MOUSE MODELS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION

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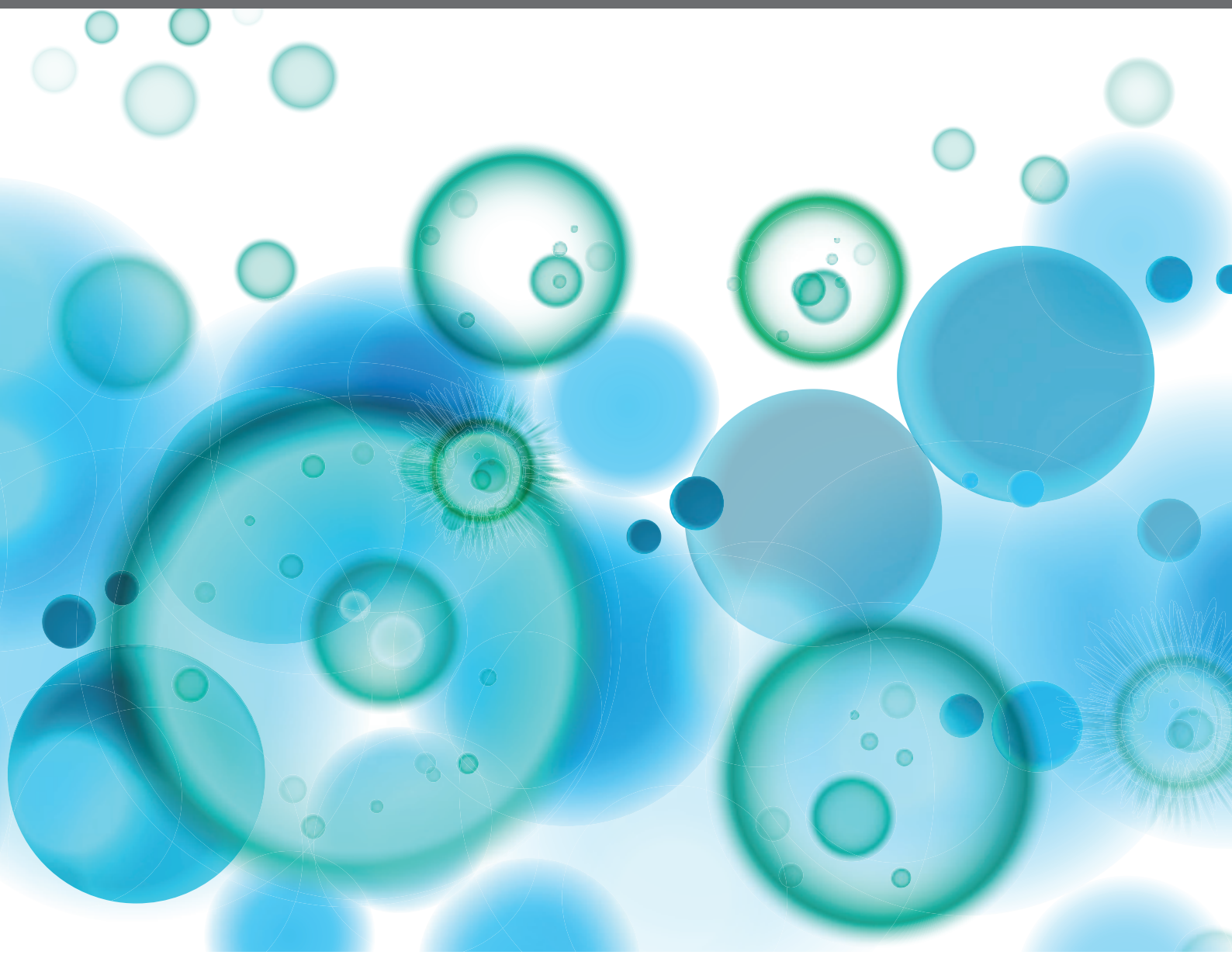

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MOUSE MODELS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Table of Contents

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EDITORIAL
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Editorial: Mouse Models of Hematopoietic Stem Cell Transplantation

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Editorial on the Research Topic

Mouse Models of Hematopoietic Stem Cell Transplantation

Bone marrow or hematopoietic stem cell transplantation (HSCT) is a curative treatment option for hematological malignancies, several non-malignant hematological disorders and severe combined immune deficiencies. However, relapse, graft-versus-host disease (GVHD) and opportunistic infections represent major complications following allogeneic HSCT which compromise the survival and quality-of-life of the recipient, limiting the wider application of this immunotherapy. Basic research has significantly advanced our understanding of the underlying mechanisms of these complications and has led to the development of promising pharmaceutical, immune and cellular therapies. Despite intensive research and development, GVHD, infection and relapse remain significant clinical problems and represent major unmet needs in HSCT.

This *Frontiers In Immunology* Research Topic presents the latest insights from preclinical studies of HSCT, highlighting opportunities and challenges, and sheds light on the future of HSCT. The collection is comprised predominantly of review articles which focus on GVHD pathophysiology, regulatory T cell-mediated immune tolerance, antigen presentation, microbiota/metabolite modulation of GVHD, intestinal immunopathology and infectious pulmonary complications, with an emphasis on the biological relevance to the clinic and translational potential.

