HLA-class-I) matched to the recipient patients is probably difficult. When allo-HLA cross-reactive T cells targeting HLA alleles expressed on cells of the patient or (organ) donor are present in the virus-specific T-cell product, acute graft vs. host disease (GVHD) or graft rejection could occur. Strikingly, only a

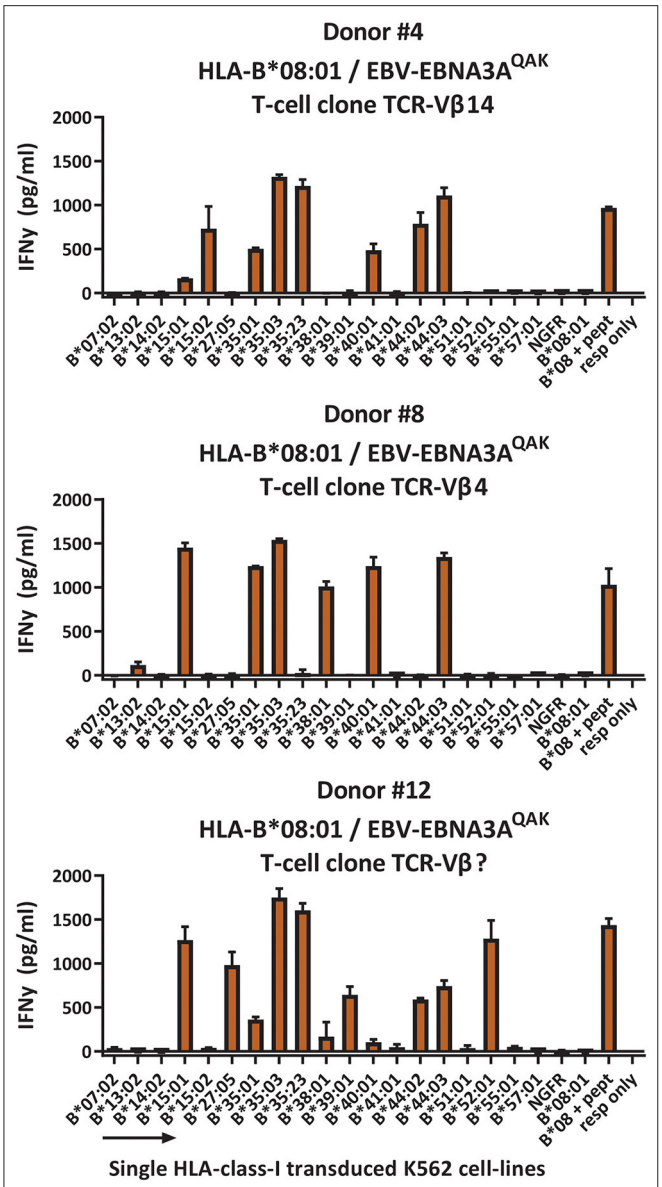

FIGURE 5 | HLA-B*08:01-restricted EBV-EBNA3A^{QAK}-specific T-cell clones recognize multiple allogeneic HLA molecules. HLA-B*08:01-restricted EBV-EBNA3A^{QAK}-specific T-cell clones showing cross-reactivity against allogeneic HLA-B*35:01 were sorted from EBV-EBNA3A^{QAK}-tetramer positive bulk T-cell populations based on expression of activation marker CD137 after stimulation with K562 cells transduced with HLA-B*35:01. All virus-specific T-cell clones showed 100% positive EBV-EBNA3A^{QAK}-tetramer staining. Six T-cell clones per donor were stimulated with a panel of single HLA-B molecule-transduced K562-cell lines (x-axis) for 16 h and IFNγ production (y-axis) was measured by ELISA to analyze which HLA molecules were being recognized. Reactivity was defined as production of >200 pg/ml IFNγ. One representative T-cell clone is shown for each donor. T-cell clones from donor #4 expressed TCR-Vβ14, T-cell clones from donor #8 expressed TCR-Vβ4 and T-cell clones from donor #12 expressed a TCR-Vβ family that could not be determined by the TCR-Vβ flow cytometry kit. Shown are means with standard deviations of 1 experiment carried out in triplicate. TCR, T-cell Receptor; Vβ, Variable Beta Chain; NGFR, Nerve growth factor receptor; Resp, responder.

very low incidence of *de novo* acute GVHD or graft rejection has been observed in clinical trials analyzing the effect of adoptive T-cell therapy with third-party donor-derived products, either in the setting of HLA-mismatched stem cell transplantation or of solid organ transplantation (18, 29). It has therefore been assumed that third-party virus-specific T cells do not mediate GVHD or graft rejection (18). It is not clear whether, the observed absence of GVHD or graft rejection in these cases was the result of: (1) no expression of the particular mismatched HLA allele recognized by the transferred virus-specific T cells, (2) removal of the *in vitro* off-target (>10% cytotoxic) virus-specific T cells from the product prior to administration to the patient and/or selection of T-cell products that do not show *in vitro* allo-HLA reactivity (18), (3) extensive culturing of the virus-specific T cells prior to adoptive transfer, leading to senescence and impaired cytokine production (30), 4) weak adhesion molecule expression (i.e., ICAM-1) by the target organ (31), (5) biased production and administration of HLA-A*02:01-restricted virus-specific T cells with an intrinsic low risk of off-target toxicity, as shown in this study, (6) low T-cell numbers of cross-reactive virus-specific T cells administered and/or limited *in vivo* proliferation, or (7) rapid rejection of the virus-specific T cells (22).

Here, we demonstrated that around 40% of HLA-A*01:01, HLA-A*02:01, or HLA-B*07:02-restricted T-cell populations recognized one or more HLA-mismatched EBV-LCLs. For each T-cell population this recognition was found to be limited to only a few HLA-mismatched EBV-LCLs and could be attributed to recognition of one or a couple of allogeneic HLA alleles. The risk for accidentally mismatching for the particular allogeneic HLA allele(s) cross-recognized by the virus-specific T cells would be low, but studies do report cases of GVHD after infusion of virus-specific T cells derived from the SCT donor (32, 33) or derived from a third-party donor (33–35). Importantly, we found that HLA-B*08:01-restricted T cells isolated from donors that were homozygous for HLA-B*08:01 or heterozygous for HLA-B*08:01 and a specific HLA-B allele (e.g., HLA-B*13:02) showed abundant allo-HLA cross-reactivity *in vitro* and are therefore likely to cause graft rejection or GVHD *in vivo*. Since in the majority of studies so far, the adoptive transfer of third-party donor-derived virus-specific T cells was focused on HLA-A*02:01- and/or HLA-B*07:02-restricted virus-specific T cells (36), the effect of HLA-B*08:01-restricted virus-specific T cells has not been extensively studied (37). Our results on the higher incidence of HLA-cross-reactivity by HLA-B*08:01-restricted compared to HLA-A*01:01, HLA-A*02:01, or HLA-B*07:02-restricted virus-specific T cells may have important value for the design of future clinical trials. Since the specificity did not contribute to the allo-HLA cross-reactivity, these results have also important value for third-party derived CAR-T cell therapies or in the field of organ transplantations. Intriguingly, studies in the field of organ transplantations show a significant increase of acute graft rejections in recipients that express HLA-B*08:01, HLA-C*07:01, and HLA-DRB1*03:01 (38, 39). These three HLA molecules are part of a common haplotype (40) and the homozygous donors

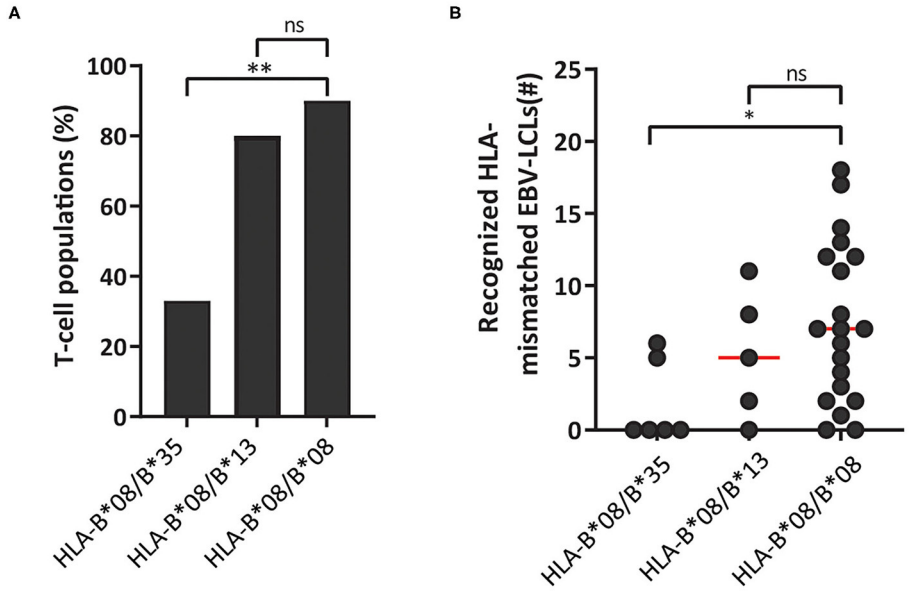

FIGURE 6 | HLA-background of donors shapes the allo-HLA cross-reactivity of HLA-B*08:01-restricted T cells. HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations isolated from HLA-B*08:01/B*35:01^{pos} donors, HLA-B*08:01/B*13:02^{pos} donors or HLA-B*08:01 homozygous donors were stimulated with a panel of HLA-mismatched EBV-LCLs for 16 h and IFNγ production was measured by ELISA. Reactivity was defined as production of >200 pg/ml IFNγ. EBV-LCLs expressing the HLA restriction molecules of the viral specificity of the respective T-cell populations were excluded. Sub-populations of T cells expressing single TCR-Vβ families derived from the initial HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations were included in the analysis of the level of reactivity against the HLA-mismatched EBV-LCL panel. **(A)** The percentages of the HLA-B*08:01-restricted EBV-BZLF1^{RAK} and EBV-EBNA3A^{QAK}-specific T-cell populations isolated from HLA-B*08:01/B*35:01^{pos} donors, HLA-B*08:01/B*13:02^{pos} donors or HLA-B*08:01 homozygous donors that recognize 1 or more HLA-mismatched EBV-LCLs were compared. **(B)** Shown are the number of HLA-mismatched EBV-LCLs (y-axis) that are recognized by HLA-B*08:01-restricted EBV-EBNA3A^{QAK} and EBV-BZLF1^{RAK}-specific T-cell populations isolated from HLA-B*08:01/B*35:01^{pos}, HLA-B*08:01/B*13:02^{pos} or HLA-B*08:01^{pos} homozygous donors. Statistical differences were assessed with a Chi-Squared Fishers Exact Test **(A)** and the Mann-Whitney *t*-test **(B)**. *$P < 0.05$; **$P < 0.01$. Red lines represent medians.

used in our study have the same haplotype, suggesting that these rejections are mediated by HLA-B*08:01-restricted T cells. Altogether, these results imply that the HLA background of the donor is important for the broadness of the allo-HLA cross-reactivity. Therefore, the most compatible HLA background of the donor should be aimed for and homozygous donors should not be used despite the lower chance of rejection.

Since we only analyzed virus-specific T cells restricted to four different HLA molecules, it remains unclear whether T cells with another HLA restriction could show similar reactivity patterns as HLA-B*08:01-restricted T cells. However, we hypothesize that these findings might only be restricted to a few HLA molecules since the peptidome of HLA-B*08:01 shows an unique pattern, that is specific for only HLA-B*08:01 and HLA-B*08:02. Based on binding data and sequence information, Sidney J. et al. classified the majority of HLA-B molecules into 9 super families (41). We hypothesized that super families with only a few HLA-B alleles, have unique peptidomes and T cells with this specific HLA background are likely to be cross-reactive against HLA molecules from other HLA super families, since negative thymic selection for these peptide-HLA complexes has not taken place. In the present study, virus-specific T cells isolated from donors that expressed HLA-B*08:01 and HLA-B*35:01 proved to be less allo-HLA cross-reactive than those from donors that were homozygous for HLA-B*08:01 or heterozygous for HLA-B*08:01 and HLA-B*13:02. We hypothesize that HLA-B*35:01 may elicit thymic negative selection for all HLA molecules present in the B07 superfamily to which it belongs (e.g., HLA-B*07:02, HLA-B*35:03, HLA-B*42:01). Being heterozygous for any of the HLA molecules from this B07 superfamily would then presumably result in the same outcome as heterozygosity for HLA-B*35:01. HLA-B*13:02 could not be assigned to a particular HLA superfamily (41), possibly explaining why it did little to the level of allo-HLA cross-reactivity of the HLA-B*08:01-restricted repertoire in our study. Therefore, if full matching for HLA-B is not possible, we propose that donors should be used that express HLA-B molecules that are part of different superfamilies to reduce the chance for a broad off-target toxicity in clinical application of third-party donor-derived T-cell products.

Altogether, our results indicate that selection of virus-specific T-cells with specific HLA restrictions and donors with specific HLA backgrounds may decrease the risk of developing GvHD or (organ) graft rejection after infusion of third-party donor-derived virus-specific T cells into patients with uncontrolled viral reactivation. Ideally, if complete HLA-class-I matching is not feasible, donor and recipient should at least be fully matched for HLA-B or matched for HLA-B alleles from the same HLA-B superfamily. Mismatching of HLA-B alleles that are unclassified should be avoided, because the peptides presented

by these HLA-molecules are unique and could mediate allo-HLA cross-reactivity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in **Supplementary Figure 1**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

WH, DL, LM, and LH, performed experiments. WH analyzed results and made the figures. WH, JF, DA, and IJ designed the research and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.630440/full#supplementary-material

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Interfering With Inflammation: Heterogeneous Effects of Interferons in Graft-*Versus*-Host Disease of the Gastrointestinal Tract and Inflammatory Bowel Disease

Eileen Haring[1,2], *Robert Zeiser*[1,2] *and Petya Apostolova*[1,2]*

[1] *Department of Medicine I, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany,* [2] *German Cancer Consortium (DKTK), Partner Site Freiburg and German Cancer Research Center (DKFZ), Heidelberg, Germany*

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Edited by:
Jaebok Choi, Washington University School of Medicine in St. Louis, United States

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Senthilnathan Palaniyandi, University of Kentucky, United States
Jan Styczynski, University of Bydgoszcz, Poland
Aleksandr Lazaryan, Moffitt Cancer Center, United States

***Correspondence:**
Petya Apostolova
petya.apostolova@uniklinik-freiburg.de

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The intestine can be the target of several immunologically mediated diseases, including graft-versus-host disease (GVHD) and inflammatory bowel disease (IBD). GVHD is a life-threatening complication that occurs after allogeneic hematopoietic stem cell transplantation. Involvement of the gastrointestinal tract is associated with a particularly high mortality. GVHD development starts with the recognition of allo-antigens in the recipient by the donor immune system, which elicits immune-mediated damage of otherwise healthy tissues. IBD describes a group of immunologically mediated chronic inflammatory diseases of the intestine. Several aspects, including genetic predisposition and immune dysregulation, are responsible for the development of IBD, with Crohn's disease and ulcerative colitis being the two most common variants. GVHD and IBD share multiple key features of their onset and development, including intestinal tissue damage and loss of intestinal barrier function. A further common feature in the pathophysiology of both diseases is the involvement of cytokines such as type I and II interferons (IFNs), amongst others. IFNs are a family of protein mediators produced as a part of the inflammatory response, typically to pathogens or malignant cells. Diverse, and partially paradoxical, effects have been described for IFNs in GVHD and IBD. This review summarizes current knowledge on the role of type I, II and III IFNs, including basic concepts and controversies about their functions in the context of GVHD and IBD. In addition, therapeutic options, research developments and remaining open questions are addressed.

Keywords: graft-versus-host disease, inflammatory bowel disease, interferon, intestine, ulcerative colitis, Crohn's disease

INTRODUCTION

The intestine poses a unique environment for the immune system. Innate and adaptive immune cells cooperate at this physiological barrier surface to maintain homeostasis and prevent infection with pathogens that are ingested with the food. An interplay between intestinal microbiota and nutritional metabolites further shapes the microenvironment. Loss of homeostasis between these factors may result in local inflammation. Two disease groups that elicit immune-mediated intestinal tissue damage are graft-versus-host disease (GVHD) and inflammatory bowel disease (IBD). These diseases develop in distinct situations. IBD is the most prevalent autoimmune condition of the intestine, while the occurrence of GVHD is limited to the specific case of a patient who has received an allogeneic hematopoietic cell transplantation (allo-HCT). Nevertheless, both diseases share similar pathophysiological mechanisms. One of them is the involvement of interferons (IFNs) as soluble mediators shaping the microenvironment. Here, we review recent literature about the role of IFNs in intestinal GVHD and IBD. We first provide an introduction about the biology of both disease groups, followed by an overview of IFN production and signaling. In the second part, we discuss the function of different IFN subtypes in preclinical models and clinical studies of GVHD and IBD.

PATHOPHYSIOLOGY OF GRAFT-*VERSUS*-HOST DISEASE

Allo-HCT is one essential curative therapy option for malignant diseases of the hematopoietic system such as leukemia or lymphoma. It is also used for the treatment of benign disorders, most predominantly immunodeficiency syndromes (1). The allograft recipient is conditioned for the graft transplantation by the administration of chemotherapy, and in some cases irradiation, followed by the intravenous infusion of allogeneic hematopoietic stem cells (HSCs). Along with HSCs, the allogeneic graft contains also pre-existing mature lymphocytes (2). These donor immune cells are able to attack residing tumor cells when the allo-HCT is performed to treat a malignant underlying disease. This process is termed graft-versus-leukemia or graft-versus-tumor effect and is essential for long-term malignancy control (3). On the other hand, the donor immune cells (especially T cells) can also harm healthy tissues in the recipient. This inflammatory process is known as GVHD and its high morbidity and mortality limit the therapeutic success of allo-HCT. Classically, GVHD presents itself in two different clinical manifestations: acute GVHD (aGVHD) and chronic GVHD (cGVHD). The main target organs in aGVHD are the liver, the skin and the gastrointestinal (GI) tract. Clinical symptoms may develop within a few weeks after allo-HCT and include a maculopapular rash, hyperbilirubinemia, cholestasis as well as voluminous diarrhea, abdominal pain and bleeding (4). In addition to the affected tissues in aGVHD, any other organ system such as oral, esophageal and ocular systems, but also hair, nails, genitalia, joint fascia and lungs can be involved in cGVHD, which occurs late (in most cases, up to one year) after allo-HCT (5). GVHD is a frequent complication of allo-HCT with 30-50% of all allo-HCT recipients being affected (4). Due to its high prevalence and the diversity of involved organs, GVHD poses a major challenge in the care of allo-HCT recipients together with the risk of infections and malignancy relapse.

The development of GVHD is a complex interplay between hematopoietic and non-hematopoietic cells, soluble mediators, metabolites and bacteria. The key cellular mediators of GVHD are the alloreactive T cells, which are contained in the donor graft and become activated by different signals during disease development. The conditioning regimen prior to allo-HCT damages tissues of the recipient resulting in the release of both danger- and pathogen-associated molecular patterns (DAMPs and PAMPs). Together with inflammatory cytokines such as TNF and IL-6, a local inflammatory environment is established (6–8). Antigen-presenting cells (APCs) get activated and present peptides from the recipient. This in turn leads to the activation and expansion of the alloreactive T cells, which recognize the host peptides as foreign based on differences in major and minor histocompatibility antigens between donor and recipient. Cellular mediators of tissue damage in the patient comprise cytotoxic $CD8^+$ T cells, NK cells as well as macrophages (7). They act together with soluble inflammatory effectors to promote local tissue destruction and further enhance inflammation (**Figure 1**). Involvement of the GI tract is associated with a high morbidity and mortality (9, 10). Intestinal epithelial cell (IEC) numbers are markedly reduced in aGVHD, and their damage leads to a loss of intestinal barrier function associated with inferior survival (11). This in turn further elevates tissue damage accompanied by bacterial transmigration and therefore strengthens the local pro-inflammatory setting during disease pathogenesis (8, 12). Besides epithelial cells, intestinal stem cells (ISCs) and Paneth cells are a major target of GVHD. ISCs are located at the bottom of the intestinal crypts where they proliferate and differentiate to regenerate all intestinal cell types. Several studies could underline that damage of the ISC is a key event in disease pathogenesis and that supporting their regeneration improves GVHD outcome (13–16). Paneth cells are located in close proximity to the ISCs. They produce antimicrobial peptides, such as lysozyme and defensins. Paneth cell number reduction in GVHD has been associated with microbial dysregulation through the reduction of intestinal α-defensins (17, 18). In humans, low Paneth cell numbers at the onset of GVHD correlated with inferior survival (19). Besides Paneth cells, L cells were recently shown to be a target of aGVHD and their loss causes a lack of the enteroendocrine hormone Glucagon-like-peptide-2 (GLP-2) (16). Another major determinant of GVHD severity is the intestinal microbiome. Multiple studies observed a loss of general bacterial diversity with a shift between beneficial and detrimental bacterial species during GVHD (20–22). Fecal microbiota transplantation has shown efficacy in patients with steroid-resistant GVHD (23–26) pointing out to the significance of microbial regulation of inflammation. Due to this complex, multi-layer pathogenesis, GVHD has proven difficult to treat in a significant number of patients.

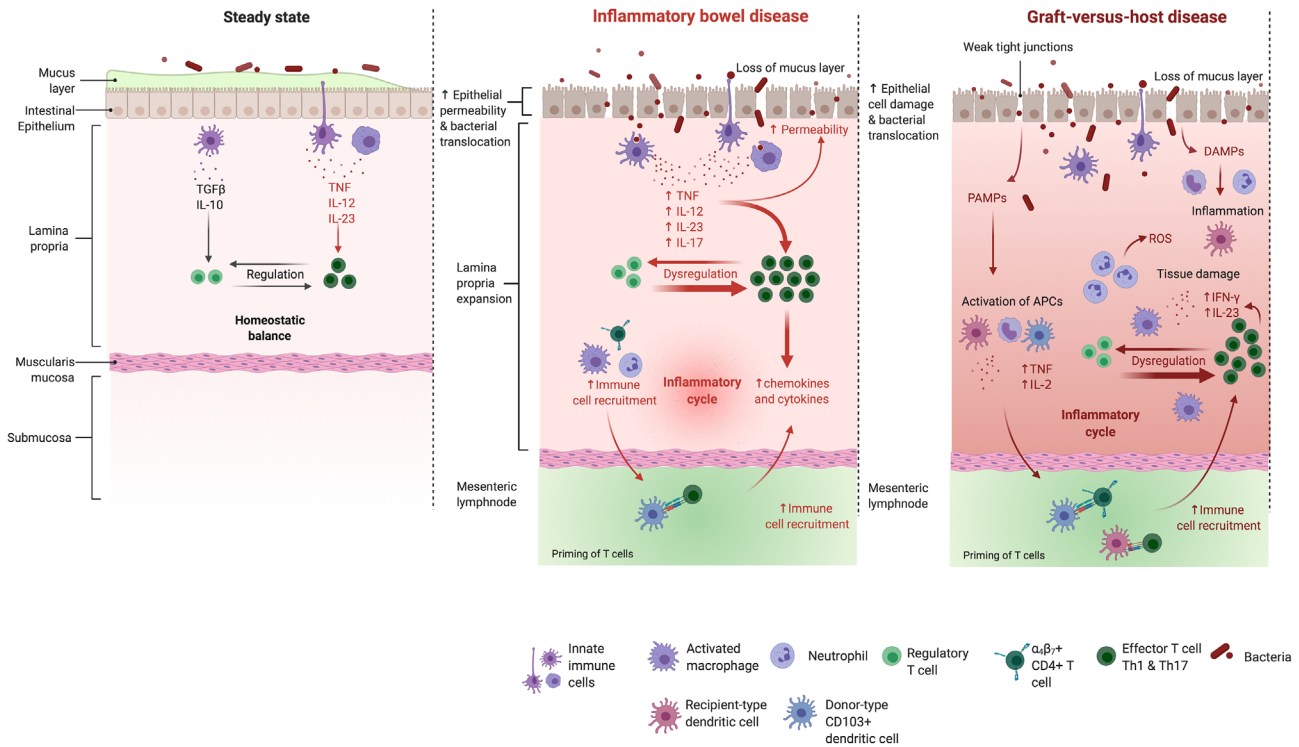

FIGURE 1 | The intestinal mucosa in the healthy bowel, in IBD and GVHD. Mechanisms maintaining the healthy intestinal barrier (e.g. a thick mucus layer and tight junctions) are disrupted in the mucosa of IBD patients. The balance between effector- and regulatory T cells gets disturbed which leads to an activation of different effector T cell subtypes and their uncontrolled migration into the inflamed intestine. Also in GHVD pathogenesis, the intestinal barrier gets disrupted. Intestinal injury due to administered conditioning regiment leads to the translocation of bacteria, PAMPs and DAMPs. Neutrophils are recruited and promote tissue damage through reactive oxygen species secretion. The costimulatory activity of host antigen presenting cells is enhanced. Donor T cells are primed, proliferate and differentiate in response to host stimulatory APCs. Th1 cytokines (IFN-γ, IL-2, and TNF) and chemokines are released in large quantities. A complex cascade including cellular mediators (e.g. cytotoxic T cells and macrophages) and soluble inflammatory effectors (e.g. TNF and IFN-γ) collectively promotes local tissue damage and further drives the inflammatory cycle. IL, interleukin; TGFβ, transforming growth factor β; TNF, tumor necrosis factor; IFN, interferon; ROS, reactive oxygen species; PAMPs, pathogen associated molecular patterns; DAMPs, danger associated molecular patterns. Adapted from "Immune response in IBD", by Biorender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

PATHOPHYSIOLOGY OF INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a group of chronic and recurrent nonspecific inflammatory autoimmune diseases of the intestinal tract. Several factors including genetic predisposition, environmental factors, the intestinal microbiome as well as immune dysregulation play a role for the development of IBD (27–29). The two main clinical presentations of IBD comprise Crohn's disease (CD), characterized by inflammation in different parts of the intestine, and ulcerative colitis (UC), which leads to persistent inflammation and ulcers limited to the colon (30, 31). CD and UC are chronic, often progressive diseases. The major clinical symptoms are chronic diarrhea, abdominal pain and bleeding, weight loss, nausea, vomiting and fatigue (32). IBD can be accompanied by a wide range of serious complications such as abscesses, fistulas and inflammation-associated colon cancer. In particular in the case of CD, extra intestinal manifestations are frequent, with skin, eyes, bones and joints being affected (33, 34).

There has been strong evidence showing that - similarly to GVHD - a loss of intestinal barrier integrity contributes to the initiation of IBD (11, 35). The barrier disruption allows translocation of microbes and microbial products which results in the engagement of pattern-recognition-receptors (PRRs) present on IECs and various hematopoietic as well as non-hematopoietic cells within the mucosa. PRR stimulation ultimately leads to the induction of an immunologic response *via* inflammasome activation and the production and release of pro-inflammatory cytokines as well as chemokines (36, 37) (**Figure 1**). Previous studies could elucidate that an imbalance between pro-inflammatory Th17 cells and anti-inflammatory regulatory T cells (Tregs) was essential in the context of IBD initiation, progress and maintenance (38–40). Proinflammatory cytokines, including TNF and IFN-γ, were shown to be key players in driving the excessive and imbalanced immune response, accompanied by harmful leukocyte infiltration and intestinal mucosal damage (41, 42). Furthermore, it was demonstrated that the microbiome played a key role in IBD onset and pathogenesis as it was seen that the development of intestinal inflammation in mice was abolished under germ-free conditions in a variety of mouse models (43). In addition to similar intestinal clinical manifestations, both GVHD and IBD also share extra

intestinal organ involvement such as bile duct damage, amongst others (37). Underlining the shared aspects of disease pathologies, corticosteroids and other immunosuppressive medication is utilized in both conditions (44, 45). Newer approaches in IBD therapy suggest that the earlier utilization of advanced therapies, including immunomodulatory drugs such as thiopurines and methotrexate effectively reduces disease progression and minimizes long-term complications for the patient (46, 47).

INTERFERON PRODUCTION AND SIGNALING

IFNs are a group of cytokines which in humans can be divided into three categories: type I IFNs (comprising IFN-α, IFN-β, IFN-ϵ, IFN-κ, and IFN-ω), type II IFNs (IFN-γ) and type III IFNs (IFN-λ1, IFN-λ2, IFN-λ3, IFN-λ4), also known and described as IFN-like molecules. Type I IFNs bind to a common cell surface receptor named type I IFN receptor, which is composed of the two subunits IFNAR1 and IFNAR2 and is expressed on all nucleated cells (48, 49). The subunits are associated with the Janus activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1. Receptor engagement by type I IFN leads to tyrosine phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT2. Together with interferon regulatory factor 9 (IRF9), both phosphorylated STAT proteins form a complex which is known as IFN-stimulated (IFN-stimulated gene (ISG) factor 3) ISGF3 complex (50, 51). This complex translocates into the nucleus and binds to IFN-stimulated response elements (ISREs) to initiate the transcription of different ISGs which mediate various biological processes (52). Aside from STAT1 and -2, type IFN I signaling can also induce STAT3-6, so that various homo- and heterodimer combinations can assemble (53). In contrast to the downstream signaling of the ISGF3 complex, which is comprised of STAT1, -2 and IRF9, the other complexes bind to another type of regulatory element: the IFN-γ-activated site (GAS) element. Various ISGs contain either only ISREs or GAS elements in their promoter regions, whereas some contain both. This shows that type I IFN signaling can induce a variety of functionally distinct target genes, although the exact mechanism behind the regulation of the various STAT engagements is not fully understood yet (51). IFN-γ, as the only type II IFN, binds to a different cell surface receptor: the type II receptor, composed of the two subunits IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2, respectively (49, 54). Here, the STAT1 homodimer is the essential transcription factor, which gets activated *via* phosphorylation. Since the STAT1 homodimer does not bind to IRF9, it is not able to bind ISREs. Therefore, type II IFN signaling only induces transcription of genes, which possess GAS elements in their regulatory regions (55–57). Finally, all type III IFNs bind to a receptor complex composed of two subunits: CRF2-12 (also designated as IFN-λR1) and CRF2-4 (also known as IL-10R2), together named 65R1. Type III INFs are the "youngest" group of IFNs and were only discovered in 2003 (58, 59). Similar to type I IFNs, signaling *via* type III IFNs induces the trimerization of the heterodimer STAT1-STAT2 with IRF9 resulting in the assembly of the ISGF3 complex. Type III IFN signaling can therefore activate ISG with ISREs or GAS elements in their regulatory region (60) (**Figure 2**). In contrast to the wide receptor expression for type I and II interferons, expression of type III interferon receptor seems to be limited to certain tissues and cell types. Keratinocytes and epithelial cells of the lung and the GI tract have been shown to express significant amounts of IFNLR1. Interestingly, so far plasmacytoid dendritic cells (pDCs) seem to be the only hematopoietic cell type which is responsive to type III IFNs (61, 62). The various impacts and functions of ISGs were recently covered in a comprehensive review by Schoggins (63).

IMMUNOREGULATORY EFFECTS OF IFNs

Type I IFNs have a wide range of functions and are produced by various cell types in response to pathogenic - mostly viral but also bacterial - infections. The functions include anti-pathogen activity as well as anti-proliferative actions. During the last decades it became also clear, that type I IFN can exert immunomodulatory actions on cells of both the innate and the adaptive immune system (54, 64). Type I IFN production is triggered by various PRRs including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) as well as NOD-like receptors (NLRs), that can be activated by sensing viral nucleic acids and other stimuli. PRR activation leads to the rapid induction of type I IFN during the early phases of viral infections before the adaptive immune response including antiviral $CD8^+$ T cells is induced and established (65). As part of the innate immune system, plasmacytoid dendritic cells (pDCs) were implied as the most predominant IFN-α producing cells (66–68). Type I IFNs indirectly affect T cell activation by inducing the maturation, migration and antigen presentation capacity of DCs to facilitate their adaptive antiviral immune response (69–74).

Natural killer (NK) cells, natural killer T cells (NKT), $CD4^+$ T helper type 1 (Th1) cells, $CD8^+$ cytotoxic T cells as well as γδ T cells are the main IFN-γ-producing cell types (75). IFN-γ plays an essential role in MHC class I and II antigen presentation pathways. It induces the upregulation of MHC class I cell surface expression which is important for the immune response against intracellular pathogens and essential for the actions of cytotoxic effects of $CD8^+$ T cells. All exact impacts of IFN-γ on genes which are associated with the MHC class I antigen presentation pathway have been reviewed by Schroder and colleagues (57). Notably, IFN-γ is the sole IFN, which is able to induce MHC class II expression on professional APCs such as DCs, macrophages and B cells. It thus plays an exclusive role in the activation of $CD4^+$ T cells *via* specific MHC class II/peptide recognition (75). During the adaptive immune response, $CD4^+$ Th1 cells as well as $CD8^+$ cells are able to secrete IFN-γ after being activated and differentiated (63). Furthermore, IFN-γ can have both immune-stimulatory as well as -suppressive roles in all stages of the tumor immunoediting process (76–78).

Type III IFNs can promote an antiviral response, which is similar to the response to type I IFNs (79). A distinct feature of both IFN types lies in the production of the respective cytokine and the distribution of the corresponding receptors. Type III IFNs are

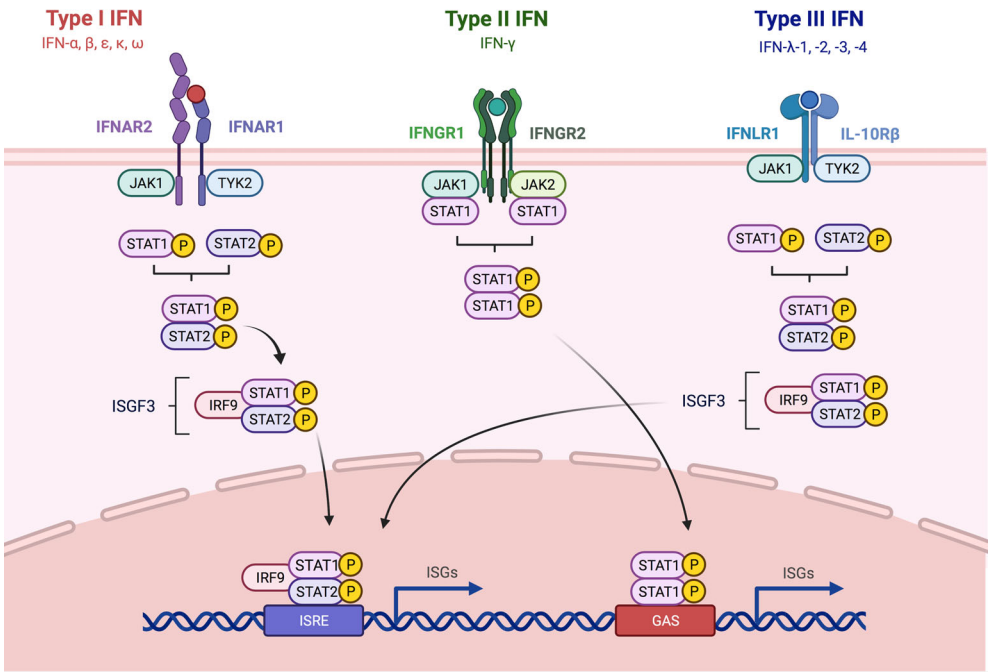

FIGURE 2 | Overview about Type I, -II and -III IFN signaling pathways. The three different types of IFNs discussed in this review signal through distinct receptor complexes on the cell surface. Type I IFNs act through the type I IFN receptor which is composed of the two subunits IFNAR1 and IFNAR2; Type II IFNs act through heterodimers consisting of IFNGR1 and 2 IFNGR2 and type III IFNs signal *via* heterodimers consisting of IL-10R2 and IFNLR1. Binding of type I and type III IFN to their respective receptor complexes triggers phosphorylation of associated JAK1 and -2, leading to the recruitment and subsequent phosphorylation of STAT1 and -2. STAT 1 and -2 form together a complex, which in turn recruits IRF9 which results in the formation of ISGF3. Engagement of type II IFN to the IFNGR1/2 complex leads to phosphorylation JAK1 and -2, and subsequently STAT1 is recruited and phosphorylated. Both IRF9 and the homodimer consisting of phosphorylated STAT1 can then translocate into the nucleus and bind to ISRE and GAS elements in the promoter region of ISGs, leading to the induction of the expression of antiviral genes. IFN, interferon; STAT, signal transducer and activator of transcription; JAK, Janus kinase; TYK, tyrosine kinase; IL, interleukin; IFNAR, interferon alpha receptor; IFNGR, interferon gamma receptor; IFNLR, interferon lambda receptor; ISGF3, interferon-stimulated gene factor 3; IRF9, interferon regulatory factor 9; ISRE, interferon-stimulated response element; GAS, interferon gamma activated site; ISG, interferon-stimulated gene. Adapted from "Interferon pathay", by Biorender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

especially important at epithelial barrier surfaces. Epithelial cells of the respiratory but also intestinal tract express high amounts of IFNLR1 demonstrating a predominant role of type III IFNs in the epithelial antiviral host defense (62, 80, 81).

ROLE OF IFNs IN THE MURINE AND HUMAN INTESTINE

IECs play a key role in balancing the intestinal immune homeostasis. They need to act tolerogenic to the vast amount of bacterial commensals but at the same time also be responsive to detrimental pathogens. In this context, there is increasing evidence that both type I and type III IFNs are important for the maintenance of the intestinal epithelial barrier integrity and the control of adaptive immune responses including antiviral responses (81, 82). In the intestine, type I IFNs are for example continuously produced by $CD11c^+$ DCs of the lamina propria (83). In contrast to that, it was shown, that murine IECs preferentially expressed type III IFNs over type I IFNs upon infection with human reoviruses and that they expressed higher levels of IFNLR1 than IFNAR1 and -2 compared to the underlying lamina propria (84, 85). This differential distribution of IFN receptors demonstrated that type III IFN IFN-λ could be seen as the very first line of defense against intestinal pathogens and might represent a nonredundant part of the innate antiviral immune response (81). Proof for that concept was established by studies, which revealed that IFN-λ signaling in IECs was protective against intestinal virus infection using mice with a conditional knock-out of IFNRL1 in the intestine. Depleting IFN-λ signaling in IECs led to an increase in intestinal virus replication and fecal shedding (86). Additionally, it was demonstrated, that administration of IFN-λ could cure intestinal virus persistence of norovirus even independent of the adaptive immune system (87). Though IFNs type III were shown to have this very essential role for the antiviral response of IECs, type I IFNs are not expendable. The same studies underlined the hypothesis, that type I IFNs, rather than protecting the IECs directly, were in fact essential for the prevention of a systemic spread of the intestinal viral infection (85, 87). Broggi and colleagues concluded, that in the intestine, type I and III IFNs acted together in a compartmentalized system. In this synergy, the type III IFN IFN-λ had the primary role in protecting the

epithelial barrier, and type I IFNs only came into action once this barrier has been penetrated by invading pathogens (88).

Nevertheless, IFN-α was found to prevent staurosporine-induced apoptosis murine model of the developing intestine *via* induction of the GTPase guanylate-binding protein-1 (GBP-1) expression, which was involved in regulating intestinal barrier function (89, 90). Using mice deficient for IFNAR1, it was demonstrated, that type I IFN signaling could determine Paneth- and goblet cell numbers in the intestine. Both epithelial cell hyper-proliferation and increased tumor burden were associated with the IFNAR1-deficient intestinal epithelium in a colitis-associated cancer model. Interestingly, intestinal cell hyper-proliferation as well as tumor promotion were reversed in the IFNAR1-deficient mice upon co-housing with WT littermates, underlining that IFNAR1 in IECs contributed to the regulation of the host-microbiome relationship which had consequences for intestinal cell regeneration as well as tumor formation (89). In the human setting it could also be demonstrated, that intestinal virus infection preferentially induced the upregulation of type III IFN to a higher extent compared to type I IFN, leading to a protective effect of type III IFN on the IECs expressing type III IFN receptors (91, 92). Recently it was discovered that, similar to the murine system, IFN-λ played an essential role in the context of epithelial cell protection during intestinal virus infection in humans. Human intestinal epithelial cells lacking IFNLR1, but not those lacking IFNAR1, showed diminished ability to control SARS-CoV-2 infection and replication in the intestine (93). Altogether, studies in both murine and human setting suggest a model, in which IECs favor type III IFN-mediated signaling over type I IFN signaling upon viral infection. This model allows an effective innate response to virus infection without triggering a systemic inflammatory process *via* type I IFN production and -signaling, thereby maintaining local intestinal gut homeostasis (91). In contrast to the protective role of type III IFNs on IECs, type II IFN IFN-γ was found to have negative effects on IECs and intestinal homeostasis (94–96). It was demonstrated, that IFN-γ produced by immune cells during mucosal immune response has destructive effects on Paneth cells (97, 98).

Two important regulators of IFN production are intestinal microbiota and their metabolites. Depletion of intestinal bacteria by antibiotic treatment reduced type I interferon responses in chicken after a challenge with influenza virus (99). In mice undergoing influenza A infection, decontamination of the gut by administration of antibiotic-containing water decreased ISG expression in stromal cells of the lung, indicating that changes of the intestinal microbiome have an impact on interferon signaling in the whole body. Interestingly, fecal transplantation was able to reverse the effects of antibiotic treatment and restored ISG expression (100). In a recent study, mice undergoing oral antibiotic treatment were also more susceptible to Chikungunya virus infection. The authors found by single-cell RNA sequencing that antibiotic treatment reduced type I IFN production by pDCs and subsequent expression of ISGs in infected monocytes. They further discovered that *Clostridium scingens*, by converting a primary bile acid into the secondary bile acid deoxycholic acid, was able to reconstitute IFN production by pDCs (101, 102). Other metabolites produced by intestinal microbiota, such as short-chain fatty acids (SCFAs), also play a significant role in colonic homeostasis and inflammation (103, 104). SCFAs can show modulatory effects on intestinal epithelial cells and neutrophils, as well as monocytes and macrophages (105). One of the most important SCFAs is butyrate, which is produced by Clostridia and Firmicutes, among others. Early on, butyrate enema therapy was found to be able to stimulate mucosal repair in experimental models of colitis in rats (106). Accordingly, several studies have been conducted highlighting the potential beneficial effect of butyrate on the course of UC in patients (107–109). In Crohn's disease, butyrate was administered orally to patients in the form of tablets. Butyrate is able to antagonize colonic inflammation (110) and has been found to reduce the production of pro-inflammatory cytokines including IFN-γ. It does this by acting as a histone deacetylase inhibitor and interfering with transcription of IFN-γ by inhibiting IFN-γ-induced tyrosine and serine phosphorylation of STAT1 (111–113). In 2019, Chen and colleagues investigated whether butyrate treatment could regulate the differentiation of T cells into Th1 and Th17 cell fates. They found that, on the one hand, promotion of both T cell subtypes was induced and differentially regulated (including promotion of IFN-γ expression in Th1 cell development), but most interestingly, expression of anti-inflammatory IL-10 was induced in both cases. Rag1-deficient mice receiving these butyrate-treated T cells showed less severe colitis compared with animals receiving untreated T cells. These data provide important details about how butyrate might be used therapeutically in IBD (114). Another interesting study from the same year examined the relationship between the microbiome, their intestinal metabloites, and interferons. Zhai and colleagues tested the ability of strains of Akkermansia muciniphila, which may exert probiotic effects in obesity and diabetes, to decrease inflammation in chronic colitis in mice. Both strains used (namely 139 and ATCC) were able to improve colonic inflammation when introduced into mice suffering from DSS-induced colitis. In addition, the levels of proinflammatory TNF as well as IFN-γ were reduced in the colon of the mice. Most importantly, they found that strain ATCC was able to induce the production of beneficial SCFAs (115). Also beyond intestinal inflammation, butyrate production by *Lachnospiraceae* was found to inhibit STING-activated type I IFN production by DCs (116). Conversely, beneficial lactic acid bacteria were shown to induce interferon type I secretion (117). Oral administration of the SCFA acetate mediated an IFN-β response by increasing ISG expression (118). These data suggest that intestinal bacteria and their metabolites have the capability to modulate interferon production and thus impact the innate immune response.

ROLE OF IFNs IN GVHD

One of the very first reports about IFNs in the context of GVHD was delivered in 1987, where Reyes and Klimpel measured the production of IFN-α/β/γ in sera of mice which were lethally irradiated and subjected to allo-BMT. They found that higher

IFN activity positively correlated with GVHD occurrence. With these observations, they paved the way for following research work regarding the influence of IFNs on GVHD development (119).