The perspective article by Teshima and Hill provides a historical overview of major experimental concepts in bone marrow transplantation discovered using murine models and the translation of these findings into clinical practice, highlighting the pivotal insights afforded by murine models.

Since the discovery of regulatory T cells (Tregs) by Sakaguchi in 1995, Tregs have been of great interest to researchers studying immune tolerance and regulation after HSCT. In this collection, Guo et al. review the biology of Tregs, their role in GVHD and GVL and the challenges related to Treg adoptive cell therapy to treat and/or prevent GVHD. The authors review Treg expansion strategies and discuss new sources of Tregs to address challenges encountered with this therapy. In the review by Ikegawa and Matsuoka, the authors detail the importance of Treg homeostasis post-HSCT and discuss strategies to manipulate this to modulate post-HSCT immunity to treat and/or prevent GVHD.

The cardinal feature of GVHD is immune-mediated tissue pathology. Gastrointestinal GVHD is the most challenging to treat and the greatest cause of GVHD-related mortality. In this collection,

Ara and Hashimoto review historical and recent advances in our understanding of the cellular and molecular mechanisms of GVHD-induced tissue damage in the gastrointestinal tract, providing important novel insights garnered from mouse models which inform translational potential. With technological advances in genomic/metagenomic sequencing and metabolomics, the ability to study intestinal microorganisms and their secreted molecules in HSCT has rapidly generated new knowledge in the field. In this collection, Fujiwara provides an in-depth review of the intestinal microbiota, metabolite and epithelial cell crosstalk critical in GVHD regulation, highlighting the complex relationships and potential microbiota-based therapeutic strategies.

Conditioning-induced toxicity, immunosuppressive therapies and alloimmunity permit opportunistic infections post-HSCT, a major cause of morbidity and mortality. Zhou and Moore review murine models of infectious pulmonary complications after HSCT (e.g. bacterial, fungal and viral) focusing on host-pathogen interactions and the important insights garnered from these studies.

The therapeutic effect of HSCT, mediated by alloimmunity towards malignant cells (graft-versus-leukemia/tumor; GVL), is compromised by detrimental alloimmunity towards recipient tissues (GVHD). Antigen presentation is central to these two processes, however the ability to delineate between these remains elusive and is considered the holy grail in HSCT. Koyama and Hill review the field and discuss recent advances uncovered by the use of antigen-specific murine models of GVHD, providing novel insights for the translational potential of targeting this pathway. Critically, the authors review antigen presentation in xenograft transplantation models used to study GVHD and GVL and discuss the controversies associated with the interpretation of findings from these models.

Chronic GVHD continues to be a major clinical problem in HSCT survivors. Song et al. review important mechanistic findings identified using murine models of chronic GVHD which reflect features characteristic of the disease in humans. The authors highlight low P-selectin glycoprotein ligand 1 expressing CD4 T cells as key players in cGVHD pathophysiology. Original research presented by Choi et al. revealed that deletion of the endoplasmic reticulum stress protein, XBP-1, reduced B cell activity and the ability to stimulate allogeneic CD4 T cells *via* a regulated IRE-1a-dependent decay pathway which led to a reduction in cGVHD. These preclinical findings demonstrate targeting XBP-1 a potentially useful strategy to ameliorate cGVHD.

Finally, Shaikh et al. present a detailed description of the development of a murine model of orthotopic mesenteric lymph node transplantation to study immune cell responses and migration in the gastrointestinal tract. The authors determined its utility in a HSCT setting and suggest potential for broader application.

In summary, murine models have proven to be instrumental for advancement of our understanding of the complications that occur following HSCT. As demonstrated in this collection, murine models of HSCT have led to the discovery of new scientific knowledge, permitted established concepts to be challenged and emerging concepts to be explored, enabled the development of new therapeutics and provided important insights that have guided clinical studies. With the development of new ideas, technologies and tools, murine models of HSCT will continue to empower academic research and translation into the clinic in the future.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Regulatory T Cells in GVHD Therapy

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Graft *versus* host disease (GVHD) is a common complication and the leading cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Pharmacological immunosuppression used in GVHD prophylaxis and treatment lacks specificity and can increase the likelihood of infection and relapse. Regulatory T lymphocytes (Tregs) play a vital role in restraining excessive immune responses and inducing peripheral immune tolerance. In particular, clinical trials have demonstrated that Tregs can prevent and treat GVHD, without increasing the risk of relapse and infection. Hence, adoptive transfer of Tregs to control GVHD using their immunosuppressive properties represents a promising therapeutic approach. To optimally apply Tregs for control of GVHD, a thorough understanding of their biology is necessary. In this review, we describe the biological characteristics of Tregs, including how the stability of FOXP3 expression can be maintained. We will also discuss the mechanisms underlying Tregs-mediated modulation of GVHD and approaches to effectively increase Tregs' numbers. Finally, we will examine the developing trends in the use of Tregs for clinical therapy.

Keywords: regulatory T cells, acute graft *versus* host disease, chronic graft *versus* host disease, hematopoietic stem cell transplantation, adoptive cellular therapy

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INTRODUCTION

Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is a curative therapy for patients with many hematological malignancies; however, graft *versus* host disease (GVHD) is a major obstacle to the utility of allo-HSCT as it contributes to subsequent mortality and morbidity. GVHD can be classified into acute (aGVHD) and chronic (cGVHD) forms and is characterized by attack of host tissues by donor lymphocytes, owing to disparities in major histocompatibility complex (MHC) molecules or minor histocompatibility antigens (mHAs), which elicit an immune response. Although the immunosuppressive agents, cyclosporine and methotrexate, are administered after allo-HSCT as prophylaxis, aGVHD incidence rates range from 20 to 80% (1), while 6 to 80% of patients develop cGVHD. Glucocorticoids are the first-line treatment for GVHD (1–3); however, only 50–80% and 40–50% of patients with aGVHD and cGVHD, respectively, respond to steroid therapy (1, 2). If patients are resistant to glucocorticoids, overall survival rates are dismal at only 5 to 30% (4). At present, there is no consensus on standard second-line treatment. This situation emphasizes the need for development of innovative therapeutic strategies to control pathological immune responses following allo-HSCT.

In recent years, with increased understanding of regulatory cell populations, cellular therapy, particularly adoptive transfer of $CD4^+CD25^+$ regulatory T cells (Tregs), has attracted more attention (5).

In 1995, Sakaguchi (6) first identified a population of $CD4^+$ cells expressing high levels of IL-2 α-chain receptor (CD25) in lymph nodes and spleens of BALB/c nu/+ mice, which could protect thymectomized mice from autoimmune disease and contribute to maintaining peripheral immune tolerance by suppressing immune responses against self- or non-self-antigens. Later, transcription factor forkhead box P3 (FOXP3) was identified as specifically expressed in Tregs and as making an indispensable contribution to controlling Treg development and suppressive function (7), deepening the understanding of this cell subset. The IL-7 receptor, CD127, is a biomarker inversely correlated with FOXP3 expression and Tregs' inhibitory activity (8). Since FOXP3 is expressed intracellularly, the combination of CD4, CD25, and CD127 has been widely used to isolate Tregs and $CD4^+CD25^+CD127^-$ T cells are now referred to as Tregs. In addition to self-tolerance, Tregs also have a vital role in inducing tolerance of alloantigens (9–11). at url breaking operator Taylor et al. (9) found that CD25-depleted $CD4^+$ cells showed increased responsiveness to allogeneic antigens and that addition of $CD4^+CD25^+$ cells had a potent capacity to regulate $CD4^+$ T cell alloresponses. Meanwhile, $CD4^+CD25^+$ cells were found to induce transplantation tolerance in experiments involving transfer of $CD4^+CD25^+$ Tregs from primarily tolerant mice to syngeneic recipients, which protected recipients from graft at url breaking operator rejection (10, 11). Based on the suppressive capacity of $CD4^+CD25^+$ Tregs toward alloreactive T cells, Cohen et al. (12) demonstrated that removal of $CD4^+CD25^+$ Tregs from the graft during transplantation accelerated the occurrence of GVHD, while addition of freshly isolated or *ex vivo*-expanded $CD4^+CD25^+$ Tregs could delay or even prevent GVHD after allo-HSCT. Tregs were found to prevent GVHD for a long time due to their survival and expansion *in vivo* after transplantation in bone marrow transplantation models (13). Besides, adoptive transfer of Tregs could accelerate the immune reconstitution after transplantation, owing to the prevention of damage of the thymic and secondary lymphoid microenvironment caused by GVHD, which was important for T cell immunity (14). Given these properties of suppressing excessive allogeneic responses, cellular therapy, based on adoptive transfer of Tregs to control GVHD has been the focus of study (12, 15–18).

T cells with immunosuppressive function are a diverse group of cells. Except for $CD4^+$ Tregs, it has been confirmed that $CD8^+$ Tregs can regulate excessive immune responses to control GVHD in animal models as well (19). Being different from $CD4^+$ Tregs, the characteristics of $CD8^+$ Tregs are controversial, and specific surface markers to isolate them have not reached agreement (20). In addition to $\alpha\beta$ T cells, $\gamma\delta$ T cells are also capable of immunoregulation (21). Regulatory $\gamma\delta$ T cells ($\gamma\delta$ Tregs) function to regulate GVHD, which can be induced by granulocyte colony-stimulating factor (G-CSF) (22). In this review, we will focus on $CD4^+CD25^+FOXP3^+$ Tregs.

Tregs have the potential to attenuate GVHD without impairing the graft *versus* leukemia (GVL) effect significantly, making the adoptive transfer of Tregs a promising strategy for treatment of GVHD (23, 24); however, translation of this phenomenon into clinical application continues to face numerous challenges, particularly the instability of Tregs and the difficulties in obtaining sufficient quantities of cells to transfer. In this review, we discuss the biological characteristics of Tregs administered in the context of GVHD. Furthermore, we focus on how to solve the problem of insufficient Treg numbers for adoptive transfer and discuss the clinical prospects for application of Tregs.