Type I IFNs

There are controversial reports about the role of type I IFNs in GVHD. In early clinical studies from the 1990s, pre-transplant exogenous type I IFN administration in humans resulted in increased GVHD occurrence and transplant-related mortality (120, 121). In contrast, several experimental studies could demonstrate, that type I IFN signaling was able to positively modulate murine GVHD outcome (121–125). In 2011, Robb and colleagues were amongst the first researchers to investigate the role of type I IFNs in GVHD and GVL. Using IFNAR1-deficient mice as recipients or donors in a murine GVHD model as well as exogenous administration of IFN-α, they found that type I IFN signaling had pleiotropic effects. These included the suppression of $CD4^+$ T cell-dependent GVHD and at the same time a paradoxical increase in $CD8^+$ T cell-mediated GVHD (122). In 2017, Fischer and colleagues elegantly showed that mice deficient for mitochondrial antiviral-signaling protein (MAVS) or stimulator of interferon genes (STING), which are innate types of PRRs that induce the expression of type I IFNs, developed worse GVHD after allo-HCT. In line with that, they could ameliorate disease outcome triggering either the RIG-I/MAVS- or the STING pathway to induce protective type I IFN signaling and maintain intestinal epithelial barrier integrity (121, 123). Consistently, several studies could demonstrate, that the administration of type I IFN or type I IFN-inducing agonists was potent in protecting mice from GVHD in a MHC-mismatched model, when given before allo-HCT (124, 125). Interestingly, intestinal microbes that produce indole and indole derivatives, mitigate GVHD development, partly by induction of IFN type I-stimulated genes (124).

Another study investigated the synergy between IL-22, known to be a key player in promoting aGVHD development, and type I IFN (126). For this, the authors used IFNAR- as well as IL-22-deficient mice as recipients of allogeneic wild-type BM cells in combination with allogeneic T cells from either IFNAR- or IL-22-deficient donors. They observed lower GVHD severity in IFNAR-deficient recipient animals when IL-22-deficient donor T cells were transferred in a major MHC mismatch model. Therefore, interference with IL-22 and type I IFN signaling could be a novel treatment approach. Additionally, the authors could connect the increased GVHD severity to elevated STAT1 activation and CXCL10 expression. It was speculated, that the synergy between donor-derived IL-22 and recipient type I IFN signaling could favor the loss of intestinal barrier integrity in aGVHD pathogenesis (126). Also in a model of systemic sclerosis (Ssc) -like cutaneous GVHD, protection was achieved by blocking type I IFN signaling *via* usage of a neutralizing Ab against IFNAR1. Notably, the central question in this study was to elucidate the role of type I IFN blocking in SSc, and the cutaneous model of GVHD was only used to mimic this disease. The authors investigated fibrogenesis, but important features such as survival rate after GVHD induction and histopathological score of the intestine were not obtained (127). Altogether, these data show that type I IFNs signaling has complex and partly opposite effects on GVHD development, depending on the preclinical model used.

Type II IFNs - Role of IFN-γ in GVHD

Over the last decades it became clear, that IFN-γ has pleiotropic effects in GVHD pathogenesis as well, depending on the examined cell type. It is well established, that intestinal damage during GVHD results in large parts from the increased release of IFN-γ and IL-12 from alloreactive Th1 T cells (128). IFN-γ induced intestinal cell apoptosis and, together with LPS originating from transmigrated bacteria, it stimulated the secretion of proinflammatory cytokines such as TNF, further supporting the inflammatory setting (129, 130). In mouse intestinal organoids, activated T cells induced tissue damage and reduction in Paneth cell and ISC numbers *via* IFN-γ signaling (131). Organoids deficient for the IFN-γ receptor remained unaffected by T cells, and *in vivo* IFN-γ administration elicited enteric inflammation (131). These data were supported by murine *in vivo* studies, where IFN-γ was described as the major mediator of ISC reduction in the colonic crypts (132). When GVHD was induced by T cells lacking IFN-γ or in mice deficient for the IFN-γ receptor in ISC, the stem cell compartment was protected (132). Collectively, these data indicate that IFN-γ has detrimental effects on the intestinal epithelium. In line with this hypothesis, already in 1989, Mowat described positive effects of the administration of an anti-IFN-γ antibody in two murine GVHD models (133).

Contrarily, a number of studies have also reported protective roles of the type II IFN in the context of GVHD. In a murine model of fully MHC-mismatched allo-BMT, IFN-γ-deficient donor $CD8^+$ T cells, but not WT donor cells, were able to induce lethal GVHD (134, 135). GVHD protection appeared to be mediated by effects of IFN-γ on T cells, either through a direct mechanism or *via* modulation of IL-12 signaling. IL-12 is essential in promoting the differentiation of naïve T cells into Th1 cells (136). IL-12 is produced by APCs and stimulates IFN-γ production by T cells as well as NK cells (137). In lethally irradiated mice, one single injection of recombinant murine IL-12 simultaneously with the BMT led to the protection of mice against aGVHD in both in fully MHC- as well as minor antigen-mismatched strain combinations (138–140). In another study, the authors pinpointed that dose as well as timing of recombinant IL-12 administration determined whether this cytokine had protective or rather detrimental effects. They found that administration of IL-12 1-12h prior to BMT resulted in protective actions of IL-12 whereas administration more than 36h after BMT completely abrogated these positive effects (141). Interestingly, in the study of Yang and colleagues from 1999, protection against GVHD was completely lost upon treatment with the neutralizing anti-IFN-γ monoclonal antibody (mAb) R4-6A2 (141). Altogether, this led to the assumption, that IFN-γ is required for the protective effects of IL-12, but is not per se responsible for GVHD induction (142). To decipher, whether recipient or donor IFN-γ was responsible for the protective effects *via* IL-12, Dey and colleagues transplanted C57/BL6 mice with allogeneic HSCs from IFN-γ KO BALB/c mice and could not achieve prolonged survival rates *via* treatment with

IL-12. This data supported the hypothesis that the IFN-γ which was mediating the protective effects of IL-12 was donor-derived. Mechanistically, the authors could show that Fas-mediated donor $CD4^+$ T cell apoptosis was one of the underlying mechanisms involved in the protective effects of IL-12 on GHVD pathogenesis (139). Apart from regulation of IL-12 signaling, a direct protective role of IFN-γ was also observed using IFN-γ KO mice. In one study, the authors could show that the dosing of conditioning regimen plays a pivotal role considering disease outcome: IFN-γ KO animals were used as donors in lethal and sublethal allogeneic BMT experiment using total body irradiation TBI as conditioning. For recipients of lethal doses of TBI, loss of donor IFN-γ was detrimental whereas recipient of sublethal doses, the loss of IFN-γ was protective (143). Consecutive studies showed that IFN-γ deficient $CD8^+$ T cells induce more severe GVHD in models with major and minor histocompatibility mismatch (134). These results were presumably based on the loss of apoptosis induced in activated $CD8^+$ T cells by IFN-γ. In line with these findings, another study could prove, that the IFN-γ receptor signaling was the major pathway responsible for the migration of both conventional- but also regulatory T cells to GVHD target organs. Altered trafficking of both T cell types was mediated by expression of CXCR3 which was connected to IFN-γ receptor signaling (144). Collectively, these reports provide evidence that IFN-γ regulates the alloreactive T cell pool and can prevent excessive T cell expansion.

The role of IFN-γ in intestinal GVHD remains controversial. Multiple studies observed that IFN-γ damages intestinal epithelial cells by inducing apoptosis and production of pro-inflammatory cytokines in the intestine. On the other hand, intact IFN-γ signaling appears important for the control of alloreacitve T cell expansion, differentiation and migration. Exploring which downstream cascades are responsible for the one or the other effect might open new avenues for targeted treatment.

Type III IFNs

Type III IFNs have only recently been discovered and therefore knowledge of their role in intestinal homeostasis and inflammation is just emerging. Epithelial cells of mucosal tissues, such as the IECs, are a major target of these type of interferons (62). Both human and murine IECs show a high responsiveness to treatment with type III IFNs. Recently, mice deficient for the IFN type III receptor (IL-28 receptor alpha subunit, IL-28Rα) showed comparable thymic regeneration potential and GVHD development as wildtype mice (145). In line with these data, IL-28A protein administration did not support recovery from irradiation-induced thymus damage (145). Nevertheless, single nucleotide polymorphisms in the IFNL4 gene in donors was associated with increased risk of non-relapse mortality in humans (146). Further studies are warranted to assess the relevance of type III IFNs in GVHD.

Modulation of IFN Signaling as a Treatment Approach in GVHD

Given the pleiotropic effects of IFNs on different cell populations involved in GVHD, it has been a challenge to develop successful clinical strategies by direct modulation of the interactions between IFN and their receptors. One indirect approach targeting IFN signaling amongst others, is the inhibition of JAK/STAT-signaling. Pre-clinical models showed, that incidence and severity of GVHD were reduced when administrating ruxolitinib, a selective inhibitor of JAK1 and -2, both being involved in the IFN-γ signaling pathway (147–149). Based on those findings, clinical trials on the potential of ruxolitinib for the treatment of glucocorticoid-refractory aGVHD showed great success and led to the approval of ruxolitinib for this indication by the Food and Drug Administration (150, 151). Another potential avenue for the use of IFN in the treatment of GVHD is related to the generation of mesenchymal stem cells (MSCs), a cell population with immunosuppressive properties. The role of IFN-γ activating MSCs has previously been described *in vitro* (152, 153). A first pilot study in patients suffering from severe steroid-resistant aGVHD could demonstrate MSCs as a promising treatment option (154). Nevertheless, development of a MSCs-based therapy for GVHD was impeded by factors such as a lack of standard protocol for the production of MSCs and the overall heterogeneity of MSCs derived from various donors and tissues (155–158). Regarding the role of IFN-γ in activating MSCs, it could be demonstrated, that MSCs primed with IFN-γ were able to reduce GVHD in NOD-SCID mice and to ameliorate survival rates when compared to animals receiving non-primed MSCs. The authors showed, that this effect was based on an induction of indoleamine 2,3-dioxygenase (IDO) *via* the IFNγ-JAK-STAT1 pathway in the MSCs, thereby enhancing their immunosuppressive properties (159). The exact mechanisms of how the various IFNs discussed in this review act in the context of GVHD remain largely unclear. It is essential to distinguish between the effects of IFNs on the hematopoietic cells of the recipient and of the donor, respectively. Furthermore, effects on the target tissues in the recipient need to be considered. Further studies are needed to elucidate the roles of IFNs in both GVHD and GVL processes after allo-HCT and to possibly make use of protective IFN administration.

ROLE OF IFNs IN IBD

Type I IFNs

In the context of genome-wide association studies, several genetic susceptibility loci for UC, CD or both were identified. These included genes which are essential key players in immunity and barrier function, amongst others. Several of those identified IBD-associated genes are involved in the type I IFN signaling pathway, for example the single nucleotide polymorphism (SNP) rs2284553, which affects the IFNAR1 gene. Other SNPs were found in the genes encoding JAK2 (rs10758669), TYK2 (rs11879191), STAT1 (rs1517352) and STAT3 (rs12942547), playing a role in several signaling pathways downstream of type I and III IFNs (28, 47, 160). Therefore, aberrations in the type I signaling network could promote an imbalanced immune response leading to induction of IBD (126). Appendicitis-appendectomy (AA) has been shown to reduce or prevent UC in adulthood, which was described in

several clinical studies (161–164), and reviewed by Koutroubakis and colleagues (165). Similar observations were made regarding the prevention and ability to decrease CD severity (161, 163, 166). Cheluvappa and colleagues developed a model of AA to identify novel therapy options for colitis amelioration. In this model, mice undergoing AA were protected from experimental colitis in and age-, bacteria- and antigen- dependent manner. They found that AA led to dampened Th17 cell activity and autophagy, but most interestingly, that AA was driving the modulation of IFN-associated molecules. Significant upregulation of the ISGs IFIT1, IFIT2 and IFIT3 in the distal colon 28 days after AA could be measured. These genes are induced by IFNs, virus infections and PAMPs, mediating immunomodulatory and antiproliferative functions as well as apoptosis induction (167–169). The authors assigned the beneficial effects of AA to this mode (170). Similar results were obtained in a study where imiquimod, a virostatic agent, induced type I IFN expression in the mucosa of the GI and was able to protect against DSS-induced colitis. Notably, no systemic IFN response could be measured. Based on their findings, the authors suggested imiquimod as a potential therapeutic approach for IBD patients (171). Other studies implied that type I IFNs rather played a dual role in the context of intestinal inflammation and recovery from colitis (172). Protective actions could be seen in a study where DCs, when stimulated with TLR9 agonists, produced type I IFNs leading to the protection against experimental colitis in RAG1-deficient mice. Consistently, administration of recombinant IFN-β led to similar protection (173). In a follow-up study, the authors could show more in detail, that the type I IFN produced by DCs was able to inhibit colonic inflammation *via* regulation of neutrophil and monocyte trafficking into the inflamed colon (174). In a T cell-induced colitis model, the protective effect of type I IFNs was attributed to its positive influence on Tregs *via* increasing their cell numbers and the maintenance of Foxp3 expression (175, 176). In contrast to that, it was seen that the local delivery of IFN-β *via* Lactobacillus into the intestine led to an exacerbation of DSS-induced colitis accompanied by increased levels of pro-inflammatory cytokines and lower numbers of Tregs in the small intestine of mice (177). It is important to underline, that the source of IFN-β in this study was a bacterial vehicle which might have diverse and different physiological effects compared to administration of pure recombinant type I IFN. Altogether, most studies suggest that type I IFNs are protective in different preclinical models of colitis.

Type II IFN

The type II IFN IFN-γ is one of the most highly upregulated cytokines found in IBD patients and in murine models of intestinal inflammation (41, 42, 178–180). It was demonstrated, that one aspect of the pathophysiological role of IFN-γ in IBD lied in its direct effects on the intestinal epithelium by influencing the homeostasis between cell proliferation and apoptosis *via* the regulation of converging of β-catenin signaling pathways. In the same study, it was observed, that TNF even increased the effects of IFN-γ, underlining a synergism between those two cytokines in the setting of intestinal inflammation (94).

Apart from that, several studies could show that IFN-γ also had significant effects on the intestinal vasculature. *In vitro*, it showed an overall antiangiogenic effect, including inhibition of proliferation, invasion and tube formation of endothelial cells *via* induction of the large GTPase guanylate binding protein-1 (GBP-1) (181–183). Based on these findings, Naschberger and colleagues could attribute GBP-1, resulting from IFN-γ upregulation in colorectal carcinoma (CRC), to an IFN-γ-dominated Th1-like immune reaction possessing potential angiostatic/antiangiogenic activity. They underlined that the microenvironment in GBP-1-positive CRC is dominated by IFN-γ, which was associated with an improved prognosis for the CRC patients (184). Interestingly, by using a neutralizing anti-IFN-γ antibody in a murine DSS-induced colitis model, it was shown, that IFN-γ exhibited an endogenous angiostatic activity in IBD and contributed to increased vascular permeability (179). In contrast to that, it was recently shown, that IFN-γ acted pathogenic in IBD by negatively impacting the vascular barrier by disruption of VE-cadherin, an adherent junction protein. By using endothelial cell-specific IFN-γ-receptor-KO mouse models, the authors of the study could show, that an endothelial-specific inhibition of the IFN-γ response led to an ameliorated outcome in DSS-induced colitis. Furthermore, IBD-associated vascular barrier dysfunction was also confirmed in human patients (185). Altogether and similar as in GVHD, IFN-γ remains a pleotropic cytokine with controversial roles in IBD pathology.

Type III IFNs

Since type III IFNs are emerging as a cytokine group with specific role on epithelial barrier surfaces, several studies tested their potential role in IBD models. First data demonstrated, that IFN-λ played a protective role in a murine model of DSS-induced colitis, thereby proposing it as an anticolitogenic cytokine (81, 126). In contrast, it was found, that levels of IFN-λ were increased in inflamed ileal tissues and sera of CD patients. This was accompanied by a loss Paneth cells. Based on those findings, the authors of this study suggested, that blocking IFN-λ or reducing its concentrations in affected patients might positively affect disease outcome (186). Further studies are required to explore the therapeutic potential of IFN-λ signaling.

Modulation of IFN Signaling as a Treatment for IBD

Studies investigating the effects of systemic administration of type IFNs to ameliorate IBD have produced controversial results. Administration of IFNs was shown to not have positive effects in the context of UC treatment (187). Overall, a Cochrane systematic literature review from 2008 investigating the efficacy and safety of type I IFN therapy (including IFN-β-1a, IFN-β-1b, IFN-α-2a, IFN-α-2b and associated PEGylated formulations) in UC showed no difference between groups of patients which were treated with type I IFNs or placebo in regards to remission achievement or symptom improvement. The authors conclude, that the data from those clinical trials do not support the use of type I IFNs to induce remission status in active UC. In accordance to the current scientific knowledge, no statistically

significant benefit regarding disease amelioration could been observed in using type I IFN for the treatment of IBDs (188).

Fontolizumab, a humanized anti-IFN-γ antibody, could not induce strong clinical responses in a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study in patients suffering from moderate to severe CD. Though well tolerated, administration only led to a significant decrease in C-reactive protein levels (189). The clinical development and further investigations on Fontolizumab in the context of IBD were stopped. Also eldelumab, an anti-INF-γ-inducible protein-10 (IP-10) monoclonal antibody, could not achieve the primary endpoint in a study in patients suffering from UC (190). Interestingly, when compared to other (auto-) immune related diseases such as rheumatoid arthritis or psoriasis, it becomes apparent, that in IBD, mainly TNF antagonizing monoclonal antibodies (mAbs), including infliximab, adalimumab and golimumab, show a beneficial effect (191). IFN signaling is mediated *via* intracellular JAKs and TYK2. It is therefore evident, that blocking these kinases could be a promising approach to cope with the elevated signaling of proinflammatory cytokines with proposed roles in mucosal immune cells in intestinal inflammation. Examples include the successful use of tofacitinib, blocking JAK3 activation and signaling *via* common γ-chain containing cytokines (IL-2,-4,-7,-9,-15 and -21) in CD and UC, and the selective JAK1 inhibitor filgotinib for Crohn's disease (192–194). This indicates that JAK inhibitors might be promising approaches for clinical therapy of IBD patients.

Regarding the therapeutic use of type III IFNs, some promising first data were collected in clinical trials for the treatment of chronic hepatitis with PEGylated forms of IFN-λ (ClinicalTrials.gov Identifier: NCT00565539). So far, there are no data available on the therapeutic potential of type III IFN administration in the context of IBD. An overview about the different IFNs and their respective role in GVHD and IBD pathogenesis can be obtained from **Table 1**.

CONCLUSION

To date, TNF is the sole proinflammatory cytokine that has been successfully targeted in IBD. Anti-TNF therapy with various anti-TNF antibodies (including infliximab, for example) is an essential backbone for the treatment of both CD and UC patients (201). Years of research and clinical success paved the way for increased interest in other cytokines and cytokine regulatory networks regarding the pathogenesis of IBD. Unfortunately, efforts in the field of anti-IFN therapy have not yet yielded promising results, as the use of fontolizumab, an anti-IFN-γ antibody, in CD patients did not result in improved disease outcome, and further investigation and development have been discontinued (189). With regard to the therapy of GVHD, IL-6 has been the best studied and targeted cytokine in this disease. Tocilizumab, an anti-IL-6 receptor antibody, has shown efficacy in steroid-refractory intestinal aGVHD as well as cGVHD (202, 203). IFN-γ in particular has been the focus of investigation in the context of GVHD. Due to divergent and pleiotropic effects of IFN-γ blockade in preclinical mouse models, no clinical studies have yet been conducted that consider direct targeting of IFN signaling pathways in intestinal GVHD.

Overall, it is clear that a highly complex and interconnected as well as -regulated cytokine network and its imbalance plays a

TABLE 1 | Overview about Type I, -II and -III IFNs and their role in GVHD and IBD pathogenesis.

	Type I IFN	Type II IFN	Type III IFN
Members	Mouse: α1, α2, α4-8, α11, α12-16, ϵ, κ, ζ Human: α1, α2, α4-8, α10, α13, α14, α16, α17, α21, β, ϵ, κ, ω	Mouse and human: γ	Mouse: λ2, λ3 Human: λ1-4
Receptor expression	Ubiquitously expressed on nucleated cells (195)	Ubiquitously expressed on nucleated cells (78)	Preferentially expressed on epithelial cells and some immune cells (e.g. DCs and neutrophils) (62, 80, 185, 196)
IFN production	In response to TLR3, RLR, cGAS and NOD1/2 stimulation (197–199)	In innate immunity: by NK- and NKT cells (75) In adaptive immunity: by $CD4^+$ Th1 cells and $CD8^+$ cells (63)	In response to TLR, RLR and Ku70 stimulation (200)
Effects in GVHD	Positive modulation of murine disease outcome (121–125) Negative effects: increased GVHD and TRM occurrences after pre-transplant administration (120)	Detrimental effects of IFN-γ on murine intestinal epithelium (129–132) IFN-γ antagonism improved GVHD outcome (133) Protective role *via* limiting the expansion of donor-derived T cells (134, 135) and donor-derived IL-12 in murine models (139, 142) Several studies report evidence that IFN-γ regulates the alloreactive T cell pool and T cell expansion (134, 144)	In humans: SNPs in IFNL4 gene in donors of HSCT associated with increased risk of non-relapse mortality (146)
Effects in IBD	Protective effects (173–176)	Detrimental effects on murine intestinal epithelium (94) Antiangiogenic effect on murine intestinal vasculature *in vitro* (181–183) In murine DSS-colitis model: angiostatic activity in IBD and contributed to increased vascular permeability (179) In humans: negative impact on intestinal barrier integrity (185)	Protective role in murine model of DSS-induced colitis (81, 126) Increased levels in inflamed intestinal tissue and sera of CD patients (186)

crucial role in the process of mucosal intestinal inflammation as well as mucosal healing. Both non-hematopoietic and hematopoietic cells of the innate and adaptive immune systems each play a central role in disease pathogenesis. In the context of GVHD, a further complication is the need to distinguish between the effect of IFNs on donor cells and, on the other hand, on recipient cells, as underscored by various preclinical models. Other factors, such as different types of MHC-mismatched BMT mouse models or even the timing of treatment in the context of IFN-cytokine network therapy, must also be considered. Further research needs to be conducted to understand why and how IFNs play such pleiotropic roles in the development and progression of both IBD and GVHD. It would be desirable to investigate the presumably positive effect of type I interferons in IBD more closely to provide the basis for eventual clinical trials. In addition, the recently discovered type III IFNs still need to be characterized in more detail, as their receptors are preferentially expressed on epithelial cells. So far, not much is known about their presumed role in signaling networks in the field of intestinal homeostasis and inflammatory processes. Ultimately, it is critical to understand better the divergent downstream signaling cascades of IFNs, and how these are connected to inflammation or tissue protection. Separating these different effects and identifying targets downstream of IFNs or their receptors might prove a promising translational approach, as seen in the example of JAK inhibition. This knowledge is essential to pave the way for more effective clinical approaches by precisely addressing the expression or functions of IFNs in intestinal inflammation.

AUTHOR CONTRIBUTIONS

EH and PA developed the overall concept for this review article. EH collected and reviewed literature, discussed the studies, and wrote the first draft of the manuscript. PA and RZ critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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LYG1 Deficiency Attenuates the Severity of Acute Graft-Versus-Host Disease *via* Skewing Allogeneic T Cells Polarization Towards Treg Cells

Huihui Liu†, Zhengyu Yu†, Bo Tang, Shengchao Miao, Chenchen Qin, Yuan Li, Zeyin Liang, Yongjin Shi, Yang Zhang, Qingya Wang, Miao Yan, Zhengyang Song, Hanyun Ren and Yujun Dong**

Department of Hematology, Peking University First Hospital, Beijing, China

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***Correspondence:**
Hanyun Ren
renhy0813@163.com
Yujun Dong
dongy@hsc.pku.edu.cn

†*These authors have contributed equally to this work*

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Acute graft-versus-host disease (aGVHD) is a lethal complication after allogeneic hematopoietic stem cell transplantation. The mechanism involves the recognition of host antigens by donor-derived T cells which induces augmented response of alloreactive T cells. In this study, we characterized the role of a previously identified novel classical secretory protein with antitumor function-LYG1 (Lysozyme G-like 1), in aGVHD. LYG1 deficiency reduced the activation of $CD4^+$ T cells and Th1 ratio, but increased Treg ratio *in vitro* by MLR assay. By using major MHC mismatched aGVHD model, LYG1 deficiency in donor T cells or $CD4^+$ T cells attenuated aGVHD severity, inhibited $CD4^+$ T cells activation and IFN-γ expression, promoted FoxP3 expression, suppressed CXCL9 and CXCL10 expression, restrained allogeneic $CD4^+$ T cells infiltrating in target organs. The function of LYG1 in aGVHD was also confirmed using haploidentical transplant model. Furthermore, administration of recombinant human LYG1 protein intraperitoneally aggravated aGVHD by promoting IFN-γ production and inhibiting FoxP3 expression. The effect of rhLYG1 could partially be abrogated with the absence of IFN-γ. Furthermore, LYG1 deficiency in donor T cells preserved graft-versus-tumor response. In summary, our results indicate LYG1 regulates aGVHD by the alloreactivity of $CD4^+$ T cells and the balance of Th1 and Treg differentiation of allogeneic $CD4^+$ T cells, targeting LYG1 maybe a novel therapeutic strategy for preventing aGVHD.

Keywords: LYG1, aGVHD, allogeneic $CD4^+$ T cells, alloreactivity, Th1 cells, Treg cells

Abbreviations: aGVHD, acute graft-*versus*-host disease; allo-HSCT, allogeneic allogeneic hematopoietic cell transplantation; BM, Bone marrow cells; Treg, Regulatory T cells; Th1, T helper 1 cells; Tc1, T cytotoxic 1 cells; rhLYG1, recombinant human LYG1 protein; LYG1, Lysozyme G-like 1; MLR, mixed lymphocyte reaction; TBI, total body irradiation; GVT, graft-*versus*-tumor.

INTRODUCTION

Acute graft-*versus*-host disease (aGVHD) is medical complication which mainly destroy host tissues including the skin, liver, colon and the lung after allogeneic hematopoietic cell transplantation (allo-HSCT), representing a major cause for morbidity and non-relapse mortality (1, 2). Alloreactive T cells were the major detrimental factors during the pathogenesis of aGVHD (3, 4). In GVHD, the donor T cells recognize the host antigens, activate, differentiate and traffic to the target organs under guidance of cytokines and chemokines, and result in inflammatory damages in the target organs (5). IFN-γ is a central regulatory cytokine in the initiation and maintenance of aGVHD due to its crucial function for $CD4^+$ Th1 cells differentiation and $CD8^+$ T cells function during the priming and expansion phase (6). Regulatory T cells (Treg) which reduces the incidence and severity of aGVHD is one of the protective factors against aGVHD (7). Due to the inhibitory characteristics, Treg cells have been widely studied for GVHD treatment in pre-clinical models and clinical trials (8, 9).

Despite considerable achievements in the treatment of aGVHD, it remains a major clinical problem for the patients undergoing allo-HSCT. Approximately 40%-60% of recipients will develop aGVHD, imposing crucial risks for long term survival (10). Because the success of allo-HSCT relies on graft-*versus*-tumor (GVT) function mediated by T cells, immunosuppressive strategies are less attractive (5). Therefore, explorations on new mechanisms and novel therapeutic strategies for aGVHD with preserving GVT responses are important and necessary.

In our previous study, we have identified and characterized a novel classical secretory protein LYG1 (Lysozyme G-like 1) through immunogenomics strategy (11). Recombinant human LYG1 protein (rhLYG1) can inhibit tumor growth by promoting the activation and IFN-γ production of tumor antigen-specific $CD4^+$ T cells (11). While LYG1 deficiency accelerated B16 and LLC1 tumor growth due to the inhibited T cell functions. However, the function of LYG1 in other immune diseases is unclear.

Given the enhanced T cell functions under rhLYG1 stimulation and the inhibited T cell functions with LYG1 deficiency, we hypothesized that LYG1 might participate in the development of GVHD. To verify the hypothesis, we explored the role and mechanisms of LYG1 during GVHD using aGVHD murine models in this study.

MATERIALS AND METHODS

Mice

Six- to eight-week-old C57BL/6 (B6, H2Kb) and BALB/c (H2Kd) and (B6 × DBA/2) F1 (BDF1, H-2b–d) mice were purchased from Vital River Laboratories. The *Lyg1* conventional knockout mice (C57BL/6 background, $Lyg1^{-/-}$) were generous gifts from Prof. Wenling Han at Peking University Health Science Center (Beijing, China). $IFN\text{-}\gamma^{-/-}$ mice (B6.129S7-IFNgtm1Ts/J) were purchased from the Model Animal Research Center of Nanjing University. Homozygous knockout ($Lyg1^{-/-}$) and the littermate wild-type (WT, $Lyg1^{+/+}$) mice were used for all related experiments. All mice were bred at the center animal laboratory of Peking University First Hospital under specific pathogen-free conditions, and all experiments were approved by the Ethics Committee of Peking University First Hospital.

Mixed Lymphocyte Reaction (MLR)

Splenocytes derived from BALB/c mice were used as stimulator cells. $CD3^+$ T cells were used as responder cells selected from splenocytes of $Lyg1^{+/+}$ or $Lyg1^{-/-}$ mice using Mouse $CD3^+$ T cell isolation kit (Biolegend, San Diego, US) according to the manufacturer's instructions. The purity of $CD3^+$ T cell was >90% assessed by flow cytometry. The responder cells (2×10^5 in 100 μl complete culture medium) labeled by CFSE were cultured with stimulator cells treated with mitomycin C (Selleck, Houston, US) for 30 minutes (5×10^5 in 100 μl complete culture medium) in 96 well plate. After 5 days of culture, the cells were analyzed by flow cytometry.

aGVHD Mouse Model

Bone marrow cells (BM) were collected by red blood cell lysis. Splenocytes were isolated by Ficoll gradient centrifugation. $CD3^+$, $CD4^+$ and $CD8^+$ T cells were sorted from splenocytes of $Lyg1^{+/+}$ or $Lyg1^{-/-}$ mice using Mouse $CD3^+$, $CD4^+$ and $CD8^+$ T cell isolation kit (Biolegend, San Diego, US) according to the manufacturer's instructions. The purities were >90% assessed by flow cytometry. Recipient (BALB/c, H2Kd) mice were conditioned with total body irradiation (TBI) at 750 cGy (60 Coγ source) on day 0 followed by allogeneic transplantation intravenously: 5×10^6 $Lyg1^{+/+}$ B6 BM (H2Kb) and 3×10^6 $CD3^+$ T cells (or 1.8×10^6 $CD4^+$ T cells or 1.8×10^6 $CD8^+$ T cells) from $Lyg1^{+/+}$ or $Lyg1^{-/-}$ splenocytes (H2Kb) (12). BM control group were given 5×10^6 $Lyg1^{+/+}$ B6 BM alone. Syngeneic transplant group (Syn) were given 5×10^6 BM (H2Kb) and 3×10^6 $CD3^+$ T cells sorting from BALB/c splenocytes (H2Kd). Haploidentical (B6→BDF1) transplant model (Haplo-HSCT): recipient ((B6 × DBA/2) F1 (BDF1, H-2b–d)) mice were conditioned with TBI at 900 cGy on day 0 followed by allogeneic transplantation intravenously: 5×10^6 $Lyg1^{+/+}$ B6 BM (H2Kb) and 2×10^7 cells from $Lyg1^{+/+}$ or $Lyg1^{-/-}$ splenocytes (H2Kb) (13). For the experiments using rhLYG1 administration, recipient (BALB/c, H2Kd) mice were conditioned with TBI at 750 cGy on day 0 followed by allogeneic transplantation intravenously: 5×10^6 B6 BM (H2Kb) and 3×10^6 $CD3^+$ B6 or $IFN\text{-}\gamma^{-/-}$ T cells (H2Kb). rhLYG1 were injected intraperitoneally in BALB/c recipients daily from day 1 to 7 after transplantation. Survival was monitored every day, recipient's weight and GVHD score were measured every week. The scoring system to evaluate the severity of aGVHD includes five clinical parameters: weight, activity, skin, fur ruffling, and posture. Individual mice were scored 0-2 for each criterion (14). Representative tissues of aGVHD target organs (liver and lung) were excised from recipients on 28 days after transplantation and subjected to histopathological scoring (15, 16). Immunohistochemical (IHC) analysis for CD4+ and CD8+ (servicebio, China) were performed on the tissues of recipient mice at 7 days post-transplantation according to the manufacturer's instructions.

GVT Mouse Model

2.5×10^4 mouse mastocytoma cell strain P815 (H2Kd) were injected intravenously into per aGVHD recipient on day 0 after allogeneic transplantation. Survival was monitored every day. The P815 was retrovirally transduced with a *luc/neo* plasmid using a protocol described previously (17). Mice that received P815-*luc/neo* were given intraperitoneal (200 mg/kg) D-Luciferin (Xenogen, Alameda, CA) and placed supine in the Xenogen IVIS bioluminescence imaging system under anesthesia with isofluorane. Pseudocolor images showing whole-body distribution of bioluminescent signal were superimposed on conventional grayscale photographs. Livers were excised from recipients died or 14 days after transplantation and tumor burden on the livers were analyzed.

Isolation of Cells and Flow Cytometry

Flow cytometry was performed using the following anti-mouse antibodies from Biolegend (Cal., US): H2Kb-FITC, CD3-APC/Cy7, CD4-PE/cy7, CD8-BV421, IFN-γ-PE, CD69-PE, CD44-FITC, CD62L-APC, T-bet-FITC. Spleens, livers and lungs were excised on day 7 after transplantation. Spleens and livers gently pressed through a cell strainer (70 μm). Livers infiltrating lymphocytes were isolated using Percoll (Living, Beijing, China). Perfused lungs were digested in RPMI-1640 medium containing type IV bovine pancreatic DNase (30 mg/ml; Sigma-Aldrich, US) and collagenase XI (0.7 mg/ml; Sigma-Aldrich, US) to obtain single-cell suspensions. Single-cell suspensions prepared from the above operation were kept on ice and blocked by incubation with anti-Fc receptor antibody. For membrane molecule analysis, cells were labeled with fluorescent conjugated antibodies at 4°C for 30 minutes followed by washes with cold PBS. For cytokine analysis, cells were stimulated with Cell Activation Cocktail (with Brefeldin A) (Biolegend, Cal., US) for 6 hours before cells were harvested for analysis. Cells were first stained with surface markers and then fixed and permeabilized with BD IntraSure Kit (BD Biosciences, NJ, US) according to the manufacturer's instructions for intracellular staining. Foxp3 and T-bet were stained using a Foxp3 Fix/Perm Buffer Set (Biolegend, Cal., US), according to the manufacturer's instructions. Flow cytometry analysis was performed on FACS Canto II (BD Biosciences, NJ, US) and analyzed with FlowJo software.

Measurements of Cytokines in Serum

The peripheral blood samples were obtained on day 7 after transplantation and clotted for 5 h at room temperature before centrifugation for 15 minutes at 2000g. The serums were collected and stored at -80°C. The serum concentrations of IFN-γ, TNF-α and IL-6 were quantitated using a mouse Th cytometric bead array kit (BD Biosciences, NJ, US) (Biolegend, Cal., US) according to the manufacturer's instructions.

Real-Time Quantitative PCR (qPCR)

qPCR was performed for quantitative analyses in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Amplifications were performed using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, MA, US). The quantification data were analyzed with ABI Prism 7000 SDS software. The expression levels of the target genes were normalized to the internal standard gene GAPDH using the comparative Ct method (ddCt). Primers used in qPCR to examine the genes:

Foxp3:
Forward Primer (5'–3') TTTCACCTATGCCACCCTTATC
Reverse primer (5'–3') CATGCGAGTAAACCAATGGTAG
CCL5
Forward Primer (5'–3') GTATTTCTACACCAGCAGCAAG
Reverse primer (5'–3') TCTTGAACCCACTTCTTCTCTG
CXCL9
Forward Primer (5'–3') AATCCCTCAAAGACCTCAAACA
Reverse primer (5'–3') TCCCATTCTTTCATCAGCTTCT
CXCL10
Forward Primer (5'–3') CAACTGCATCCATATCGATGAC
Reverse primer (5'–3') GATTCCGGATTCAGACATCTCT
GAPDH
Forward Primer (5'–3') CACCAACTGCTTAGCCCCC
Reverse primer (5'–3') TCTTCTGGGTGGCAGTGATG

Statistical Analysis

Survival curve was analyzed using Kaplan-Meier method. Differences between groups in survival studies were determined using log-rank test. A student t test was applied for the other studies. Independent experiment was performed 3 times. The results in the repeats were similar in this study. $p < 0.05$ is considered statistically significant. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Analyses were performed using GraphPad Prism 7.

RESULTS

LYG1 Deficiency Inhibited Alloreactivity of CD4$^+$ T Cells *In Vitro*

Firstly, we examined whether LYG1 affected the alloreactivity of CD4$^+$ T cells *in vitro* by MLR assay. The expression of the activation maker CD69 on CD4$^+$ T cells were decreased in *Lyg1*$^{-/-}$ mice compared with the *Lyg1*$^{+/+}$ mice (**Figure 1A**), so was for the IFN-γ production (**Figure 1B**). While the percentages of Treg cells gated on CD4$^+$ T cells were higher in *Lyg1*$^{-/-}$ group than *Lyg1*$^{+/+}$ group (**Figure 1C**). The control group (without stimulating cells) had not response (**Figure 1S**). Whereas there were no differences in the expression of CD69, IFN-γ and Foxp3 between *Lyg1*$^{+/+}$ and *Lyg1*$^{-/-}$ mice prior to the culture (**Figure 1D**). These results suggest that LYG1 deficiency restrains the alloreactivity of CD4$^+$ T cells *in vitro*.

LYG1 Deficiency in Donor T Cells Alleviated aGVHD

We adopted a major MHC mismatched aGVHD model to examine the role of LYG1 in the development of aGVHD (**Figure 2A**). There were no differences in distribution of

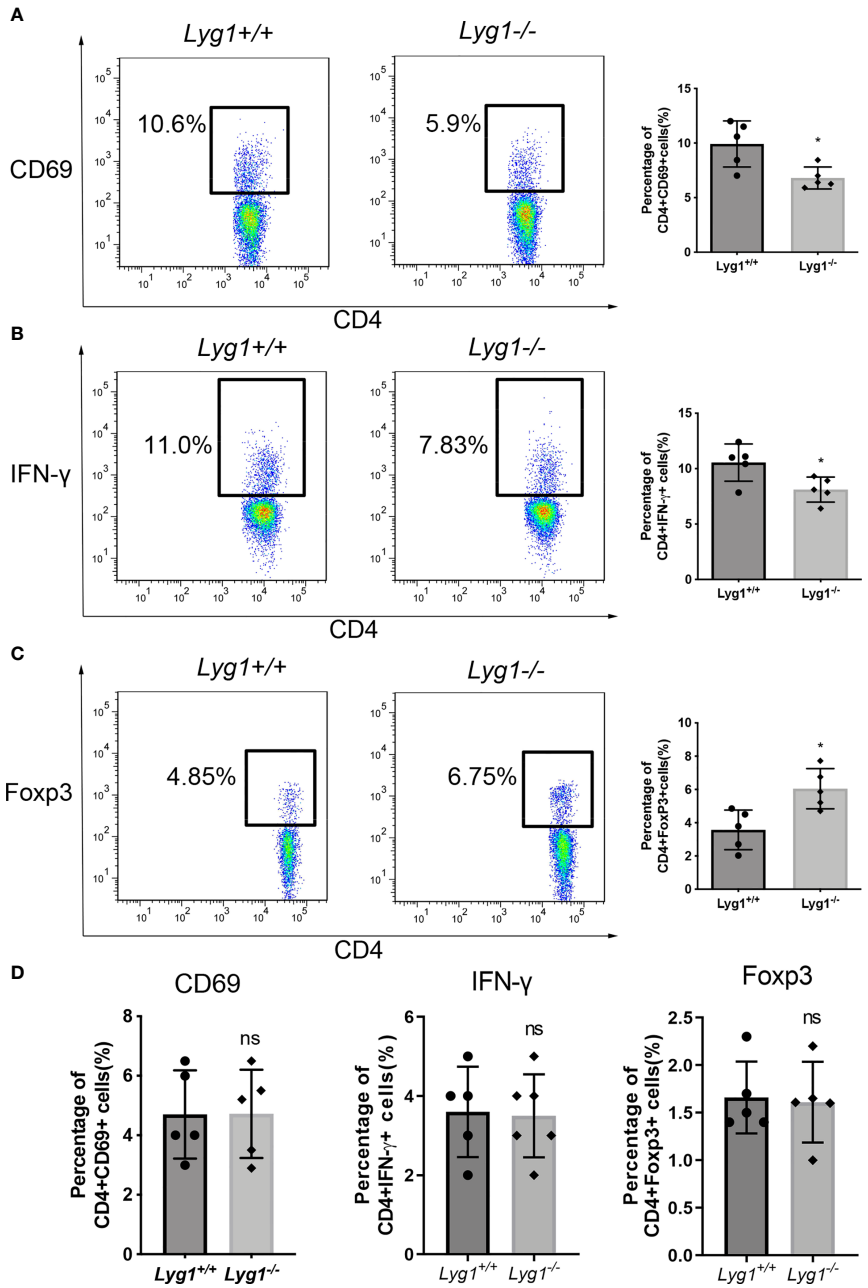

FIGURE 1 | LYG1 deficiency inhibited alloreactivity of $CD4^+$ T cells *in vitro*. In MLR assay, $CD3^+$ T cells from $Lyg1^{+/+}$ or $Lyg1^{-/-}$ mice as responder cells were cultured with mitomycin treated splenocytes from BALB/c mice as stimulator cells. After 5 days, CD69 expression **(A)**, Th1 **(B)** and Treg **(C)** in the responder $CD4^+$ T cells were detected by flow cytometry analysis. **(D)** The expression of CD69, IFN-γ and Foxp3 gated on $Lyg1^{+/+}$ and $Lyg1^{-/-}$ $CD4^+$ T cells prior to the culture. Independent experiment was performed 3 times. n = 5 per group. Representative plots gated on H2Kb+CD4+ T cells are shown and statistical results are expressed as the mean ± SD, *p < 0.05 compared with $Lyg1^{+/+}$ group. ns, no significance.