THE THEORETICAL BASIS OF TREGS' ADMINISTRATION TO TREAT GVHD

Tregs' Definition and Function

Tregs account for only 5–10% of $CD4^+$ T cells in peripheral blood, but they are essential for maintenance of immunological tolerance (6, 9–12). FOXP3 is specifically expressed in Tregs and acts as a major regulator that controls their development and stability (7, 25). Inactivating mutations or specific deletion of FOXP3 causes a lethal autoimmune syndrome due to a deficiency of Tregs (7). Tregs can be roughly divided into two groups according to their developmental origin. First, thymic Tregs (tTregs), also known as nTregs, are generated when $CD4^+$ single-positive thymocytes encounter self-antigen stimuli in the thymus during development; T cell receptors (TCRs) expressed on tTregs mainly recognize self-antigens, which means tTregs have advantages in preventing autoimmune disease (26, 27). The other type of Tregs develops from naïve $CD4^+$ T cells in the periphery following antigen encounter, through exposure to appropriate cytokines, such as transforming growth factor-β (TGF-β) and IL-2 (28–31). When this pathway occurs *in vivo*, the resulting $FOXP3^+$ Treg cells are referred to as peripherally induced Tregs (pTregs), whereas, when it takes place *in vitro*, they are termed induced Tregs (iTregs) (28–31). Relative to tTregs, pTregs are considered to play an important role in maintaining mucosal tolerance, as the TCRs expressed on pTregs can also be specific for foreign antigens from commensal bacteria (32). In mice, neuropilin-1 (NRP1) is selectively expressed on nTregs rather than pTregs, whether they are generated *in vivo* or *in vitro*, and can be used to distinguish nTregs from pTregs (33). Based on analysis of NRP1, Yadav et al. (33) found that $NRP\text{-}1^{lo}$ Tregs have similar ability to suppress autoimmune responses as $NRP\text{-}1^{hi}$ Tregs, but that the function of $NRP\text{-}1^{lo}$ Tregs was compromised in inflammatory and lymphopenic environments, relative to that of $NRP\text{-}1^{hi}$ Tregs. Unfortunately, no cellular markers have been found to distinguish nTregs and pTregs in humans. Currently, the evaluation of Treg-specific demethylated region (TSDR), a conserved CpG-rich region within the FOXP3 locus, is the only way to distinguish nTregs, and the stability of FOXP3 expression is positively correlated with DNA demethylation at the TSDR (34). The TSDR is completely demethylated in nTregs, but iTregs exhibit incomplete demethylation of TSDR and are prone to losing FOXP3 expression and suppression ability (34), which may explain their instability in inflammatory environments.

FOXP3 Stability in Tregs

FOXP3, which is regarded as a Treg lineage-specific factor, acts as a master regulator of gene expression in Tregs and exerts

regulatory functions at the transcriptional, epigenetic, and post-transcriptional levels (35). Continuous FOXP3 expression is indispensable for maintenance of the Tregs' immunosuppressive phenotype; however, under certain proinflammatory conditions, Tregs are prone to losing FOXP3 expression and transdifferentiating into pathogenic T cells, also referred to as ex-Tregs (36). Therefore, it is particularly important to enhance FOXP3 expression in Tregs to ensure that they continue to exert immunosuppressive activity under post-transplantation conditions. Our laboratory and others found that liver kinase b1 (LKB1) is essential for maintaining the stability of FOXP3 expression and the suppression capacity of Tregs in murine models by deletion of the gene encoding LKB1 specifically in Tregs (37–40). We also found that LKB1 may prevent STAT4-mediated methylation of the TSDR, ensuring stable FOXP3 expression (37). Furthermore, we observed dramatically decreased expression of FOXP3 in human Tregs owing to knockdown of the *LKB1* gene (40). MicroRNAs (miRNAs) can govern the expression of protein-coding genes at the post-transcriptional level (41). Cobb et al. found that miRNA profiles expressed in Tregs were distinct from those in conventional $CD4^+$ T cells and confirmed that eliminating miRNAs by conditional deletion of Dicer (an RNAse III enzyme needed to generate functional miRNA) could influence the development of Tregs in the thymus, reduce the number of Tregs in the periphery, and down-regulate FOXP3 expression (42). Moreover, mice lacking Dicer were prone to developing immune pathology (42). Nevertheless, the detailed mechanisms underlying interactions between miRNAs and FOXP3 are not fully understood. MiR-146b can restrain FOXP3 protein levels by targeting TNF receptor-associated factor 6 (TRAF6) and suppressing the NF-κB pathway (43). Conversely, MiR-146b antagomirs function to promote the proliferation and suppressive ability of Tregs (43). Further, miR-4281 interacts directly with the TATA-box motif in the FOXP3 promoter, thereby strongly increasing FOXP3 expression (44). Similarly, miR-142-3p knockdown upregulates FOXP3 expression and enhances the anti-apoptotic and suppressive function of Tregs (45). Ectopic expression of FOXP3 can confer partial Tregs miRNA profiles, demonstrating that FOXP3 may control Treg-specific miRNA expression (42). Hence, miRNAs may have important roles in regulating Tregs' biology and potentially represent strong targets for intervention against GVHD.

Characteristics of GVHD

The clinical manifestations of aGVHD include an exaggerated inflammatory response, usually involving the skin, intestine, and liver, which occurs within the first 100 days after allo-HSCT (46). Ferrara categorized the occurrence of aGVHD into three continuous phases (47): First, the activation of antigen presenting cells (APCs) by proinflammatory cytokines and danger-associated molecular pattern molecules (DAMPs), which originate from damaged host tissues in response to primary disease and conditioning regimens (47, 48); Second, after encountering activated APCs, donor T cells are rapidly expanded and differentiated into effector T cells (Teffs); Finally, Teffs migrate to target organs with the help of chemokines, causing further damage to host tissues (47, 48). Compared with aGVHD, the mechanisms underlying cGVHD are not clearly understood. The manifestations of cGVHD are similar to those of autoimmune diseases, involve more organs than aGVHD, and usually develop more than 100 days after allo-HSCT (47). Overall, GVHD can be considered as an imbalance between the effector and regulatory arms of the immune system and is characterized by an overproduction of inflammatory cytokines (49, 50).

Numbers of Tregs in the peripheral blood and target organs decline in the inflammatory environment of GVHD, which, in turn, increases GVHD severity (51, 52). Conversely, when Tregs are co-transferred in equal numbers with $CD4^+$ T cells, GVHD can be modestly inhibited in animal models (53). Furthermore, improving early reconstitution of Tregs can prevent GVHD by inhibiting the rapid oligoclonal proliferation of pathogenic $CD4^+$ Teffs (54). Understanding the mechanisms underlying immune tolerance induction by Tregs can provide information about potential therapeutic targets of GVHD.

Role of Tregs in GVHD

In general, Tregs can regulate and suppress excessive responses to alleviate GVHD using both contact and non-contact-dependent mechanisms, which can be classified into four categories: cytolysis, secretion of inhibitory cytokines, metabolic disruption, and targeting of dendritic cells (DCs) (55) (**Figure 1**). Perforin and granzyme B secreted by Tregs can kill Teffs directly (55). Furthermore, inhibitory cytokines, such as interleukin-10 (IL-10), IL-35, and TGF-β, which are expressed by Tregs, are required for Tregs' function (48, 55–57). IL-2 is indispensable for the homeostasis of both Tregs and Teffs. Tregs consume high amounts of IL-2 in local sites, since CD25 (the IL-2 receptor alpha chain) is highly expressed by Tregs, and this may lead to IL-2 starvation of Teffs (48, 55). The reciprocal relationship between Tregs and DCs performs a vital and complex function in controlling GVHD. In addition to killing reactive T cells *via* cell–cell contact, Tregs can act on multiple target cells, particularly DCs. Moreover, Tregs appear to have a more stable association with DCs than that with $CD4^+$ T helper (T_H) cells in NOD mouse models, which prevents subsequent interaction between DCs and T_H cells (58). Thus, the interaction between Tregs and DCs may have a core role in the mechanisms underlying Tregs-mediated immune suppression (58, 59). DCs are considered the most powerful APCs and have a dual role in GVHD development (60–65); host DCs or *de novo* generated donor DCs both contribute to T-cell priming by presenting alloantigens in the context of HLA molecules, as well as providing secondary signals to promote full T cell activation (60–63). Tregs constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA-4), the affinity of which for CD80/86 expressed on DCs is superior to that for CD28, hindering complete T-cell activation *via* blocking the binding of CD28 and CD80/86 (66). Further, Tregs can facilitate the removal and degradation of CD80/86 from DCs *via* CTLA-4 through the process of trans-endocytosis (67). In addition, immunosuppressive cytokines, such as IL-10, released by Tregs can interfere with DC activation and antigen presentation (68). Tregs can hamper DC

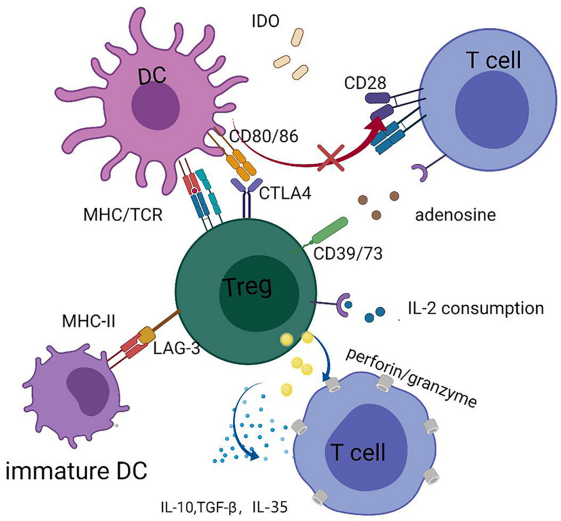

FIGURE 1 | Tregs can secrete perforin and granzyme B to kill Teffs directly and secret IL-10, IL-35, and TGF-β, inhibitory cytokines to suppress functions of Teffs; Tregs can cause IL-2 starvation of Teffs *via* highly expressing CD25. Tregs exert the function of suppression by interaction with DCs, such as downregulating the expression of CD80/CD86 in DCs and interfering with the maturation of DCs. Tryptophan is vital for the survival of Teffs and Tregs can enhance the expression of indoleamine 2, 3-dioxygenase (IDO) in DCs, which accelerates the decomposition of tryptophan. Adenosine triphosphate (ATP) is a pro-inflammatory factor, and CD39/73 expressed on Tregs can transform ATP to adenosine, an anti-inflammatory factor.