T cells subsets, including naive (the most dominant subset), central memory and effector $CD4^+$ T cells and $CD8^+$ T cells, from $Lyg1^{+/+}$ and $Lyg1^{-/-}$ mice before adoptive transfer (**Figure S2**). As shown in **Figures 2B, C**, the control mice in BM group (only transplantation of BM) and Syn group did not induce aGVHD. Comparing with recipients receiving $Lyg1^{+/+}$ T cells, recipients receiving $Lyg1^{-/-}$ T cells showed significantly higher long-term survival rates (**Figure 2B**), less weight loss (**Figure 2C**),

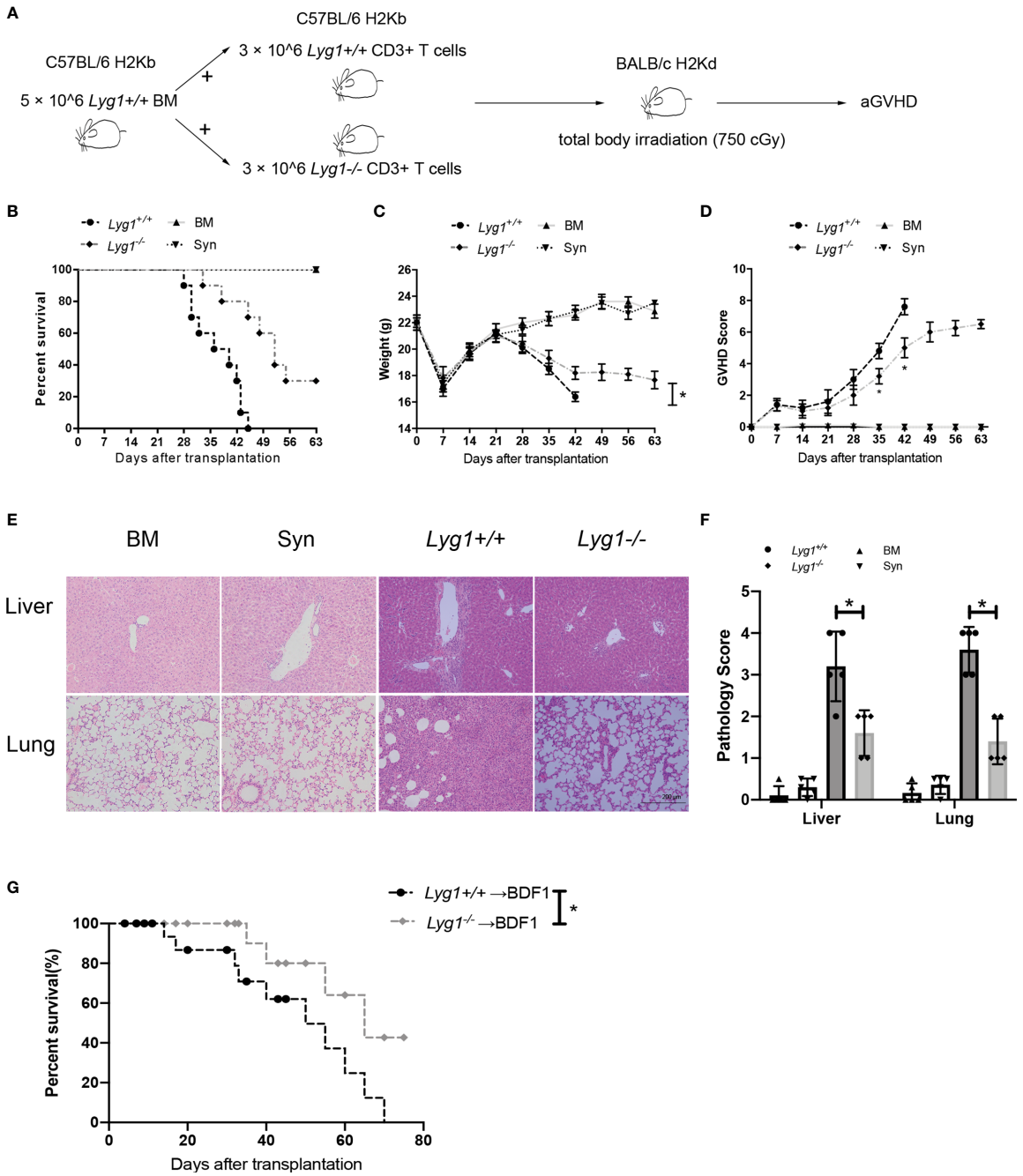

FIGURE 2 | LYG1 deficiency in donor T cells alleviated aGVHD. Lethally irradiated BALB/c mice were reconstituted with 5 × 10^6 *Lyg1*$^{+/+}$ BM and 3 × 10^6 T cells from *Lyg1*$^{+/+}$ mice (*Lyg1*$^{+/+}$ group) or *Lyg1*$^{-/-}$ mice (*Lyg1*$^{-/-}$ group). BM control (BM group) were given 5 × 10^6 *Lyg1*$^{+/+}$ BM alone. **(A)** The diagram illustrating the experimental procedure. Survival **(B)**, weight **(C)** and aGVHD scores **(D)** were monitored. **(E)** Histological examination (×200 magnification) of liver and lung in four groups were analyzed on day 28 after transplantation. **(F)** Histologic scores of liver and lung were shown. **(G)** Survival of BDF1 recipients given transplants with 5 × 10^6 *Lyg1*$^{+/+}$ BM and 2 × 10^7 splenocytes from *Lyg1*$^{+/+}$ mice (*Lyg1*$^{+/+}$ group) or *Lyg1*$^{-/-}$ mice (*Lyg1*$^{-/-}$ group). Independent experiment was performed 3 times. Data pooled: 3 experiments (n = 10 for *Lyg1*$^{+/+}$ group and *Lyg1*$^{-/-}$ group, n = 5 for BM group). Results are expressed as the mean ± SD, *p < 0.05 compared with *Lyg1*$^{+/+}$ group.

and lower aGVHD clinical scores (**Figure 2D**) after 3 weeks since the allogeneic transplantation. Pathology revealed that mice receiving Lyg1$^{-/-}$ T cells showed dramatically reduced inflammation in the livers and lungs compared with those receiving Lyg1$^{+/+}$ T cells (**Figure 2E**). The histological grades of livers and lungs were significantly decreased in recipients receiving Lyg1$^{-/-}$ donor T cells (**Figure 2F**). There was no pathological lesion and inflammation in BM group and Syn

group (**Figures 2E, F**). We also examined the LYG1 effect on aGVHD using haplo-HSCT model. The mice receiving Lyg1$^{-/-}$ T cells also exhibited a higher survival rate than did the control mice (**Figure 2G**). These results proved that LYG1 deficiency in donor T cells decreased aGVHD mortality and severity.

LYG1 Deficiency in Donor T Cells Dampened the Function of Allogeneic CD4$^+$ T Cells in Spleens

First, we examined the donor chimerism in the spleen of recipient mice on day 7 after transplantation. Nearly 98% of H2Kb+ donor cells can be observed in recipient spleens in *Lyg1*$^{+/+}$ and *Lyg1*$^{-/-}$ aGVHD groups (**Figure 3A**), suggesting LYG1 deficiency in donor T cells did not affect the engraftment of donor cells. To explore potential regulation mechanisms for LYG1 in aGVHD, we investigated the activation and differentiation of H2Kb+ donor T cells from recipients receiving either *Lyg1*$^{+/+}$ or *Lyg1*$^{-/-}$ T cells. We did not observe notable difference in the absolute number (data not shown) and ratio of CD4$^+$ or CD8$^+$ T cells between the two groups (**Figure 3B**); however, we noticed significant decrease of CD69 expression on CD4$^+$ T in mice receiving *Lyg1*$^{-/-}$ T cells (**Figures 3C, D**), suggesting reduced activation of CD4$^+$ T cells in these mice. The effector T cells (CD44^{hi}CD62L^{lo}) also decreased in mice receiving *Lyg1*$^{-/-}$ T cells (**Figures 3E, F**). The IFN-γ producing CD4$^+$ (Th1) and IFN-γ producing CD8$^+$ T cells (Tc1, T cytotoxic 1) were significantly reduced in recipients of *Lyg1*$^{-/-}$ T cells compared with those of *Lyg1*$^{+/+}$ group (**Figures 3G, H**). T-bet is a master regulator for Th1 differentiation and IFN-γ production (18).Then we examined T-bet and found the percentages of T-bet on CD4$^+$ T cells were lower in mice receiving *Lyg1*$^{-/-}$ T cells (**Figure 3I**).

Treg cells have been shown to be capable of reducing the severity of aGVHD by restraining immoderate immune activation and maintaining immune homeostasis (19). We found that the proportions of Treg cells (Foxp3+ gated on CD4$^+$ T cells) in spleens were dramatically enhanced from recipients received *Lyg1*$^{-/-}$ donor T cells (**Figure 3J**), suggesting LYG1 deficiency in donor T cells promotes Treg differentiation. Interestingly, the proportions of Foxp3+ population gated on CD8$^+$ T cells increased in spleens in *Lyg1*$^{-/-}$ group than *Lyg1*$^{+/+}$ group (**Figure 3K**). The mRNA expression of Foxp3 in spleens were also higher in recipients received *Lyg1*$^{-/-}$ donor T cells (**Figure 3L**).

We found the similar results in the haplo-HSCT model, LYG1 deficiency in donor T cells decreased the expression of CD69 and IFN-γ, but increased the expression of FoxP3 on T cells (**Figure S3**). Whereas the BM and Syn control group had a lower T cells response (**Figure S4**).

LYG1 Deficiency in Donor T Cells Inhibited Allogeneic CD4$^+$ T Cells Infiltration in aGVHD Target Organs

We also evaluated the lymphocytes in livers and lungs, the representative target organs of aGVHD. Similarly with spleens, the infiltrating lymphocytes were almost H2kb+ donor cells (data not shown). A significant reduction of CD4$^+$ T cells in livers and lungs were observed in mice receiving *Lyg1*$^{-/-}$ donor T cells compared with the *Lyg1*$^{+/+}$ group determined by IHC (**Figure 4A**). CD8$^+$ T cells infiltration in aGVHD target organs also reduced slightly in *Lyg1*$^{-/-}$ group (**Figure 4B**). The T cells infiltration in livers and lungs were rarely detected in BM and Syn control group. The decrease of T cells infiltrating in livers and lungs suggested that LYG1 deficiency might change the expression of chemokines that recruited T cells. Therefore, we examined the expression of CCL5, CXCL9, CXCL10 in livers and lungs. LYG1 deficiency inhibited significantly the mRNA expression of CXCL9 and CXCL10, but had no obvious effect on CCL5 expression (**Figures 4C, D**).

LYG1 Deficiency in Donor T Cells Inhibited the Function of Allogeneic CD4$^+$ T Cells in GVHD Target Organs

LYG1 deficiency reduced the number of T cells infiltrating in GVHD target organs, whether it affect allogeneic T cells function? Therefore, we investigated the activation and differentiation of donor T cells in livers and lungs from recipients receiving either *Lyg1*$^{+/+}$ or *Lyg1*$^{-/-}$ T cells. Similarly, the CD69 and IFN-γ expression of CD4$^+$ T cells and CD8$^+$ T cells decreased in *Lyg1*$^{-/-}$ recipient livers and lungs compared with the *Lyg1*$^{+/+}$ groups (**Figures 5A–D**). The percentages of Foxp3+ population gated on CD4$^+$ T cells and CD8$^+$ T cells were higher in livers and lungs in *Lyg1*$^{-/-}$ group than *Lyg1*$^{+/+}$ group (**Figures 5E, F**). The expression of Foxp3 in mRNA level in livers and lungs also increased in recipients received *Lyg1*$^{-/-}$ donor T cells (**Figures 5G, H**).

LYG1 Mediated GVHD Development Mainly Through CD4$^+$ T Cells

To test whether the effects of LYG1 on GVHD mediated through CD4$^+$ T cells or CD8$^+$ T cells, we performed GVHD models using purified CD4$^+$ T cells or CD8$^+$ T cells as grafts, respectively. As illustrated in **Figures 6A, F**, the reduction of aGVHD lethality by LYG1 deficiency was observed in CD4$^+$ T cells transplant, but not CD8$^+$ T cells transplant. LYG1 deficiency in CD4+ T cells transplant significantly reduced the activation of CD4$^+$ T cells and IFN-γ and T-bet expression, but increased Treg ratio (**Figures 6B–E**), but not in CD8$^+$ T cells transplant (**Figures 6G–I**). Taken together, the results suggested that LYG1-mediated GVHD development mainly depended on CD4$^+$ T cells, but not CD8$^+$ T cells.

rhLYG1 Aggravated aGVHD *via* Promoting IFN-γ Production and Inhibiting Foxp3 Expression

Furthermore, we used the purified rhLYG1 to evaluate the role of LYG1 in aGVHD model. As shown in **Figures 7A, B**, rhLYG1 significantly accelerated and exacerbated the death and weight loss compared with PBS control. Higher clinical aGVHD scores were seen in rhLYG1 group than in control group (**Figure 7C**). The IFN-γ production of CD4$^+$ T cells and CD8$^+$ T cells (Th1 and Tc1 cells) were significantly higher than PBS control in spleens (**Figures 7D, E**). The mRNA expression of FoxP3 in spleens decreased in mice treated with rhLYG1 compared with PBS (**Figure 7F**). The IFN-γ concentrations in serum from mice treated with rhLYG1 were higher than that from

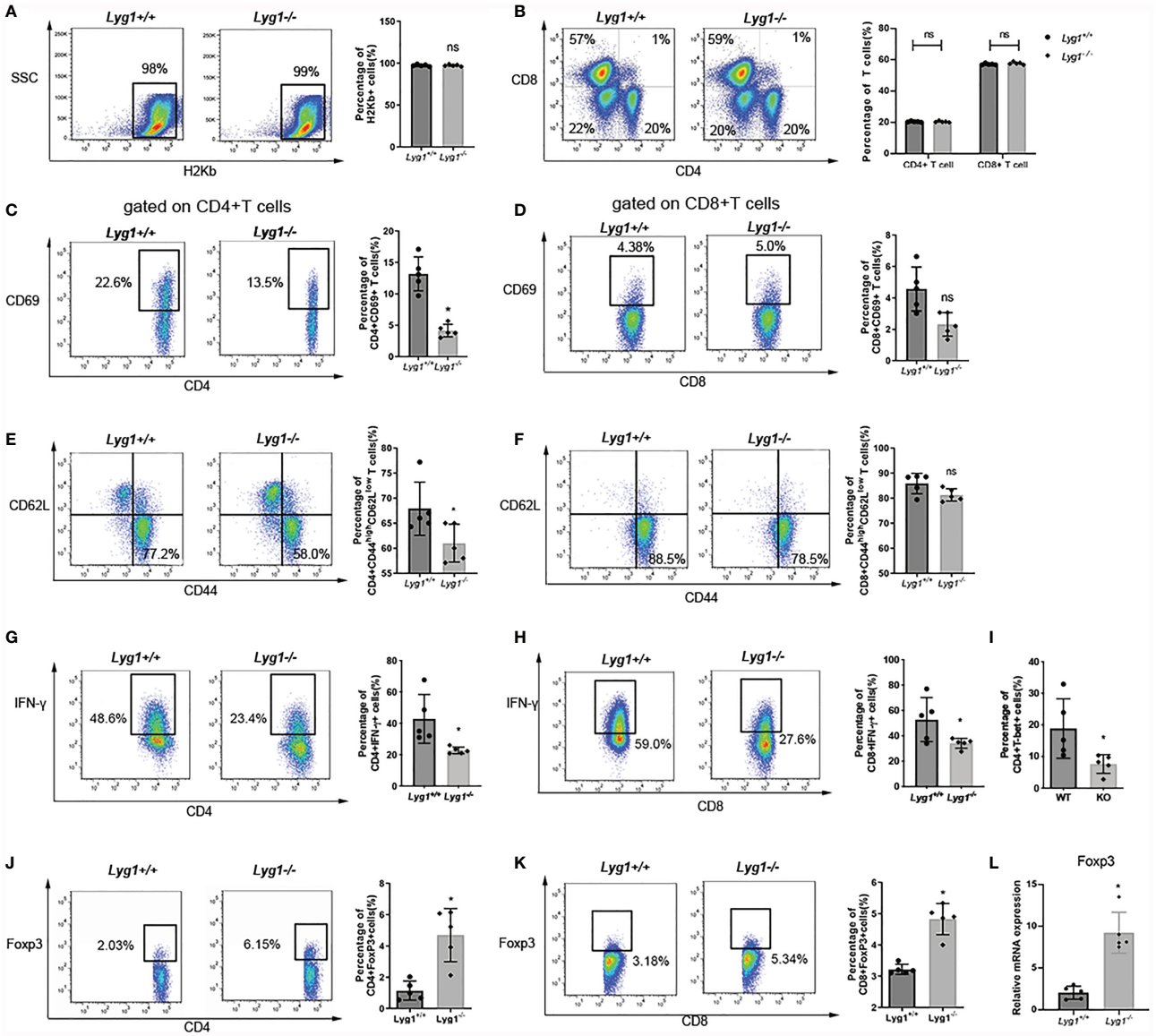

FIGURE 3 | LYG1 deficiency reduced allogeneic T cells function in spleens. Splenocytes of recipient mice were isolated on day 7 after transplantation and analyzed by flow cytometry and qPCR. **(A)** The percentages of H2Kb+ cells in living splenocytes. **(B)** The percentages of $CD4^+$ T and $CD8^+$ T cells in H2Kb+ splenocytes. **(C, D)** The percentages of CD69 expression in $CD4^+$ T cells and $CD8^+$ T cells. **(E, F)** The expression of effector ($CD44^{hi}CD62L^{lo}$) phenotype gated on $CD4^+$ T and $CD8^+$ T cells. **(G, H)** The percentages of IFN-γ expression in $CD4^+$ T cells and $CD8^+$ T cells. **(I)** The percentages of T-bet expression in $CD4^+$ T cells. **(J, K)** The percentages of Treg in $CD4^+$ T cells and $CD8^+$ T cells. The percentages of **Figure 3 (C–K)** were all gated on $H2Kb^+CD4^+$ cells or $H2Kb^+CD8^+$ cells. **(L)** Foxp3 expression of splenocytes were examined by qPCR. Independent experiment was performed 3 times. The results in the repeats were similar. n = 5 per group. Representative plots are shown and statistical results are expressed as the mean ± SD, *$p < 0.05$ compared with $Lyg1^{+/+}$ group. ns, no significance.

PBS group (**Figure 7G**). We also detected the IFN-γ, TNF-α and IL-6 in serum in the above GVHD models and found that the concentrations of IFN-γ, TNF-α and IL-6 reduced in recipients received $Lyg1^{-/-}$ donor T cells compared with recipients received $Lyg1^{-/-}$ donor T cells, especially for IFN-γ (**Figure S5**). Further we verified the role of IFN-γ in the effects of LYG1 on GVHD using $IFN\text{-}\gamma^{-/-}$ T cells as grafts. As showed in **Figures 7H–J**, with the deficiency of IFN-γ, the effect of rhLYG1 aggravating aGVHD was partially abrogated, which reconfirmed the crucial role of IFN-γ in LYG1-mediated GVHD development.

LYG1 Deficiency in Donor T Cells Preserved GVT Response

To determine whether the reduction of aGVHD lethality by LYG1 deficiency would affect GVT activity, mouse mastocytoma cell strain P815 (H2Kd) were injected intravenously on day 0 to

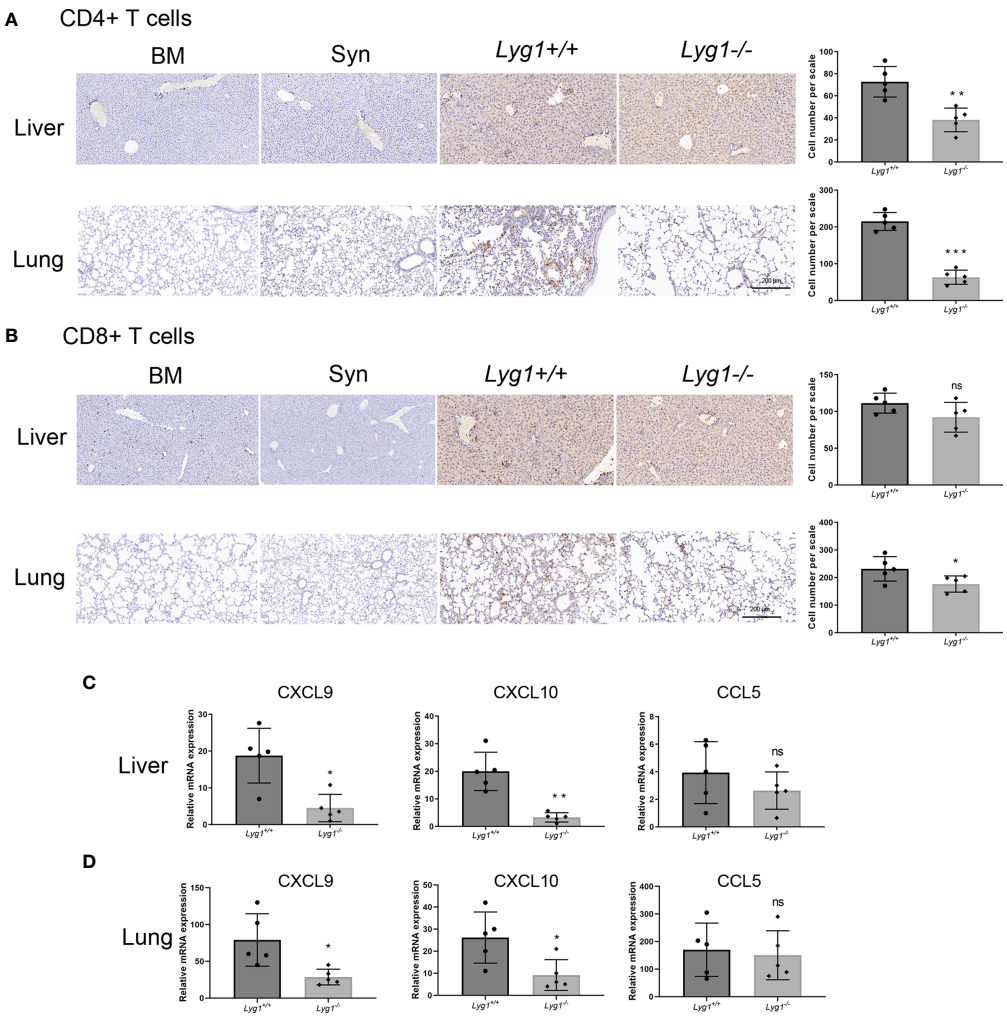

FIGURE 4 | LYG1 deficiency inhibited allogeneic CD4$^+$ T cells infiltration in aGVHD target organs. The samples of livers and lungs were excised at day 7 after transplantation and stained with antibodies CD4 and CD8. **(A, B)** The infiltration of CD4$^+$ T cells and CD8$^+$ T cells in livers and lungs in BM, Syn group, or mice receiving *Lyg1*$^{+/+}$ and *Lyg1*$^{-/-}$ donor T cells determined by IHC assay (×200 magnification). The left is one representative section per group. The right is the number of CD4$^+$ T cells and CD8$^+$ T cells per scale in the livers and lungs. **(C, D)** Chemokines expression were examined by qPCR in lymphocytes isolated from livers and lungs. Independent experiment was performed 3 times. The results in the repeats were similar. n = 5 per group. Representative sections are shown and statistical results are expressed as the mean ± SD, *p < 0.05, **P < 0.01 and ***P < 0.001 compared with *Lyg1*$^{+/+}$ group. ns, no significance.

generate murine GVT model. The mice receiving *Lyg1*$^{-/-}$ T cells exhibited a higher survival rate, lower tumor signal and lower tumor burden than that of the mice receiving *Lyg1*$^{+/+}$ T cells and BM cells (**Figures 8A–C**). Furthermore, there was no evident GVHD as the time of death in GVT model mice. The results suggested that targeting LYG1 might be an alternative to ameliorating aGVHD without impairing GVT function.

DISCUSSION

In this study, the role and mechanisms of LYG1 in aGVHD were explored. We demonstrated that mice receiving *Lyg1*$^{-/-}$ donor T cells alleviated aGVHD, increased long-term survival rates, showed less weight loss, lower GVHD clinical pathological scores and milder tissues damages, than mice receiving *Lyg1*$^{+/+}$ donor T cells in CD3$^+$ or CD4$^+$ T cells transplanting-major MHC mismatched aGVHD model and in haplo-HSCT model. Additionally, rhLYG1 intraperitoneally administration aggravated aGVHD severity, which confirmed the results established in the *Lyg1*$^{-/-}$ mice. Furthermore, we discovered that LYG1 deficiency in donor T cells can decrease infiltration of alloreactive CD4$^+$ T cells in aGVHD mice target organs, inhibit alloreactive of CD4$^+$ T cells and Th1 differentiation, promote Treg differentiation of allogeneic CD4$^+$ T cells *in vitro* and *in vivo*.

Donor-derived CD4$^+$ T cells are particularly important in the pathogenesis of aGVHD. A large number of clinical trials have

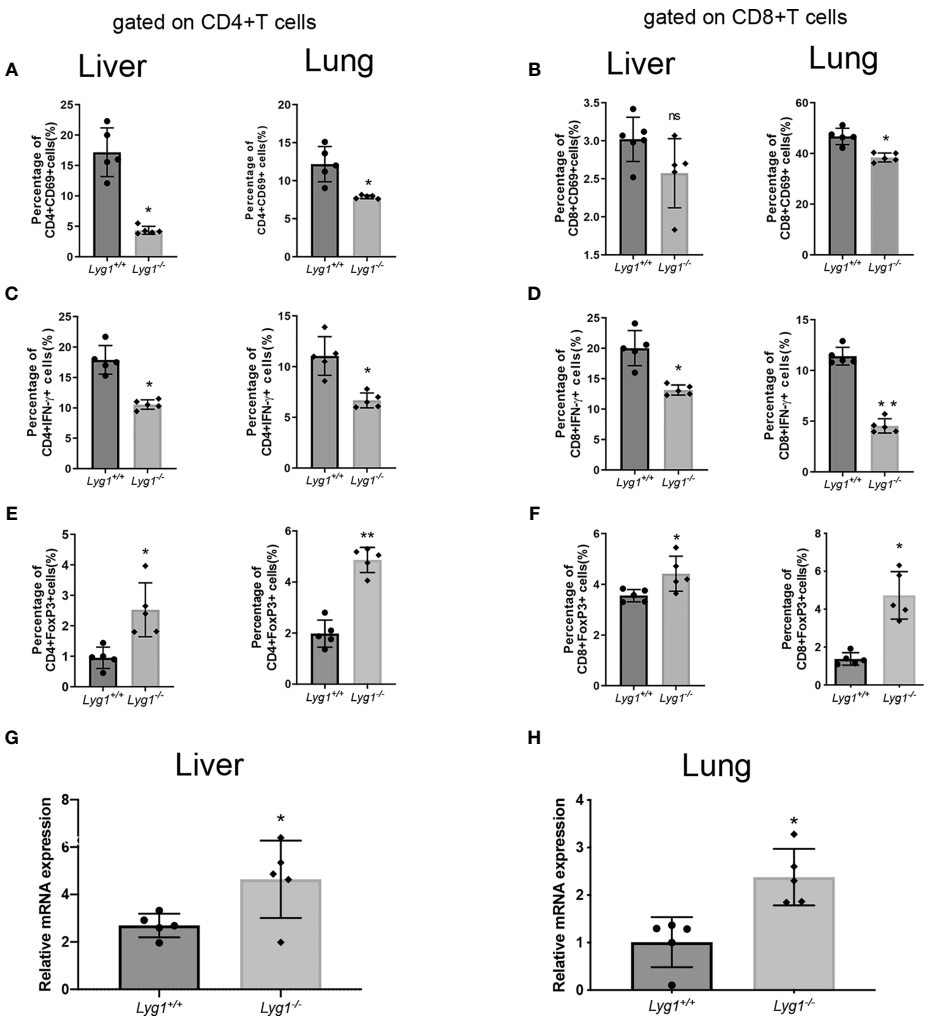

FIGURE 5 | LYG1 deficiency inhibited allogeneic T cells function in livers and lungs. Lymphocytes were isolated from livers and lungs of recipient mice on day 7 after transplantation and analyzed by flow cytometry and qPCR. **(A, B)** The percentages of CD69 expression in $CD4^+$ T cells and $CD8^+$ T cells. **(C, D)** The percentages of IFN-γ expression in $CD4^+$ T cells and $CD8^+$ T cells. **(E, F)** The percentages of Treg in $CD4^+$ T cells and $CD8^+$ T cells. The percentages of **Figure 5 (A–F)** were all gated on H2Kb+CD4+ cells or H2Kb+CD8+ cells in lymphocytes isolated from livers and lungs. **(G, H)** Foxp3 expression of lymphocytes isolated from livers and lungs were examined by qPCR. Independent experiment was performed 3 times. The results in the repeats were similar. n=5 per group. Statistical results are expressed as the mean ± SD, *p < 0.05 and **P < 0.01 compared with $Lyg1^{+/+}$ group. ns, no significance.

taken $CD4^+$ T cells as a potential target for GVHD treatment (3). Firstly we proved that LYG1 mediated GVHD development mainly through $CD4^+$ T cells, but not $CD8^+$ T cells by using purified $CD4^+$ and $CD8^+$ T cells as grafts. In aGVHD, alloreactive $CD4^+$T cells are directed by chemokines and migrate to target tissues and organs where they cause tissue injury (20–22). CXCL9, CXCL10-CXCR3 interactions has been linked to activated T cell trafficking to aGVHD target organs in humans and mice (16). Our previous study found that rhLYG1 administration in mice can enhance the expression of T cell chemokines, including CCL5, CXCL9 and CXCL10, and infiltration of T cells in tumors (11). In this study, the decreased infiltration of allogeneic $CD4^+$ T cells in the livers and lungs of mice that received $Lyg1^{-/-}$ T cells, which maybe related that LYG1 deficiency inhibited the expression of CXCL9 and CXCL10, explained partially that LYG1 deficiency in donor T cells suppressed aGVHD.

Another mechanism for LYG1 deficiency alleviating GVHD was able to inhibit IFN-γ production of donor derived T cells. IFN-γ plays an important promoting role in the alloreactivity of donor derived T cells in aGVHD (23). However, other studies have found that IFN-γ played a protective role against aGVHD, which depended on the time phase of IFN-γ production in allo-HSCT (23–25). Exogenous injection of IL-12 or IL-18 increased the expression of IFN-γ, thereby inducing the expression of Fas in donor T cells, leading to activation-induc,ed cell death, reducing donor T cells responses to host antigens and finally attenuating aGVHD (26, 27). Our previous studies have shown

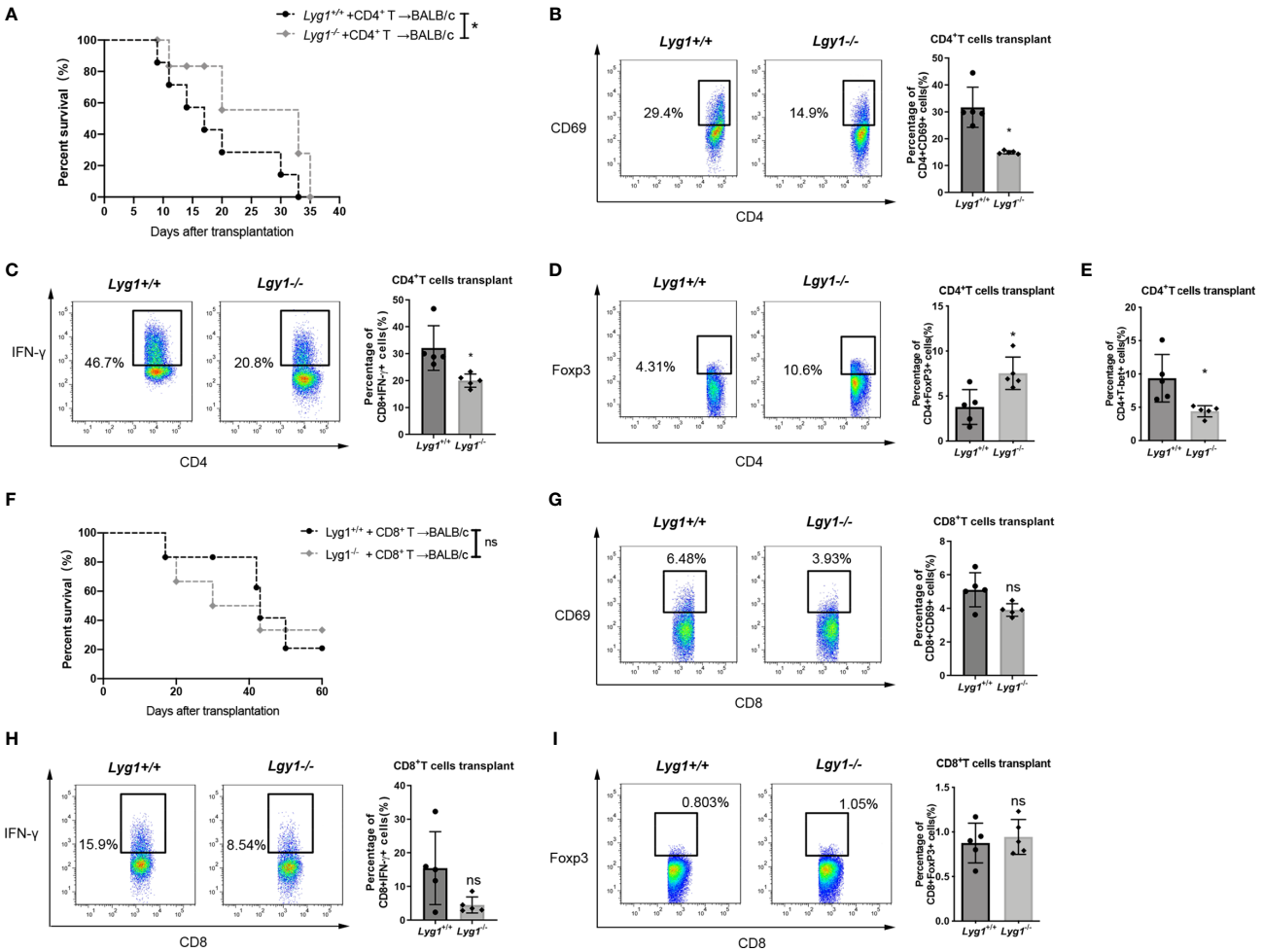

FIGURE 6 | LYG1 mediated aGVHD development mainly through CD4+ T cells. Survival of mice receiving $CD4^+$ **(A)** and $CD8^+$ **(F)** donor T cells were monitored. Data pooled: n = 10 for $Lyg1^{+/+}$ group and $Lyg1^{-/-}$ group **(A, F)**. **(B–E)** The expression of CD69, IFN-γ, Foxp3 and T-bet gated on $CD4^+$ T cells. **(G–I)** The expression of CD69, IFN-γ and Foxp3 gated on $CD8^+$ T cells. n = 5 per group **(B–E, G–I)**. Representative plots are shown and statistical results are expressed as the mean ± SD, *p < 0.05 compared with $Lyg1^{+/+}$ group. ns, no significance.

that rhLYG1 can promote antigen specific activity and IFN-γ production of T lymphocytes in tumor models (11). In this study, we found inhibited activation of $CD4^+$ T cells and IFN-γ expression of allogeneic T cells in mice receiving $Lyg1^{-/-}$ donor T cells, whereas rhLYG1 administration aggravated aGVHD severity through promoting IFN-γ production of allogeneic T cells, more importantly, the absence of IFN-γ in donor T cells could partially abrogate rhLYG1-induced GVHD development, corroborating that the effect of LYG1 on aGVHD were mainly mediated by IFN-γ.

Treg cells play a significant role in maintaining tolerance in aGVHD by limiting T cell function (28). Many studies have proven that therapeutic modulation or adoptive transfer of Treg can directly prevent GVHD (29). $CD8^+Foxp3^+$ T cells, a Treg subpopulation, can be induced and ameliorate GVHD in mouse models (30). In our study, LYG1 deficiency led to the enhanced proportions of Treg cells *in vitro*. Similarly, the absence of LYG1 in donor T cells increased the proportions of allogeneic Treg ($CD4^+Foxp3^+$ T cells and $CD8^+Foxp3^+$ T cells) in different GVHD models *in vivo*. These results provided another explanation that LYG1 deficiency in donor T cells alleviated GVHD. Importantly, $CD8^+Foxp3^+$ Treg cells display cytotoxic activity which can suppress tumor during GVHD (31). These results explained partially if not fully that LYG1 deficiency in donor T cells suppressing GVHD while preserving GVT effect.

Our study demonstrated that LYG1 deficiency in donor T cells suppressed Th1 cells and promoted Treg cells differentiation in aGVHD model. Th cell differentiation is regulated by multiple cytokines and transcription factors. In the absence of IL-6, TGF-β stimulates a transcriptional program in naive $CD4^+$ T cells with Foxp3 up-regulation and leads the evolvement of Treg cells (32).TNF-α blockade was shown to increase Foxp3 expression

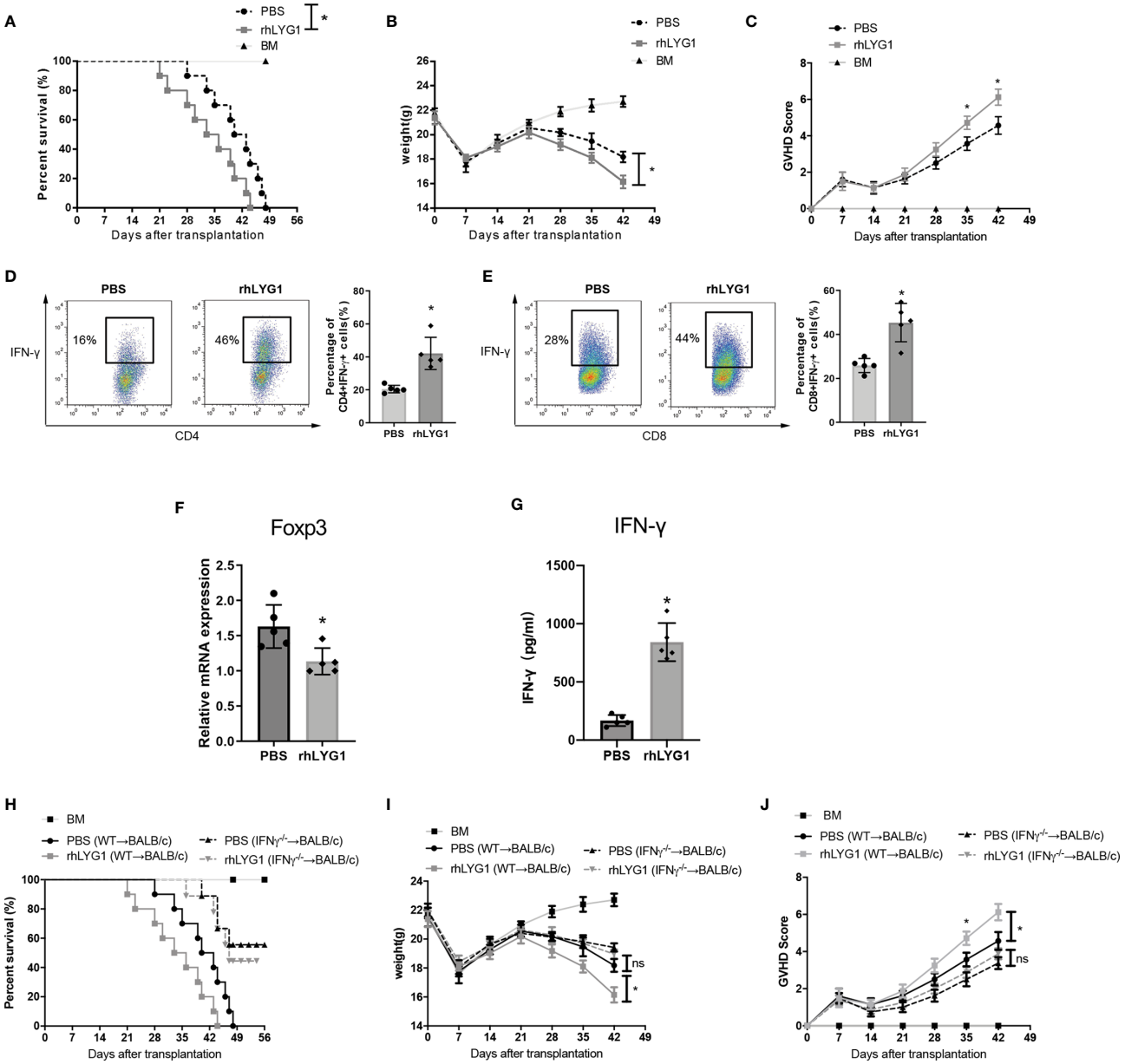

FIGURE 7 | rhLYG1 aggravates aGVHD *via* promoting IFN-γ production and inhibiting Foxp3 expression. **(A–C)** Lethally irradiated BALB/c mice were reconstituted with 5 × 10^6 B6 BM and 3 × 10^6 B6 $CD3^+$ T cells, rhLYG1 (20 μg per mice) or PBS was injected intraperitoneally (i.p.) each day on days 0 to +7 after transplantation. Survival **(A)**, weight **(B)** and aGVHD scores **(C)** were monitored. **(D, E)** The percentages of IFN-γ positive gated on H2Kb+CD4+ T cells and H2Kb+CD8+ T cells in spleens at day 7 after transplantation. **(F)** Foxp3 expression of splenocytes were examined by qPCR at day 7 after transplantation. **(G)** The concentrations of IFN-γ in serum at day 7 after transplantation. **(H–J)** Lethally irradiated BALB/c mice were reconstituted with 5 × 10^6 B6 BM and 3 × 10^6 *IFN-γ*$^{-/-}$$CD3^+$ T cells, rhLYG1 (20 μg per mice) or PBS was injected intraperitoneally (i.p.) each day on days 0 to +7 after transplantation. Survival **(H)**, weight **(I)** and aGVHD scores **(J)** were monitored. **(A–C, H–J)** Data pooled: n = 10 for PBS group and rhLYG1 group, n = 5 for BM group. **(D–G)** Independent experiment was performed 3 times. The results in the repeats were similar. n = 5 per group. Representative plots are shown and statistical results are expressed as the mean ± SD, *p < 0.05 compared with PBS group. ns, no significance.

in patients with RA (33, 34). In this study, we found that the absence of LYG1 in donor T cells reduced the production of IL-6 and TNF-α in different GVHD models. Therefore, we speculated that LYG1 deficiency promoted Treg cells differentiation by inhibiting IL-6 and TNF-α. T-bet is a transcriptional activator of IFN-γ and orchestrates the cell-migratory program by directly controlling expression of the chemokine receptors CXCR3 (18). We showed that the absence of LYG1 decreased the expression of T-bet and CXCL10 in GVHD models. It was supposed that LYG1 deficiency suppressed Th1 cells polarization *via* inhibiting T-bet

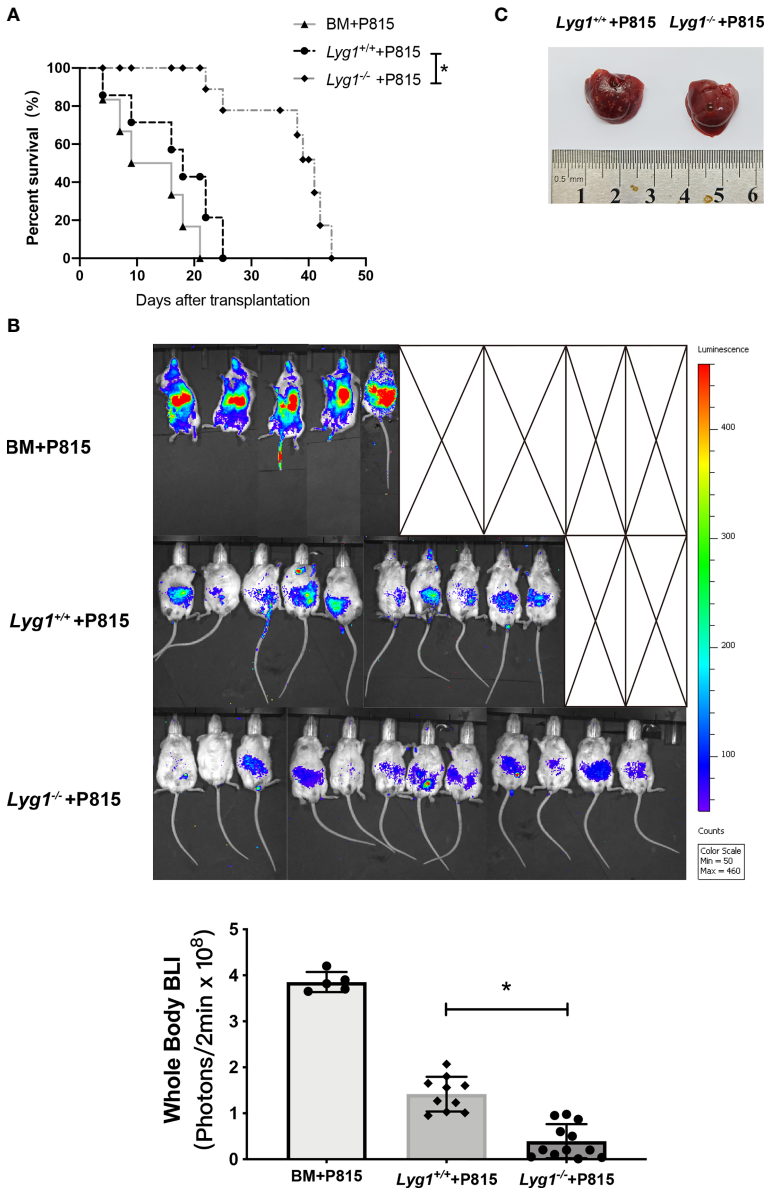

FIGURE 8 | LYG1 deficiency in donor T cells preserved GVT response. Lethally irradiated BALB/c mice were reconstituted with 5 × 10^6 B6 BM with or without 3 × 10^6 T cells from *Lyg1*$^{+/+}$ or *Lyg1*$^{-/-}$ mice, followed by 2.5 × 10^4 P815 cells (H2Kd) injected intravenously. **(A)** Survival after transplantation was monitored. **(B)** Tumor growth was monitored using bioluminescence imaging on day 14. Bioluminescence was quantified using whole body with Living Image software. Whole body images are shown and statistical results of average bioluminescence intensities are expressed as the mean ± SD, *p < 0.05 compared with *Lyg1*$^{+/+}$ group. **(C)** Livers were excised when the mice died or on day 14 after transplantation. n = 12 for *Lyg1*$^{+/+}$ or *Lyg1*$^{-/-}$ mice group, n = 10 for BM group. *p < 0.05 compared with *Lyg1*$^{+/+}$ group.

pathway related with CXCL10-CXCR3 axis, which was consistent with this report (35).

As a secretory protein, the cell sources of LYG1 are unclear. In this study, we demonstrated the role of LYG1 in aGVHD using WT BM and *Lyg1*$^{+/+}$ or *Lyg1*$^{-/-}$ T cells as the graft, but not KO BM and *Lyg1*$^{+/+}$ or *Lyg1*$^{-/-}$ T cells as the graft. Because only transplantation of allogeneic BM did not induce aGVHD, we excluded the effects of LYG1 derived from of BM in aGVHD mouse model we used in this study. Second, the recipients and its irradiation conditions of WT and KO groups were all the same, so we excluded the impacts of LYG1 derived from recipients in aGVHD mouse model we used. More importantly, rhLYG1 aggravated the aGVHD severity by promoting IFN-γ production and inhibiting Foxp3 expression, providing orthogonal validation for the results established using the *Lyg1*$^{-/-}$ mice.

In summary, we demonstrate LYG1 regulates aGVHD *via* altering the alloreactivity of $CD4^+$ T cells and the balance of Th1 and Treg differentiation of allogeneic $CD4^+$ T cells. Our study indicates that LYG1 may be a novel target in

aGVHD by mitigating aGVHD without impairing GVT effect. The therapeutic effect of targeting LYG1 is required in future investigations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Peking University First Hospital. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

HL contributed conception and design of the study, performed the most experiments of this study and wrote the paper. ZY performed the MLR assay and part of aGVHD models. BT, SM, and CQ helped to establish aGVHD mouse models. YL, ZL, YS, and YZ performed part of flow cytometric analysis. QW, MY, and ZS performed part of qPCR experiments. HR and YD contributed conception and design of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.647894/full#supplementary-material

Supplementary Figure 1 | Gating strategies for flow cytometry analyses presented in **Figure 1**. **(A–C)** The expression of CD69 **(A)**, IFN-γ **(B)** and Foxp3 **(C)** in isotype (ISO) (left) and negative control without stimulating cells (right).

Supplementary Figure 2 | Gating strategies for flow cytometry analyses in T cells before adoptive transfer presented in **Figure 2 (A)**. **(B, C)** Representative flow cytometry plots and frequencies of naive ($CD44^{lo}CD62L^{hi}$), central memory ($CD44^{hi}CD62L^{hi}$), effector ($CD44^{hi}CD62L^{lo}$) in $CD4^+$ T cells and $CD8^+$ T cells respectively. n=5 per group. **(D, E)** The percentages of $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells in BM cells from donor mice before adoptive transfer.

Supplementary Figure 3 | LYG1 deficiency reduced allogeneic T cells function in haploidentical transplant model. Splenocytes of recipient mice were isolated on day 14 after transplantation and analyzed by flow cytometry. **(A, B)** The percentages of CD69 expression in $CD4^+$ T cells and $CD8^+$ T cells. **(C, D)** The percentages of IFN-γ expression in $CD4^+$ T cells and $CD8^+$ T cells. **(E, F)** The percentages of Foxp3 expression in $CD4^+$ T cells and $CD8^+$ T cells. n=5 per group.

Supplementary Figure 4 | Gating strategies for flow cytometry analyses presented in **Figure 3**. The expression of CD69 **(A, B)**, CD44 and CD62L **(C, D)**, IFN-γ **(E, F)** and Foxp3 **(G, H)** in $CD4^+$ T cells and $CD8^+$ T cells respectively in ISO (left), BM control (middle) and Syn control (right).

Supplementary Figure 5 | The concentrations of IFN-γ, TNF-α and IL-6 in serum in aGVHD models. **(A)**The concentrations of IFN-γ, TNF-α and IL-6 in serum at day 7 after transplantation in major MHC mismatched aGVHD model. **(B)**The concentrations of IFN-γ, TNF-α and IL-6 in serum at day 14 after transplantation in haploidentical model. **(C, D)** The concentrations of IFN-γ, TNF-α and IL-6 in serum at day 7 after transplantation in purified $CD4^+$ T cells or $CD8^+$ T cells. n=4-5 per group. Statistical results are expressed as the mean ± SD, *p< 0.05 compared with $Lyg1^{+/+}$ group.

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CORRECTION
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Corrigendum: LYG1 Deficiency Attenuates the Severity of Acute Graft-Versus-Host Disease *via* Skewing Allogeneic T Cells Polarization Towards Treg Cells

Huihui Liu†, Zhengyu Yu†, Bo Tang, Shengchao Miao, Chenchen Qin, Yuan Li, Zeyin Liang, Yongjin Shi, Yang Zhang, Qingya Wang, Miao Yan, Zhengyang Song, Hanyun Ren and Yujun Dong**

Department of Hematology, Peking University First Hospital, Beijing, China

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Antoine Toubert, Université Paris Diderot, France

***Correspondence:**
Hanyun Ren
renhy0813@163.com
Yujun Dong
dongy@hsc.pku.edu.cn

†These authors have contributed equally to this work

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Keywords: LYG1, aGVHD, allogeneic CD4+ T cells, alloreactivity, Th1 cells, Treg cells

A Corrigendum on

LYG1 Deficiency Attenuates the Severity of Acute Graft-Versus-Host Disease *via* Skewing Allogeneic T Cells Polarization Towards Treg Cells

By Liu H, Yu Z, Tang B, Miao S, Qin C, Li Y, Liang Z, Shi Y, Zhang Y, Wang Q, Yan M, Song Z, Ren H and Dong Y (2021). Front. Immunol. 12:647894. doi: 10.3389/fimmu.2021.647894

In the original article, there was a mistake in **Figure 3F** as published. **Figure 3F** contains a duplicated FACS plot in Lyg1-/- group. We used the graph of Lyg1-/- group as that of Lyg1+/+ group by mistake. The corrected **Figure 3** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

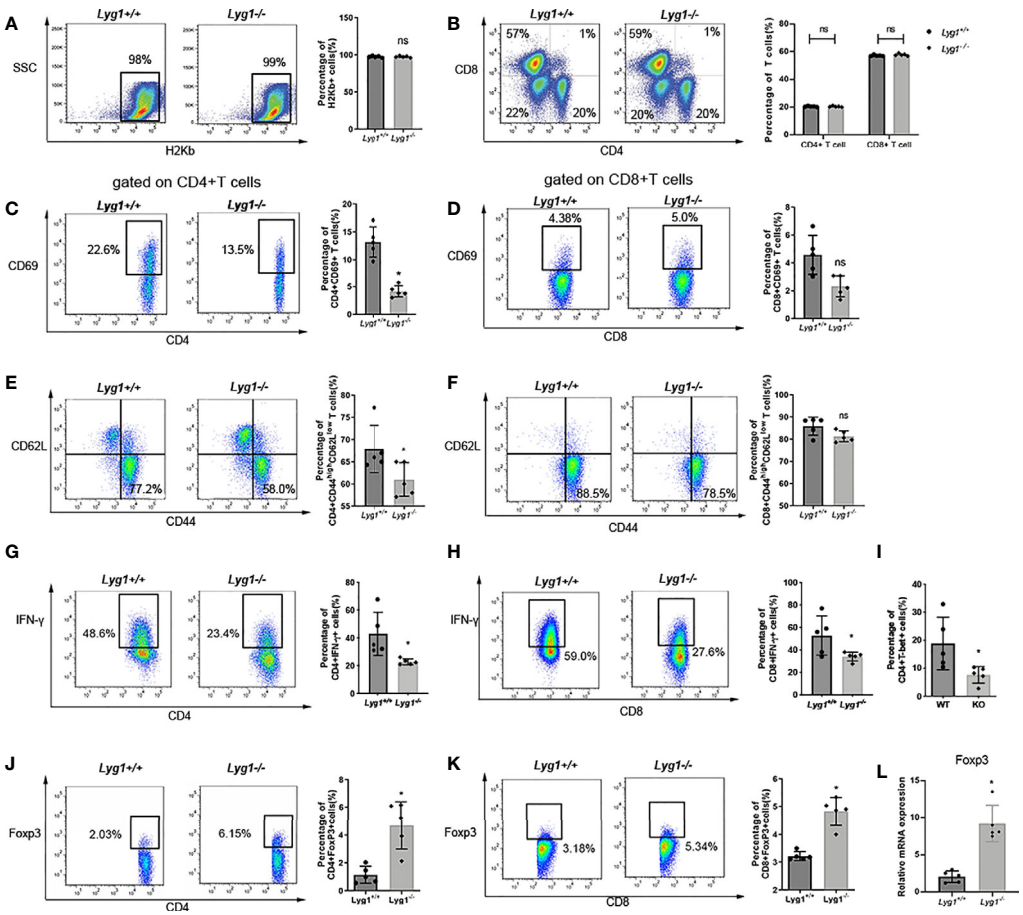

FIGURE 3 | LYG1 deficiency reduced allogeneic T cells function in spleens. Splenocytes of recipient mice were isolated on day 7 after transplantation and analyzed by flow cytometry and qPCR. **(A)** The percentages of H2Kb+ cells in living splenocytes. **(B)** The percentages of CD4+ T and CD8+ T cells in H2Kb+ splenocytes. **(C, D)** The percentages of CD69 expression in CD4+ T cells and CD8+ T cells. **(E, F)** The expression of effector (CD44hiCD62Llo) phenotype gated on CD4+ T and CD8+ T cells. **(G, H)** The percentages of IFN-g expression in CD4+ T cells and CD8+ T cells. **(I)** The percentages of T-bet expression in CD4+ T cells. **(J, K)** The percentages of Treg in CD4+ T cells and CD8+ T cells. The percentages of Figure 3 **(C–K)** were all gated on H2Kb+CD4+ cells or H2Kb+CD8+ cells. **(L)** Foxp3 expression of splenocytes were examined by qPCR. Independent experiment was performed 3 times. The results in the repeats were similar. n = 5 per group. Representative plots are shown and statistical results are expressed as the mean ± SD, *$p < 0.05$ compared with Lyg1+/+ group. ns, no significance.

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Efficiency and Toxicity of Ruxolitinib as a Salvage Treatment for Steroid-Refractory Chronic Graft-Versus-Host Disease

Dong Wang[1,2†], *Yin Liu*[1,2†], *Xiaoxuan Lai*[1,2†], *Jia Chen*[1,2], *Qiao Cheng*[1,2*], *Xiao Ma*[1,3], *Zhihong Lin*[4], *Depei Wu*[1,2*] *and Yang Xu*[1,2*]

[1] National Clinical Research Center for Hematologic Diseases, Jiangsu Institute of Hematology, Key Laboratory of Thrombosis and Hemostasis of Ministry of Health, The First Affiliated Hospital of Soochow University, Suzhou, China, [2] Institute of Blood and Marrow Transplantation, Collaborative Innovation Center of Hematology, Soochow University, Suzhou, China, [3] Department of Hematology, Soochow Hopes Hematonosis Hospital, Suzhou, China, [4] Soochow Yongding Hospital, Department of Affiliated Renji Hospital of Shanghai Jiao Tong University of Medicine, Suzhou, China

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Edited by:
Jaebok Choi,
Washington University School of Medicine in St. Louis, United States

Reviewed by:
Robert Zeiser,
University of Freiburg, Germany
Jacob Rozmus,
British Columbia Children's Hospital, Canada

***Correspondence:**
Yang Xu
yangxu@suda.edu.cn
Qiao Cheng
chengqiao555111@163.com
Depei Wu
drwudepei@163.com

[†]These authors have contributed equally to this work and share first authorship

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Graft-versus-host disease (GVHD), especially steroid-refractory GVHD, remains a life-threatening complication after hematopoietic stem cell transplantation (HSCT). The effect of the JAK1/2 kinase inhibitor ruxolitinib on treating steroid-refractory acute GVHD has been verified by the REACH1/2 study; however, its safety and efficacy in patients with steroid-refractory chronic GVHD (SR-cGVHD) remain unclear. In this retrospective study, 70 patients received ruxolitinib as a salvage therapy for SR-cGVHD. Twenty-four weeks after ruxolitinib treatment, the overall response rate (ORR) was 74.3% (52/70), including 34 patients who achieved complete remission (CR) and 18 who achieved partial remission (PR). The main adverse event was cytopenia, which occurred in 51.4% (36/70) of patients. After ruxolitinib treatment, the percentage of CD4 cells increased from 18.20% to 23.22% (P<0.001), while the percentages of NK ($CD16^+CD56^+$) cells and regulatory T cells ($CD4^+CD127^{\pm}CD25^+$) decreased (P<0.001, P<0.001). Among the B cell subsets, the proportion of total B cells approximately tripled from 3.69% to 11.16% (P<0.001). Moreover, we observed a significant increase in IL-10 levels after ruxolitinib treatment (P=0.025) and a remarkable decrease in levels of suppression of tumorigenicity 2 (ST2) from 229.90 ng/ml to 72.65 ng/ml. The median follow-up after the initiation of ruxolitinib treatment was 401 (6-1076) days. The estimated one-year overall survival rate of the whole group was 66.0% (54.4–77.6%, 95% CI), and the one-year overall survival rate of patients with mild and moderate cGVHD was 69.6% (57.4–81.8%, 95% CI), which was better than that of patients with severe cGVHD (31.3%, 0.0–66.2%, 95% CI) (P=0.002). Patients who achieved a CR and PR achieved better survival outcomes (84.5%, 73.9–95.1%, 95% CI) than those who showed NR to ruxolitinib treatments (16.7%, 0–34.3%, 95% CI) (P<0.001). At the final follow-up, cGVHD relapse occurred in six patients after

they reduced or continued their ruxolitinib doses. Collectively, our results suggest that ruxolitinib is potentially a safe and effective treatment for SR-cGVHD.

Keywords: hematopoietic stem cell transplant, ruxolitinib, steroid-refractory chronic graft-versus-host disease, overall response rate, overall survival

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) has been one of the most important therapies for hematological malignancies. However, graft-versus-host disease (GVHD) remains an unremovable barrier, leading to late morbidity and mortality (1). Corticosteroids are the first-line treatment for GVHD. Unfortunately, more than 50% of patients with chronic GVHD (cGVHD) fail to achieve remission (2). Despite various clinical trials, no global consensus has been reached regarding second-line therapy for cGVHD (3).

Ruxolitinib, an oral JAK1/2 kinase inhibitor, was approved for intermediate-or high-risk myelofibrosis in 2011 (4) and for polycythemia vera with an inadequate response to or intolerance to hydroxyurea in 2014 (5). In addition, the JAK/STAT signaling pathway plays an important role in immune cell activation and tissue inflammation during GVHD (6, 7). Researchers have already confirmed the effect of ruxolitinib, which reduces the incidence and severity of aGVHD while preserving graft-versus-leukemia effects in preclinical models (8–10). Afterwards, ruxolitinib was subsequently reported to have shown encouraging outcomes in curing patients with aGVHD (11–14). On May 24, 2019, ruxolitinib was approved by the Food and Drug Administration (FDA) as a treatment for steroid-refractory aGVHD (SR-aGVHD) in adult and pediatric patients aged 12 years and older (15).

In 2015, Zeiser et al. first reported that ruxolitinib produced encouraging results in cGVHD therapy (16). In 2020, Zeiser et al. reported that ruxolitinib showed superior efficacy to the best available therapy (BAT) in a phase 3 trial of patients with SR-cGVHD. However, no large-scale study has focused on the efficiency and toxicity of ruxolitinib in the treatment of cGVHD among Chinese people. Here, we report a single-center retrospective study of 70 patients who received ruxolitinib as a salvage therapy for steroid-refractory cGVHD (SR-cGVHD) in our center between March 2017 and December 2019 to evaluate the safety and efficacy of ruxolitinib after HSCT.

METHODS

Study Subjects and Data Collection

In this retrospective study, data from 70 patients who received HSCT between September 2009 and September 2019 and developed SR-cGVHD between March 2017 and December 2019 at the First Affiliated Hospital of Soochow University were collected for analysis. This study was conducted in accordance with the *Declaration of Helsinki* and approved by the ethics committee of the First Affiliated Hospital of Soochow University.

Inclusion and Exclusion Criteria

Patients who underwent HSCT and developed SR-cGVHD at the First Affiliated Hospital of Soochow University were included in the study. When devising inclusion and exclusion criteria, the REACH3 study was used as a reference. Inclusion criteria were as follows: 1) aged > 12 years; 2) complete hematopoietic reconstitution (absolute neutrophil counts > $1.0*10^9$/L and platelet counts > $25*10^9$/L) after HSCT; and 3) a diagnosis of SR-cGVHD according to the NIH criteria (17), including no response to a minimum of 1 mg/kg/day of prednisone therapy after 1 week, as well as disease persistence without improvement after treatment with prednisone at > 0.5 mg/kg/day or 1 mg/kg/every other day for at least 4 weeks or an increase to a prednisolone dose to > 0.25 mg/kg/day after 2 unsuccessful attempts to taper the dose. The exclusion criteria were as follows: 1) relapse of underlying disease before the use of ruxolitinib for treatment, 2) uncontrolled infections or severe organ damage not related to cGVHD, and 3) enrollment in other clinical studies of cGVHD treatments at the start of the research.

Conditioning Regimens for HSCT

The conditioning regimen for patients diagnosed with aplastic anemia (AA) was the FCA-based conditioning regimen, including IV fludarabine at 30 mg/m^2/d on days -9 to -6, IV cyclophosphamide (CTX) at 50 mg/m^2/d on days -5 to -2 and IV anti-thymocyte globulin (ATG) at 3.0 mg/kg^2/d on days -5 to -2. Other patients who received HLA-matched sibling, unrelated or haploidentical transplantation were administered a Bu/Cy-based regimen consisting of oral semustine at 250 mg/m^2/d on day -10, IV cytarabine at 4 g/m^2/d on days -9 to -8, IV busulfan at 4 mg/kg/d from day -7 to day -5, and IV CTX at 1.8 g/m^2/d from days -4 to -3.

GVHD Prophylaxis

Patients who underwent HLA-matched sibling transplantation received a GVHD prophylaxis strategy consisting of cyclosporin A (CsA) and methotrexate (MTX). The GVHD prophylaxis strategy for unrelated or haploidentical transplantation patients consisted of CsA, MTX, mycophenolate mofetil (MMF) and ATG or ALG. CsA was administered at a dose of 3 mg/kg/day by continuous infusion over 24 h from day -10 until patients were able to switch to the oral formulation, with a target blood concentration ranging from 200 to 300 ng/ml. MTX was administered intravenously at a dose of 15 mg/m^2 on day +1 and 10 mg/m^2 on days +3, days +6 and days +11. MMF was administered at an oral dose of 250 mg twice daily from day -10 until day +30. ATG/ALG was administered intravenously at a dose of 2.5 mg/kg/d from day -5 to day -2.

Clinical Definitions

cGVHD was diagnosed and graded according to the 2014 National Institute of Health (NIH) criteria (17). We assessed the treatment efficacy 24 weeks after the initiation of ruxolitinib therapy. Treatment responses to ruxolitinib were defined according to a previous study (16). The overall response rate (ORR) was defined as the percentage of patients assessed as achieving a complete response (CR) or partial response (PR). CR was defined as the absence of any manifestation related to cGVHD, and PR was defined as improvement in at least one specific target organ without deterioration in any other organ according to the NIH consensus (18). Events for failure-free survival (FFS) included relapse or recurrence of underlying disease or death due to underlying disease, nonrelapse mortality (NRM) and addition or initiation of another systemic therapy for cGVHD. Disease relapse was defined as morphological or cytogenetic evidence of disease with pretransplantation characteristics or morphological evidence without pretransplantation characteristics. NRM included mortality of patients who did not die due to the progression of underlying diseases.

Laboratory Studies and Analysis of Lymphocyte Subsets

Blood samples were collected from all patients 1-3 months before and after ruxolitinib treatments, at least once per time window, for the detection of different lymphocyte subsets using flow cytometry. Blood samples were collected in EDTA anticoagulant tubes and processed within an hour for multiparameter flow cytometry analyses. Phenotyping of T cells, B cells, NK cells and other cell types was performed. Samples were stained with the following antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD16, anti-CD56, anti-CD69, anti-CD25, anti-CD127, anti-CD27 and Ig-D. $CD19^+CD3^-$ cells were defined as total B cells, $CD19^+CD27^-IgD^+$ cells were defined as naive B cells, $CD19^+CD27^+IgD^+$ cells were defined as marginal zone B cells and $CD19^+CD27^+IgD^-$ were defined as classical traditional B cells.

Safety and Adverse Events

Safety was assessed by monitoring the occurrence, duration, and severity of adverse events. Adverse events were assessed according to the Common Terminology Criteria for Adverse Events, version 4.03 (https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03/CTCAE_4.03_2010-06-14_QuickReference_8.5x11.pdf).

Statistical Analysis

Our results were analyzed using SPSS 22.0 software. Normally distributed data were analyzed with Student's t test, and nonparametric comparisons of two means were performed using the Mann-Whitney U test or the chi-square test. In the risk factor analysis, a logistic regression model was used. Time to CR, PR, NR and overall survival (OS) were defined as the time from ruxolitinib treatment to the event. Spearman's rank correlation analysis was used. OS was analyzed using the Kaplan–Meier methodology. Comparisons were performed using the log-rank test. Cumulative incidence analysis was used to assess the incidence of relapse and NRM. A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics

A cohort of 70 patients were enrolled in this study. All patients received HSCT between September 2009 and September 2019 and developed cGVHD between March 2017 and December 2019. The detailed information is outlined in **Table 1**. The

TABLE 1 | Clinical characteristics of patients with steroid-refractory chronic graft-versus-host disease.

N (%)	
Age (median, range)	35 (13-63)
Sex	
Male	42 (60.0%)
Female	28 (40.0%)
Diagnosis	
Acute myeloblastic leukemia	24 (34.3%)
Acute lymphoblastic leukemia	24 (34.3%)
Chronic myeloblastic leukemia	5 (7.1%)
Chronic lymphoblastic leukemia	1 (1.4%)
Myelodysplastic syndrome	9 (12.9%)
Aplastic anemia	4 (5.7%)
Non-Hodgkin lymphoma	3 (4.3%)
Status at HSCT	
CR	41 (58.6%)
PR	2 (2.9%)
SD	12 (17.1%)
Others	15 (21.4%)
Type of transplant	
Matched donor	29 (41.4%)
Haploidentical donor	41 (58.6%)
Graft Source	
Peripheral blood stem cells	32 (45.7%)
Bone marrow + Peripheral blood stem cells	38 (54.3%)
Transplanted cell count (median, range)	
MNC ($10^{\wedge 8}$/kg)	11.4 (3.43-29.96)
CD34 ($10^{\wedge 6}$/kg)	3.80 (2.00-21.22)
GVHD prophylaxis	
CsA + MTX	29 (41.4%)
CsA + MTX + MMF	41 (58.6%)
Days of reconstitution after HSCT (median, range)	
NE > 1.0*10^9/L	12 (10-23)
PLT > 20*10^9/L	13 (8-80)
Complications	
Bacterial Infections	49 (70.0%)
Hemorrhagic cystitis	11 (15.7%)
CMV infection	10 (14.3%)
EBV infection	5 (7.1%)
aGVHD	
None	28 (40.0%)
Grade 1-2	22 (31.4%)
Grade 3-4	20 (28.6%)

HSCT, hematopoietic stem cell transplantation; CR, complete remission; PR, partial remission; SD, steady disease; MNC, mononuclear cell; CsA, cyclosporin A; MTX, methotrexate; MMF, mycophenolate mofetil; ATG, anti-thymocyte globulin; NE, neutrophil; PLT, platelet; CMV, cytomegalovirus; EBV, Epstein-Barr virus; aGVHD, acute graft-versus-host disease.

median age of the patients was 35 years (range 13-63 years). Acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) were the most common underlying diseases. Matched donor transplantation was performed on 29 patients including 27 patients with related donor and 2 patients with unrelated donor, and haploidentical donor transplantation was performed on 41 patients. In this study, 32 patients received grafts of peripheral blood stem cells alone, and others received grafts combining bone marrow and peripheral blood stem cells. The median counts of transplanted mononuclear cells and $CD34^+$ cells were 11.4*10^8/kg (range 3.43-29.96) and 3.80*10^6/kg (range 2.00-21.22), respectively. After HSCT, the median times of neutrophil and platelet reconstitution were 12 (range 10-23) days and 17 (range 8-80) days, respectively. The most commonly occurring complication after transplantation was bacterial infections, followed by hemorrhagic cystitis and virus infections. Forty-two patients had previously experienced acute GVHD, and 4 of them had been treated with ruxolitinib.

cGVHD Grade and Organ Classification

The median time of cGVHD occurrence after HSCT was 317 days (range 101-3078). Twenty-three patients (32.9%) had mild cGVHD, 38 (54.3%) had moderate cGVHD, and 9 (12.8%) had severe cGVHD. Multiple organs were involved in 33 (47.1%) patients. By analyzing the targeted organs, as shown in **Table 2**, we found that the most commonly involved organ was the skin, which was affected in 28 (40.0%) patients, and the skin had the highest percentage of severe cGVHD (39.3%, 11/28). Lung, liver and gut cGVHD occurred less frequently than skin cGVHD, and severe symptoms occurred in 27.3% (6/22), 32.0% (8/25) and 30.0% (6/20) of patients, respectively. Eye cGVHD occurred in only 9 patients, and it was graded as mild or moderate. Kidney and joint cGVHD were very rarely observed in this study. In addition, skin cGVHD mostly occurred in the haploidentical HSCT group (21/41, 51.2%), while lung cGVHD was mostly common in the matched HSCT group (13/29, 44.8%). For patients who had previously been diagnosed with aGVHD, 14.3% (6/42) were graded into severe cGVHD, while the percentage of patients who had not experienced aGVHD was only 10.7% (3/28) (P=0.048).

TABLE 2 | Characteristics of steroid-refractory chronic graft-versus-host disease.

N (%)	
Days from transplantation to cGVHD	
Median (range)	317 (101-3078)
cGVHD grade at baseline	
Mild	23 (32.9%)
Moderate	38 (54.3%)
Severe	9 (12.8%)
Organ affected by cGVHD	
Eye	9 (12.9%)
Mouth	6 (8.6%)
Skin	28 (40.0%)
Lung	22 (31.4%)
Liver	25 (35.7%)
Kidney	2 (2.9%)
Gut	20 (28.6%)
Joint	5 (7.1%)
Previous lines of therapy	
Steroids alone	19 (27.1%)
Steroids and others	51 (72.9%)

cGVHD, chronic graft-versus-host disease.

Treatment Efficacy

All patients received ruxolitinib (10-20 mg/d) as salvage therapy for cGVHD. Response rates were evaluated 24 weeks after ruxolitinib initiation. As shown in **Figure 1A**, after 24 weeks, the ORR to ruxolitinib therapy in patients with SR-cGVHD was 74.3% (52/70), including 34 patients with a CR (48.6%) and 18 with a PR (25.7%). Except for kidney and joint cGVHD cases that were too few to be analyzed, the mouth was the organ with the best response at 83.3% ORR, and the skin was the organ that achieved the highest CR of 60.7%. The ORR in patients with liver cGVHD was the lowest at only 64.0%. For patients diagnosed with different severity grades, we found that patients with severe cGVHD showed a worse ORR than patients with mild cGVHD (44.4% vs 82.6%, P=0.034) or moderate cGVHD (44.4% *vs* 76.3% P=0.063) (**Figure 1B**). After 24 weeks of treatment, we reevaluated the cGVHD severity in every patient and discovered significant reductions in the grades of cGVHD at baseline and after 24 weeks of therapy in most organs (**Figure 1C**). Next, we compared the days from ruxolitinib initiation to response among different organs, and the median time for patients with liver cGVHD to achieve remission was longer than that of other patients (125 days *vs* 49 days, P=0.019) (**Figure 1D**).

Steroid and Other Combination Treatments

At the initiation of ruxolitinib treatment, all patients were receiving steroid treatments. The median dose of steroid was 1mg/kg/d (rang 0.5-2). After 4 weeks of ruxolitinib treatment, 16 patients have stopped steroid treatments and 8 patients were capable to reduce their steroid doses owing to improved symptoms. 24 weeks after ruxolitinib treatments, 18 patients have been dead, 38 patients were finally able to withdraw steroid treatments and 14 patients were still with steroids treatments with median dose of 1mg/kg/d (rang 0.5-2). In these 14 patients, 9 patients showed no response to ruxolitinib treatments and 5 patients were steroid dependent.

Besides steroid treatments, some immunosuppressor treatments were also involved. In total, 40 patients were receiving different immunosuppressor treatments at the start of ruxolitinib treatments, including tacrolimus (TAC) in 21 patients, cyclosporin A (CsA) in 12 patients and mycophenolate mofetil (MMF) in 7 patients. After 24 weeks treatments, immunosuppressors were discontinued in 31 patients and 2 patients were still receiving TAC for treatments.

Adverse Events

Cytopenia was the most common adverse event occurring after ruxolitinib treatments (36/70, 51.4%). Anemia was the most common form, and thrombocytopenia was the second most common form. However, severe thrombocytopenia (grade III

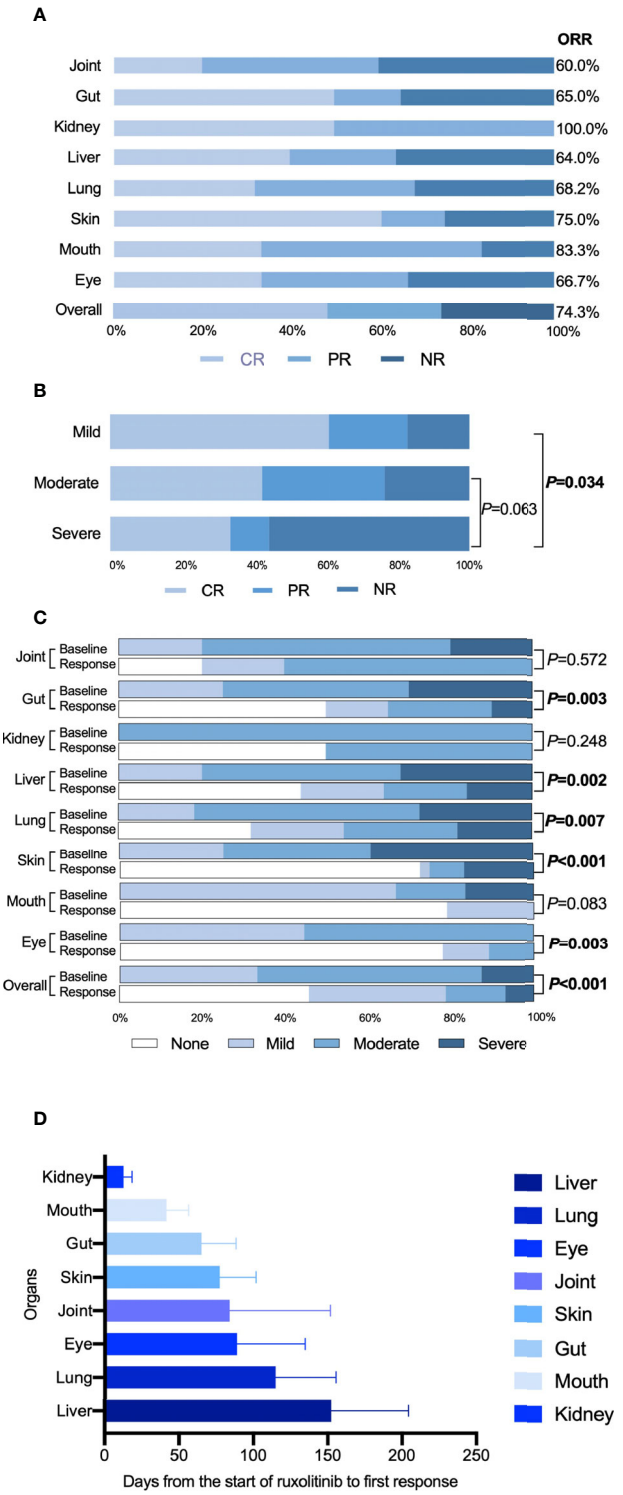

FIGURE 1 | Treatment efficacy of ruxolitinib. **(A)** Response rates of different organs. **(B)** Comparison of treatment efficacy among patients with mild, moderate and severe cGVHD. **(C)** Comparison of the cGVHD grades of different organs before and 24 weeks after ruxolitinib treatments. **(D)** Time, in days, from the start of ruxolitinib administration to the response of different organs.

or IV) was observed in 15 of 28 patients, while severe anemia (grade III or IV) was observed only in 8 of 29 patients. Cytomegalovirus (CMV) reactivation occurred in 8 patients, while Epstein-Barr virus (EBV) and herpes infections occurred in 2 patients (**Table 3**). Viral reactivation was quickly controlled by antiviral therapy, and no other complications were observed.

Immune Function

We analyzed different lymphocyte subsets during the 3 months before and after ruxolitinib treatments. The median date of the collected sample before and after ruxolitinb treatments were 54 days (range 28-88) and 63 days (range 34-94) respectively. A correlation analysis between age, lymphocyte subsets, and cytokines was performed to exclude the effect of age on different lymphocyte subsets and cytokine levels, and only naïve B cells had a negative correlation with age (**Supplementary Table S1**). CD4 lymphocytes were increased after treatment from 18.20% to 23.22% ($P<0.001$). The same trend was observed in the DP cell ($CD4^+CD8^+$) group, which increased from 0.50% to 0.68% ($P=0.026$). The numbers of both regulatory T cells ($CD4^+CD127^{\pm}CD25^+$) and NK cells ($CD16^+CD56^+$) decreased by approximately half after ruxolitinib treatment ($P<0.001$ for both) (**Figure 2A**). By analyzing the B cells of some patients, we made the novel discovery that the proportion of total B cells among lymphocytes nearly tripled from 3.69% to 11.16% ($P<0.001$). In a detailed analysis of various B cell subsets, no significant differences were observed among naïve B cells, marginal zone B cells (MZ B) and classical traditional B cells (**Figure 2B**).

In addition, we examined the levels of inflammatory cytokines in patients during treatment. We observed a significant increase in IL-10 levels from 3.02 pg/ml to 5.04 pg/ml ($P=0.025$). Moreover, we detected decreased levels of suppression of tumorigenicity 2 (ST2), a definite predictor of aGVHD, decreased by over 66% from 229.90 ng/ml to 72.65 ng/ml after ruxolitinib treatment ($P=0.027$) (**Figure 2C**).

For a more detailed analysis, we compared the variations among the skin, liver, lung and gut. In these four organs, the trends of variation in different cell subsets were basically the same (**Supplementary Figure S1**). Regarding cytokines, patients with skin cGVHD presented a significant decrease in IL-6 levels

TABLE 3 | Adverse effects of ruxolitinib treatment on patients with steroid-refractory chronic graft- versus-host disease.

	N (%)
Total	50 (71.4%)
Cytopenia	36 (51.4%)
Anemia	29 (41.4%)
Leukopenia	21 (30.0%)
Thrombocytopenia	28 (40.0%)
Liver function damage	6 (8.6%)
Kidney function damage	1 (1.4%)
CMV infection	8 (11.4%)
EBV infection	2 (2.9%)
Herpes virus infection	2 (2.9%)
TMA	7 (10.0%)

CMV, cytomegalovirus; EBV, Epstein-Barr virus; TMA, thrombotic microangiopathy.

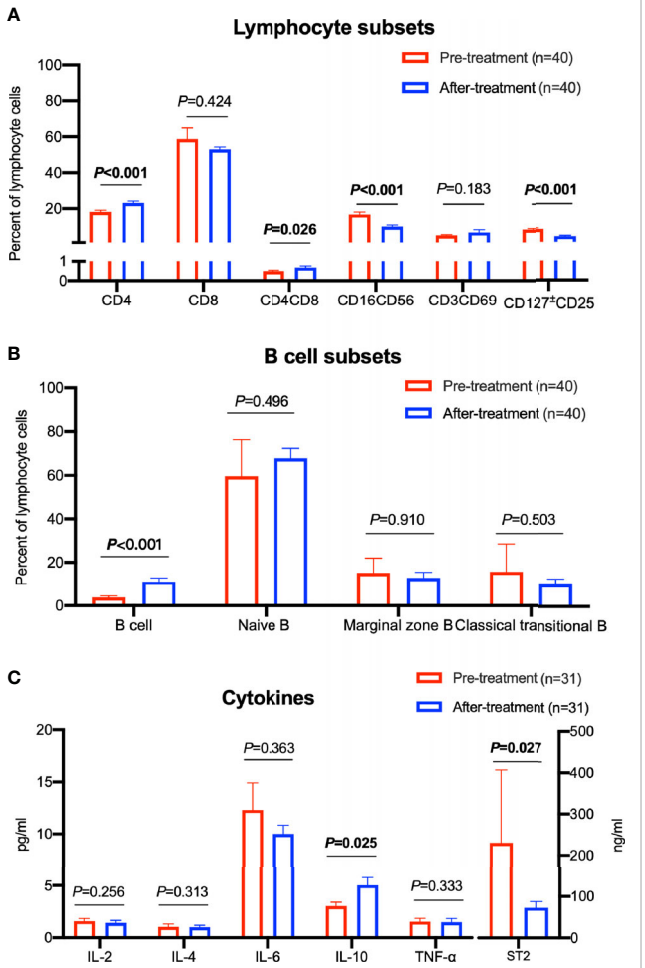

FIGURE 2 | Comparison of different lymphocyte subsets and cytokine levels before and after treatment with ruxolitinib. **(A)** Comparison of different lymphocyte cell subsets. **(B)** Comparison of different B cell subsets. **(C)** Comparison of different cytokine levels. Paired sample t test was used for the analysis. The numbers of patients are indicated in each graph.

(P=0.008) and an increase in IL-10 levels (P=0.014) after ruxolitinib treatment. However, significant differences were not observed among patients with liver, lung and gut cGVHD (**Supplementary Figure S1**).

Long-Term Outcomes

The median follow-up time of this study was 401 days (range 6-1076 days) after the initiation of ruxolitinib. The one-year estimated survival rate of the whole group was 66.0% (54.4–77.6%, 95% CI) (**Figure 3A**). The FFS estimate of the study at one year was 60.4% (48.2–72.6%, 95% CI) (**Figure 3B**). At the one-year follow-up, the estimated survival rate of patients with mild and moderate cGVHD was 69.6% (57.4–81.8%, 95% CI), which was better than that of patients with severe cGVHD (31.3%, 0.0–66.2%, 95% CI) (P=0.002) (**Figure 3C**). Patients who achieved CR and PR achieved better survival outcomes (84.5%, 73.9–95.1%, 95% CI) than those who showed NR to ruxolitinib treatments (16.7%, 0–34.3%, 95% CI) (P<0.001) (**Figure 3D**). cGVHD relapse occurred in six patients after decreases in the ruxolitinib dose or discontinuation, among which 3 patients responded to the restart of ruxolitinib therapy and achieved a response later, while the others died from cGVHD progression.

Twenty-five patients had died by the last follow-up date. Approximately half of the deaths were associated with underlying disease progression (11/25). Others included uncontrolled severe cGVHD (4/25), thrombotic microangiopathy (4/25) and complicated infections or multiple organ dysfunction syndrome (4/25). The cumulative incidence of NRM at the one-year follow-up was 20.0% (0.0-31.8%, 95% CI) (**Figure 3E**). For patients with mild and moderate cGVHD, the one-year NRM was only 16.7% (6.7-26.7%, 95% CI). However, for severe cGVHD patients, NRM at the one-year follow-up was up to 62.5% (22.7-100.0%, 95% CI) (**Figure 3F**).

DISCUSSION

cGVHD remains one of the major hurdles to the success of HSCT. Although corticosteroid treatment has saved millions of lives of patients with cGVHD, no consensus on second-line treatments has been established for patients with SR-cGVHD. Ruxolitinib, a JAK1/2 kinase inhibitor, was first reported in a 2015 multicenter retrospective survey by Zeiser et al. (16) to have exerted satisfactory therapeutic effects on SR-cGVHD, supported by a favorable ORR of 85.4%. Among other commonly used second line cGVHD treatments, extracorporeal photopheresis (ECP) treatments were reported to achieve an ORR of 56.0% in a randomized controlled study (19) and an ORR of 67.0% in a retrospective multicenter study (20) of patients with cGVHD. In a large retrospective study including 269 patients with SR-cGVHD by Axt et al. (21), the ORRs of calcineurin inhibitors, MMF, mTOR inhibitors and ECP were all lower than 60.0%. Ibrutinib, a Bruton tyrosine kinase inhibitor, showed a 67.0% ORR for patients with cGVHD in a multicenter, open-label study (22). Some researchers recruited only patients with moderate and severe cGVHD into study, while many studies included patients with mild to severe cGVHD (20, 21, 23–25). In our single-center retrospective survey conducted among 70 patients diagnosed with mild, moderate and severe cGVHD, the median follow-up time was 401 (range 6-1076) days. Up to the final follow-up time, 74.3% of patients had responded to ruxolitinib, of whom 48.6% and 25.7% achieved CR and PR, respectively. A comparable ORR was reported in studies by Abedin et al. (26), Modi et al. (24) and Khoury et al. (27). Many investigators also evaluated the ORR of ruxolitinib at different time points. Abedin et al. (26) assessed the treatment efficacy at 28 days after the use of ruxolitinib; nevertheless, the ORR was only 63%. In the investigation of Modi et al. (24), treatment efficacies were evaluated at two time points. After six months of ruxolitinib therapy, the authors observed a CR in 10% of patients and PR in 37% of patients, while after 12 months, the results differed only slightly, with a CR observed in 13% of patients and PR in 30% of patients. In 2020,

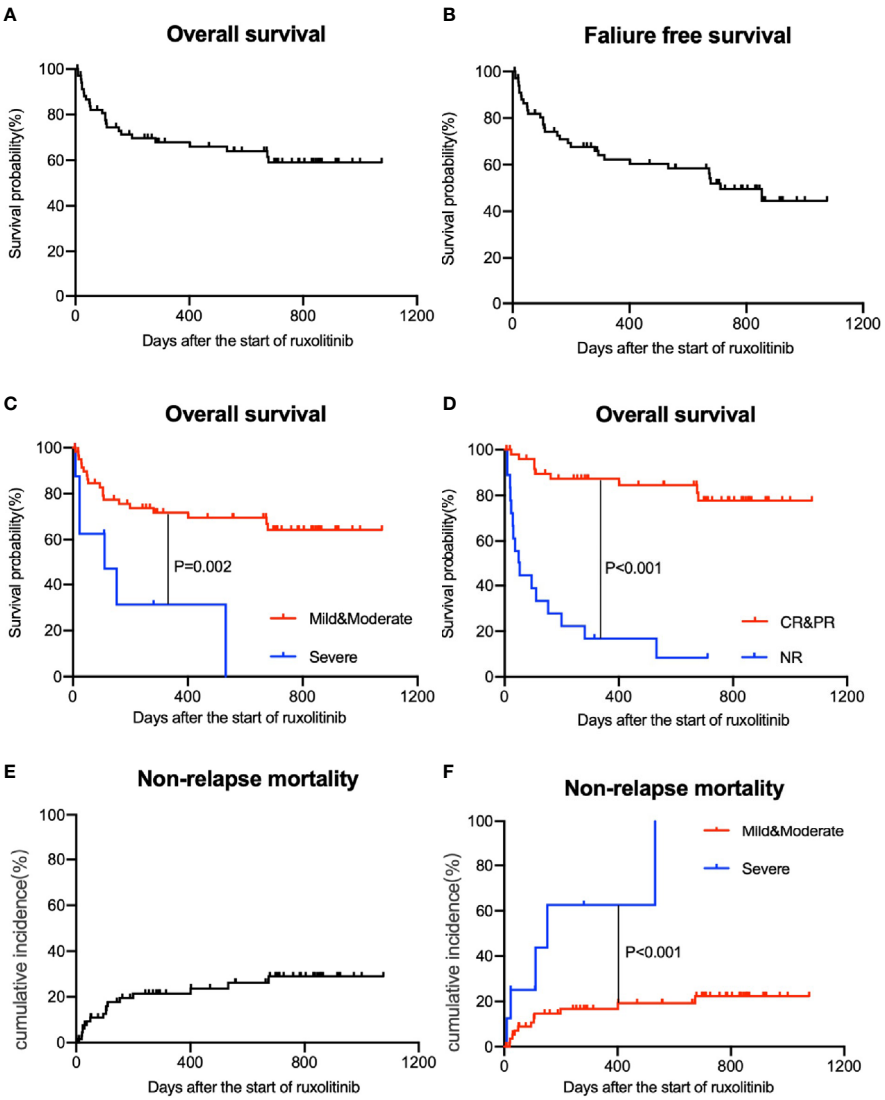

FIGURE 3 | Overall survival (OS) and nonrelapse mortality (NRM) of patients with cGVHD. **(A)** OS of all patients. **(B)** Failure-free survival (FFS) of all patients. **(C)** Comparisons of OS among different grade groups. **(D)** Comparisons of OS among different treatment efficacy groups. **(E)** NRM of all patients. **(F)** Comparisons of NRM among different grade groups.