maturation, rendering them deficient in priming T cell activation, and lymphocyte activation gene 3 (LAG-3), expressed by Tregs, may play a dominant role in the process of DC maturation (69–72). Recently, Mavin et al. (70) discovered that human Tregs can modulate DC function through disturbing their reprogramming during maturation, which may involve the NF-κB signaling pathway and WNT5A expression. In addition to influencing the activity of DCs themselves, Tregs may alter DC function; for example, Treg-treated DCs tend to skew $CD4^+$ naïve T cell polarization and impair $CD8^+$ Teffs function in inducing GVHD (70). In contrast, DCs can induce donor T cell tolerance by promoting the expansion and function of Tregs, thus protecting them from GVHD (60, 73). Our laboratory found that LKB1 has an important role in DCs to increase the number of Tregs, which has also been confirmed in other studies (74–76). Granulocyte-macrophage colony-stimulating factor (GM-CSF) functions to increase $CD4^+CD8^-$ DC numbers, thus preferentially inducing Tregs expansion, and alleviating tissue damage in a cGVHD mouse model (77). The relationship between DCs and Tregs intricately controls GVHD, and further research is warranted to inform the development of new strategies to prevent GVHD.

Tregs in GVL

T cells originating from donors can eliminate remaining tumor cells, hence allo-HSCT is considered the only curative therapy for numerous malignant hematological diseases. This also means that immunosuppressive Tregs controlling GVHD by inhibiting the initial activation of alloreactive T cells may compromise the GVL effect, thereby increasing the risk of relapse and infection. Experiments in several animal models have revealed that Treg therapy can suppress GVHD while maintaining GVL, thus separating GVHD from GVL (78, 79). This may involve Treg-mediated inhibition of excessive donor T cell proliferation and downregulation of serum proinflammatory cytokine levels, while not interfering with the activation of conventional T cells (Tcons), particularly the ability of $CD8^+$ T cells to kill tumors (78); however, some preclinical experiments have also demonstrated that $CD4^+$ iTregs can partially impair GVL in a mouse model, with animals suffering short-term leukemia relapse (80, 81). The combination therapy of $CD4^+$ iTregs and $CD8^+$ iTregs may provide a new way to solve the problem because the GVL effect can be preserved by $CD8^+$ iTregs, and $CD4^+$ iTregs function to attenuate GVHD in the meanwhile, which achieves the effect of one plus one being greater than two (81). Excitingly, Treg-based therapy has seldom been found to have a detrimental influence on the risk of relapse and infection in clinical trials (18, 82–84).

TREGS' EXPANSION FOR GVHD THERAPY

Expansion *Ex Vivo*

As mentioned above, Tregs account for a small proportion of $CD4^+$ T cells in the peripheral blood. Therefore, the numbers of Tregs freshly isolated from donors are far from sufficient to cater for clinical infusion requirements. Furthermore, the purity of Tregs isolated from donors is sub-optimal, as there is a lack of specific markers to distinguish Tregs from Tcons. Currently, Tregs isolated by leukapheresis from healthy donors and expanded by exposure to αCD3/αCD28 beads and IL-2 stimulation are a common source for cellular therapy in the clinic (85) (**Figure 2**). Tregs expanded by using good manufacturing practices–compatible protocol were confirmed to prevent GVHD and retain GVL effectively in animal models (86). The approach of automated clinical-grade expansion of Tregs *ex vivo* has been developed to improve the purity and quantity of the infused Tregs (85, 87). Nevertheless, the technology to isolate and expand Tregs is complex and costly; hence, new strategies to produce sufficient functional Tregs are required.

Although iTregs are intrinsically unstable, adoptive transfer of iTregs may also function to control GVHD (53, 88, 89). Notably, iTregs can overcome the problem of lack of sufficient cell quantity, as they can be manufactured abundantly *ex vivo*. Further, alloantigen-specific iTregs are considered more effective than polyclonal Tregs as they directly target specific antigens (29, 88). Nevertheless, iTregs are prone to lose FOXP3 expression, particularly under inflammatory conditions, which limits their therapeutic activity in GVHD (29, 90). There have been attempts to optimize iTregs' stability (**Figure 2**). Kasahara et al. (88) found

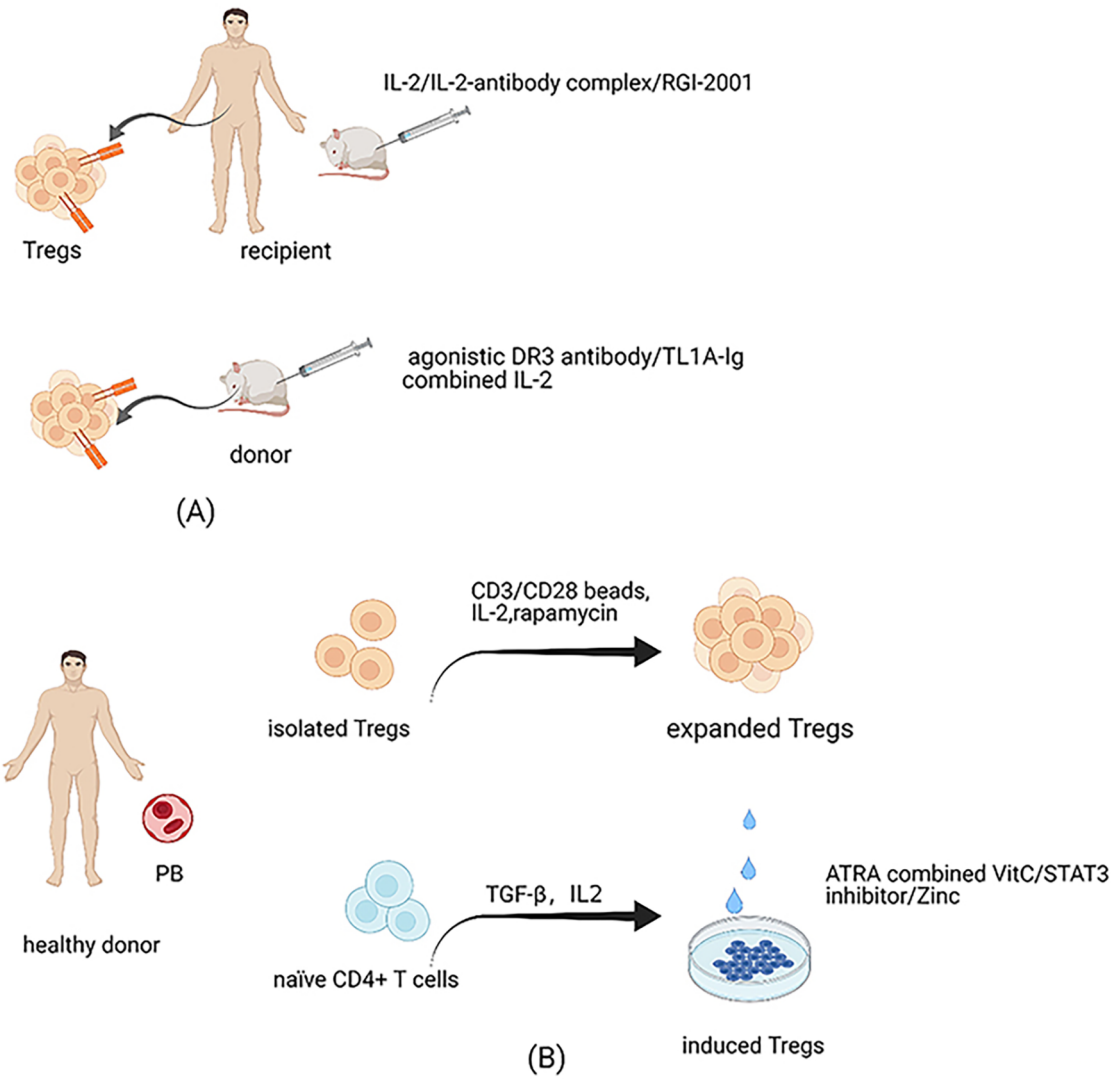

FIGURE 2 | Treg expansion. **(A)** Expansion of Tregs in recipients and donors. Administration of low-dose IL-2 can expand Tregs of hosts preferentially *in vivo* since a high-affinity IL-2 receptor is expressed highly on Tregs. Considering IL-2 can also enhance the activation of Teffs, antibody-IL-2 complex is created, which can electively expand Tregs *in vivo* due to altering the structure of IL-2. Besides, RGI-2001 also has the function to accelerate the expansion of Tregs *in vivo*. The effectiveness of these cells has been tested in human and animal models. Reagents to expand Tregs of donors have been administrated in animal models, such as agonistic antibody against αDR3 and TL1A-Ig combined with IL-2. **(B)** Expansion of Tregs isolated from donors *in vitro* and methods to improve iTregs' stability. Tregs isolated from healthy donors and expanded *in vitro* are the most common source at the expense of complex technologies and costly prices. Because of the unstable properties of iTregs, methods have been tried to improve its stability, such as the combination of all-trans retinoic acid and vitamin C, administration of STAT3 inhibitor, and the supplementation of Zinc.

that stable iTregs could be generated using a combination of all-trans retinoic acid and vitamin C. Vitamin C-treated iTregs significantly alleviated GVHD symptoms in murine models compared with untreated iTregs. Further, iTregs treated with STAT3 inhibitor exhibited dramatically enhanced suppression ability and achieved stability *via* high levels of FOXP3 demethylation (91). Furthermore, STAT3 phosphorylation-inhibited iTregs have potent function in preventing xenogeneic GVHD by reducing excess immune responses caused by alloreactive T cells (92). Zinc supplementation may also increase iTreg numbers while maintaining their stability by prolonging FOXP3 expression (93). Enhancing iTregs' stability will solve the problem of insufficient Treg quantity for the requirements of clinical application.