Zeiser et al. reported their findings from the phase 3 randomized REACH3 study of ruxolitinib compared with BAT in patients with SR-cGVHD. Ruxolitinib resulted in a significantly higher ORR at week 24 than BAT (49.7% *vs* 25.6%, $P<0.0001$), and it was the first agent to show superior efficacy to BAT in a phase 3 trial of patients with SR-cGVHD.

In the present study, mouth cGVHD had the highest ORR to ruxolitinib therapy, and skin cGVHD had the highest CR, a comparable result to the research conducted by Hurabielle et al. (28), who focused on sclerodermatous cGVHD independently. Moreover, in most studies, the mouth and skin were always the best-responding organs. The liver and lung were reported to be the organs with the worst response to ruxolitinib therapy (28–30). Additionally, the ORR in the gut, liver and lung was the lowest, and patients with liver and lung cGVHD had the longest response times in this study. Moreover, Moiseev et al. (29) and Streiler et al. (31) both reported that ruxolitinib significantly improved the respiratory function of patients with cGVHD, reduced steroid requirements and stabilized lung function in patients with bronchiolitis obliterans as a manifestation of cGVHD.

The safety of ruxolitinib treatment was also important. Hemocytopenia was the most common adverse event observed in this study of patients with cGVHD, consistent with previously reported data. In addition, Moiseev et al. (29) claimed that the severity of neutropenia and thrombocytopenia was affected by CMV reactivation ($P=0.07$), treatment with ganciclovir ($P=0.0006$), and a higher initial steroid dose ($P=0.0017$). González Vicent et al. (25) also determined that the incidence

of neutropenia was related to the appearance of CMV and treatment with ganciclovir. In the majority of published articles, the incidence of CMV activation was reported to be greater than 10% (32, 33). However, in the present study, a low risk of reactivating CMV, EBV or herpes virus infections was observed, and reactivation was quickly controlled by antiviral therapies. Additionally, liver and kidney toxicities were uncommon in all published articles, including articles published by our group (24, 28, 29, 34). One possible reason for the low occurrence of adverse effects in this study might be the relatively low dose of ruxolitinib.

As reported before, the JAK1/2 inhibitor ruxolitinib influences the immune response after HSCT (6, 7, 10). In preclinical research, ruxolitinib has been reported to reverse dysregulated T helper cell responses and control autoimmunity resulting from signal transducer and activator of transcription 1 (STAT1) gain-of-function mutations (35). Vicent et al. (25) discussed the variations in the immune system before and after patients with cGVHD received ruxolitinib treatments, in which ruxolitinib was associated with increased numbers of $CD4^+$ T cells and B cells and decreased numbers of NK cells and $CD4^+$ Tregs. Notably, we observed increased numbers of $CD4^+$ and $CD8^+$ DP cells after ruxolitinib treatments. DP cells are a well-described T cell developmental stage within the thymus; in patients with cGVHD, a higher percentage of DP cells indicates better thymus function and less GVHD damage (36, 37). B cells play an indispensable role in the occurrence and development of cGVHD (38, 39); however, few researchers have analyzed the changes in specific B cell subsets before and after ruxolitinib treatment. Studies from both McManigle (40) and Yehudai-Ofir (41) reported that CD27 is normally expressed on B cells and that CD27-positive B cells are proportionally increased in patients with cGVHD. In the present study, the percentage of CD27-negative naïve B cells increased, while the percentages of MZ B cells and classical traditional B cells, which were both CD27-positive, decreased after treatment. Among cytokines, we detected an increase in the levels of IL-10, a definite inhibitory mediator of GVHD (42), after ruxolitinib treatments. In further analyses, the level of the proinflammatory factor IL-6 was decreased in patients with skin cGVHD after ruxolitinib treatments, consistent with published data (43, 44). However, these variations were not observed in patients with liver, lung and gut cGVHD, whose ORRs were lower than patients with skin cGVHD.

Additionally, ST2 has been previously reported to be a specific indicator of aGVHD (45, 46). In 2015, Reichenbach et al. (47) analyzed animal GVHD models and reported that ST2 was upregulated on murine alloreactive T cells and that ST2 levels increased as experimental GVHD progressed. Compared with wild-type (WT) donor T cells, $ST2^{-/-}$ donor T cells displayed a marked reduction in GVHD lethality. In our study, ST2 expression also fluctuated with the severity of cGVHD.

Notably, the median follow-up time in our study was 401 (range 6-1076) days, the one-year estimated survival rate was 66.0% (54.4–77.6%, 95% CI), and the one-year estimated FFS rate was 60.4% (48.2–72.6%, 95% CI). In our study, patients with severe cGVHD experienced a significantly shorter OS and higher NRM than patients with mild and moderate diseases. The OS of patients with mild and moderate cGVHD was 69.6%, probably because approximately two-thirds of these patients had moderate cGVHD. Considering the relatively long follow-up time compared with the studies by Zeiser et al. (16) and Moiseev et al. (27), we propose that our study describes an encouraging survival benefit for patients with SR-cGVHD.

Several limitations also existed in our study. Besides the retrospective nature of this study, it was also difficult to properly account for the effects of concurrent immunosuppressive therapies including corticosteroids and calcineurin inhibitors on the clinical course of cGVHD in addition to the effect of ruxolitinib.

Interestingly, in addition to salvage therapy for SR-cGVHD, ruxolitinib showed excellent performance as a prophylactic agent for GVHD in place of calcineurin inhibitors. Kröger et al. (48) reported on 12 patients who used ruxolitinib during the peritransplantation period. The incidence of grade II–IV aGVHD on day +100 was only 8%, and no NRM was recorded. In the study designed by Zhao et al. (49), after the replacement of a calcineurin inhibitor with ruxolitinib once patients showed intolerance or contraindication to CsA or TAC, only two of ten patients developed aGVHD, and 3 patients developed cGVHD after tapering or stopping ruxolitinib. Moreover, in July 2020, Saraceni et al. (50) reported that patients with cGVHD who were diagnosed with severe coronavirus disease 2019 (COVID-19) were successfully treated with ruxolitinib.

Collectively, the results of this study support ruxolitinib as a safe and effective option as a second-line treatment for patients with SR-cGVHD, with a high ORR of 73.4% and impressive outcomes. Further multicenter studies enrolling a larger number of participants should be conducted in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethical committee of the First Affiliated Hospital of Soochow University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DWu and YX designed the study. XM, QC, and ZL contributed to the collection of data. DWang analyzed the data. DWang, YL, and XL discussed and interpreted the results. DWang wrote the

manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.673636/full#supplementary-material

Supplementary Figure 1 | Comparison of different lymphocyte subsets and cytokine levels in different organs before and after treatment with ruxolitinib. **(A)** Comparison of different lymphocyte cell subsets in different organs. **(B)** Comparison of different B cell subsets in different organs. **(C)** Comparison of different cytokine levels in different organs. Paired sample t test was used for the analysis. The numbers of patients are indicated in each graph.

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REVIEW
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The Effects of Interferons on Allogeneic T Cell Response in GVHD: The Multifaced Biology and Epigenetic Regulations

Chenchen Zhao[1], *Yi Zhang*[2*] *and Hong Zheng*[1*]

[1] Penn State Cancer Institute, Penn State University College of Medicine, Hershey, PA, United States, [2] Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia, PA, United States

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for hematological malignancies. This beneficial effect is derived mainly from graft-versus-leukemia (GVL) effects mediated by alloreactive T cells. However, these alloreactive T cells can also induce graft-versus-host disease (GVHD), a life-threatening complication after allo-HSCT. Significant progress has been made in the dissociation of GVL effects from GVHD by modulating alloreactive T cell immunity. However, many factors may influence alloreactive T cell responses in the host undergoing allo-HSCT, including the interaction of alloreactive T cells with both donor and recipient hematopoietic cells and host non-hematopoietic tissues, cytokines, chemokines and inflammatory mediators. Interferons (IFNs), including type I IFNs and IFN-γ, primarily produced by monocytes, dendritic cells and T cells, play essential roles in regulating alloreactive T cell differentiation and function. Many studies have shown pleiotropic effects of IFNs on allogeneic T cell responses during GVH reaction. Epigenetic mechanisms, such as DNA methylation and histone modifications, are important to regulate IFNs' production and function during GVHD. In this review, we discuss recent findings from preclinical models and clinical studies that characterize T cell responses regulated by IFNs and epigenetic mechanisms, and further discuss pharmacological approaches that modulate epigenetic effects in the setting of allo-HSCT.

Keywords: type I interferon, IFN- γ, GVHD, epigenetic regulation, alloreactive T cells

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Jaebok Choi,
Washington University School of Medicine in St. Louis, United States

Reviewed by:
Cameron Scott Bader,
Stanford University, United States
Daniel Peltier,
University of Michigan, United States

***Correspondence:**
Yi Zhang
yi.zhang@temple.edu
Hong Zheng
hzheng@pennstatehealth.psu.edu

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) provides the long-term effective and curative treatment for patients with hematological malignancies. The therapeutic benefit of allo-HSCT is primarily attributed to the graft-versus-leukemia (GVL) effect, which is mainly mediated by infused donor T cells (1). However, these allogeneic T cells can also cause harmful graft-versus-host disease (GVHD) (2–4). Acute GVHD is a major risk for non-relapse mortality in the first 200 days after allo-HSCT (5). Therefore, maintaining the beneficial GVL effect while reducing GVHD is the holy grail of allo-HSCT.

Upon stimulation by host antigen-presenting cells (APC), infused donor T cells are activated to undergo robust proliferation and effector differentiation (2, 6). These APCs express high levels of antigen-presenting molecule MHC class II and costimulatory molecules (e.g., CD80, CD86), which are required to activate allogeneic T cells and promote expansion of activated T cells, respectively. Many cytokines, such as IL-2 and IL-12, are important for instructing these activated T cells to differentiate into effector cells mediating host tissue injury (7, 8). Notably, interferons (IFN) have an essential role in regulating T-cell activities during GVHD (9, 10). Type I (mainly IFN-α/β) and type II (IFN-γ) are two major IFNs that mediate pathophysiologic changes during infection, cancer and autoimmune diseases (11–15). IFN-γ is primarily derived from T helper 1 (Th1) $CD4^+$ T cells and cytotoxic $CD8^+$ T cells once adaptive immunity develops, whereas IFN-α can be produced by plasmacytoid dendritic cells (pDCs) (16, 17). Both IFN-γ and IFN-α are pivotal regulators of alloreactive T cell responses that mediate GVHD (18–20). However, optimal control of GVHD by modulating IFN signaling remains challenging. IFN signaling is complex and frequently context-dependent: it can lead to distinct effects at different times or stages of a disease course. IFNs regulate T cell functions by regulating a group of intracellular transcription programs. Epigenetic regulations of molecules in the IFN signaling pathway and the interferon-stimulated genes (ISG) are crucial for T cell activity (21, 22). This review focuses on how IFNs regulate alloreactive T cell responses and what role epigenetic regulation plays in this process.

EFFECTS OF IFNs ON T CELL DIFFERENTIATION AND FUNCTION DURING GVHD

Type I IFNs

Type I IFNs contain a subgroup of highly related polypeptides that have proven essential in regulating innate and adaptive immunity (23). Approximately 12-14 types of IFN-α and one type of IFN-β, IFN-ϵ, IFN-κ, and IFN-ω have been identified (24). Intriguingly, although type I IFNs are structurally divergent, only one form of heterodimer receptor, IFNAR, has been found. Thus, all type I IFNs activate the same receptor and many subsequent cell-signaling activities are shared. IFN-α and IFN-β are well defined and are the main subtypes from the immunological perspective. Virtually all cell types reserve the ability to produce variable level of IFN-β, whereas pDCs are the main source of IFN-α (23). Host tissue injuries triggered by conditioning regimens, such as preparative irradiation and chemotherapy, induce damage-associated molecular patterns (DAMP) and foreign pathogen-associated molecular patterns (PAMP). Type I IFNs are among the early cytokines whose production is triggered by the host and donor APCs after the detection of these danger signals by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleic acid sensors that located on or within the cytosol of cells (25) (**Figure 1**). IFN-α/β can exert antiviral and antitumor activity by up-regulating MHC-I and subsequently promoting antigen presentations.

IFN-α/β has context-dependent roles in $CD4^+$ T cell activation, differentiation and survival (26). IFN-α is associated with $CD4^+$ T cell activation and contributes to the IFN-γ-mediated Th1 response (27). In contrast, IFN-α/β may suppress Th2 differentiation of human $CD4^+$ T cells. Importantly, IFN-α regulation of T cell differentiation appears to be context-dependent. Dichotomous T cell polarization towards either Th1 or T_{FH} was recently observed depending on the IFN-α exposure at different times (28, 29). In a colon-targeted GVHD murine model, IFN-α signaling prevented donor $CD4^+$ T cell proliferation and differentiation, resulting in alleviating colon tissue damage (30, 31). Regulatory T cells (Tregs) play essential roles in controlling immune tolerance after allo-HSCT (32). Adoptive transfer of Treg ameliorates GVHD and improves survival in a murine model (33). However, IFN-α/β has shown some controversial impacts on Tregs. Some studies suggested that overexpression of IFN-α significantly reduced the frequency of Tregs in the tolerogenic tumor environment (34). Other studies suggested that IFN-α stimulation may increase differentiation of $CD4^+CD25^+FOXP3^+$ Tregs (iTregs) (35) (**Figure 1**).

IFN-α/β signaling is essential for antigen-driven $CD8^+$ T cell responses. First, differentiation of effector $CD8^+$ T cell was associated with decreased IFNAR but increased IL-12 receptor, whereas augmented IFNAR favors the development of central memory T (T_{CM}) cell (36, 37). In IFNAR deficient mice, $CD8^+$ T cells lose the ability to become memory T cells during lymphocytic choriomeningitis virus (LCMV) infection (37, 38). Paradoxically, withdrawal of IFN-α monotherapy in clinical chronic myeloid leukemia resulted in elevated frequency of peripheral $CD8^+$ T_{CM} cells (39). Given that many different types of cells express IFNAR, the difference in regulating memory formation between IFNAR deficiency and IFN-α monotherapy may be attributable to both direct and indirect mechanisms. Second, the activation of IFN-α/β signaling in T cells could benefit cytokine secretion and cytolytic activity. In mice, injection of IFN-α incited substantial primary $CD8^+$ T responses through cross-priming by DCs that were independent of $CD4^+$ T-cell help (40, 41). IFN-α/β signaling plays a co-stimulatory role in $CD8^+$ T activation and slows the death of activated T cells (42, 43). Moreover, direct activation of granzyme B transcription through IFN-α/β in effector $CD8^+$ T cells contributes to tumor suppression as well as autoimmunity (44, 45). Consistent results were found in the context of GVHD that both CD8-dependent GVHD and GVL effects were enhanced through IFN-α/β signaling (30). In addition, despite IFN-α/β signaling induces transient attrition of bystander naïve T cells in the wake of T-cell response, it can rapidly activate nonspecific bystander memory $CD8^+$ T cells. Activation of memory T cells contributes to rapid production of proinflammatory cytokines including IFN-γ (**Figure 1**) (46–48).

Clinically, recombinant IFN-α has been used alone or in combination with donor lymphocytes infusions or other cytokines such as granulocyte-macrophage colony-stimulating factor to establish GVL effects in patients with minimal residual

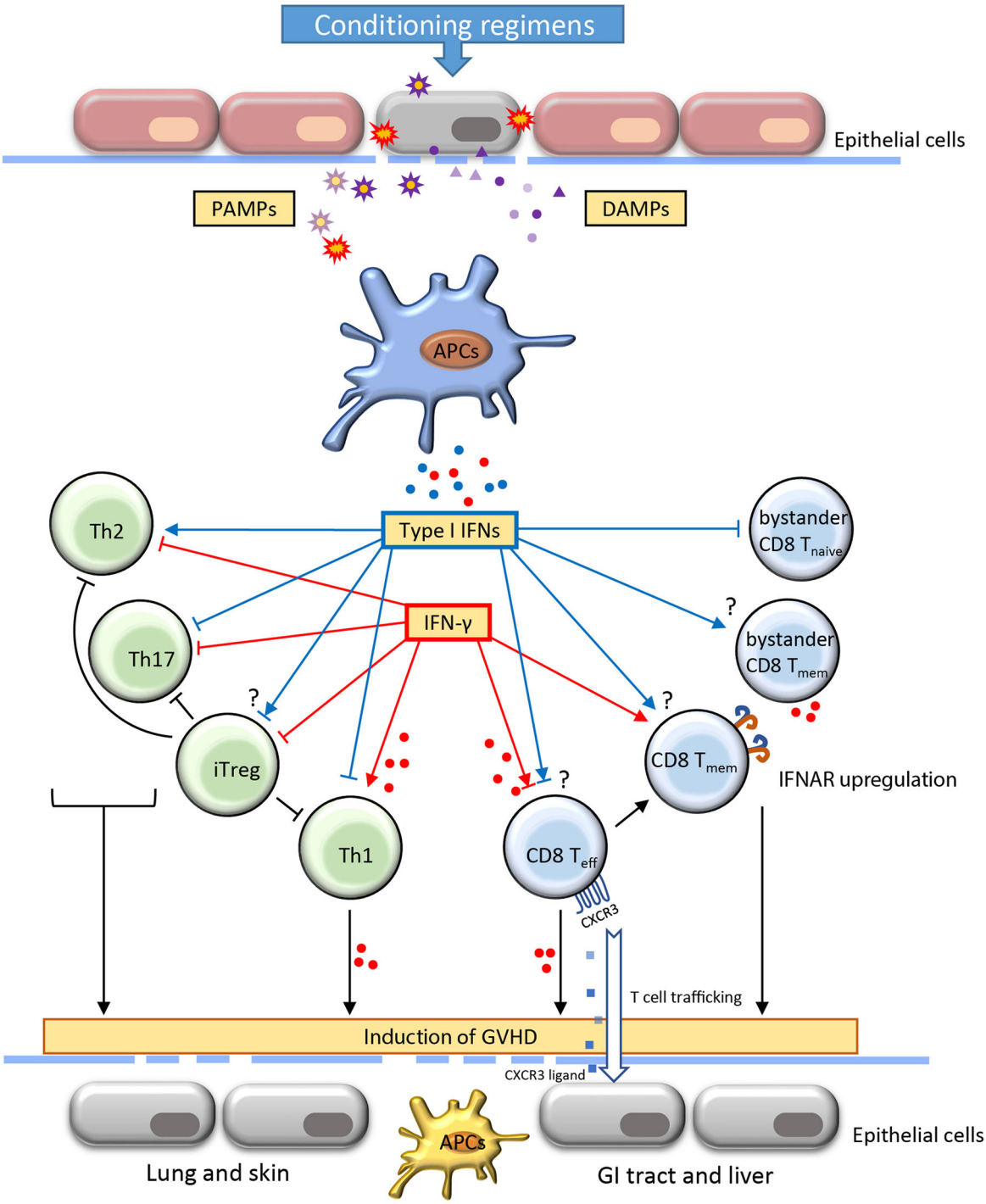

FIGURE 1 | Role of type I IFNs and IFN-γ in the development of acute graft-*versus*-host-disease. Acute graft-*versus*-host disease (GVHD) is often initiated with the destruction of the epithelial barrier through the conditioning regimens including irradiation and chemotherapy. The signal of pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) released from damaged cells and microbiota induce the activation of antigen-presenting cells (APCs). Consequently, the production of IFNs by APCs interact with the alloreactive T cells and regulate their activation, differentiation, function and contraction. The proliferation of Th1 cells and effector CD8 T cells result in increased secretion of IFN-γ. The induction of activated alloreactive T cells and cytokines further affect the resident APCs and host tissues contributing to extensive functional incapability and damages of different organs. The IFNs play a critical role in orchestrating T cell activities throughout the induction and effector phase of GVHD. The blue and red dots indicate the IFNs (type I IFNs and IFN-γ, respectively) secreted by adjacent cells. CXCR, CXC chemokine receptors; GI, gastrointestinal; Th, T helper cells; iTreg, induced regulatory T cells; T_{eff}, effector T cell; T_{eff}, memory T cell.

disease or relapse after allo-HSCT (49–53). Studies using animal models demonstrate the effectiveness of IFN-α treatment against leukemia cells. A most recent trial showed that the proportion of granzyme positive $CD8^+$ effector and effector memory subsets is positively correlated with GVHD incidence (53). This suggests that IFN-α-induced $CD8^+$ T cells may be a double-edged sword against both malignant and normal cells.

In response to IFN-α/β signaling, the trimolecular interferon-stimulated gene factor 3 (ISGF3), which comprises signal transducer and activator of transcription (STAT) 1, STAT2 and interferon regulatory factor (IRF) 9, leads to most of the cellular effects of IFN-α/β (23). The dysregulation of ISGF3 results in aberrant T cell functions. For example, the absence of IRF2, a negative regulator of ISGF3, induces hyperresponsiveness of $CD8^+$ T cells and promotes spontaneous inflammatory skin lesion in mice (54). Furthermore, in a LCMV infection mouse model, the STAT1 deficiency leads to a $CD4^+$ T cell-mediated lethal disease. This effect is independent of IFN-γ, but it coincides with exaggerated proinflammatory cytokine production as well as increased frequency of LCMV-specific $CD4^+$ and $CD8^+$ T cells (55). These observations suggest that the effects of IFN-α/β signaling are not only divergent on $CD4^+$ or $CD8^+$ T cells, but also highly dependent on the pathophysiological backgrounds. Of note, several molecules in the downstream of the IFN-α/β pathway, including Janus Kinase (JAK) 1, STAT1 and STAT3, are shared with IFN-γ signaling, which indicates the possibility of crosstalk between the two signals in the transcriptional level.

IFN-γ

IFN-γ promotes $CD4^+$ T cell differentiation towards Th1 lymphocytes and drives $CD8^+$ T cell expansion and differentiation towards both effector and memory cells (**Figure 1**). Early studies in GVHD models suggest that IFN-γ contributes as a pathogenic factor to alloreactive responses. For instance, high serum levels of IFN-γ correlated with increased severity of GVHD after allo-HSCT (56). IFN-γ induced apoptosis of intestinal epithelial crypt cells, leading to extensive erosion of intestinal epithelium and GVHD propagation (57, 58). Genetic deletion of IFNGR in T cells prevents lethal GVHD while preserving the robust GVL effect (59). Furthermore, evidence from live-cell imaging reveals that both motility and cytotoxicity of $CD8^+$ T cells are enhanced in alloreactive tissue due to autocrine/paracrine IFN-γ (60). The expression of CXCR3 induced by IFN-γ signaling is one of the mechanisms that drive the T cells to the sites of GVHD target organs (59).

Intriguingly, some studies suggested that IFN-γ may negatively regulate alloreactive T cells and prevent tissue damages. Evidence from IFN-γ knockout mice shows that IFN-γ could be protective against GVHD depending on the extent of conditioning in mouse models (18, 61). Infusion of IFN-γ-null donor T cells increased mortality of GVHD compared to that of wild-type T cells (62). One possible reason might be that IFN-γ is required for normal T cell contraction since IFN-γ deficiency would lead to delayed apoptosis of $CD8^+$ T cell population, leading to prolonged inflammation (63–65). In addition, PD-L1, which is considered as an inhibitory checkpoint molecule in infections and tumors, was identified as a positive contributor to T cell-mediated GVHD in the murine model, as decreased inflammatory cytokines and increased apoptosis were observed in both *Pdl1*$^{-/-}$ allogeneic $CD4^+$ and $CD8^+$ T cells. Of note, both *Ifngr*$^{-/-}$ $CD4^+$ and $CD8^+$ donor T cells showed impaired PD-L1 expression, suggesting that loss of IFN-γ signaling mitigates tissue damages in GVHD *via* the PD-L1 pathway (66).

Interestingly, manipulation of IFN-γ signaling in alloreactive T cells results in variable lesions in GVHD target organs. IFN-γ produced by alloreactive T cells is the primary mediator contributing to the apoptosis of intestinal stem cells and intestinal damage (57). In addition, both clinical and preclinical studies suggest that IFN-γ-producing Th1 cells mediate damages in the gastrointestinal (GI) tract (67), whereas IFN-γ KO model results in exacerbated skin and lung injury (68). In the absence of IFN-γ signaling, alloantigen-primed $CD4^+$ T cells showed decreased capacity to produce IFN-γ-secreting Th1 cells while skewing toward both Th2 and Th17 cells (68). Further studies are needed to define the correlation between the effects of IFN-γ on alloreactive T cells and the consequence of GVHD.

The binding of IFN-γ to the receptor, IFNGR1 and IFNGR2 complex, induces recruitment and phosphorylation of receptor-associated JAK1/2, which triggers subsequent signaling pathways predominantly through STAT1 (**Figure 2**). Interestingly, TCR stimuli initiate the translocation of STAT to IFNGR1-rich regions of the membrane similar to IFN-γ ligation (69). Blocking the JAK1/2 molecule significantly abrogates the polarization and proliferation of activated T cells as well as downregulates activation markers, such as CD69 and CD25, and reduces the production of proinflammatory cytokines (70). In light of the suppressive effect on T cell responses, the JAK inhibitors were reported to control GVHD in both mice and humans. Recently, ruxolitinib was approved for the treatment of steroid-refractory acute GVHD. JAK inhibitors mitigate GVHD *via* pleiotropic effects on T cells. For example, ruxolitinib mitigates acute GVHD by reducing CXCR3 expression, which results in less T-cell infiltrates in target organs (59, 71, 72), and by decreasing IFN-γ and IL17A production in $CD4^+$ T cells (73). Similarly, another JAK1/2 selective inhibitor, baricitinib, can abrogate IFN-γ and IL-6 signaling in $CD4^+$ T cells and significantly decrease Th1 and Th2 cell differentiation while augmenting the frequency of Tregs (74). In addition to the reduction of GVHD, baricitinib could also improve GVL (74). Despite these promising observations, the transcriptional regulations of the downstream genes in T cells are yet to be found.

Notably, as the IFNGR can be expressed on almost all cell types, the generated IFN-γ from activated allogeneic T cells could have a remarkable influence on the innate immunity and break the homeostasis of the surrounding tissues. For instance, IFN-γ signaling acts as a 'super-activator' of macrophages, inducing transcriptional activation of proinflammatory genes (e.g., IL-6 and TNF-α) and enhancing antigen presentation. Furthermore, recent evidence suggests that IFN-γ directly inhibits the proliferation of hematopoietic stem/progenitor cells (HSPCs) and their generation of pDCs that can induce immune tolerance

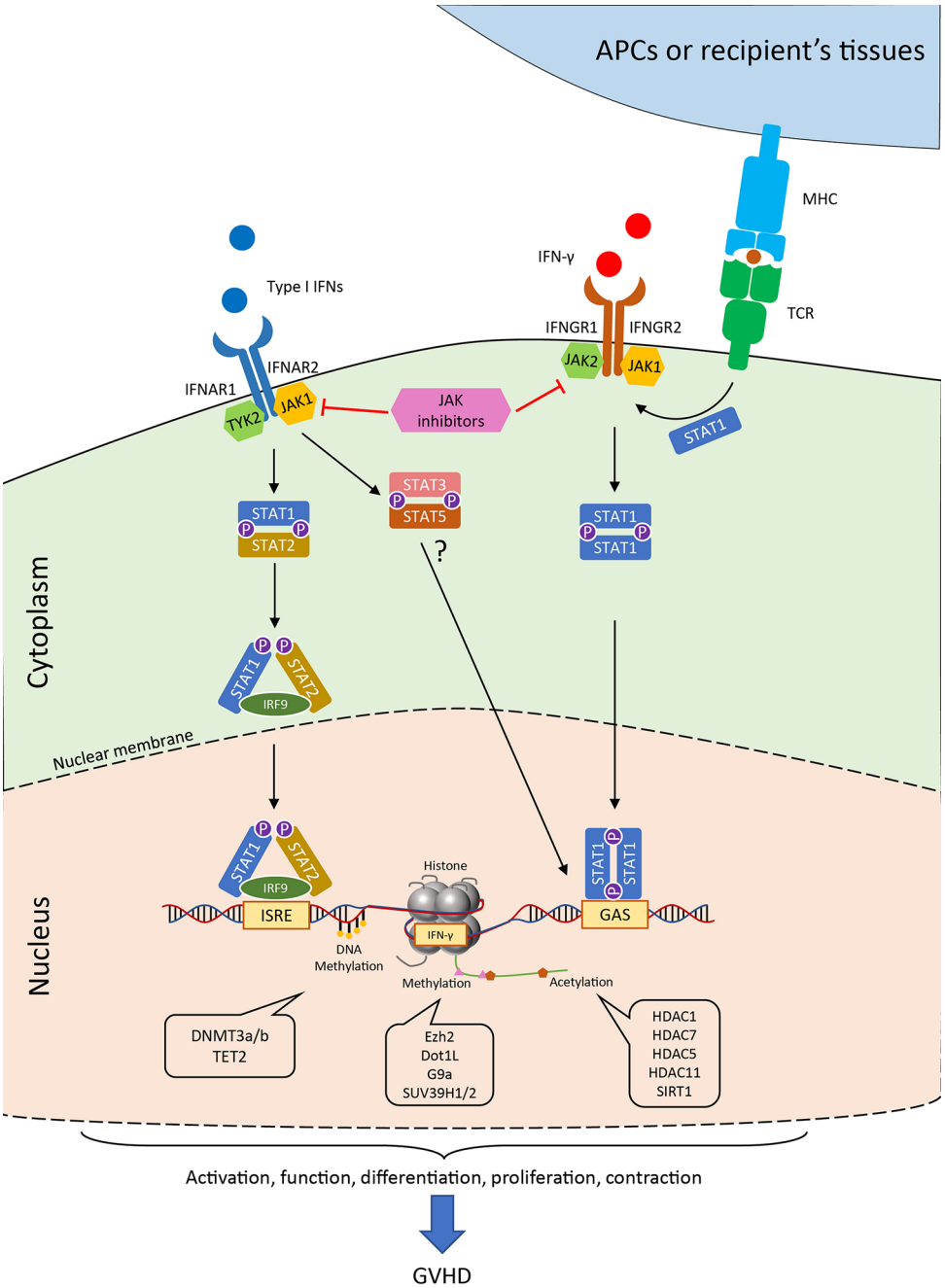

FIGURE 2 | Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways and the epigenetic regulations of IFN signaling in T cell. Generally, the binding of type I IFN to the receptor initiates the engagement of IFNAR1 associated Tyk2 protein tyrosine kinase and the IFNAR2 associated JAK1 protein tyrosine kinase. The signal further passes to the phosphorylation and the heterodimerize of STAT1 and STAT2, which together with IRF9 form the ISGF3 complex in the cytoplasm. ISGF3 translocates into the nucleus and binds to IFN-stimulated response elements (ISREs) found in most of IFN-stimulated genes (ISGs). Alternatively, STAT3 and STAT5 heterodimer are also observed after IFNAR activation in the absence of STAT1. Canonical IFN-γ signaling occurs through IFNGR and activates the JAK1/2 kinases, which further induce the phosphorylation of STAT1. The STAT1 homodimer can directly move into the nucleus and binds to gamma-activated sequence (GAS) sites. The activation of T-cell receptor could also help the co-localization of STAT1 to IFNGR-rich regions of the membrane. The transcription of the downstream genes as well as the production of IFN-γ are tightly regulated by numerous epigenetic enzymes, which control the modification of the DNA and histones. These regulators critically control the T cell activities in the process of GVHD, which allows for possible therapeutic interventions. JAK, Janus kinase; STAT, signal transducer and activator of transcription; IFNAR, interferon alpha receptor; IFNGR, interferon gamma receptor; ISREs, IFN-stimulated response elements; GAS, gamma-activated sequence; TCR, T cell receptor; MHC, major histocompatibility complex; IRF, interferon regulatory factor; HDAC, histone deacetylase; SIRT, sirtuin.

against GVHD (20). On the other hand, exposure to IFN-γ reduces the proliferation of intestinal epithelial cells and further induces apoptosis by modulation of the AKT/β-catenin and Wnt/β-catenin pathway (75). Thus, although IFN-γ plays dichotomous roles in the regulation of T cells, its proinflammatory damage to the tissues in GVHD is generally acknowledged (**Figure 1**).

Type III IFNs

Recent studies have discovered other members of type III IFNs, including IFN-λ1 (IL-29), IFN-λ2 (IL28A), IFN-λ3 (IL-28B) and IFN-λ4 (76–78). They participate in the antiviral activities similar to the type I IFNs, but primarily in barrier tissues such as mucosal epithelial cells (79, 80). Although IFN-α/β and IFN-λ engage different receptors, they induce similar downstream signaling pathways through the phosphorylation of STAT1/2 and the subsequent transcription factor ISGF3 (81). However, our knowledge of IFN-λ in GVHD is limited. An initial study found that IFN-λ2 did not significantly modulate GVHD mortality in a murine model upon deleting its receptor (*Ifnlr1*$^{-/-}$) or administration of recombinant IFN-λ2 (82). Intriguingly, a most recent study revealed that *Ifnlr1* deletion led to exaggerated damages in the GI tract and recombinant IFN-λ treatment reduced GVHD lethality (83). Deletion of *Ifnlr1* led to increases of donor T-cell expansion and serum IFN-γ levels, however, it did not affect the proliferation and apoptosis of alloreactive T cells. Interestingly, the effect of IFN-λ on T cells seemed to be indirect since the T-cell expansion was influenced by early engraftment, which was related to IFN-λ signaling in NK cells (83). Further studies of IFN-λ in the modulation of GVHD the underlying mechanisms are warranted.

EPIGENETIC REGULATION OF IFN EXPRESSION AND FUNCTION IN T CELLS

The epigenomic signatures, including DNA methylation on cytosine nucleotides, histone modifications and chromatin accessibility, reflect previous and present gene expression, and can positively or negatively regulate future transcription according to environmental stimuli. The labeled or 'bookmarked' chromatin organized as 'epigenetic code' that can be recognized by protein complexes called 'readers'. It is also closely controlled by enzymes, called 'writers and erasers', that are able to manipulate different modifications mounted on specific residues (84). The major contributors comprise DNA methyltransferases (DNMTs) or DNA demethylases on DNA level; histone acetyltransferase (HATs), histone deacetylase (HDACs), histone methyltransferases (HMTs) and lysine demethylases (KDM) on histone level. Other epigenetic mechanism includes the microRNAs (miRNAs), which negatively control target gene expression post-transcriptionally *via* interaction with the complementary sequences. It has been well established that IFN-signaling can generate 'interferon epigenomic signatures' and reprogram cell response (21).

During antigen-driven immune responses, such as GVHD, T cells are located at the downstream of type I IFN signaling as they receive this signal from innate immune cells (20, 85). Although many regulators and pathways of type I IFN signaling from innate cells may be interchangeable in T cells, the cell type and context-dependent mechanisms have yet to be characterized. As discussed earlier, the canonical signal of type I IFN depends on the activation of STAT1 and STAT2, which leads to the activation of ISGs by transcription factor IRF9 (also known as ISGF3) (23). Notably, multiple pathways co-exist in the downstream of IFNAR1/2 and control T cell immune responses (**Figure 2**). For example, IFN-α/β induced STAT1 is responsible for the suppression of CD8$^+$ T cell expansion, whereas STAT3 and STAT5 mediate antiapoptotic and mitogenic effects in T cells in the absence of STAT1 (86).

The epigenetic regulation of ISGs in T cells is far less documented compared with studies in innate immune cells. Evidence has been widely found on innate immune cells that ISG promoters are associated with increased level of histone acetylation, which in part mediated by STAT1 and STAT2 (87), STAT1/2 promotes histone acetylation after IFN-α/β stimulation in T cells as well. Early study has linked IFN-α signaling with histone hyperacetylation at the granzyme B and eomesodermin (Eomes) loci during CD8$^+$ T cell differentiation (88). Similarly, IFN-α/β signaling enhances H3K4me3 and H3K9ac (transcription permissive) at the promoter region of Eomes and activates it in an IRF9-dependent manner (89). Interestingly, T-bet is found to counteract aberrant IFN-α/β signaling during Th1 cell development by repressing ISGs such as *Isg15*, *Mx1*, Oasl1a, etc. Deletion of T-bet results in accumulation of STAT2 and elevation of transcription active mark H3K27ac at ISGs activated by IFN-β (90), highlighting the complexity of interactions between the extrinsic cytokine influence and the intrinsic regulation of cell development. In addition, the epigenetic modulation of T cells in responding to IFN-α/β is likely to be context-dependent. For example, although STAT1 mRNA levels are both increased in lupus and normal CD8$^+$ T cells with IFN-α stimulation, the signature of hypomethylated DNA sites in lupus CD8$^+$ T cells facilitates the upregulation of HLA-DRB1 in a STAT1-signaling-dependent manner (91). In addition to histone and DNA modification, the miRNA-155 downregulates the T-cell responsiveness to IFN-α/β *via* IFNAR-STAT pathway. Despite the direct targets of miRNA-155 were not defined, the loss of microRNA-155 results in impaired antiviral CD8 T cell response (92). To reconcile these paradoxical and the highly context-dependent effects of IFN-α/β on T cells, it will be important to map how the ISGs are regulated on the epigenetic level in alloreactive T cells during GVHD.

Compared to IFN-α/β, the epigenetic regulation of IFN-γ is much more complicated. IFN-γ not only promotes antigen-driven T cell differentiation, but also is the major mediator for tissue injury. Much work has investigated the epigenetic regulation of *Ifng* locus in T cells. The CpG dinucleotide at *Ifng* promoter in naïve CD8$^+$ T cells is substantially methylated and undergoes demethylation when these CD8$^+$ T cells are activated. Memory CD8$^+$ T cells retain relative hypomethylated status to enable a rapid gene expression for the re-activation in the future (93, 94). Similar regulation can be found in Th1 CD4$^+$

T cells. The CpG for *Ifng* promoter in naïve $CD4^+$ T cells is mostly methylated, but only approximately 50% of CpG regions are methylated in resting memory cells (95). Conversely, in the lineages other than Th1 development, their suppression of IFN-γ can also be achieved in part with DNA methylation (96). The ten-eleven translocation (TET) 2 enzyme, which mediates DNA demethylation, positively regulates *Ifng* transcription by promoting 5-hydroxymethylcytosine level in $CD4^+$ T cells (97). From another view, histone modifications act in accordance with the gene regulation of DNA methylation. For example, the *Ifng* promoter region in Th1 cells is associated with hyperacetylation of histones H3 and H4, but not in Th2 cells (98). Additionally, the *Ifng* suppression in Th2 cells is accompanied by repressive H3K9me3, which is governed by enzyme SUV39H1. Loss of this enzyme results in skewed lineage stability (99).

EPIGENETIC PROGRAMS AND PHARMACOLOGICAL MODULATIONS THAT CONTROL IFNs IN ALLOGENEIC T CELLS DURING GVHD

The function and differentiation of T cells are closely intertwined with IFN expression. Epigenetic processes, including DNA methylation, histone modification and chromatin remodeling, are the key mechanisms that control T cell differentiation and function (100, 101). Multiple epigenetic enzymes have been identified to regulate the production and subsequent effect of IFNs in allogeneic T cells (102–104). A number of chemical compounds that selectively inhibit these enzymes are made available, and their effect on IFN signaling and therapeutic potentials are under active investigation (104–106). Since there are very limited reports studying the epigenetic regulation of type I IFNs in the GVHD context, our continual discussion will focus on the epigenetic effects on IFN-γ.

DNA Methylation

DNA methyltransferase (DNMT) 3a or DNMT3b contributes to *de novo* DNA methylation, resulting in genetic silencing. In T cells, DNMT3a expression is regulated by TCR signaling (107). The promoter of *Ifng* locus remains hypomethylated during Th1 differentiation from naïve $CD4^+$ T cells, albeit *de novo* DNA methylation at *Ifng* promoter is observed in other commitments, such as Th2, Th17 and iTreg cells (108). DNMT3a is responsible for maintaining the silence of *Ifng* gene in non-Th1 lineages (109). Accordingly, deletion of DNMT3a after T cell activation selectively reduces the level of *Ifng* methylation (107), and allows significant IFN-γ production from non-IFN-γ producing $CD4^+$ T cells (109). During secondary contact with antigen, DNA demethylation at the IFN-γ promoter takes place in memory T cells in order to facilitate rapid effector responses (94). Furthermore, the functional exhaustion of the $CD8^+$ T cells couples with persistent DNA hypermethylation at *Ifng* loci, even if the cells are treated with anti-PD-1 blockade. Inhibition of DNA methylation by hypomethylating agent together with anti-PD-L1 significantly promotes IFN-γ secretion by exhausted $CD8^+$ T cells (110). The above studies suggest that inhibition of DNMT may promote alloreactive T cell activities in GVHD by suppressing DNA methylation and subsequently enhance IFN-γ production.

Although hypomethylating agents globally alter DNA methylation levels, their influence on gene expression in T cells shows preference. Compelling evidence indicates that both IFN-γ and FOXP3 locus are demethylated by Azacytidine (Aza) (94, 111, 112). In vitro studies revealed that Aza and decitabine could directly induce FOXP3 expression in T cells, whereas IFN-γ gene expression along with other cell-cycle related genes were significantly down-regulated by Aza (104). Consistently, decitabine significantly suppressed differentiation of naïve $CD4^+$ T cells into Th1 subsets but not Tregs (113). These findings applied to the alloreactive T cells in GVHD. It has been observed that Aza mitigates GVHD in murine models by converting alloreactive $CD4^+CD25^+FOXP3^-$ cells to suppressive $CD4^+CD25^+FOXP3^+$ Tregs and directly increase Treg proliferation. In addition, the frequency of IFN-γ-producing $CD4^+$ T cells was significantly decreased (114–116). The inhibition of naïve $CD4^+$ T cell proliferation by decitabine is also accompanied by the elevation of the TET2, an enzyme that acts opposite to DNMTs, which promote DNA demethylation (113).

Despite the above *in vitro* data supporting that hypomethylating agents up-regulate Treg and suppress conventional $CD4^+$ T cells (117, 118), post-transplantation Aza treatment in patients with high risk of AML and MDS shows no significant differences in terms of overall survival and GVHD incidence in patients compared to the control arm (119). It is possible that additional epigenetic mechanisms are involved in the IFN-γ regulation of alloreactive T cells. For example, in DNMT3a-null Th2 or Th17 cells, decreased level of DNA methylation at the *Ifng* loci correlated with low level of H3K4 and high H3K27 methylation, which permits and inhibits DNA transcription, respectively (109).

Histone Methylation

Histone methylation is predominantly restricted to the N-terminal tails of H3 and H4 histones and is usually presented by one, two, or three lysine residues (120). The effects of histone methylation on gene expression are loci-specific. Genes that bound by H3K4, H3K36 and H4K20 are more likely to be actively transcribed, whereas H3K9, H3K27 and H3K79 are usually associated with gene suppression (121–124). The histone methylation level at each site is controlled by one or a set of HMTs and KDMs (120, 124). Thus, the activities of these enzymes are the key factors that determine gene transcription.

$CD4^+$ and $CD8^+$ T cells display unique patterns of histone methylation landscapes at *Ifng* locus based on the stages of cell differentiation. Once activated by TCR signaling or specific cytokines, the histone methylation markers of T cells are dynamically catalyzed by their dedicated enzymes. During the quiescent stage of naïve T cells, the *Ifng* promoter of both $CD4^+$ and $CD8^+$ T cells are occupied with repressive H3K27me3 but low level of permissive H3K4me3 (125, 126). Upon activation, *Ifng* region of both $CD4^+$ and $CD8^+$ T cells loses H3K27me3 markers (125, 127, 128). However, effector $CD8^+$ T cells gain H3K4me3 at *Ifng* locus (127, 129). $CD4^+$ Th1 cell differentiation increases both H3K4me3 and H3K9me2 (permissive and

repressive, respectively), whereas $CD4^+$ Th2-cells rapidly extinguish H3K9 methylation by STAT6 and GATA-3 dependent mechanisms (130).

Ezh2 is a crucial enzyme that catalyzes H3K27 methylation and remarkably silences target genes to facilitate T cell differentiation and function. During Th2 cell development, Ezh2 is recruited with STAT6 and GATA3 to the *Ifng* locus and is responsible for the silencing of *Ifng* locus through H3K27 methylation (130). *In vitro* studies revealed that Ezh2 affected $CD4^+$ T cell differentiation depending on the context of the extracellular environment. For instance, Ezh2-deficiency could enhance the $CD4^+$ T cell production of either IFN-γ or IL-4, depending on the cell-inducing cytokines *in vitro*, such as IL-12 or IL-4, respectively (131–133). Further, both T-bet and Eomes are required for the regulation of IFN-γ production by Ezh2 (131). The role of Ezh2 in GVHD is complex. In an MHC-mismatched B6 anti-BALB/c GVHD murine model, loss of Ezh2 in donor T cells resulted in impaired IFN-γ production and reduced GVHD. Specifically, Ezh2 promoted Th1 development by stimulating *Ifng*, *Tbx21* and *Stat4* expression (102). Similar results could also be found from Th1 cells in aplastic anemia, in which Ezh2 directly activated *Tbx21* transcription by direct binding to its promoter (134). Contradictive results are also found with $CD8^+$ T cells. Ezh2 inhibition resulted in increased frequency of IFN-γ producing tumor-infiltrating $CD8^+$ T cells (135), whereas Ezh2-deficient $CD8^+$ T cells exhibit an impaired ability to produce IFN-γ in a virus infection model (136). How to explain the discrepancy observed in these studies remains elusive. In addition, Ezh2 could co-localize with FOXP3 and assist in silencing the IFN-γ expression (137). Consistently, the absence of Ezh2 resulted in defective Treg differentiation, which could further contribute to autoimmune colitis (132).

Dot1L, a solo H3K79 methyltransferase, has been recently identified to regulate T cell activation and polarization. In general, H3K79 methylation strongly correlates with active gene transcription (138, 139), but exceptions are also reported (140, 141). When T cells were cultured in Th1 cell-polarizing conditions, IFN-γ production was enhanced by Dot1L inhibition with a small molecule inhibitor (SGC0946) at the beginning of polarization and was associated with the reduction of H3K79me2. Interestingly, the proliferative capacity was not affected (142). These observations indicate that Dot1L may play a negative role in regulating Th1 cell differentiation and IFN-γ production. Another group recently used a T-cell-specific Dot1L-deficient infection mouse model and observed that the repressive effect of IFN-γ production by Dot1L was T-bet dependent. In this study, the enhanced IFN-γ secreting ability *via* Dot1L inhibition (with chemical probe SGC0946) in Th2 cells was abrogated by T-bet deletion (143). However, the opposite phenomenon was observed in GVHD setting. Inhibition of Dot1L with the same chemical probe attenuated xenogeneic GVHD by globally suppressing T cell activation-induced genes, in which IFN-γ production was significantly reduced (103). Of note, this effect was only observed in T cells with low-avidity TCR interaction. Therefore, Dot1L inhibition increased the TCR stimulation threshold and was controlled in an ERK phosphorylation-dependent manner (103). The inconsistent findings among different studies are likely due to the different roles of Dot1L in the regulations of upstream and downstream of IFN-γ signaling. Similar to the data found in $CD4^+$ T cells, Dot1L also remarkably controls the differentiation of $CD8^+$ T cells. Dot1L-deficiency resulted in the induction of memory-like transcriptome feature in antigen inexperienced $CD8^+$ T cells. Furthermore, these cells were functionally impaired as they were incompetent to produce IFN-γ upon stimulation with anti-CD3 and anti-CD28 antibodies (144). In addition, using the approach of genetic Dot1L deletion and a specific inhibitor, EPZ004777, the repression of Dot1L resulted in inhibition of H3K79me2 in $CD8^+$ T cells that associated with increased $CD8^+$ T cell apoptosis and suppressed IFN-γ and TNF-α secretion. Besides, the methionine metabolism in the microenvironment also affects the methylation status of H3K79 in $CD8^+$ T cells, further promoting the dysregulation of the immune response (145). However, genetic approaches are required to define the precise role of Dot1l in T cells.

Both G9a and SUV39H1/2 contribute to the methylation of the H3K9 site. However, G9a catalyzes H3K9 residue to mono- or dimethylation (H3K9me/me2), whereas SUV39H1/2 is responsible for di- to tri-methylation (H3K9me3) (146, 147). These enzymes could be found in multiple repressive complexes that promote transcription inhibition. Importantly, the heterochromatin protein 1α (HP1α) directly recognizes and binds to H3K9me3 and initiates the chromatin remodeling by forming heterochromatin (148). Despite their similarity in histone modification, G9a and SUV39H1/2 are remarkably divergent in epigenetic regulation of IFN and its subsequent effects on T cell functions. During Th2 development, G9a facilitates the transcriptional silence of *Ifng* locus since increased IFN-γ production was observed in G9a deficiency $CD4^+$ T cells along with a decreased level of H3K9me2 (149). However, given that G9a deficiency and inhibition do not affect the development of Th1 cells as well as their capacity in secreting IFN-γ both *in vitro* and *in vivo*, G9a is currently considered dispensable for Th1 cell response (149, 150). Similarly, the ability to produce IFN-γ in $CD8^+$ T cells is not affected in G9a knockout cells, but G9a is crucial to repress helper T lineage genes after the activation of $CD8^+$ T cells (151). These studies indicate a moderate role of G9a in epigenetic control of IFN-γ. In addition, although not verified in T cells, evidence suggests that the downstream of IFN-α/β signaling and ISGs are negatively regulated by G9a (152). On the other hand, SUV39H1-H3K9me3-HP1α pathway also contributes to Th2 stability by decorating H3K9me3 at *Ifng* promoter (99). Less is known whether this signaling redundancy may mutually compensate for both G9a and SUV39H1 when activated *via* different upstream pathways.

In addition, SETDB1, which belongs to the SUV39H family, is responsible for H3K9me3 deposition at specific promoters. Adoue et al. demonstrated that SETDB1 was required to maintain IFN-γ silencing in Th2 cells. Instead of directly catalyzing H3K9me3 on the target gene, SETDB1 represses adjacent endogenous retrovirus location that affects the

transcription of Th1 genes (153). This study reveals the spatial regulation of histone modification in the epigenetic control of Th cell differentiation. In contrast to the inhibitory regulation of IFN-γ in $CD4^+$ T cells, wild-type $CD8^+$ T cells exhibited a higher ability to produce IFN-γ compared to SUV39H1-conditional knockout mice that infected with *L. monocytogenes*. SUV39H1 was responsible for silencing stem/memory gene programs while enhancing the functions of effector cells in $CD8^+$ T cells (154).

Histone Acetylation

Unlike methylation, histone acetylation uniformly assists gene transcription because the acetyl group neutralizes the positive charge on the histones, thereby reducing the electrostatic force between histone and the negatively charged DNA molecules (155). On the other hand, together with methylation, phosphorylation and other covalent modifications, histone acetylation also takes part in the formation of 'epigenetic code', which allows the recognition by the protein complexes that help amplify the gene transcription (156). HAT and HDAC regulate acetylation status on both H3 and H4 histones.

The anti-inflammatory properties of the HDAC inhibitors have long been recognized by numerous experimental and clinical studies, including GVHD. Early studies that first linked histone acetylation with GVHD revealed that the panoramic HDAC inhibitor (pan-HDACi) suberoylanilide hydroxamic acid (SAHA) ameliorate and delayed the development of GVHD and reduced the serum level of proinflammatory cytokines such as IFN-γ and TNF-α following allogeneic bone marrow transplantation (157, 158). Although the STAT1 phosphorylation was inhibited during this process, the T cell proliferation and cytotoxic responses in GVL activity remained intact. Several clinical trials using SAHA as prophylactic treatment after allo-HSCT also observed reduction of GVHD in patients (159, 160). These trials revealed higher Treg cell numbers in the peripheral blood after HDACi administration as well as lower GVHD-related biomarkers, such as ST2 and Reg3α, and the proinflammatory cytokine IL-6 in the plasma. Another clinical trial uses pan-HDACi panobinostat, combined with glucocorticoids, as primary treatment for acute GVHD demonstrates an enhanced H3 acetylation in both $CD4^+$ and $CD8^+$ T cells (161). However, in contrast to the mouse model, the level of plasma IFN-γ was not significantly changed in patients (160, 161). It will be interesting to determine whether functional changes of T cells are controlled by HDACi and correlate with clinical responses and outcomes of patients.

Accumulating evidence from recent studies discovered the regulation of IFN-γ by specific HDAC members in T cell responses with or without allogeneic antigens. HDAC1-deficiency does not affect the development and effector functions but increases the STAT1 activity in $CD4^+$ T cells, which results in the elevated level of IFN-γ production in activated Th1 cells (162, 163). Similar effects were also detected in effector $CD8^+$ T cells (164), indicating a negative role of HDAC1 in controlling IFN-γ transcription. Besides, HDAC7 and SIRT1 may synergize with HDAC1 in repressing T cell activation and IFN-γ production *via* separate pathways (165, 166). On the contrary, HDAC5 and HDAC11 positively regulate IFN-γ production. Both HDAC5 deletion in $CD8^+$ T cells and HDAC11 knockout in $CD4^+$ and $CD8^+$ T cells induce increased IFN-γ production upon anti-CD3 and anti-CD28 activation *in vitro* (167, 168). In addition, potent GVHD in the murine model can be induced in HDAC11 KO mice. Both $CD4^+$ and $CD8^+$ T cells with HDAC11 deletion are hyperresponsive to alloantigen and associated with increased expression of Eomes and T-bet (167).

Of note, inconsistent results can be found among studies. These discrepancies may partially attribute to the fact that some HDACs can both have histone and non-histone targets, which increases the complexity of gene regulation by introducing indirect effects. For example, genetic deletion and inhibition of SIRT1 reduces T-cell alloreactivity and promotes the function of iTreg through the enhancement of p53 acetylation, leading to the attenuation of GVHD (169).

Compared to the extensive investigations exploring the features of HDACs, the regulatory role of HAT in IFN related T cell responses are not well understood. In Th2 cells, the HAT p300 is recruited by Gata3 and Chd4 complex to promote the transcription of Th2 cytokine, whereas HDAC2 is recruited in the Gata3-Chd4-NuRD complex to suppress *Tbx21* and the subsequent IFN-γ expression (170). Moreover, the CREB-binding protein and p300 complex regulates the differentiation of human Treg *via* H3K27 acetylation. Although much evidence has been found in innate immune cells that p300 and other HATs essentially regulate STAT-ISG signaling and type I IFN production, our understanding of the epigenetic control of HATs remains low in regard to functional regulation of IFN in T cells. Especially, further studies of HATs should be conducted to validate the roles of both HATs and HDACs in GVHD models.

CONCLUSION AND PERSPECTIVES

Although extensive efforts have been made in defining the roles of IFNs in alloreactive T cells, the mechanisms underlying the effects of IFNs in the setting of GVHD remain largely unknown. Much of our knowledge about the IFN-related regulations in T cells mostly come from infection, tumor and autoimmune diseases. However, considering the release of DAMP and PAMP, anti-leukemia effect and the exposure of alloreactive antigens in patients after allo-HSCT, the T cell response in GVHD and GVL scenarios reflect combined effects of these conditions. In addition, the effect of IFNs in different organs and tissues, which have distinct microenvironments, may also affect T cell response. Development of novel genetic approaches is important to further dissect the impact of IFNs on T cell alloimmunity and tumor immunity.

The advancement in epigenetics of T cell biology opens a unique way to understand the molecular mechanisms of IFN regulation. Much effort has been made to identify key epigenetic enzymes and pathways that affect IFN expression in T cells. However, most of the effects of the enzymes are still unknown in the context of GVHD. It will be interesting to determine the

connection between the inhibitors of epigenetic enzymes and the outcomes of GVHD models and clinical patients. Future studies mapping epigenetic mechanisms of IFN regulations in allogeneic T cells may be beneficial to elucidate how IFN modulates GVHD and GVL.

AUTHOR CONTRIBUTIONS

CZ conceived the manuscript and drew the figures. YZ and HZ discussed the concepts and critically revised the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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GVHD Pathogenesis, Prevention and Treatment: Lessons From Humanized Mouse Transplant Models

Nicholas J. Hess[1], *Matthew E. Brown*[2] *and Christian M. Capitini*[1,3]*

[1] Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States,
[2] Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States,
[3] University of Wisconsin Carbone Cancer Center, Madison, WI, United States

Graft-*vs*-host disease (GVHD) is the most common cause of non-relapse mortality following allogeneic hematopoietic stem cell transplantation (HSCT) despite advances in conditioning regimens, HLA genotyping and immune suppression. While murine studies have yielded important insights into the cellular responses of GVHD, differences between murine and human biology has hindered the translation of novel therapies into the clinic. Recently, the field has expanded the ability to investigate primary human T cell responses through the transplantation of human T cells into immunodeficient mice. These xenogeneic HSCT models benefit from the human T cell receptors, CD4 and CD8 proteins having cross-reactivity to murine MHC in addition to several cytokines and co-stimulatory proteins. This has allowed for the direct assessment of key factors in GVHD pathogenesis to be investigated prior to entering clinical trials. In this review, we will summarize the current state of clinical GVHD research and discuss how xenogeneic HSCT models will aid in advancing the current pipeline of novel GVHD prophylaxis therapies into the clinic.

Keywords: graft-versus host disease, xenogeneic transplantation, humanized mouse models, hematopoietic stem cell transplantation, T cells

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Philippe Saas, U1098 Interactions Hôte-Greffon-Tumeur & Ingénierie Cellulaire et Génique (INSERM), France
Qifa Liu, Southern Medical University, China

***Correspondence:**
Christian M. Capitini
ccapitini@pediatrics.wisc.edu

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INTRODUCTION

Since the first successful allogeneic hematopoietic stem cell transplant (HSCT) was performed in 1956 by E. Donnall Thomas, its use has grown exponentially (1). While allogeneic HSCT is a curative approach for many malignant and non-malignant diseases, a majority of patients will develop life-threatening complications highlighted by graft-*vs*-host disease (GVHD) or relapse (in the malignant disease setting) within three years post-transplant (2, 3). GVHD is defined by the recognition and reactivity of the donor immune cells for recipient antigens (alloreactivity) that eventually leads to organ-specific pathologies to develop (classically the skin, gastrointestinal tract and liver). Importantly, the balance between too much and too little alloreactivity if often what determines a patients probability of developing GVHD (too much) or relapse (too little) (4, 5). As such, the ability to predict and control the graft-*vs*-host (GVH) response underscores the highly complicated goal for HSCT research (6).

The application of allogeneic HSCT as the first true immunotherapy was not fully appreciated until T cell-depleted (TCD) grafts were investigated as a means to eliminate GVHD (7, 8). While TCD grafts were successful in decreasing GVHD to extremely low frequencies, TCD grafts were also associated with unacceptable rates of infections, poor engraftment, Epstein Barr virus (EBV)-reactivation-induced lymphoproliferative disease and elevated rates of relapse (8, 9). From this observation, the field began to acknowledge the novel graft-*vs*-leukemia (GVL) activity the donor cells have in controlling malignant disease. While αβ T cells (which will be the main focus of this review) have been the primary focus of many studies, ongoing studies are also exploring the role of NK cells and γδ T cells as donor-lymphocyte infusions (DLI) to treat/prevent relapse post-HSCT (NCT01823198, NCT01904136, NCT03533816) (9, 10). The application of these cell populations in DLI is extremely exciting because they are naturally cytotoxic and do not cause GVHD, though their inability to form memory responses remains a major hurdle for long-term disease surveillance.

Overall relapse rates of patients undergoing allogeneic HSCT have remained fairly unchanged in the past few decades. However, multiple advancements have been made including: improved human leukocyte antigen (HLA) class I/II genotyping leading to more precise HLA compatible grafts (11), the establishment of reduced-intensity and non-myeloablative conditioning regimens for older patients (12), the introduction of alternative graft sources (G-CSF mobilized peripheral blood and umbilical cord blood) (13, 14), novel T-cell specific prophylaxis drugs (15, 16) and most recently, the widespread use of post-transplant cyclophosphamide as a method of *in vivo* allo-reactive T cell depletion (17, 18) have all significantly improved allogeneic HSCT outcomes.

Research into the fundamental mechanisms of T cell activation following allogeneic HSCT have also advanced tremendously in the past few decades thanks to the genetic tractability and feasibility of using murine models. Through these means, the field has identified numerous pathways/targets that contribute to the GVH reaction with many currently being studied in clinical trials. Unfortunately though, many of these targets will not translate into the clinic; while a less-than-perfect success rate in clinical trials is to be expected, many of these failures are most likely a result of fundamental differences in murine and human immunology (19). Thus, there remains an "open niche" in the field for a mouse-to-human translational model system to help identify and triage targets for clinical trials.

In this review, we will highlight research investigating GVHD using humanized mouse models and discuss how the growing use of humanized mice have the potential to revolutionize the field. To do this, we will highlight each signal of the three-signal hypothesis of T cell activation (T cell receptor, cytokines and co-stimulation) individually and contrast the relative insights each model system (murine, humanized and clinical) has made toward understanding how each signal impacts the development and pathology of GVHD.

HUMANIZED MICE FOR GVHD RESEARCH

With the development of the NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mouse in 2005 by Leonard Shultz, it became possible to transplant human immune cells and/or immune-progenitors into these mice to study aspects of human immunology/hematopoiesis (20). Shortly, these mice allow for human immune cell persistence due to several key mutations including: a NOD background specific SIRPα mutation that allows binding to human CD47 to prevent phagocytosis; a SCID mutation that prevents T/B cell development; and a null IL2Rγ (common γ chain or CD132) mutation that prevents signaling from cytokine receptors utilizing the IL2Rγ chain (which includes IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21) (20). Overall, these mutations result in a lymphopenic mouse that lacks T/B cells (due to the SCID mutation) and NK cells (due to the lack of IL-15 signaling required for NK cell development) which were the primary mediators of human cell rejection after xenotransplantation (20). Alternatively, while the myeloid, granulocytic and non-hematopoietic cells (e.g. endothelial and stromal cells) compartments of NSG mice are "blind" to the presence of human cells (due to the SIRPα mutation), they are still present at similar frequencies as wild-type BALB/c mice and are fully capable of sensing and responding to damage-associated-molecular-patterns (DAMPs). Furthermore, these remaining cell populations remain essential components of xenogeneic transplants through their ability to present host murine antigens (Signal 1) and produce inflammatory cytokines (Signal 3).

Since the creation of the NSG mouse, several additional "next-generation" immune-deficient mice capable of humanization have been developed including the NSG-SGM3, MISTRG, NBSGW, NOG, NRG and NSG-HLA-A2 mice (21–26). Importantly, the term "humanized" has sometimes become synonymous with human cell "engraftment", with the latter term generally reserved for model systems studying human hematopoiesis or *de novo* generation of human immune lineages that are capable of self-renewal and long-lasting human cell immune reconstitution. To better distinguish these studies from human transgene-alone (e.g., HLA-A2, hACE2) without human cell/tissue transplantation, model systems incorporating human immune cells engrafted into these host strains are now referred to as "human immune system" or "HIS" mice.

Alternatively, immune-deficient mice can also be used to study the human T cell response to xenogeneic antigens. These studies are often much shorter in duration, with the outcome either death due to GVHD or short/moderate persistence of human T cells that are not *de novo* generation. Peripheral blood-humanized mice (PBL-Hu) are commonly used for these studies for these studies though modern versions also utilize isolated T cells or T cell subsets from primary human HSCT graft tissue including bone marrow, G-CSF mobilized peripheral blood or umbilical cord blood. Additionally, human cancer cell lines and patient-derived cancers (commonly referred to patient-derived-

xenograft or PDX models) can be transplanted into immunodeficient mice prior to human graft tissue to examine the GVL effect. In this review, we will describe these transplant-related versions of humanized mice research with the general term of "xenogeneic transplant" model systems.

THE TCR : MHC INTERACTION: SIGNAL 1

Of the three T cell activation signals, signal 1 remains arguably the most important and mandatory for successful activation. The strength of any T cell response is due in part to the diversity of the T cell receptor (TCR) repertoire and their ability to recognize non-self-antigens (27–29). Unlike current immunotherapies that target one or two antigens (i.e. monoclonal antibodies, CAR-T cells and BiTES), the T cell response after an allogeneic HSCT has the capacity to target tens to hundreds of different antigens that prevent the cancer from escaping though antigenic escape and allow for long-term prevention of relapse (30, 31). Unfortunately, this phenomenon is not limited to cancer-associated-antigens with T cell responses against host-antigens often developing into GVHD (32). This section is dedicated to understanding how antigenic targeting by the TCR can instruct both the GVL and GVH responses.

Different T Cell Populations Influence GVHD Development

T cells are broadly divided along two separate lineages; the CD4 *versus* CD8 lineage represent modulatory and cytotoxic functions respectively; and the naïve (CD45RA) *versus* memory (CD45RO) lineages denoting antigen-inexperienced or -experienced respectively. While there are numerous other sub-populations of T cells (some of which will discussed in the "Extracellular Messengers: Signal 3" section), this review will focus on the T cell lineages highlighted above.

In murine models of allogeneic HSCT, two independent groups have shown that murine memory T cells are not able to mediate GVHD (33–35). These groups theorized that since memory T cells are already antigen-experienced, there is a low likelihood of them having additional cross-reactivity with a host allo-antigen. Cross reactivity of memory T cells to allo-antigen though has been detected when viral-specific memory T cells were cultured with mismatched HLA molecules, though these studies also highlighted that these viral-specific memory T cells did not cause GVHD in a cohort of 153 patients, 73 of which had an HLA mismatch (36, 37). This may be due to a suboptimal TCR signal of the cross-reactive HLA leading to anergy or an abortive T cell response.

Two recent phase I studies have transitioned this work to investigate naïve T cell depleted or $CD8^+$ memory T cells for donor-lymphocyte infusions (DLI) respectively (38, 39). Both of these studies showed that DLI infusions with their respective T cell populations were safe, feasible and were associated with a low incidence of acute GVHD (aGVHD). Despite these observations, a recent phase II study analyzing the usage of naïve T cell depleted grafts compared to historical controls showed no difference in grade II-IV aGVHD. One limitation of this study though is that the naïve T cell depleted arm received calcineurin inhibitor (CNI) monotherapy for aGVHD prophylaxis (compared to CNI plus methotrexate) and a more myeloablative conditioning regimen than the historical controls. The study also reported that only 3/35 patients developed grade III aGVHD and all patients were steroid-responsive (40). There was no difference in engraftment rates or EBV/cytomegalovirus (CMV) reactivation showing that naïve T cell depleted grafts do not suffer from the same complications as T cell depleted grafts (9). The use of naïve T cell depleted grafts is also currently under investigation in a phase II trial comparing four different GVHD prophylaxis regimens (NCT03970096).

In regard to the role that CD4 and CD8 T cell lineages have in GVHD, very few clinical studies have investigated this directly. One randomized double-blind phase II study performed in 1994 selectively depleted CD8 T cells from 19 bone marrow grafts transplanted into HLA-identical sibling donors with CNI monotherapy for GVHD prophylaxis. The overall incidence of grade II-IV in the CD8-depleted arm was 20% and 80% in the 17 control patients (41). While this study highlights the importance of the CD8 lineage in GVHD pathogenesis, to our knowledge no further studies have followed up on this observation.

Human T Cell Reactivity After Xenogeneic Transplantation

With the clinical observations noted above, one question was if transplantation of human cells into NSG mice (xenogeneic transplantation) can model these same T cell responses. Initially, it was unknown whether human TCRs could even recognize murine major histocompatibility complex (MHC) complexes and if they did, if the result be a GVHD-like disease (20, 42). In an elegant study, Brehm et al. showed that human T cells transplanted into γ-irradiated NSG mice developed acute signs of GVHD that included liver, lung and skin pathology followed by extreme weight loss and death. Furthermore, they showed that human T cells transplanted into NSG mice lacking both MHC class I and II expression did not develop GVHD, persisted and were able to reject an allograft of human islet cells (43). In another study, the use of CNI was able to ablate xenogeneic GVHD development (44). These studies confirm that human TCR can recognize both murine class-I and -II MHC and that a successful TCR signal is required to initiate a successful GVH (i.e. xenogeneic) response (20, 42–44) (**Table 1** and **Figure 1**).

The other constituent of the human TCR to murine MHC complex are the human CD4 and CD8 molecules responsible for binding and stabilizing the TCR : MHC interaction. In one study, researchers showed that insertion of the human CD4 gene into mice deficient in murine CD4 was sufficient to restore the murine CD4 population (45). In a separate study, another group using biochemical analyses showed that human CD8 can bind to murine $H2K^b$, initiate killing of cells infected with a target antigen and that this interaction can be blocked with a CD8 antibody (46). These group of studies support the hypothesis that the human TCR complex is compatible with murine MHC to elicit antigen-specific immune responses (**Table 1** and **Figure 1**).

TABLE 1 | List of mouse to human cross-reactive molecules.

Murine Component	Human Component	Cross-Reactivity	Reference
TCRComplex			
MHC Class I	TCR	Yes	43
MHC Class I	CD8	Yes	46
MHC Class II	TCR	Yes	43
MHC Class II	CD4	Yes	45
Cytokine Receptors			
IL-2	IL-2R	Yes*	56
IL-3	IL-3R	No	21-26
IL-4	IL-4R	No	56, 85
IL-6	IL-6R	No	86
IL-7	IL-7R	Yes	54
IL-10	IL10-RA	?	n/a
IL-12	IL12R	Yes	87-88
IL-15	IL-15R	Yes	56
IL-17A	IL-17R	?	n/a
IL-23	IL23R	Yes	87-88
IFNα/β	IFNAR	No	99-100
IFNy	IFNGR	No	99-100
Type III Interferons	IFNLR1/IL10RB	Yes	101
M-CSF (CSF1)	M-CSFR (CD115)	No	21-26
GM-CSF (CSF2)	GM-CSFR (CD116)	No	21-26
G-CSF (CSF3)	G-CSFR (CD114)	Yes	21-26
TNFα	CD120a	Yes	21-26
FLT3L	FLT3	Yes	21-26
TGF-β	TGF-βR1-3	Yes	21-26
SCF	CD117	Yes	20
SDF-1	CXCR4	Yes	20
TNF Receptor Superfamily (TNFRSF)			
OX40L (CD252)	OX40 (CD134)	?	n/a
FASL (CD178)	FAS (CD95)	?	n/a
CD70	CD27	?	n/a
4-1BBL (CD137L)	4-1BB (CD137)	?	n/a
CD40	CD40L (CD154)	?	n/a
Immunoglobulin Superfamily (IgSF			
B7 (CD80/86)	CD28	Yes	128
B7 (CD80/86)	CTLA-4 (CD152)	Yes	128
PD-L1 (CD274), PD- L2 (CD273)	PD-1 (CD279)	?	n/a
ICOSL (CD275)	ICOS (CD278)	?	n/a

Next, it was determined that T cells can become xenoreactive after transplantation. Several studies have shown that human T cells develop into an effector memory population ($CD45RO^+$, $CD27^+$, $CCR7^-$, $CD62L^-$) shortly after transplantation with very few naïve ($CD45RA^+$) T cells detected (47, 48). Importantly, most of these studies were conducted with primary human peripheral blood that contains very few hematopoietic stem and progenitor cells (HSPCs) such that *de novo* T cell generation cannot occur. Additionally, the thymus of NSG mice atrophies shortly after birth and is completely absent by 4-6 weeks of age, negating the likelihood of *de novo* T cell production in these model systems. The same effector memory phenotype has also been identified in several primary human T cells clones taken from GVHD patients (49–52). While there has not been a study directly investigating the capacity of isolated human memory T cells to mediate GVHD in a xenogeneic transplant model, studies using human umbilical cord blood T cells (which are all naïve $CD45RA^+$ T cells) also detect a universal transition to an effector memory phenotype several weeks after transplantation (47).

Interestingly though, this same effector memory transition was detected when human T cells were transplanted into MHC class-I and -II deficient mice, who did not develop GVHD (43). This suggests that this phenotype is not solely antigen-driven and may in fact be caused by homeostatic proliferation (53). Homeostatic proliferation arises when T cells are transplanted into a lympho-deplete environment high in IL-2 and IL-7, which occurs in HSCT patients and in NSG mice. Though in the latter case, murine IL-2 requires 5-10 times the concentration for equivalent activation of human T cells while murine IL-7 is fully cross-reactive (54–56). The potential mechanisms and consequences of homeostatic proliferation in immunodeficient mice have been reviewed previously (53). While an effector memory phenotype is associated with GVHD in xenogeneic mice and clinical GVHD samples, the cause of this phenotype may most likely be homeostatic proliferation and thus not a valid marker of alloreactive (or xenoreactive) T cells.

Lastly, one xenogeneic transplant study has investigated the role of CD4 and CD8 T cells in GVHD. This study showed that isolated CD8 T cells but not CD4 T cells were necessary for xenogeneic GVHD (44). While we await further studies dedicated to exploring the specific pathologies and activation pathways used by human CD4 versus CD8 T cells in xenogeneic transplantation, these limited but highly interesting studies suggest that CD8 T cells and not CD4 T cells may be the more prominent T cell lineage to study in terms of GVHD pathology.

The Clonal Response to Xeno-Reactive Antigens

Pioneering studies on the role of antigen-presenting-cells (APCs) in GVHD development have shown that host hematopoietic and non-hematopoietic APCs are responsible for alloreactive antigen presentation (57–60). This was further confirmed in two xenogeneic transplant studies that used isolated human T cells. These studies showed that even in the absence of human (donor) APCs, GVHD still occurs at similar frequencies as in unmanipulated human grafts (43, 47). Additionally, when γ-irradiated NSG mice are used for xenogeneic transplantation, they develop GVHD almost universally. In contrast, when non-irradiated NSG mice are used, GVHD is less prevalent/delayed and highly manipulatable based on the cell dose, graft tissue, graft composition and the host inflammatory status (see Extracellular Messengers: Signal 3 for further discussion) (47). These observations highlight several unique possibilities in terms of the specific antigenic stimulation experienced by donor T cells.

Antigenic stimulation in T cell is generally described by the type of HLA mismatching that occurs between the donor and host. MHC mismatches occur due to a complete mismatch of the HLA allele. In murine models, this is often either a C57BL/6 or BALB.B strain (both express $H\text{-}2^b$) into a B10.BR ($H\text{-}2^k$), C3H ($H\text{-}2^k$) or BALB/c ($H\text{-}2^d$) strain. In the clinic, this occurs when there is a defined HLA mismatch at one or more of the HLA loci (see "The Importance of HLA Matching"). Minor histocompatibility mismatches occur between donor and host despite matching HLA loci and are thought to be caused from variations within individual HLA loci (i.e. allelic diversity in

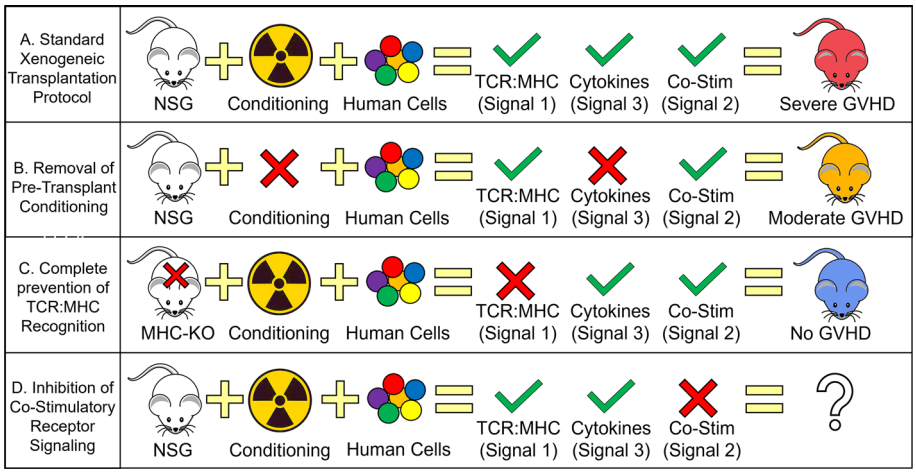

FIGURE 1 | Human T cell Requirements for GVHD Development During Xenogeneic Transplantation. Schematic depicting the relative contribution of each T cell activation signal toward the development of GVHD. **(A)** Standard xenogeneic transplant protocols provide all three T cell activation signals, human TCR to murine MHC recognition, pro-inflammatory cytokine secretion from genotoxic conditioning (i.e. γ-irradiation) and human CD28 to murine B7 cross-reactivity (with possible contributions from other co-stimulatory proteins) to cause severe GVHD. **(B)** Removing the presence of pro-inflammatory cytokines by not conditioning NSG mice prior to transplant results in only a slight decrease in GVHD severity with clinical data using tocilizumab/ruxolitinib also showing modest effects on GVHD mitigation. **(C)** Complete prevention of human TCR recognition of murine MHC (by knocking out murine MHC) eliminates all signs of GVHD. The widespread adoption of calcineurin inhibitors (e.g. tacrolimus) for GVHD prophylaxis also supports the important role of TCR : MHC interactions though in the case of clinical calcineurin inhibitors, only a partial inhibition is achieved. **(D)** Blocking co-stimulatory signaling remains the only T cell activation signal not investigated with xenogeneic transplant studies and is only recently entered the clinical domain. Severe GVHD is generally described as achieving ≥ 70% lethality with $3E^6$ PB-MNC with moderate GVHD ranging from 30-70% lethality with the same dose of human cells.

humans) and from the expression of non-classical HLA peptides. While the T cell reactive antigen in minor histocompatibility mismatches is almost certainly due to variations in the presented peptide structure, it is unclear if the primary antigen in MHC mismatches is the mismatched HLA peptide itself or the unique repertoire of peptides it can present compared to the host genotype.

In the case of xenogeneic transplantation studies, NSG mice express the $H2^{g7}$ haplotype consisting of HLA-K^d, -D^b, IA^{g7}, IE^{null} which would represent MHC mismatches at three different loci (two MHC class I and one MHC class II locus) (20). Assuming NSG mice express the same repertoire of murine antigens, one hypothesis would be that a similar TCR clonality would develop post-xenotransplantation. In one study, investigators showed that TCR diversity was indeed reduced 14 days after xenotransplantation when compared to the initial sample, but it remained surprisingly diverse overall (48). Interestingly though, there was a very low overlap between $CD4^+$ and $CD8^+$ T cell clonotypes shared between NSG mice receiving the same donor graft which prevented the authors from correlating specific clonotypes with GVHD. The authors surmised that this may be due to the presence of xeno-reactive T cells existing at a low frequency in the starting human peripheral blood graft (48). This may also help explain the observation from another study that showed an LD_{50} from GVHD after transplantation into non-conditioned NSG mice of $3E^6$ peripheral blood mononuclear cells (47). If xeno-reactive T cell clones are indeed rare, this may explain the variability in lethal GVHD seen even when the same human graft tissue is transplanted into identical NSG mice as each mouse could receive a different set of human T cell clones. It has been approximated that ~$1E^{11}$ T cell circulate through a human body with ~$1E^9$ unique clonotypes present (61). While this is much lower than the estimated maximum number of clonotypes that could exist (~$1E^{15}$), it is also much higher than in mice, thought to be around $2E^6$ different clonotypes (61).

Despite the difficulty in overcoming the diversity of the human T cell clonality, two studies (one in humans and the other in mice) suggest that the field may be able to elucidate xeno-reactive (or allo-reactive) T cell clones in the future. One study investigated the TCRβ repertoire from 15 different allogeneic HSCT patients with various degrees of HLA mismatching (62). All patients were diagnosed with gastrointestinal tract (GI) GVHD with the cohort further divided by those having steroid-refractory GVHD (SR-GVHD) and those that were steroid-responsive. They reported that although each patient had a unique TCRβ clonal structure with little overlap between patients, SR-GVHD patients had a more conserved TCRβ clonality that steroid-responsive patients (62). Furthermore, they showed that over time, the same T cell clones identified in the GI tract expanded in the blood of SR-GVHD patients but not the steroid-responsive patients (62). A separate study performed in mice revealed that the T cell clonality in the GI tract after transplant was dependent on the host mouse strain (63). The authors took C57BL/6 graft tissue and performed syngeneic (into C57BL/6), minor histocompatibility mismatch (into BALB.B) or two different MHC mismatch transplants (B10.BR and BALB/c). In each case, the resulting T cell clonality was different among each host strain with

the authors able to predict the recipient mouse strain based on the overall clonal architecture (63). While it remains unclear if the dominant antigen in xenogeneic transplantation (or MHC mismatches in general) is directed against the specific mismatched HLA peptide or the antigens it presents, these studies highlight the possibility of using TCRβ diversity (i.e. Vβ spectratyping and/or TCRB sequencing) as a measurement of GVHD responses (64).

The Importance of HLA Matching

T cell development in the thymus is a carefully orchestrated process to ensure that mature T cells have HLA affinity (positive selection) while minimizing reactivity to host antigens (negative selection). As a result, each individual's TCR repertoire at any given time reflects their own unique HLA genotype. For example, due to the diversity of alleles and variation within those alleles, other than identical twins, it is unlikely more than 10 to 100 people in the world express the exact same immunopeptidome and thus have the exact same clonal TCR architecture though the presence of public TCRs (shared TCR clones between individuals) still occurs quite frequently (27–29).

This highlights the importance of having high quality HLA-matching for allogeneic HSCT, as even a minor variation in HLA could introduce a multitude of alloreactive antigens capable of being recognized by the donor T cell population. Currently, the standard of care is 8/8 allele matching (HLA-A, -B, -C and -DRB1) with 10/10 matches (which include HLA-DQ) becoming increasingly common (11). Additionally, our knowledge of permissive and non-permissive HLA-DPB1 alleles, which is dependent on the relative overlap of the immunopeptidome continues to grow (65, 66). Although genotyping is becoming increasingly sensitive to allelic variation, GVHD can still develop in 10/10 matched unrelated donors suggesting that a deeper understanding of the mismatches in HLA class Ib alleles (HLA-E, -F, -G and -H), HLA-DM (despite their relatively low expression and allelic diversity) and millions of minor histocompatibility antigens (e.g. H-Y) may be necessary to fully understand a patient's susceptibility/probability of developing GVHD (11).

Over the years though, there have been several studies that have isolated specific alloreactive T cell clones from GVHD patients. Expansion of these clones, primarily from the skin or blood, against host cells revealed HLA-restricted cytotoxic $CD4^+$ and $CD8^+$ T cell clones (49–51). Furthermore, the TCRα/β usage was extremely diverse across patients and even in clones directed against the same HLA allele within an individual patient (49). The clones isolated in these studies were shown to target HLA-A, -B and HLA-DR, -DQ and -DP mismatches (49–51, 67). While the majority of these studies were completed before 2000 and did not have the capabilities of modern-day sequencing technology, they nevertheless highlight the capacity of GVH responses to develop against mismatched HLA alleles.

Organ Specificity in GVHD

In the clinic, aGVHD manifests primarily with gastrointestinal tract, skin and/or liver pathogenesis (32). In xenogeneic transplant models, the same repertoire of organs are affected in addition the lungs which in the clinic is normally restricted to chronic GVHD pathology (20, 43, 47, 48). While most of the same organs are affected, the severity and prevalence of each organ's pathology is altered. Liver, lung and gastrointestinal tract pathology is common with the skin having variable responses (in contrast to skin being the most common organ affected in the clinic) (20, 43, 47, 48). Additionally, when NSG mice are not conditioned prior to transplant, there is no gastrointestinal tract pathology observed (47).

The idea that GVHD is an organ-specific disease is currently under investigation and remains unresolved. Using an MHC-matched, minor histocompatibility mismatched murine model with 2-photon microscopy, one study suggested that CD4 and CD8 T cells were relatively stationary in GVHD target organs, with few T cells entering or egressing out of the tissues after initial pathology was established. This study also showed that tissue residency of the T cells was dependent on the direct interactions with tissue-resident APCs (68). In contrast, another study in rhesus macaques used serial intravascular staining and scRNA-seq to show that alloreactive T cells were identifiable in the blood and developed a transcriptional signature of tissue invasiveness (i.e. ITGB2, CD74 and others). They surmised that alloreactivity may develop in the circulation/lymph system before tissue residency is established, though the timing and the site of initial T cell activation are still to be fully supported (69).

EXTRACELLULAR MESSENGERS: SIGNAL 3

While signal 2 (co-stimulatory proteins) would classically be discussed next, there are substantially more basic and clinical studies investigating the role of cytokines in GVHD. As such, this review will follow a similar path as the field and discuss the role signal 3 has on GVHD before signal 2.

Cytokines are often deemed accessory to optimal T cell activation, though it is clear that they play important roles in directing and shaping the T cell immune response (70, 71). They also represent systemic mediators of inflammation capable of interacting with almost every organ in the body (70). Due to their relative abundance in the circulation, they have been much easier to study and as a result, have been and remain at the forefront of GVHD prophylaxis research.

Influence of Conditioning on GVH Responses

The role of conditioning regimens on the outcomes of HSCT have changed drastically since the first bone marrow transplantation (12). HSCT was originally designed to "rescue" a patient's immune system after an otherwise lethal dose of irradiation and/or chemotherapy. High dose irradiation/chemotherapy was given to eliminate residual leukemia from the body but had the side-effect (among others) of destroying the patients HSPCs (1). While high dose irradiation/chemotherapy, now called myeloablative conditioning (MAC), is still used today, the field has generally trended toward the use of lesser (less damaging) forms of MAC conditioning, thanks in part to

the observation that there is substantial anti-leukemic activity from donor cells (31, 72).

The use of both reduced intensity (RIC) and non-myeloablative conditioning (NMA) regimens has expanded the use of allogeneic HSCT to older patients who otherwise would not of survived MAC and children with non-malignant disease who do not require such intensity from their conditioning regimen (12). Additionally, the intensity of conditioning regimen is directly correlated with relapse and GVHD rates in patients. NMA/RIC regimens have higher rates of relapse but decreased frequencies of GVHD when compared broadly (6, 12). As such, no specific conditioning regimen has emerged superior to another, with most clinics operating on patient or disease specific criteria as to which conditioning regimen a patient receives. These observations though highlight two questions; what is the modern-day purpose of conditioning and is conditioning required for GVHD to develop?

In regard to the role of conditioning in GVHD development, there are two metrics that are strongly associated with GVHD development. The first is not directly related to conditioning but involves the mismatching of the donor/recipient HLA (see the Importance of HLA Matching above) in which case mismatched transplants generally receive a harsher conditioning regimen to facilitate engraftment. The second is the degree of host damage which is also associated with a more myeloablative conditioning (12). This is highlighted by the MAGIC consortium that have used the serum biomarkers sST2 and REG3α, both released by damage host cells, as predictors of non-relapse mortality (NRM) and SR-GVHD (73, 74). In addition to sST2 and REG3α, necrotic and pyroptotic (two inflammatory forms of cell death) host cells release a variety of damage-associated-molecular-patterns (DAMPs) that activate the innate immune response (e.g. ATP, mtDNA, HSPs and HMGB1) leading to a cytokine storm of pro-inflammatory cytokines (e.g. IL-1α/β, IL-6, IL-8 and IL-12) (70, 71). As a result, most of the prospective GVHD prophylaxis clinical trials have focused on these cytokines to prevent GVHD development. Interestingly though, several xenogeneic transplant studies have shown that GVHD develops irrespective of conditioning the host (via γ-irradiation) though at a much reduced frequency (21, 47). Furthermore, the penetrance of GVHD can be modified with the addition of LPS to mimic the inflammatory environment post-conditioning (47). These early xenogeneic studies suggest that inflammatory cytokines have a modulatory role in shaping the GVHD response but may not be required for its development.

Now that the graft-*vs*-leukemia (GVL) effect by donor graft tissue is well accepted, do allogeneic HSCTs require conditioning? Conditioning serves two purposes beyond that of killing residual leukemia cells (and non-leukemic cells). The first is to remove the host HSPCs. Both MAC and RIC regimens are sufficient to eliminate the host HSPCs while NMA do not offer complete elimination of host HSPCs (12). As a result, NMA regimens often suffer from mixed chimerism and may require a DLI to maintain donor engraftment (12). The second purpose is to suppress/deplete enough of the host immune cells that the donor cells (specifically the HSPCs) are eliminated before engraftment in the bone marrow can occur (12). As a result, there has recently been a movement in the field to develop targeted and/or non-genotoxic conditioning regimens (75).

To our knowledge, there are currently six different antibody/antibody-drug-conjugate (ADC) based conditioning regimens in development. All of these candidates use either the immune cell marker CD45 or the HSPC-specific marker CD117 to target immune cells for clearance, sparing non-immune cells from any off-target damage. Of the two CD117 antibody-based conditioning regimens, JSP191 and MGTA-117, JSP191 has progressed the furthest so far. JSP191, formerly known as AMG191 or SR-1, has had extensive pre-clinical studies performed showing its ability to deplete both mouse and human HSPCs in a dose-dependent manner that allows for adoptive transfer of allogeneic HSPCs (76–79). JSP191 is now currently in a phase I clinical trial for use in severe-combined immunodeficiency disorder (SCID) patients prior to transplant (NCT02963064). MGTA-117 is a CD117 antibody conjugated to amanitin, a potent inhibitor of RNA polymerase II/III, that plans on starting a phase I/II clinical trial in relapse/refractory AML/MDS patients in late 2021. The four CD45 antibody based conditioning regimens are conjugated to either iodine^{131} (β/γ emitter), astatine^{211} (α emitter), yttrium^{90} (β emitter) or saporin (non-radioactive) (80, 81). Of these, the CD45-iodine^{131} candidate is part of the IOMAB-B phase III clinical trial investigating its use in older AML patients followed by NMA conditioning (NCT02665065) and the CD45-astatine^{211} or 211^At-BC8-B10 antibody has a phase I/II trial ongoing to determine the optimal dose before allogeneic HSCT in patients with AML/ALL/MDS or mixed-phenotype acute leukemia (NCT03128034). As these targeted/non-genotoxic antibody-based conditioning targets progress, it will be important for the field to monitor how the reduction in host damage affects the GVHD penetrance after transplant.

The Role of Cytokines in the GVH Response

One implication for the growth in targeted/non-genotoxic antibody-based conditioning regimens is that the normal cytokine storm fueled by the release of DAMPs from necrotic and pyroptotic cells will be diminished. While it is assumed these type of conditioning regimens will be better tolerated by the patient and reduce the number of NRM deaths, it is unclear how it will affect the frequency and severity of GVHD. In almost all model systems of allogeneic HSCT, the host is conditioned (by γ-irradiation) prior to transplant. This is both a requirement for a successful HSCT in murine, canine, porcine and non-human primate models and mimics what occurs in the clinic. Interestingly, many studies using the immunodeficient NSG mouse have continued this protocol of conditioning prior to transplant despite no longer being a requirement to study GVHD (75) (**Figure 1**). Since NSG mice are genetically pre-conditioned (i.e. lack all adaptive immune cells and have impaired innate immune responses), human cells can be adoptively transferred without prior conditioning (this is true for the study of human T cell responses though we acknowledge that for human HSPC engraftment, conditioning is almost always required) (47, 82).

In the studies performing xenogeneic transplantation in non-conditioned NSG mice, GVHD development can still occur even in the absence of host damage/cytokine storm. Thus, these limited number of studies suggests that inflammatory cytokines are not required for GVHD initiation though they undoubtedly influence the frequency, severity and pathology of the disease. For example, by studying which murine cytokines are cross-reactive with their cognate human receptors (in addition to which human cytokines are produced by activated human T cells), we may be able to investigate the role of individual cytokines in influencing disease pathology. A non-exhaustive list of murine cytokines and their cross-reactivity on human cells are highlighted in **Table 1**.

As mentioned above, the pathology of xenogeneic GVHD resembled that in the clinic though will a few important differences. The liver is one of the dominant organs affected during xenogeneic GVHD with skin GVHD occurring infrequently and mostly associated with the use of peripheral blood grafts (47, 48). From murine models, we know that liver and gastrointestinal tract GVHD (interestingly, GI GVHD is absent in non-conditioned NSG mice) is dominated by a TH_1 response while skin GVHD is dominated by a TH_{17} response (21, 83, 84). The lineage commitment of T cells to the TH_1 lineage is controlled by IL-12 while the TH_{17} lineage is controlled, in part, by IL-6 (70). Human T cells from xenogeneic mice are almost exclusively TH_1 bias with only a small TH_{17} fraction observed and no TH_2 population suggesting that the lineage commitment of the human T cells is skewed by some mechanism (47, 48, 85). Interestingly, while murine IL-12 is fully cross-reactive with the human IL-12 receptor, murine IL-6 and IL-4 are not cross-reactive, potentially explaining one mechanism by which human T cells become TH_1 biased after xenotransplantation (56, 85–88).

The interferon family, specifically IFNγ, is arguably the most ubiquitous cytokine secreted by activated T cells and has been shown to have direct effects on GVHD pathology. In addition to being a feed-forward signal for T cell activation, IFNγ also has direct effects on HSPCs. While acute stimulation of human or murine HSPCs can result in robust myelopoiesis (e.g., in an infection), chronic IFNγ signaling results in the exhaustion and depletion of HSPCs progenitor populations (89–91). Specifically, IFNγ has been shown to sterically block the engagement of thrombopoietin (TPO) with its receptor c-MPL (90, 91). Transplantation with IFNγ-R1 KO bone marrow relieved this HSPC suppression in addition to suppressing T cell activation and GVHD (89). Less is known though about the role of type I interferons (IFNα/β) in GVHD. In several murine studies, type I interferons, specifically type I interferon receptor knockout and exogenous IFNα administration were able to prevent gastrointestinal tract GVHD by suppressing donor $CD4^+$ T cell proliferation (92). These effects were also shown to be dependent on the activation of both MAVS and STING for full effect (93). Interestingly, the phosphorylation of STAT1, which also downstream of both type I and type II interferon receptors is generally considered to be pathogenic in regards to gastrointestinal GVHD (94–96). Phosphorylation of STAT1 in plasmacytoid dendritic cells (pDCs) causes them to drive TH_{17} differentiation with increases in both of these cell populations detected in the gastrointestinal biopsies of human GVHD patients (94). The presence of IL-22, secreted by TH_{17} cells in the gastrointestinal tract has also been shown to synergize with type I interferon signaling to enhance STAT1 phosphorylation and exacerbate GVHD (95). Additionally, it has been shown that the knock-out of STAT1 in donor CD4 T cells leads to the expansion of regulatory T cells, while knock-out of STAT1 in non-T cells leads to the expansion of STAT3+ pDCs and a reduction in GVHD severity (96, 97). Thus, it is currently unclear if the true role of type I interferons in GVHD is protective through the activation of MAVS and STING or harmful through the activation of STAT1. Type III interferons, such as IFNλ (IL-29), were recently shown to be protective against severe gastrointestinal GVHD in a mouse model of HSCT (98). Furthermore, pegylated IL-29 as able to enhance the survival of intestinal stem cells which protected against gastrointestinal damage (98). Despite the active roles for type I-III interferons in murine GVHD, only the type III interferons are cross-reactive between mice and humans, suggesting that while they may play an active role in GVHD pathology their role in GVHD development may be limited (99–101).

Cytokine-Directed GVHD Prophylaxis in the Clinic

Despite the idea from non-conditioned xenogeneic transplant studies that cytokines may not be essential for GVHD development, they have been one of the most heavily investigated potential mechanisms for GVHD prevention and treatment. Surprisingly though, there has not yet been a clinical study identifying any of these inflammatory cytokines as biomarkers of the GVH response (102, 103). To date, the best biomarkers for HSCT are sST2 and REG3α, all of which are not secreted by immune cells (73, 74, 104). The release of sST2 is mediated by damaged endothelial stromal cells and REG3α is secreted by damaged intestinal epithelium cells. As such, while sST2 and REG3α have been used by the MAGIC consortium to predict NRM and SR-GVHD, they are representative markers of host damage and do not measure the degree of immunological activation from auto-reactive T cells in the host (73, 74, 104).

Overall, antibody and/or cytokine regimens for the treatment and/or prevention of GVHD have been met with mixed results. Cytokine therapies involving IL-1RA, IL-2 or an IL-1 decoy receptor have all failed to show efficacy in large phase III clinical trials (105–107). Antibodies against CD25 or TNFα have also failed to enhance the treatment of SR-GVHD compared to best available treatments (108–110). A promising antibody therapy discovered so far is tocilizumab, a humanized monoclonal antibody against the IL-6R. In several phase I/II clinical trial, tocilizumab showed efficacy in treating SR-GVHD, chronic GVHD and lowering the overall incidence of grade II-IV acute GVHD when administered early (111–113). Though in a more recent phase III randomized, double-blind trial (ACTRN12614000266662), tocilizumab given at day -1 resulted in a non-significant trend in the reduction of grade II-IV aGVHD and no improvement in long-term survival (114).

One hypothesis as to why antibody-directed therapies have not yet shown promise in the treatment/prevention of GVHD is because GVHD is a multi-faceted disease, influenced by a variety of cytokines secreted after condition and that the blocking of just one pro-inflammatory cytokine isn't sufficient for efficacy (115, 116). As a result, tyrosine-kinase-inhibitors (TKIs) are now involved in multiple different clinical trials involving GVHD (**Table 2**). TKIs benefit from being able to suppress the signaling of multiple different cytokines at once through the inhibition of the JAK-STAT pathway (115, 116). In this mechanism, TKIs benefit from their broad suppressive profile though as a result, they have also been shown to have more adverse-events and a shorter half-life that antibody based therapies (115).

The primary targets of TKIs used for GVHD are JAK1, JAK2 and BTK (115). While there are subtle differences in their use, JAK1/JAK2 broadly mediate the signaling of >20 cytokine receptors and BCR signaling in the case of BTK. This is due to the shared use of common signaling domains among cytokine receptors (115) (**Table 2**). While there are multiple TKIs FDA approved for a variety of diseases, there are only two currently FDA approved for GVHD related treatment. Ruxolitinib is a JAK1/2 inhibitor and the focus of the ongoing REACH trials (117). Recently, ruxolitinib was FDA approved for the treatment of SR-GVHD, the first new drug for SR-GVHD in the last 30 years after showing efficacy in a phase III trial (REACH II) (118, 119). Ruxolitinib is currently now in a phase III trial for the treatment of chronic GVHD (cGVHD) (REACH III). Another TKI, ibrutinib, which targets BTK, has already been approved for the treatment of cGVHD (120). Future studies comparing both ruxolitinib and ibrutinib in the treatment of cGVHD will yield important insights into redundant and non-redundant tyrosine kinase signaling during disease pathogenesis. Other TKIs in clinical trials include the selective JAK2 inhibitor, pacritinib, which is currently in a phase II clinical trials (NCT02891603). The authors reported that the pacritinib/sirolimus/tacrolimus prophylactic regimen was safe with its efficacy in preventing grade II-IV GVHD the subject of the phase II trial (121, 122). Another JAK1/2 inhibitor, baricitinib, is also investigating its efficacy in cGVHD patients through an ongoing phase I/II clinical trial (NCT02759731). Fostamatinib is an inhibitor of SYK, a B cell specific tyrosine kinase similar to BTK and is also being investigated in a phase I trial for the treatment of cGVHD (NCT02611063). While many of these TKIs have shown promising results, the failure of the JAK1 inhibitor itacitinib to meet its primary endpoints in the treatment of SR-GVHD as part of the GRAVITAS-301 phase III trial highlights the need for additional studies into the specific roles each kinase has in mediating GVHD pathology (123).

From both the clinical trial data and xenogeneic transplant studies, it is clear that cytokines have a major impact on GVHD pathogenesis. Pro-inflammatory cytokines secreted by innate immune cells have been well-studied in directing T cell adaptive responses (e.g. IL-12 promoting TH_1 and IL-6 promoting TH_{17}) with the family of TKI taking advantage of the promiscuous use of shared JAK proteins to suppress a broad number of cytokine signals. One criticism of TKIs though is the inability to modulate which cytokines are affected. For example, STAT3 activation downstream of the IL-6R promotes TH_{17} differentiation but STAT5 activation is also known to promote T_{reg} development (70, 124, 125). For this reason, drugs such as the ROCK2 inhibitor (belumosudil or KD025), which recently completely a phase II study for the treatment of cGVHD and is under review for FDA approval, may be ahead of its time. Pre-clinical studies have showed that belumosudil inhibits TH_{17} differentiation and promotes T_{reg} development through the inhibition/activation of STAT3 and STAT5 respectively (126, 127). Lastly, it is important to note that to date, there has not been a cytokine-directed-antibody or TKI therapy that has shown efficacy in preventing the development of grade II-IV aGVHD in the clinic. While the field will continue to investigate these classes of drugs, past studies and xenogeneic transplant models suggest that the role of cytokines in GVHD pathogenesis may only be efficacious as treatments of established GVHD after clinical symptoms have arose.

TABLE 2 | JAK usage among the common cytokine receptor families.

Cytokine Receptor Family	Cytokines Affected	JAK Usage
Type I Cytokine Receptors		
Common γ Chain (CD132)	IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21	JAK1, JAK3
Common Chain β (CD131)	IL-3, IL-5, GM-CSF (CSF2), EPO, TPO	JAK2
gp130 (CD130)	IL6, IL-11, IL-12, IL-23, IL-27, LIF, OSM	JAK1, JAK2, TYK2
Type II Cytokine Receptors		
Interferon αβ Receptor	IFNα/β	JAK1, TYK2
Interferon γ Receptor	IFNγ	JAK1, JAK2
Type III Interferons	Type IIIIFN	JAK1, TYK2
IL-10 Receptor	IL-10, IL-20, IL-22, IL-28	JAK1
Other Cellular Receptors		
BCR	B-cells	BTK

STIMULATORY/INHIBITORY LIGANDS/ RECEPTORS (SIGNAL 2)

So far in the review, we have shown that TCR ligation with allogeneic and/or xenogeneic antigen presented by HLA peptides is essential for GVHD development. We have also highlighted studies suggesting that cytokines may not be essential for the initiation of GVHD (i.e. the reactivity to allogeneic/xenogeneic antigens) but most likely are integral components of pathology and the perpetuation of disease. This leads to our last section on the role of co-stimulatory and co-inhibitory ligands on GVHD pathogenesis.

Co-Stimulatory/Inhibitory Signaling During Xenogeneic Transplantation

Co-stimulatory receptors are generally divided into two superfamilies' based on their extracellular domains, the immunoglobulin superfamily (IgSF) and the TNF receptor

superfamily (TNFRSF). Importantly, their signaling pathways rely on adaptor proteins (e.g. TRADD, TRAF, FADD), MAP kinase signaling (e.g. ERK, JNK, P38) and transcription factor activation that is distinct from both TCR and cytokine signaling pathways (Zap70/PI3K and JAK/STAT respectively) (70).

While the importance of co-stimulation is well-known in murine models of GVHD, the relative contribution of each receptor/ligand pair on human GVHD is not as clear (70, 124). While xenogeneic transplant studies are now well-established and have the ability to investigate the importance of each co-stimulatory protein, that research has been hindered by not knowing which co-stimulatory proteins are cross-reactive between species are which are not (**Table 1**). Since GVHD develops in non-conditioned NSG mice that lack a cytokine storm, we believe there must be a subset of co-stimulatory proteins that are in-fact cross-reactive. To date, the only known proteins with cross-species reactivity is human CD28 and CTLA-4 for murine CD80/86 (B7-1 and B7-2 respectively) (128) (**Table 1**). Interestingly, one study showed that the infusion of a CTLA-4-Ig fusion protein, a well-documented inhibitor of human T cell activation, at the time of xenogeneic transplantation could prevent GVHD from developing in NSG mice (44). Furthermore, infusion of the CTLA-4-Ig fusion protein at the onset of GVHD was also able to rescue a subset of mice from death (44).

Of the many co-stimulatory ligand/receptor pairs that have shown efficacy in murine models, only OX40 (CD134), CD40L (CD154) and ICOS (CD278) have been shown to be either upregulated or maintain a high level of expression on human T cells after xenogeneic transplantation (47, 129). Of these three, only ICOS has been studied directly for its efficacy to prevent GVHD in xenogeneic transplantation. In this study, an antibody directed against human ICOS was injected at the time of transplant and was able to prevent lethal GVHD in 60% of mice (compared to 100% lethality in the control mice). This study though also noted that they were unable to control GVHD when the ICOS antibody was injected at later time points (130, 131). As the use of xenogeneic transplantations continues to grow, future studies investigating the role of each co-stimulatory protein on human T cells will be essential in our understanding of human GVHD pathogenesis (**Figure 1**).

Co-Stimulatory Protein Based GVHD Prophylaxis in the Clinic

Currently, the most promising agent for GVHD prevention is Abatacept, a CTLA4 fusion protein currently being used in several clinical trials (132). In one recent phase II trial (ABA2), Abatacept in combination with standard calcineurin inhibitor plus methotrexate prophylaxis reduced the incidence of grade III-IV GVHD from 14.8% to 6.8% in 8/8 matched URD as part of a randomized double-blind placebo-controlled arm (133). Additionally, this study reported a decrease from 30.2% to 2.3% grade III-IV GVHD in a smaller 7/8 matched URD population compared to a nonrandomized matched cohort (133). This trial, which used an Abatacept dosing schedule of day -1, +5, +14 and +28 is now being extended as part of the ABA3 trial (NCT04380740) where all patients will be given the same four doses of Abatacept treatment followed by randomization and either another four doses of Abatacept or placebo. A second ongoing single arm, multi-center phase II study, ASCENT, is investigating an eight dose Abatacept treatment on pediatric patients with serious non-malignant hematological diseases undergoing mismatched URD transplants (NCT03924401).

The only other co-stimulatory protein based GVHD prophylaxis treatment currently under investigation is BMS-986004, a CD40L blocking antibody that is currently in a phase 1/2 open label trial (NCT03605927). The aim of this trial is to determine the safety of intravenous injection of BMS-986004 every two weeks starting on day +13 in conjunction with tacrolimus and sirolimus based GVHD prophylaxis and determine the efficacy in preventing grade II-IV aGVHD. In summary, while co-stimulatory proteins have been well-studied in murine models of HSCT, they have been vastly understudied when it comes their efficacy on human T cells, either in xenogeneic transplant models or the clinic. While the field of TKI for GVHD is highly exciting, these early studies also suggest that harnessing the power of co-stimulatory/inhibitory receptors may be the optimal target for future novel GVHD prophylaxis targets.

CONCLUSIONS

While murine and clinical investigations into GVHD will remain workhorses in the field, it is clear that humanized mouse models are becoming increasingly utilized and offer a unique model system to directly investigate human T cell biology. Xenogeneic transplantation has already provided us insights into the importance of TCR: MHC interactions, the necessity of pro-inflammatory cytokines and a novel tool to investigate the role of co-stimulatory ligands in mediating GVHD development (**Figure 1**).

In addition to the GVHD treatments discussed above targeting specific T cell activation signals, cellular therapies for GVHD benefit from being able to target multiple pathways at once. Cellular therapies like Treg and mesenchymal stromal cell (MSC) were both studied in xenogeneic transplant models before moving into late stage clinical trials (134, 135). While the suppressive mechanism(s) identified for each cellular therapy are distinct, one common theme is the secretion of anti-inflammatory cytokines (e.g. IL-10, IL-35, TGF-β and PGE2) with only a limited role identified for inhibitory ligand/receptors (134, 135). Phase I trials using these adoptive therapies have shown promising but mixed results, most likely due to the inefficiencies and irregularities involved with ex vivo expansion of these cells (NCT04678401) (136). The large success of these therapies in both mouse models and xenogeneic transplant studies though suggests that future GVHD therapies may benefit from actively promoting anti-inflammatory cytokine production in addition and/or instead of solely blocking pro-inflammatory cytokines with xenogeneic transplant models serving as an excellent test-bed for such studies.

Abatacept remains the best example of the role xenogeneic transplantations will have in the future as the CTLA-4-fusion protein was first tested in xenogeneic transplantation models before moving into the clinic, where it has now become a highly promising candidate for preventing GVHD though its role in treating established GVHD remains uncertain (132, 133). Additionally, xenogeneic transplant models have questioned the role of pro-inflammatory cytokines in the development of GVHD (47, 75). While it is clear that the degree of host damage influences GVHD frequency/severity, it is unclear if cytokines are responsible for mediating this causation (12, 75). This hypothesis is supported tangentially by both xenogeneic transplant and clinical data suggesting that there may be a temporal switch in the importance of co-stimulatory proteins and cytokines with their effect mediated early and late post-transplant respectively (47, 107, 108, 110, 114). Lastly, xenogeneic transplant models have revealed to the field the variability of human clonal T cell responses even among inbred NSG mice (48, 62, 63). While the possibility of developing a computational model capable of predicting xeno- or allo-reactivity against a defined set of HLA molecules remains daunting, it will most likely be completed first in xenogeneic transplant model systems before transitioning to the clinic.

Their remains a plethora of exciting research possibilities that are now feasible thanks to the development of xenogeneic transplant model systems. Their feasibility will only grow as we learn more about the cross-reactivity of specific cytokines and co-stimulatory ligand/receptor pairs. With the rapid increase in single-cell-RNA-sequencing (scRNA-seq) and TCR-sequencing capabilities, researchers will be able to delve deeper into the clonality and unique gene expression patterns associated with human T cell responses post-transplant as well as the importance of KIR typing/mismatches in GVHD and GVL (31, 72). Additionally, the addition of proteosome inhibitors such as ixazomib and bortezomib, both of which are being studied in the context of cGVHD, and the generation of NSG mice expressing human HLA alleles, may aid in our investigations into the nature of xeno-reactive antigens (137). The goal of developing xenogeneic transplant models was to offer researchers a model system capable of studying human immune responses and to serve as a bridge from murine studies to clinical trials. In the end, we believe xenogeneic transplant studies have met this need and will continue to advance the field of GVHD research in the decades to come.

AUTHOR CONTRIBUTIONS

NH drafted the manuscript and performed the literature search. MB and CC revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Janus Kinase Inhibitors and Cell Therapy

Amer Assal[1] *and Markus Y. Mapara*[1,2]*

[1] Department of Medicine, Bone Marrow Transplantation and Cell Therapy Program, Columbia University Irving Medical Center, New York, NY, United States, [2] Columbia Center for Translational Immunology, Columbia University, New York, NY, United States

Cellular therapies such as allogeneic hematopoietic stem cell transplantation (HSCT) and immune-effector cell therapy (IECT) continue to have a critical role in the treatment of patients with high risk malignancies and hematologic conditions. These therapies are also associated with inflammatory conditions such as graft-*versus*-host disease (GVHD) and cytokine release syndrome (CRS) which contribute significantly to the morbidity and mortality associated with these therapies. Recent advances in our understanding of the immunological mechanisms that underly GVHD and CRS highlight an important role for Janus kinases (JAK). JAK pathways are important for the signaling of several cytokines and are involved in the activation and proliferation of several immune cell subsets. In this review, we provide an overview of the preclinical and clinical evidence supporting the use of JAK inhibitors for acute and chronic GVHD and CRS.

Keywords: JAK - STAT signaling pathway, graft-versus-host disease (GvHD), cytokine release syndrome (CRS), transplantation, cell therapy (CT)

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Robert Zeiser,
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***Correspondence:**
Markus Y. Mapara
mym2111@columbia.edu

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) continues to grow as a field owing to its curative potential for a variety of hematologic conditions and malignancies (1). Recent advances in immune effector cell therapy (IECT) using chimeric antigen-receptor T (CART) cells have introduced new possibilities and challenges in the treatment of patients with hematologic malignancies (1). Graft-*versus*-host disease (GVHD), both acute and chronic, is a common complication of HSCT and contributor to morbidity and mortality thus limiting its therapeutic potential (2). GVHD incidence, both acute and chronic, is >50% and 7-9% of deaths post-transplant are attributed to GVHD in matched sibling HSCT and 9-10% in unrelated donor HSCT (2–4). Acute GVHD (aGVHD) occurs when donor-derived T cells in the donated graft recognize host antigens as foreign (5). The target antigens of donor-derived T cells include human leukocyte antigen (HLA) molecules, both class I and class II (5). HLA proteins are highly polymorphic and encoded by the major histocompatibility complex (MHC) genes. Donor T cells may also recognize host minor histocompatibility antigens (mHA) contributing to aGVHD. Chronic GVHD (cGVHD) pathophysiology is more complex to model and study; a proposed model suggests that cGVHD is caused by early inflammation due to tissue injury, followed by chronic inflammation, thymic injury and dysregulated B and T cells all leading to tissue repair with fibrosis (6). Several factors have been shown to increase the risk of GVHD and these include donor/recipient HLA mismatch, increased age, sex, conditioning regimen intensity, and donor graft source whether mobilized peripheral blood stem cells or bone marrow (7, 8).

Immune suppression with corticosteroids, with or without a calcineurin inhibitor or sirolimus, remains the mainstay of treatment for both acute and chronic GVHD, which has changed very little over the past 40 years (9–11). One of the limitations affecting reproducibility and generalizability of GVHD clinical trial results has been a lack of consistency in diagnosing and grading GVHD (12). The efficacy of corticosteroids in the treatment of aGVHD is limited with response rates ranging from 30 to 64% (9, 13). Treatment related mortality remains high even in responders and is markedly increased in steroid refractory aGVHD (SR-aGVHD) (14). cGVHD outcomes are also poor despite treatment as the majority of cases require multiple lines of therapy and only a third of cases achieve long term remissions off of immune suppression (15).

Similarly, IECT is complicated by cytokine release syndrome (CRS), which is an inflammatory condition that can be life-threatening and require intensive care (16, 17). The incidence of CRS varies by the cell product used as well as by the malignancy treated. Patients with acute lymphoblastic leukemia (ALL) have a higher reported rate of CRS than large B cell lymphoma (LBCL) in their respective registration trials with tisagenlecleucel (18, 19). Axicabtagene ciloleucel, which contains a CD28 costimulatory domain rather than 4-1BB used in tisagenlecleucel, also reported higher rates of CRS in the registration trial (20). Risk factors suggested for the development of CRS include higher disease burden, higher cell dose infused, lymphodepleting chemotherapy selection, cell product used, a low pre-treatment platelet count, and the CD4/CD8 T cell ratio (16, 17). Comparison of CRS rates across trials can be challenging owing to different CRS grading systems, however increased adoption of the American Society of Transplant and Cellular Therapy (ASTCT) grading schema may help address this (21). The pathogenesis of CRS is related to the activation of CART cells as well as other immune cells such as those of the monocyte/macrophage lineage (16, 17). Elevation in several cytokines and inflammatory mediators are noted during CRS contributing to endothelial activation, capillary leak, and coagulopathy. Treatment of CRS includes supportive care measures for lower grades, and tocilizumab, an IL-6 receptor antagonist that is the only FDA-approved therapy for CRS, for grades 2 or greater. Corticosteroids are also used for higher grade CRS particularly when it is associated with neurotoxicity (22). Optimizing the toxicity and financial impact of IECT remains a challenge as more centers move towards outpatient administration (23).

Targeting the Janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathway through JAK inhibition has emerged as a promising therapeutic strategy for GVHD and CRS. Insights into the pathogenesis of GVHD demonstrate a necessary role for signaling through the JAK/STAT pathway, particularly STAT1 and STAT3 (24–27). This is supported by clinical efficacy of JAK inhibitors in the treatment of acute and chronic GVHD (28–32). The FDA approval of ruxolitinib, a JAK1/2 inhibitor, represents a major advance in the treatment of SR-aGVHD (31). Furthermore, the JAK/STAT pathways are critical for cytokine signaling suggesting a potential role for JAK inhibition in the management of CRS (33). The JAK1 inhibitor itacitinib is currently being studied for CRS prophylaxis in recipients of IECT (34). In this review, we will present an overview of the role of JAK/STAT pathways in GVHD and inflammatory conditions relevant to cell therapies such as CRS and present recent clinical developments in the field.

JAK/STAT PATHWAY IN GVHD

The identification of several cytokines as key players in the pathogenesis of GVHD (such as interferon-γ (IFN-γ), tumor necrosis factor (TNF) suggested early on that targeting pathways involved the in signal transduction of these cytokines may be promising targets for therapeutic intervention. Early evidence linking GVHD and cytokine signaling through the JAK/STAT pathways was found by expression profiling studies (35) and results from our groups detecting activation of STAT1/3 activation in GVHD target organs (24, 25) and in donor T cells (26) in murine models of GVHD. In addition, HDAC-inibition -dependent mitigation of GVHD was associated with reduced STAT1 activation (24). Our laboratory was the first to show that disruption of the JAK/STAT1 signaling pathway in donor T cells prevented development of GVHD in minor Ag-mismatched GVHD and mitigated GVHD in fully-MHC mismatched GVHD (MA) (26). Furthermore, we could show that the observed effect was achieved by blocking IFNγ-R signaling rather than IFNα-R signaling (25).

Reduced alloantigen induced activation and proliferation was seen in STAT1-deficient donor T cells, and correlated with CD4+CD25+Foxp3+ Treg expansion (26). Our findings were confirmed and further expanded upon by the labs of Choi and DiPersio showing similar results using IFN-γ-receptor knock-out donors demonstrating that pharmaceutical targeting of JAK1/2 signaling is highly effective in preventing GVHD while retaining GVL-responses (36, 37). Thus, ruxolitinib treatment was found to ameliorate GVHD in MHC-mismatched murine models (36–38). Decreased T cell expansion as well as a higher frequency of CD4+Foxp3+ Tregs and lower frequencies of central memory T cells were observed in treated mice (38, 39). *In vitro* studies of CD4+ T cells stimulated with allogeneic dendritic cells (DC) showed decreased T cell expansion and cytokine production in presence of ruxolitinib treatment and CD4+ STAT3 phosphorylation (38). Baricitinib, another JAK1/2 inhibitor, was also shown to be effective in blocking GVHD in MHC-mismatched murine models as well as treating ongoing GVHD (40).

Another aspect of JAK/STAT involvement in GVHD pathogenesis involves its role in chemokine-mediated T cell trafficking to target organs. IFN-γ receptor deficient conventional T cells were found to be defective in trafficking to target organs and exhibited reduced CXCR3 expression, a phenotype that was replicated by the use of ruxolitinib or momelotinib as JAK1/JAK2 inhibitors (37). Further work by the same group demonstrated a preservation of the graft-*versus*-leukemia effect in 2 different murine MHC-mismatched allogeneic HSCT models using either a myeloid or lymphoid murine leukemia models (36). Similar results were reported by another group where ruxolitinib-treated mice exhibited decreased T cell and macrophage migration to the skin, small

intestine, and liver (41). Decreased expression of CXCR3 on splenic CD4+ and CD8+ T cells was also observed.

The JAK/STAT pathway also has a role in modulating APCs. Ruxolitinib and the JAK1/JAK3 inhibitor tofacitinib suppressed the inflammatory phenotype of macrophages isolated from patients with rheumatoid arthritis (42). IFN-α and IFN-γ mediated STAT1 and STAT3 phosphorylation was blocked by JAK inhibitors in macrophages. Furthermore, TNF-dependent STAT1 activation, STAT1 expression and IFN-dependent genes were blocked by JAK inhibitors. JAK1/2 inhibition with ruxolitinib was also shown to affect DC function by impairing monocyte differentiation, DC activation and DC-dependent T cell activation (43). JAK/STAT inhibition may be particularly effective in patients with a MicroRNA-146a CC polymorphism which leads to lower levels of miR-146a and subsequently increased JAK/STAT pathway signaling and MHC II expression in DC (44). Baricitinib was also shown to exert effects on antigen presenting cells (APC) as decreased expression of MHC II, CD80/86 and PD-L1 was noted on recipient CD11c+ and B220+ APCs (40). Neutrophils, which are the first cells to reach sites of tissue injury after conditioning chemotherapy, migrate to the mesenteric lymph nodes, increase expression of MHC II, and may present antigen to T cells (45). JAK/STAT inhibition with ruxolitinib may attenuate the role of neutrophils in mediating GVHD (45).

While significant evidence supports the role of multi-kinase inhibitors that target more than 1 JAK protein, selective JAK1 or JAK2 inhibition has also been shown to be effective in GVHD models. Itacitinib, a selective JAK1 inhibitor currently being studied in clinical trials, has been shown to inhibit weight loss and improve GVHD scores without impacting engraftment in mismatched MHC mouse models (30). On the other hand, JAK2-/- donor T cells also lead to attenuated GVHD without impacting graft-*versus*-leukemia effect (46). JAK2-/- T cells exhibit decreased Th1 polarization and increased Treg and Th2 polarization. Pacritinib, a JAK2 selective inhibitor, significantly reduced GVHD in murine models, induced a Th2 polarization in human T cells, and spared Tregs.

Considering the role of the JAK/STAT pathways in T cell activation and expansion, APC function, and Tregs expansion, JAK inhibitors are well positioned to also have a role in cGVHD treatment. Tregs frequency is reduced in patients with cGVHD (47), and treatment with low dose IL-2 ameliorated cGVHD in patients with glucocorticoid-refractory cGVHD which was associated with Treg expansion and an increased Treg to conventional T cell ratio (48). Patients with active cGVHD have lower frequencies of circulating T follicular helper cells which are skewed towards a highly activated profile and also have higher levels of CXCL13 (49). Furthermore, in a murine model of sclerodermatous cGVHD, donor macrophages mediated cGVHD-like manifestations (50). Recent experimental evidence support the role of JAK1/2 in cGVHD as inhibition with ruxolitinib was shown to attenuate cGVHD in a murine sclerodermatous murine model where a decrease in the frequency of effector CD4+ T cells and CD11b+ macrophages, and IFN-γ producing CD4+ T cells was noted as well as an expansion in Tregs (51). Ruxolitinib suppressed IFN-γ production by CD4+ T cells and monocyte chemoattractant protein (MCP)-1 from CD11b+ macrophages, the proliferation of these cells, as well as the migration of a macrophage cell line in response to IFN-γ.

JAK/STAT PATHWAY AND CRS

As the JAK/STAT pathways play an important role in immune function and modulation, targeting these pathways in hyperinflammatory conditions such as CRS is a reasonable consideration. The activation of several immune cell subsets is responsible for the cytokine profile of CRS. Elevations in IFN-γ, IL6, IL8, soluble interleukin 2 receptor (sIL2R)-α, sgp130, soluble IL6 receptor (sIL6R), MCP1, MIP1α, MIP1β, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were noted in ALL recipients of IECT who developed severe CRS (52). Interestingly, a nearly identical pattern of cytokine elevation was noted in patients with hematophagocytic lymphohistiocytosis (HLH). IL-6 production is derived from monocytes in response to CART cell recognition of their targets (53, 54). IL-1 secretion preceded IL-6 secretion in a mouse of IECT and IL-1 blockade is emerging as a promising strategy for CRS and neurotoxicity management (54–56).

Considering the similarity in the pathophysiology of CRS and HLH, lessons learned from experimental and therapeutic studies in HLH may be applicable to CRS. Ruxolitinib administration improved survival and physiological parameters in murine models of HLH, decreased levels of phosphorylated STAT1 in peripheral blood white blood cells, decreased serum levels of IL-6, TNF-α, MCP-1, CXCL10, and soluble IL-2 receptor, and reduced tissue infiltration (57). Ruxolitinib was found to act in INF-γ dependent and independent pathways in another study which showed similar findings including a lowering CD8+ T cell and neutrophil infiltrations of organs, dampening CD8+ T cell activation, and decreased production of TNF-α, IFN-γ by T cells, and lower levels of TNF-a, IL-6, IL-12, CXCL10, IL-1b, GM-CSF, MIP-1a and G-CSF (58). In a study of multiple models of hyperinflammation, ruxolitinib was effective in reducing inflammation including a murine model of HLH as in the preceding study, and reduced IL-6 production by macrophages *in vitro* (59). Ruxolitinib may also sensitize CD8+ T cells to dexamethasone which is commonly used as therapy in hyperinflammatory syndromes (60). Ruxolitinib has since been successfully used to treat patients with HLH (61–63), although cases of relapsed disease in lymphoma-associated HLH were also reported in the setting of ruxolitinib treatment (64). These experimental and clinical findings support the targeting of JAK/STAT pathways in hyperinflammatory syndromes including potentially CRS which overlaps with HLH in its pathophysiology (52). Itacitinib, a JAK1 selection inhibitor, was studied *in vitro* and *in vivo* in IECT models (65). Itacitinib successfully reduced cytokine levels associated with CRS in a murine model of hyperinflammation, reduced IL-6 production by macrophages *in vitro* and *in vivo*, reduced cytokines

production by CART cells, and had no impact on CD8+ T cell or CART cell expansion or target lysis at lower doses that are pharmacologically relevant. A recent report studied ruxolitinib as CRS prophylaxis in patients with relapse-refractory acute myeloid leukemia who were being treated with a CD123 x CD3 bispecific molecule (66). Cytokine analysis showed a significant reduction in levels of IL-4, IL-12p70, IL-13, IL-15, IL-17A, IFN-α2, but higher levels of GM-CSF. However, the incidence and severity of CRS events were similar. Only a small number of patients were treated with ruxolitinb (10 patients). Itacitinib is currently being studied as CRS prophylaxis in an ongoing phase II study (34).

CLINICAL EFFICACY OF JAK INHIBITORS IN THE TREATMENT OF GVHD

Studies in Refractory GVHD

In light of supporting preclinical evidence and the lack of effective alternatives, JAK inhibitors were used as salvage therapies in GVHD with great success (28, 38). Of the earliest reports of JAK inhibitors for the treatment of GVHD were published by Zeiser et al. who described outcomes after ruxolitinib therapy for GVHD in patients from multiple stem cell transplant centers across Europe and the United States. 54 SR-aGVHD and 41 steroid-refractory cGVHD (SR-cGVHD) patients were given ruxolitinib. The overall response rate (ORR) was 81.5% in SR-aGVHD including 46.3% complete responses (CR) with a low rate of GVHD relapse of 6.8% (28). Impressive 6-month-survival of 79% (67.3–90.7%, 95% CI) was reported. In SR-cGVHD patients, an ORR of 85.4% was observed with a low rate of relapse (5.7%). The 6-month survival in this group was 97.4% (92.3%–100%, 95% CI). Regarding adverse events, cytomegalovirus (CMV) reactivation rate of 33.3% and 14.6% was noted in SR-aGVHD and SR-cGVHD patients respectively. A case of CMV-retinitis was reported, and all CMV cases were subsequently controlled by antiviral medication. Cytopenias were observed in 55.5% and 17% of SR-aGVHD and SR-cGVHD patients, respectively. Severe cytopenias (grades 3 and 4) were found in 33.3% and 7.3% SR-aGVHD and SR-cGVHD respectively. This was confounded by the presence of cytopenias preceding ruxolitinib therapy. A low malignancy relapse rate of 9.3% in SR-aGVHD and 2.4% SR-cGVHD patients was noted.

Itacitinib, which is an investigational tyrosine kinase inhibitor selective for JAK1, has been studied in aGVHD. Itacitinib was used in the first registered study of a JAK inhibitor in patients with acute GVHD (INCB 39110-108) where patients with steroid-naïve or steroid refractory aGVHD were randomized 1:1 to received either 200 mg (n=14) or 300 mg (n=15) daily dose (30, 67). In this phase I study, only 1 DLT was observed which was thrombocytopenia attributed to GVHD progression in a patient with pre-existing thrombocytopenia. The most common non-hematologic treatment emergent adverse event (TEAE) was diarrhea (48.3%) although 79% of those patients had GI GVHD at baseline. GI hemorrhage was reported in 3 and 2 patients at the 200 mg and 300 mg dose groups respectively. 1 patient had 2 CMV infections. Most commonly reported hematologic TEAEs were anemia (37.9%), decreased platelet count (27.6%), thrombocytopenia (24.1%). Grade 3-4 thrombocytopenia was reported in 2 and 3 patients on the 200 mg and 300 mg dose groups respectively. Sepsis was the most common infection AE occurring in 2 and 3 patients on the 200 mg and 300 mg dose groups respectively. Four patients, all in the 200 mg dose group, had CMV infection. The most common itacitinib-related TEAEs were anemia and decreased platelet counts which occurred more in the 300 mg dose group. Day 28 ORR in all patients for the 200 mg and 300 mg dose levels was 78.6% and 66.7% respectively. Day 28 ORR for steroid-naïve and steroid refractory patients were 75.0% and 70.6% respectively. Median duration of response was not reached for steroid-naïve aGVHD patients and 386 days for SR-aGVHD patients. In steroid-naïve aGVHD patients, 6- and 12- month OS was 75.0% and 58.3% respectively, whereas in the SR-aGVHD group, 6- and 12-month OS rates were 47.1% and 41.2% respectively.

Ruxolitinib was studied prospectively for the treatment of SR-aGVHD in an open-label phase II study (REACH1) (29). Ruxolitinib was given at a dose of 5 mg twice daily, with the possibility to increase to 10 mg twice daily in the absence of cytopenias. Ruxolitinib could be tapered after 6 months of therapy in patients who had discontinued corticosteroids for 8 weeks and had achieved a CR or very good partial remission (VGPR). Day 28 ORR, the primary endpoint of the study, was 54.9% (95% CI, 42.7%-66.8%), where 26.8% achieved a CR, 9.9% achieved a VGPR, and 18.3% a partial response (PR). When analyzed by GVHD grade, ORR of 82.6%, 41.2% and 42.9% were observed in patients with grade II, III, and IV SR-aGVHD, respectively. The median time to first response was 7.0 days (range, 6-49). Median duration of response at 6 months was 345 days. The 6- and 12-month overall survival (OS) rates were 51.0% and 42.6% respectively. Adverse events observed were in line with expectations for patients with SR-aGVHD being treated with ruxolitinib. Hematologic treatment-emergent adverse events (TEAE)s were frequent, with the most common hematological TEAEs being anemia (64.8%), thrombocytopenia (62.0%) and neutropenia (47.9%). There were 2 cases of thrombotic microangiopathy. Infections occurred in 80.3% of patients, with the most frequent being CMV, where rates of infection, viremia, and retinitis were (12.7%), (5.6%), and (1.4%), respectively. Fatal treatment-related TEAEs included sepsis and pulmonary hemorrhage (1 subject each). These findings have since led to the FDA approval of ruxolitinib for SR-aGVHD (68).

The REACH1 study was subsequently followed up by the REACH2 study, which was a multicenter, open-label, randomized phase III study comparing ruxolitinib to best available therapy (BAT) in SR-aGVHD. 154 patients received ruxolitinib and 155 were assigned to the control arm. 49 patients (32%) crossed over to the ruxolitinib arm on or after day 28. ORR at day 28 was significantly higher in the ruxolitinib arm (62% *vs* 39%, p<0.001). CR rates were also higher (34% *vs*. 19%).

Responses were more durable (40% *vs* 22% at day 56) and incidence of loss of response at 6 months was lower with ruxolitinib (10% *vs* 39%). Response rates were highest in grade II disease, although the odds ratio for response with ruxolitinib was highest in patients with grade IV disease at baseline (53% *vs*. 23%; odds ratio, 3.76; 95% CI,1.24 to 11.38). Failure-free survival (FFS) and OS were also significantly longer in the ruxolitinib arm (5.0 *vs* 1.0 months and 11.1 *vs* 6.5 months, respectively). The most common adverse events in the treatment *vs*. control arm were thrombocytopenia (33% *vs* 18%), anemia (30% *vs*. 28%) and CMV infection (26% *vs*. 21%). Grade 3 infections up to day 28 were reported in 34 patients (22%) who received ruxolitinib and in 28 patients (19%) in the control arm. Median time to first infection of grade 3 severity was 0.8 months in the ruxolitinib arm and 0.7 in the control arm. At the data cutoff date, incidence of grade 3 or higher bleeding was 12% *vs* 7% in the ruxolitinib and control arms respectively. Severe adverse events (SAE)s by day 28 were reported in 38% of patients in the ruxolitinib arm and 34% in the control arm. These data confirm results from the REACH1 study and ruxolitinib is now standard of care for SR-aGVHD.

The REACH3 study evaluated ruxolitinib in SR-cGVHD and was presented at the 2020 annual meeting of the American Society of Hematology (32). It was an open-label, randomized phase III trial comparing ruxolitinb to BAT. A total of 329 pts were randomized, 165 received ruxolitinib and 164 received BAT. 61 patients (37.2%) crossed over the ruxolitinib arm. The primary endpoint was ORR at week 24. Ruxolitinib was superior to best available therapy with an ORR of 49.7% *vs* 25.6% (odds ratio, 2.99; $P < 0.001$) and the CR rate was higher with ruxolitinib as well (6.7% *vs* 3.0%). Key secondary endpoints also showed superiority of ruxolitinib, where FFS was improved in the ruxolitinib group (median FFS, >18.6 *vs* 5.7 months; HR, 0.37 [95% CI, 0.27-0.51]; $P < 0.001$), and improved response rate on the modified Lee symptom score (defined as a 7 point or greater reduction in symptom score) (24.2% *vs* 11.0%; odds ratio, 2.62; $P = 0.001$). Rates of AEs were comparable in both arms. The most common AEs of grade 3 or higher in both arms (ruxolitinib *vs* best available therapy) included thrombocytopenia (15.2% *vs* 10.1%), anemia (12.7% *vs* 7.6%), neutropenia (8.5% *vs* 3.8%) and pneumonia (8.5% *vs* 9.5%). Infections of any type occurred in 63.6% of ruxolitinib treated patients and 56.3% of best available therapy patients. FDA approval of ruxolitinib for SR-cGVHD is anticipated.

Baricitinib, another JAK1/2 inhibitor approved for rheumatoid arthritis, was used in a phase I/II study in patients with SR-cGVHD (69). No DLT was observed with the 2 mg dose of baricitinib. Possibly treatment-related AEs included upper respiratory infection in 13 patients, neutropenia in 6, hypophosphatemia in 12, and hypertriglyceridemia in 5. Notable viral reactivation included 6 patients with CMV, 7 patients with Epstein-Barr virus (EBV) and 5 patients with BK viruria; none of which required treatment. One patient was diagnosed with post-transplant lymphoproliferative disorder (PTLD) within 1 cycle on therapy who had EBV viremias and lymphadenopathy at enrollment. 11 SAEs were reported, of which 5 were possibly drug-related, and there were no deaths on study. ORR at 6 months was 63% and ORR at any time reached 90%. 1- and 2-year FFS was 74% and 37%, respectively.

Several published studies also support these findings, where ruxolitinib was given to refractory GVHD patients and are summarized in **Table 1**. These includes reports in adults (71–74, 76, 77, 79, 82) and pediatric patients (70, 75, 77–82), SR-aGVHD (70, 71, 73, 75, 77–81) as well as SR-cGVHD (71–82).

Studies in Upfront GVHD Therapy

Ruxolitinib has been used in combination with corticosteroids as upfront treatment for aGVHD in a prospective study of patients receiving haploidentical transplants (83). 32 patients were treated, and day 28 CR rate was 96.9%. Response rates were significantly higher than those observed in a group of matched historical controls treated with corticosteroids alone. cGVHD rates were low with a 1-year and 2-year cumulative incidence rates of 9.4% and 13.8%, respectively. Estimated 1-year OS was 73.4%. aGVHD recurred in 31.2% of patients, mostly in the setting of taper of immunosuppressive medications. Ruxolitinib dose was initially 5 mg twice daily, but later reduced in the study protocol for patients receiving azoles due to a high incidence of cytopenias. CMV reactivation was seen in 78.1% of patients, with 2 cases of CMV encephalitis, one of them proved fatal. EBV viremia was detected in 87.5% of patients, and 2 patients developed PTLD. Other notable infections included a case of pulmonary aspergillosis and a case of Pneumocystis jirovecii pneumonia, both successfully treated. Grade 3-4 thrombocytopenia occurred in 3 patients, all before the protocol-recommended dose reduction of ruxolitinib. Subsequent patients developed reversible thrombocytopenia that did not require a dose reduction. No neutropenia was observed and 2 cases of thrombotic microangiopathy were observed that resolved after reduction of calcineurin inhibitor.

GRAVITAS-301 was a placebo-controlled, randomized, phase III study of corticosteroids with or without itacitinib as upfront treatment for aGVHD (84). Randomization was 1:1 where 219 patients received itacitinib and 220 received placebo. The study failed to meet its primary endpoint, which was a statistically significant improvement of the day 28 ORR (itacitinib *vs* placebo, 74% *vs* 66%, p=0.08). Post-hoc analysis however of the day 28 CR rates showed a significant improvement for itacitinib *vs* placebo when stratified by aGVHD risk status (odds ratio, 1.66; 95% CI, 1.14–2.44; P=0.008). Median time to first response was 8 days in both groups. Median duration of response was also similar. Notably, the 6-month estimates of non-relapse mortality were similar in both groups (itacitinib *vs* placebo, 18% *vs* 19%). At median follow-up of 267 days, the 1-year OS estimated with 70% for itacitinib and 66% for placebo. Treatment-related AEs were also similar in both groups.

Studies in GVHD Prophylaxis

Majority of the preclinical data in mouse models described above studied the ability of JAK inhibition to prevent GVHD, whereas clinical studies focused on treating refractory disease, which is

TABLE 1 | Clinical studies of JAK inhibitors for the treatment of refractory GVHD.

Reference	Agent	Target	Study Type	Indication	N	Response	Survival
Spoerl et al. (38)	Ruxolitinib	JAK1/2	Pilot	SR- aGVHD	6	100%	NA
Zeiser et al. (28)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	95	SR-aGVHD 81.5% SR-cGVHD 85.4%	6-mo SR-aGVHD 79% 6-mo SR-cGVHD 97.4%
Khandelwal et al. (70)	Ruxolitinib	JAK1/2	Retrospective	SR-aGVHD	13	45% (n=11)	7/13 alive at median follow up of 401 days
Maldonado et al. (71)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	3 5	Overall ORR 85%	NA
Khoury et al. (72)	Ruxolitinib	JAK1/2	Retrospective	Steroid dependent-cGVHD	19	100%	NA
Abedin et al. (73)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	19 24	84% 83%	6-mo FFS SR-aGVHD 58% SR-cGVHD 88%
Ferreira et al. (74)	Ruxolitinib	JAK1/2	Retrospective	SR-cGVHD	20	75%	11-mo 67%
Gonzalez Vicent et al. (75)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	13 9	77% 89%	6-mo OS SR-aGVHD 30 +/-15% SR-cGVHD 100%
Modi et al. (76)	Ruxolitinib	JAK1/2	Retrospective	SR-cGVHD	46	1-yr 58%	1-yr FFS of 54.2%
Escamilla Gomez et al. (77)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	23 56	69.5% 57.1%	6-mo SR-aGVHD 47% 1-yr SR-cGVHD 81%
Uygun et al. (78)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD, overlap syndrome and SR-cGVHD	13 1 15	84.6% 100% 80.0%	90% alive at end of study
Dang et al. (79)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	10 28	100% 82.1%	NA
Jagasia et al. (29)	Ruxolitinib	JAK1/2	Phase II	SR-aGVHD	71	Day 28 ORR 54.9%	6-mo 51.0%
Zeiser et al. (31)	Ruxolitinib	JAK1/2	Phase III	SR-aGVHD	309 (ruxolitinib 154)	Day 28 ORR 62%	6-mo 59.5% *vs* 50.3%
Shroeder et al. (30)	Itacitinib	JAK1	Phase 1	Steroid-naïve aGVHD and SR-aGVHD	12 17	Day 28 ORR 75.0% 70.6%	6-mo 75.0% 47.1%
Zeiser et al. (32)	Ruxolitinib	JAK1/2	Phase III	SR-cGVHD	329 (ruxolitinib 165)	Week 24 ORR 49.7% *vs* 25.6%	FFS >18.6 mo *vs* 5.7 mo
Holtzman et al. (69)	Baricitinib	JAK1/2	Phase I/II	SR-cGVHD	20	63%	1-yr FFS 74%
Yang et al. (80)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	17 36	64.7% 80.6%	6-mo SR-aGVHD 92.3% SR-cGVHD 100%
Mozo et al. (81)	Ruxolitinib	JAK1/2	Retrospective	SR- aGVHD and SR-cGVHD	8 12 (1 patient treated for both acute and chronic)	87% 91%	1-yr SR-aGVHD 64.8%% 2-yr SR-cGVHD 76.4%
Wang et al. (82)	Ruxolitinib	JAK1/2	Retrospective	SR-cGVHD	20	70%	90% alive at end of study

NA, not applicable.

in line with the clinical development of agents for novel indications. Studies using JAK inhibitors in the prophylaxis setting are emerging, however. One study of 12 patients with myelofibrosis continued ruxolitinib therapy until stable engraftment (85). Only 1 case of aGVHD was reported before day +100, however 4 patients developed aGVHD after taper of cyclosporine. All patients were alive at the time of analysis. CMV reactivation occurred in 5 patients, 1 of whom developed CMV colitis. All responded to treatment with ganciclovir. 2 patients discontinued therapy due to cytopenias. A reduction in levels of inflammatory cytokines was reported as well. Another study administered ruxolitinib to calcineurin inhibitor intolerant patients as aGVHD prophylaxis (86). 10 patients were enrolled into this pilot study. After ruxolitinib initiation, only 1 patient developed grade II skin aGVHD, and 1 patient developed severe aGVHD after day +100. 2 patients developed cGVHD after ruxolitinib taper. CMV reactivation was reported in 4 patients, and EBV viremia was reported in 3 patients. None developed CMV disease or PTLD. Finally, a study employed post-transplant cyclophosphamide with ruxolitinib as a calcineurin-free GVHD prophylaxis regimen (87). 20 patients with primary or secondary myelofibrosis were enrolled. 1 patient experienced primary graft failure and 2 patients died before engraftment. Dose reduction in ruxolitinib was required in 11 patients due to severe poor graft function. Overall, the regimen was well tolerated with 30% grade 3-4 non-hematolgic toxicity, 45% viral reactivation rate, and severe sepsis reported in 15% of patients. Incidence of grade II–IV aGVHD was 25%, grade III-IV aGVHD was 15%. No severe cGVHD cases were reported, and moderate cGVHD occurred in 20% of patients. Only 2 patients required systemic steroids. The 2-year OS and event-free survival were 85% and 72% respectively.

The GRAVITAS-119 trial is a single arm phase I study of itacitinib in combination with calcineurin inhibitor based interventions for the prophylaxis of GVHD (88). The primary endpoint was day 28 hematologic recovery. 65 patients were enrolled, all patients achieved hematologic recovery which included 1 patient with myelofibrosis who achieved neutrophil engraftment by day 31. 2 patients developed secondary graft failure. In 63 evaluable patients, cumulative incidence of grade III-IV aGVHD was 4.8% and 1 year GVHD-relapse-free survival (GRFS) was 38.5%. The addition of itacitinib was well tolerated; the most common grade 3-4 hematologic AEs included thrombocytopenia (49%) and anemia (31%). CMV reactivation occurred in 26% of patients, and 12% had EBV infection. No cases of PTLD were reported. 1 patient developed invasive bronchopulmonary aspergillosis. The most common reasons for itacitinib discontinuation were AEs (22%) and relapse (17%). 15 patients in the per-protocol population died, 2 of which were due to infections and 1 due to intracranial hemorrhage. Other ongoing studies for GVHD prophylaxis using itacitinib include a phase I study in patients receiving haploidentical transplants (NCT03755414, www.ClinicalTrials.gov), and a phase IIa study of patients receiving reduced-intensity conditioning (NCT04339101 www.ClinicalTrials.gov). A phase I study with baricitinib for GVHD prophylaxis is ongoing as well (NCT04131738, www.ClinicalTrials.gov).

CONCLUSIONS

JAK inhibitors are well positioned as therapies for complications common after cellular therapies such as GVHD in the setting of HSCT, and CRS in the setting of IECT. The JAK/STAT pathway is involved in the signaling of several cytokines that are critical to the pathogenesis of GVHD and CRS as described above. JAK inhibition has been shown to ameliorate the pathogenic T cell and macrophage proliferation and activation in experimental models and enhance Treg function and proliferation, results which have now been translated to successful clinical studies in refractory GVHD. Most importantly, JAK inhibition does not seem to interfere with the graft *versus* leukemia effect or the activity of CART cells used in IECT which is a common concern with the blunting of immune activity (36, 40, 65). Results from further studies in the upfront or prophylactic setting are highly anticipated, despite the negative results from the GRAVITAS-301 study (84).

Despite the success of ruxolitinib in the treatment of SR-aGVHD and SR-cGVHD, adverse events remain common and the response rates are far from perfect. Other JAK inhibitors may prove more efficacious or less toxic especially as they may differ in the off-target effects. Combination therapies with agents that target other pathways such as CD28:CD80/86 constimulation with abatacept (89), Rho-associated kinase 2 with belumosudil (90), or CSF-1R blockade with axatilimab (91) may also prove beneficial as we refine our understanding of the pathogenic pathways controlling development of GVHD.

AUTHOR CONTRIBUTIONS

AA wrote the manuscript. MM wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Targeting the CD27-CD70 Pathway to Improve Outcomes in Both Checkpoint Immunotherapy and Allogeneic Hematopoietic Cell Transplantation

Forat Lutfi[1], *Long Wu*[2], *Sarah Sunshine*[3] *and Xuefang Cao*[2,4*]

[1] Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland Medical Center, Baltimore, MD, United States, [2] Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland Baltimore, Baltimore, MD, United States, [3] Department of Ophthalmology and Visual Sciences, Marlene and Stewart Greenebaum Comprehensive Cancer, University of Maryland Medical Center, Baltimore, MD, United States, [4] Department of Microbiology and Immunology, School of Medicine, University of Maryland Baltimore, Baltimore, MD, United States

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Edited by:
Jaebok Choi,
Washington University School of Medicine in St. Louis, United States

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Antonio Pierini,
University of Perugia, Italy
Peng Li,
Guangzhou Institutes of Biomedicine and Health (CAS), China

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

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Immune checkpoint inhibitor therapies and allogeneic hematopoietic cell transplant (alloHCT) represent two distinct modalities that offer a chance for long-term cure in a diverse array of malignancies and have experienced many breakthroughs in recent years. Herein, we review the CD27-CD70 co-stimulatory pathway and its therapeutic potential in 1) combination with checkpoint inhibitor and other immune therapies and 2) its potential ability to serve as a novel approach in graft-*versus*-host disease (GVHD) prevention. We further review recent advances in the understanding of GVHD as a complex immune phenomenon between donor and host immune systems, particularly in the early stages with mixed chimerism, and potential novel therapeutic approaches to prevent the development of GVHD.

Keywords: CD27, CD70, immunotherapy, allogeneic hematopoietic cell transplant (alloHCT), graft-*versus*-host disease (GVHD)

INTRODUCTION

Allogeneic hematopoietic cell transplant (alloHCT) provides the greatest probability for long-term cure in many hematologic malignancies where few other effective therapeutic options exist. However, despite the obvious life-saving benefits of alloHCT, graft-*versus*-host disease (GVHD), a significant toxicity of alloHCT, can be devastating and lead to multi-system tissue damage including the skin, liver, GI tract, and eyes potentially leading to significant morbidity and mortality including liver failure, systemic sclerosis, and severe ocular surface disease (1, 2). The treatment paradigm in alloHCT has evolved rapidly in the last three decades, largely due to a better mechanistic understanding of the complex interactions between donor and host immune cells and host organ systems. This understanding has revolutionized care and dramatically improved patient outcomes. This is well demonstrated by a retrospective analysis comparing alloHCT recipients with grade III and IV acute GVHD from 1997-2006 and 2007-2012 where 12-month treatment related mortality decreased from 58% to 38% in this period of time (3). These improved

clinical outcomes have occurred as a result of an improved understanding of the pathogenesis of GVHD. However, despite advances, GVHD remains a significant cause of morbidity and non-relapse related mortality in alloHCT.

The framework of classical acute GVHD occurring in the first 100 days of transplant due to alloreactivity driven by donor T-cells has more recently been supplanted by a more robust understanding involving the intricate interplay of donor and host immune cells with host tissue (4). The initiation phase of GVHD is believed to be mediated by both surviving host and donor Antigen Presenting Cells (APCs) (5, 6). The insult of conditioning chemotherapy and Total Body Irradiation (TBI) has been shown to cause significant changes in hematopoiesis, activation of host APCs, and host tissue damage, leading to an inflammatory environment, which sets the stage for the development of acute GVHD (7–10). This inflammatory milieu includes cytokine release in both hematopoietic and non-hematopoietic compartments, leading to both host and donor T-cell activation and proliferation and alloreactivity which subsequently damages host tissue as GVHD manifests (9–13). A multitude of diverse therapies to alter these underlying mechanisms of GVHD have been adopted into standard clinical practice. As the current standard of care, this has included post-transplant T-cell depletion with cyclophosphamide as well as corticosteroids, calcineurin and Inosine-5′-monophosphate dehydrogenase (IMPDH) inhibitors, and Janus kinase inhibitors; while many others, including checkpoint inhibitors (CPI) and co-stimulatory pathways have also been investigated in GVHD models (6, 14–18).

Another realm of treatment modality in the arena of cancer therapy that has revolutionized the field has been the adoption of CPI therapies, which are now utilized in the treatment of a diverse array of advanced stage malignancies, from non-small cell lung cancer to classical Hodgkin's lymphoma (19, 20). Despite their successes in a diverse array of malignancies, overall response to CPI therapy remains low, with reported response rates of 12-24% in solid tumors to date (21, 22). The adoption of CPI therapy is based on the premise of the importance of the immune system, particularly the tumor microenvironment and more specifically cytotoxic CD8+ T-cells, in regulating tumor pathogenesis and progression. An important mechanism of tumor immune escape is the attenuation of cytotoxic T-cell activity and proliferation by T-cell exhaustion. Exhaustion occurs by a multifactorial etiology due to persistent tumor antigen exposure, loss of effector cytokine secretion/stimulation [Interleuken-2 (IL-2), Interferon (IFN)-gamma), immunosuppressive cell types (e.g. myeloid derived suppressor cells (MDSCs)], and immunophenotypic changes, including increased checkpoint inhibitor expression [programmed death receptor-1 (PD-1), cytotoxic T-lymphocyte antigen number 4 (CTLA-4), T-cell immunoglobulin mucin-3 (TIM-3), and Lymphocyte-activation gene 3 (LAG-3)] (23, 24).

While CPI targeting agents derive their function by countering an inhibitory signal, an alternate and possibly synergistic approach has been agonizing T-cell stimulatory co-signaling pathways. Co-stimulatory pathways are broadly speaking, either part of the B7/CD28 or tumor necrosis factor (TNF) family (25). Clinically significant co-signaling pathways include CD26, CD27, CD28, CD40, 4-1BB (CD137), OX40 (CD134), glucocorticoid-induced TNF receptor family-related protein (GITR), herpes virus entry mediator (HVEM) (CD270), and inducible T-cell co-stimulator (ICOS) (26–29). Although a significant oversimplification, this is analogously described as CPI therapy being akin to "pulling the foot off of the brake", while agonizing co-signaling pathways are "pressing down on the accelerator" (See **Figure 1**).

Thus far, the clinical use of co-stimulatory signaling pathways have lagged behind that of CPIs. However, given the need for improved response rates in those undergoing CPI therapy, the use of co-stimulatory pathways has been explored as a potential therapeutic intervention to increase responses. Additionally, the co-stimulatory receptors CD28 and 4-1BB (CD137) have been utilized in the development of both experimental and commercially available second generation chimeric antigen receptor T-cell (CAR-T) therapies leading to significantly greater activation, expansion, and persistence of CAR-T cells (30, 31). More recently, these pathways have also been studied

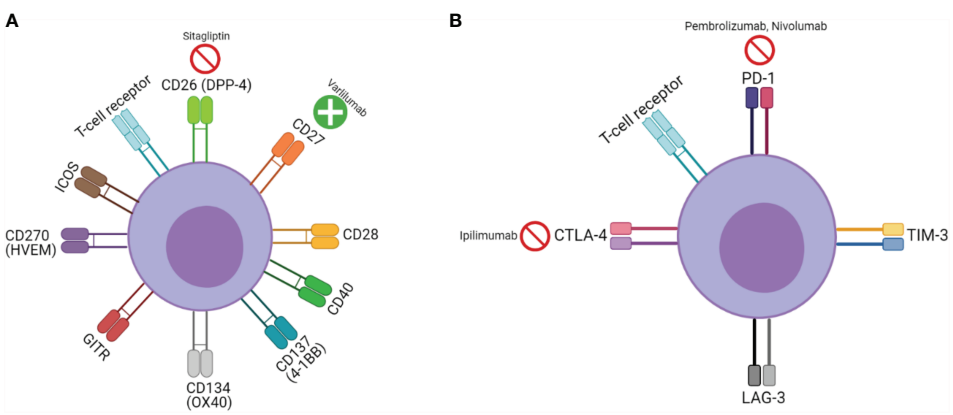

FIGURE 1 | T-cell signaling, function, and pharmacologic targets: **(A)** Co-stimulatory receptors and **(B)** inhibitory receptors; represents inhibitor, represents agonist.

and exploited as potential therapeutic targets for attenuating GVHD. Ultimately, however, the concern remains that any immunosuppressive GVHD-targeted therapy may adversely impact the graft-*versus*-tumor (GVT) effect as there is a strong correlation between incidence and severity of GVHD and disease free survival (32).

Thus, it is critical to identify co-stimulatory pathways which when blocked decrease GVHD but do not interfere with GVT. One potential way to decrease the incidence of GVHD would be by inhibiting a co-stimulatory receptor thereby attenuating CD4+ and CD8+ cytotoxic T-cell activity. CD26 has been studied in both pre-clinical and clinical models, while the CD27-CD70 pathway has been studied extensively in pre-clinical murine models. In murine models, inhibition of CD26 [also known as dipeptidyl peptidase-4 (DPP4)] by a monoclonal antibody has been demonstrated to decrease GVHD incidence without compromising GVT (33). In a small, non-randomized clinical trial, the diabetic medication and DPP4 inhibitor, sitagliptin, was administered from day -1 to day +14 of alloHCT, resulting in a low incidence (5%) of grades II-IV GVHD followed to day +100 (34). CD27-CD70 has also been studied in murine and cellular models. Cao et al. and colleagues demonstrated that antagonism of the host CD27-CD70 co-stimulatory pathway significantly increased, rather than decreased, the development of murine GVHD (35, 36).

Herein, we conduct an in-depth review of the CD27-CD70 pathway and its application in both GVHD attenuation following alloHCT and its use in the treatment of numerous malignancies in combination with CPI therapies.

CD27-CD70 PATHWAY

CD27, a member of the TNF receptor superfamily is constitutively expressed on naive T-cells, memory B-cells, NK-cells, and hematopoietic stem cells (HSCs) and progenitor cells (37–40). CD27 is a transmembrane phosphoglycoprotein expressed on both CD4+ and CD8+ T-cells with increased expression upon T-cell activation and shedding from the cellular surface and formation of soluble CD27 (sCD27) upon activation (41, 42). CD70 (CD27L), the only ligand for CD27, is a tightly regulated transmembrane glycoprotein expressed on both B and T-lymphocytes and APCs (43). CD70 has structural similarity to other TNF superfamily members (TNFα, FasL, receptor activator of NF-κB ligand (RANKL), TNF-related apoptosis-inducing ligand (TRAIL), 4-1BBL, CD30L, and CD40L) (44). Upon binding of CD70, CD27 is bound to TNF receptor-associated factors (TRAFs) leading to intracellular signaling which potentiates survival and activation of T, B, and natural killer (NK) cells *via* Traf2 and Traf5 signaling and activation of the NF-κB pathway (45). The interaction of CD27-CD70 is tightly regulated to prevent overexpression and subsequent excessive lymphocyte activation. In a normal physiologic state, CD70 is only expressed in the thymus and lamina propria (46). However, stimulation by interaction with toll-like receptor (TLR) ligands and dendritic cells (DCs), the most prominent of APCs, results in increased expression of CD70 on DCs, albeit transiently (47). Although exceedingly rare, human CD27 deficiency has been associated with Epstein-Barr virus (EBV) associated lymphoproliferative disorders [lymphoma and hemophagocytic lymphohistiocytosis (HLH)], and recurrent infections (48, 49).

Under pro-inflammatory conditions (infection, malignancy, autoimmune conditions) CD27-CD70 activity is increased, leading to proliferation and survival of lymphocytes with multiple downstream effects (50). CD27-CD70 signaling has also been shown to promote B-cell activation and terminal differentiation to plasma cells, increase cytotoxic CD8+ T-cell activity, promote TNFα production by T-cells, and increase NK-cell activity with production of IFNγ and IL-2 (44). In response to IFN-γ secretion due to CD27-CD70 stimulation, C-X-C motif chemokine ligand 10 (CXCL10) [also known as interferon gamma-induced protein 10 (IP-10)] has been demonstrated to increase the CD8+ T-cell effector pool (51). Additionally, CD27 expression was noted in a subset of IFNγ producing γδ T-cells following infection, while CD27 negative γδ T-cells did not produce IFNγ, suggesting a role for CD27 in regulation of interferon and specific cytokine production in immune responses (52). The CD27 co-stimulatory response has also been shown to be key for acute effector CD8+ T-cell expression of IL-7Rα, an important cytokine for the generation of CD8+ T-memory cells (53).

In the bone marrow, HSCs are a heterogeneous population serving as precursors to all myeloid and lymphoid lineage cell types (54). In contrast to their mature counterparts, HSCs have limited surface antigen expression and lack lineage specific cell surface markers. However, interestingly, HSCs have been shown to exhibit high CD27 expression (90% of HSCs in murine models express CD27) (38, 55). In murine *in vitro* models, CD27 agonism of bone marrow progenitor cells decreased monocytic differentiation and overall inhibited leukocyte differentiation, while in competitive transplantation assays CD27 agonism decreased donor B and T lymphocytes, suggesting the CD27-CD70 pathway's ability to influence hematopoiesis and immune cell differentiation (56).

CD27-CD70 FOR CANCER IMMUNOTHERAPY

At the time of writing, the study of the CD27-CD70 pathway in GVHD remains confined to murine and cellular models, with ongoing studies seeking to better understand the effect of CD27 agonism on donor hematopoietic cell differentiation, engraftment, and GVT effect. However, a CD27 agonizing monoclonal antibody, varlilumab, has been extensively studied both in *in vitro* and *in vivo* in phase I/II clinical trials for a number of hematologic and solid tumor types, including Hodgkin's lymphoma, non-Hodgkin's lymphoma (NHL), glioblastoma, melanoma, renal cell carcinoma, prostate adenocarcinoma, colorectal adenocarcinoma, and ovarian cancer (57–60). (See **Table 1** for further details of previous and ongoing registered clinical trials.) The rationale behind these trials has been to study the impact of CD27 agonism alone as a T-cell co-stimulator as well as to determine if it functions in a synergistic

manner in combination with checkpoint inhibitor therapy and cancer vaccines to improve antineoplastic response. Additionally, many B-cell lymphomas express CD27, which may serve as a direct target in a fashion similar to CD20 targeting with Rituximab. In multiple *in vitro* and murine tumor models, PD1/PDL1 blockade in combination with an agonist CD27 monoclonal antibody was shown to enhance CD8+ cytotoxic T-cell expansion and function in an IL-2 dependent manner with gene expression changes promoting T-cell proliferation (66). In various syngeneic tumor murine models, varlilumab was shown to have two predominating anti-tumor mechanisms of action by its co-stimulatory effect and Treg depletion (67).

The recent development of a bispecific antibody, CDX-527, has sought to improve the efficacy of the CD27 agonism and PD1/PDL1 blockade by combining CD27 agonism with cross-linking through PDL1 and Fc receptors (68). CDX-527 was demonstrated to have potent T-cell activation by increasing IL-2 and IFNγ production and anti-tumor activity to CD27-expressing lymphoma cells in an immunodeficient mouse model, with comparable anti-tumor activity to separate CD27 agonizing and PDL1 inhibiting monoclonal antibodies. Similarly, a hexavalent TNF receptor agonist (HERA) targeting CD27 has been developed and demonstrated to cause an increased proliferative response to CD4+ and CD8+ T-cells when compared to CD27L *in vitro* with healthy human T-cells and *in vivo* in murine models (69).

In addition to combination with checkpoint inhibitor therapy, the combination of anti-CD20 and CD27 agonizing monoclonal antibodies has been investigated in an immunocompetent murine B-cell lymphoma and B-chronic lymphocytic leukemia models with a 100% tumor remission rate noted at 100 days (70). The combination antibody group was noted to have significantly increased CD8+ cytotoxic T-cells and Treg cells compared to CD20 monoclonal antibody alone. Additionally, the combination was shown to promote tumor infiltration and activation of myeloid cells and macrophages towards an anti-tumor phenotype. The efficacy of this combined therapy is currently being investigated in humans in the RIVA study, a phase IIa open-label clinical trial of patients with relapsed/refractory CD20+ B-cell lymphomas (64).

In the limited clinical trials to date, the CD27 agonizing monoclonal antibody, varlilumab, as monotherapy and with PD1/PDL1 checkpoint inhibitor therapy (nivolumab, atezolizumab), has resulted in varying degrees of objective clinical responses in a subset of cancer patients enrolled. This has included complete remission in Hodgkin's lymphoma and partial responses in ovarian, colorectal, and squamous cell cancer of the head and neck (see **Table 1**). Furthermore, it was well tolerated with limited, predominately grade 1-2 toxicities (fatigue, nausea, and thrombocytopenia) reported at all dose levels up to 10mg/kg in trial subjects (57, 71). In ovarian cancer patients, the combination therapy of varlilumab and nivolumab resulted in increased tumor expression of PD-L1 and CD8+ tumor infiltrating lymphocytes in 61% and 58% of patients, respectively (61). Upon administration to trial subjects, soluble CD27 plasma concentrations were significantly increased in a dose-dependent fashion. Cytokines were also increased in a dose-independent manner, indicative of an inflammatory response, particularly IL-12, monokine induced by IFNγ (CXCL9), MIP-1β (CCL4), and monocyte chemoattractant protein-1 (CCL2). In *in vitro* studies of T-cell isolates from healthy volunteer peripheral blood mononuclear cells (PBMCs) treated with varlilumab revealed that both CD4+ and CD8+ T-cells were stimulated (although with a greater emphasis on CD8+ activation), which was accompanied by upregulation of other co-stimulatory pathways (4-1BB, OX40, GITR, and ICOS) along with the inhibitory PD1 pathway (72).

CD27 agonism alone and with an PD1 checkpoint inhibitor has also been explored as a potential mechanism of increasing the efficacy of tumor-specific peptide vaccines by enhancing CD4+ helper T-cell and CD8+ cytotoxic T-cell response following vaccination (73, 74). Clinical trials are currently underway combining varlilumab with 6MHP, a vaccine of six melanoma peptides; ONT-10, a peptide vaccine incorporating MUC1 tumor antigen, a TLR-4 agonist, and PET lipid A in breast and ovarian malignancies; and IMA950, a multi-peptide vaccine with 11 glioma-associated antigens.

While varlilumab has yet to obtain an FDA indicated approval for use, six clinical trials with varlilumab are actively recruiting patients with B and T-cell lymphomas, neurologic malignancies, melanoma, and non-small cell lung cancer (**Table 1**).

CD27-CD70 IN alloHCT AND GVHD

Traditionally, the prevailing thought behind the etiology of GVHD rested solely with donor immune cells, particularly T-cells becoming activated upon alloreactivity to host antigens. However, more recently, the complex interaction between donor and host immune systems leading to GVHD has been noted, particularly in the early stages of alloHCT, where a mixed chimerism exists (75, 76). While the pre-alloHCT conditioning regimen clears the peripheral blood of most host T-cells, they often persist for many months in the tissues most effected by acute GVHD—the skin and gastrointestinal tract. The role of persistent host T-cells mediating acute GVHD by interaction with donor APCs has been noted in murine models and in alloHCT transplant patients with increased IFNγ–secreting CD4+ T-cells in skin GVHD biopsies compared to healthy controls, as well as an increased monocyte population with upregulation of chemoattractant receptors and IFN-response genes (IFITM1 and GBP1) compared with healthy controls (77). Conversely, the interaction between host APCs and donor T-cells had been reported earlier to be associated with the development of acute GVHD (7, 11). These findings underscore the complexity of immune interactions between a diverse array of both donor and host immune cells that may ultimately result in the development of GVHD (see **Figure 2**).

The most commonly employed conditioning regimens in alloHCT are given with myeloablative or reduced intensity/non-myeloablative intensity consisting of a combination of myelotoxic chemotherapeutic agents with or without TBI (78–80). The conditioning regimen acts as a profound insult to the marrow microenvironment leading to increased cytokine and interferon levels. This also impacts the function of HSCs, akin to emergency hematopoiesis seen in other stressful states such as severe infection and radiation exposure where pro-inflammatory

TABLE 1 | Clinical trials with CD27 agonizing monoclonal antibody.

Study Title:	Trial identifier:	Status:	Sponsor:	Phase:	Conditions:	Intervention:	Results[+]:	Adverse Events[++]:	Related Publications:
A Dose Escalation and Cohort Expansion Study of Anti-CD27 (Varlilumab) and Anti-PD-1 (Nivolumab) in Advanced Refractory Solid Tumors	NCT02335918	Completed	Celldex Therapeutics	I/II	Squamous Cell Carcinoma of the Head and Neck, Ovarian Carcinoma, Colorectal Cancer, Renal Cell Carcinoma, Glioblastoma multiforme	varlilumab and nivolumab	Colorectal cancer- 2/41 patients PR, 7/41 patients SD Ovarian cancer- 5/49 patients PR, 19/49 patients SD Squamous Cell of the Head and Neck- 1/3 patients PR	Colorectal cancer- 3/42 patients with mixed motor sensory neuropathy, pneumonitis, elevated ALT) Ovarian cancer- 2/66 patients with acute kidney injury, hepatitis, small bowel obstruction	(61, 62)
A Study of CDX-1127 (Varlilumab) in Patients With Select Solid Tumor Types or Hematologic Cancers	NCT01460134	Completed	Celldex Therapeutics	I	CD27 Expressing B-cell Malignancies (Hodgkin's Lymphoma, Chronic Lymphocytic Leukemia, Mantle Cell Lymphoma, Marginal Zone B Cell Lymphoma, Any T-cell Malignancy, Solid Tumors (Metastatic Melanoma, Renal (Clear) Cell Carcinoma, Hormone-refractory Prostate Adenocarcinoma, Ovarian Cancer, Colorectal Adenocarcinoma, Non-small Cell Lung Cancer), Burkett's Lymphoma, Primary Lymphoma of the Central Nervous System	CDX-1127 (varlilumab)	Hodgkin's Lymphoma- 1/10 patients CR, 1/10 patients with SD Non-Hodgkin Lymphoma- 3/18 patients SD	Any adverse event-9/34 patients Grade 2 cytopenias- 3/34 patients Grade 2 fatigue- 5/34 patients Grade 2 neurologic symptoms- 2/6 Grade 2 hypotension- 1/34 patients	(57)
Study of ONT-10 and Varlilumab to Treat Advanced Ovarian or Breast Cancer	NCT02270372	Completed	Cascadian Therapeutics Inc.	I	Advanced Breast Carcinoma, Advanced Ovarian Carcinoma	ONT-10 and varlilumab	None Posted	None Posted	None Posted
A Study of Varlilumab and IMA950 Vaccine Plus Poly-ICLC in Patients With WHO Grade II Low-Grade Glioma (LGG)	NCT02924038	Recruiting	Nicholas Butowski, MD,University of California San Fransisco	I	Glioma, Malignant Glioma, Astrocytoma, Grade II, Oligodendroglioma, Glioma, Astrocytic, Oligoastrocytoma, Mixed	IMA950 vaccine, poly-ICLC vaccine, and varlilumab	None Posted	None Posted	None Posted
Nivolumab With or Without Varlilumab in Treating Patients With Relapsed or Refractory Aggressive B-cell Lymphomas	NCT03038672	Recruiting	National Cancer Institute	II	Numerous subtypes of Non-Hodgkin lymphoma	varlilumab and nivolumab	None Posted	None Posted	(63)
A Combination of Rituximab and Varlilumab Immunotherapy in Patients With B-cell Lymphoma (RIVA)	NCT03307746	Recruiting	University Hospital Southampton NHS Foundation Trust	I/II	CD20+ B-Cell Lymphoma	varlilumab and rituximab	None Posted	None Posted	(64)
Atezolizumab and Varlilumab in Combination With Radiation Therapy for NSCLC	NCT04081688	Recruiting	Rutgers, The State University of New Jersey	I	Refractory Lung Non-Small Cell Carcinoma, Stage IV Lung Cancer	varlilumab, atezolizumab, and stereotactic radiation therapy	None Posted	None Posted	None Posted
Vaccination With 6MHP, With or Without Systemic CDX-1127, in Patients With Stage II-IV Melanoma	NCT03617328	Recruiting	Craig L Slingluff, Jr MD,University of Virginia	I/II	Melanoma	CDX-1127 (varlilumab), 6MHP, Montanide ISA-51, polyICLC	None Posted	None Posted	None Posted
DC Migration Study to Evaluate TReg Depletion In GBM Patients With and Without Varlilumab (DERIVE)	NCT03688178	Recruiting	Gary Archer Ph.D., Duke University	II	Glioblastoma	Human CMV pp65-LAMP mRNA-pulsed autologous DCs, temozolomide, varlilumab, Td, 111In-labeled DCs, Unpulsed DCs	None Posted	None Posted	None Posted
A Study of Varlilumab (Anti-CD27) and Ipilimumab and CDX-1401 in Patients With Unresectable Stage III or IV Melanoma	NCT02413827	Terminated	Celldex Therapeutics	I/II	Unresectable Stage III or Stage IV Melanoma	varlilumab and ipilimumab; varlilumab, ipilimumab, CDX-1401, and poly-ICLC	None Posted	None Posted	None Posted
A Study of Varlilumab (Anti-CD27) and Sunitinib in Patients With Metastatic Clear Cell Renal Cell Carcinoma	NCT02386111	Terminated	Celldex Therapeutics	I	Carcinoma, Renal Cell, Urogenital/Urologic Neoplasms	varlilumab and sunitinib	None Posted	None Posted	None Posted
A Study of Varlilumab and Atezolizumab in Patients With Advanced Cancer	NCT02543645	Terminated	Celldex Therapeutics	I/II	Carcinoma, Renal Cell, Urogenital/Urologic Neoplasms, Melanoma, Triple negative breast cancer, Head and neck cancer, Non-small cell lung cancer	varlilumab and atezolizumab	None Posted	None Posted	None Posted
Pilot Study of SBRT and CDX-1127 in Prostate Cancer (Prostate-04)	NCT02284971	Terminated	James Larner, MD, University of Virginia	I	Prostate cancer	Stereotactic Body Radiation and varlilumab	None Posted	None Posted	None Posted

(Continued)

TABLE 1 | Continued

Study Title:	Trial identifier:	Status:	Sponsor:	Phase:	Conditions:	Intervention:	Results[+]:	Adverse Events[++]:	Related Publications:
A Study of Glembatumumab Vedotin as Monotherapy or in Combination With Immunotherapies in Patients With Advanced Melanoma	NCT02302339	Terminated	Celldex Therapeutics	II	Melanoma	glembatumumab vedotin, glembatumumab vedotin and varlilumab, glembatumumab vedotin and PD-1 targeted checkpoint inhibitor, gembatumumab vedotin and CDX-301	1/31 patients with objective response in glembatumumab vedotin and varlilumab group	14/34 with serious adverse event reported in glembatumumab vedotin and varlilumab group	(65)

Results generated from search for "CD27 antibody" and "varlilumab." Publications listed by google scholar, PubMed, and clinicaltrials.gov reported publications.
+CR, Complete Response; PR, Partial Response; SD, Stable Disease; PD, Progressive disease per RECIST 1.1 criteria.
++ Adverse events graded per National Cancer Institute–issued Common Terminology Criteria for Adverse Events version 4.0.

signals (IFNα/β, IFNγ, TNFα, IL1-R, IL-5, and IL-6) encourage HSC response and subsequent downstream maturation and differentiation (10, 12, 13). In a study of the bone marrow microenvironment in 28 patients undergoing alloHCT for hematologic malignancies, dramatic changes were noted over the course of one year. In six patients undergoing a myeloablative conditioning regimen, bone marrow samples were obtained on the day of transplantation (day 0) to determine the effect of conditioning, which demonstrated a statistically significant increase in Tregs and a 30-fold increase in IFNγ concentration (9). However, the concentration of IL-2, IL-6, IL-10, and IL-17A were not significantly different, while IL-1b, IL-4, IL-11, and TNFα were mostly undetectable. By day +100 (the timeframe for classical acute GVHD), the percentage of Tregs and concentration of IFNγ was comparable to healthy donors, suggesting a normalization of the bone marrow microenvironment by day +100.

Collectively, these findings suggest the importance of alterations in the bone marrow microenvironment following the noxious insult of the conditioning regimen leading to emergency hematopoiesis and the complex interaction of host and donor immune cells which may persist for many months following alloHCT, during the time acute GVHD is most likely to occur.

Given its ability to broadly influence hematopoietic differentiation and lymphocyte activity, the CD27-CD70 pathway presents itself as an attractive and novel target in the development of a future GVHD targeted therapy. Similar to the inhibition of CD26, it has been hypothesized that inhibition of CD27 would result in attenuated GVHD, namely by decreasing cytotoxic T-cell alloreactivity. However, in murine models, the administration of an anti-CD70 monoclonal antibody following alloHCT resulted in significantly increased GVHD in a dose dependent fashion (35). This was an unexpected finding, suggesting an alternative and more vital mechanism relating to the pathogenesis and development of GVHD. In further study, while APC-expressed CD70 provides a co-stimulatory signal, T-cell-expressed CD70 serves an inhibitory role in T-cell response, akin to CPIs PD-1 and TIM-3, leading to decreased inflammatory response and GVHD in murine models (36). To better elucidate the mechanism of the CD27-CD70 pathway and its impact on GVHD pathogenesis, cytokines associated with GVHD were measured in CD70 knockout host mice which showed significantly higher levels of pro-inflammatory IFNγ, TNFα, IL-2, and IL-17 when compared to WT mice (see **Figure 3**) (35). This was noted to result in significant changes in host and donor immunophenotype with expansion of donor, but not host, CD4+ and CD8+ T-cells. Furthermore, CD70 knockout was studied in host hematopoietic and non-hematopoietic compartments, with CD70 knockout in hematopoietic compartments shown to result in greater GVHD, indicating that CD70 expression in host hematopoietic cells was the main contributor to the development of GVHD in these models. Meanwhile, interestingly, T-cell derived CD70 was shown to have an inhibitory role by inhibiting allogeneic CD4+ and CD8+ T-cell responses *via* caspase-dependent T-cell apoptosis and upregulation of inhibitory immune checkpoint inhibitor pathways (36). Thus, based on these findings, the

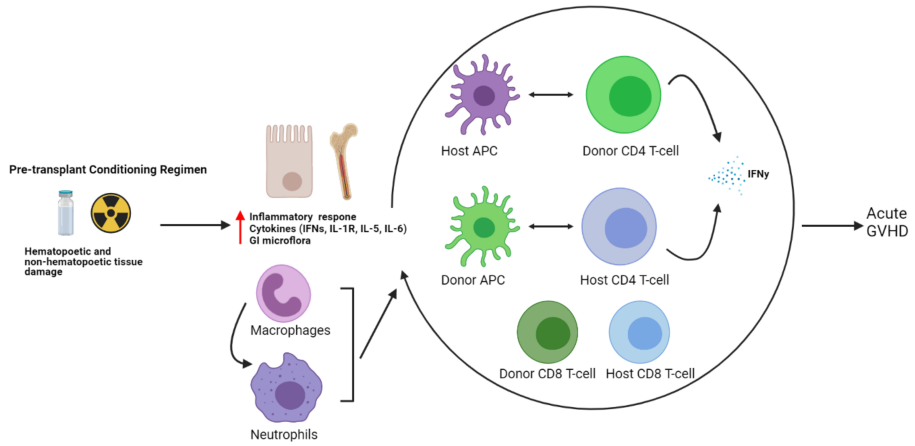

FIGURE 2 | Host and donor immune cell interactions and pathogenesis of acute GVHD. The current understanding of acute GVHD pathogenesis involves a complex interaction of host and donor immune cells.

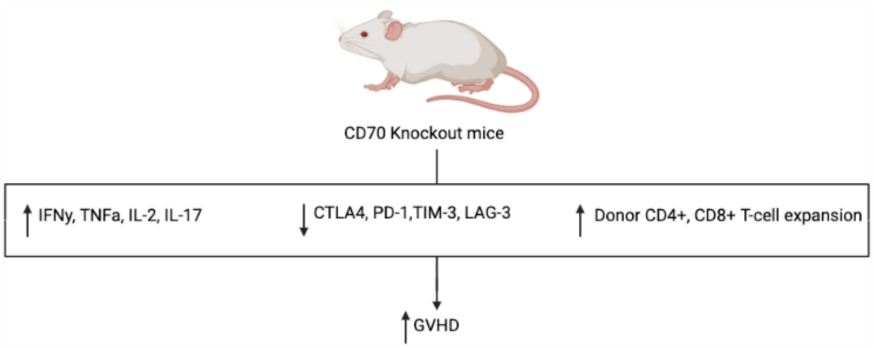

FIGURE 3 | Mechanism of increased GVHD in CD70 knockout mice. Compared to WT control, CD70 knockout mice have significantly more GVHD with increased inflammatory cytokines, decreased CPI expression, and increased expansion of donor T-cells.

CD27-CD70 pathway has multiple immunomodulating effects, both activating and inactivating, depending on the environment and cell type expressing CD27 or CD70. This further suggests that the CD27-CD70 pathway also has an impact on host hematopoiesis and immune cell differentiation, impacting the development of GVHD, perhaps by promoting a decrease in inflammatory cell types in favor of less inflammatory ones, although more studies are required to develop an understanding of the underlying mechanisms.

CONCLUDING REMARKS

More recently, with the evolution of CPI and other T-cell concentrated therapies in other fields of Oncology, co-stimulatory mechanisms involved in the activation and proliferation of T-cells have been explored. Of notable importance, agonism of the co-stimulatory CD27-CD70 pathway, a member of the TNF superfamily, has been studied as a potential therapeutic intervention as an oncologic therapy for multiple tumor cell types as well as a therapeutic intervention to attenuate GVHD. Thus, agonism of the CD27-CD70 pathway presents itself as a novel future therapeutic target, particularly with the availability of a CD27 agonizing monoclonal antibody that has completed phase I/II study and been shown to be quite safe and well tolerated with minimal high-grade toxicities reported.

AUTHOR CONTRIBUTIONS

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

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