Expansion *In Vivo*

Fujioka et al. (94) found that a low ratio of Tregs to $CD4^+$ T cells in the early stage after allo-HSCT can predict impending

aGVHD. Our laboratory results also suggest that Tregs from patients with aGVHD become defective in terms of their stability, survival, and suppressive function (40). Expansion of Tregs in recipients after allo-HSCT may be a promising strategy to alleviate GVHD (**Figure 2**). It is established that IL-2 is vital for the development, proliferation, and activity of Tregs (95), which can react to low concentrations of IL-2 by expressing high levels of a high-affinity IL-2 receptor (CD25). Administration of low-dose IL-2 in cGVHD patients was associated with preferential expansion of Tregs *in vivo* and attenuated the symptoms in certain patients (96, 97). Notably, IL-2 can also target many other cells, including promoting conventional T cell activation, and the appropriate effective dose for controlling GVHD is difficult to manage (95). Trotta et al. (98) generated a human antibody against IL-2 and combination with the antibody altered IL-2 conformation. The antibody–IL-2 complex could considerably and selectively expand Tregs *in vivo* and was effective in protecting mice from GVHD. Recently, Hirai et al. (99) innovatively engineered an orthogonal IL-2/IL-2 receptor (IL-2R) pair. The results demonstrated that transduced Tregs with orthogonal IL-2R were selectively expanded by orthogonal IL-2 stimulation without increasing other T cell subsets in murine models. Besides, this method did not reduce the function of Tregs and improved graft tolerance. Infusion of RGI-2001, a liposomal formulation carrying α-galactosylceramide dramatically improved the survival of mice with aGVHD by enhancing the expansion of alloantigen specific $CD4^+CD25^+$ Tregs *in vivo*; notably, administration of RGI-2001 did not abrogate GVL (100). Further, a clinical trial of RGI-2001 showed that the incidence of severe GVHD was lower in responders than in non-responders (101).

In addition to expanding Tregs in hosts, methods to increase the number of donor Tregs for transfer *in vivo* have been attempted (**Figure 2**). An agonistic antibody against death receptor 3 (αDR3) has been found to enhance $CD4^+FOXP3^+$ Treg proliferation *in vivo* when administered to the donor, thus increasing the percentage of Tregs in the graft (102–104). Further, αDR3-treated Tregs demonstrated potent proliferation and suppression abilities when transferred to host mice. The severity of GVHD in recipient mice receiving grafts from αDR3-treated donors was significantly less than that in isotype-treated donors (102–104). Similarly, TL1A-Ig (a soluble fusion protein from the natural ligand of tumor necrosis factor superfamily receptor 25 (TNFRSF25)) combined with IL-2 led to marked Treg expansion in donors *via* respective targeting of TNFRSF25 and CD25 *in vivo*. Adoptive transfer of Tregs from donors treated with TL1A-Ig/IL-2 effectively protected recipients from GVHD (16, 17). Furthermore, preclinical trial data suggest that GVHD after allo-HSCT can be controlled in patients with low numbers of Tregs stimulated by TL1A-Ig combined with IL-2, as this method of amplification can enhance Tregs' suppressive activity (16). Reagents found to increase Tregs' numbers and promote their inhibitory function in the donor may be used to develop new strategies for expanding Tregs *ex vivo* in the future.

NEW SOURCES OF TREGS FOR GVHD

Third-Party Tregs

In animal models and clinical trials, donors have been the most common source of Tregs used to modulate GVHD. As $CD4^+CD25^+FOXP3^+$ Tregs are scarce in the peripheral blood and HLA-matched donors are not always available, the efficacy and safety of Tregs from other sources have been evaluated for use in GVHD control (105). Tregs derived from a third-party [umbilical cord blood (UCB)] could confer protection from GVHD in a xenogeneic GVHD mouse models (106). Mice prophylactically injected with UCB-derived Tregs achieved better GVHD scores and overall survival rates than those receiving peripheral blood mononuclear cells (PBMCs) only (106). Another study found that Tregs derived from third-party mice could inhibit GVHD development compared with those originating from donors or hosts. Although the optimal therapeutic effect was observed in mice treated with donor Tregs, third-party Tregs could still be considered as a promising alternative source (105). Large numbers of Tregs can be isolated from pediatric thymuses, which are generally removed during cardiac surgery (107). Expanded thymic Tregs manifest stable FOXP3 expression and even maintain inhibitory ability under inflammation conditions. Furthermore, thymic Tregs more effectively protected mice from GVHD than Tregs derived from peripheral blood in xenogeneic GVHD mouse models. Therefore, pediatric thymuses may become an alternative source of functional Tregs (107).

CAR Tregs

Compared with polyclonal Tregs, alloantigen-specific Tregs, particularly those with chimeric antigen receptors (CARs), have the advantage of being able to achieve specific immunosuppression using fewer cells (108). CAR technology confers Tregs with superior ability to identify whole proteins expressed in cells, dispensing with HLA molecule restriction. MacDonald et al. (108) created alloantigen-specific Tregs using a CAR targeting HLA-A2, which is commonly mismatched in transplantation, and the A2-CAR Tregs maintained phenotypic stability and suppressive function. Surprisingly, the A2-CAR Tregs prominently delayed GVHD development and improved mouse survival compared with polyclonal Tregs in xenogeneic GVHD models. Further, owing to their larger numbers in circulation and considerable proliferation rates, $CD4^+$ T cells can be transduced to express both A2-CAR and FOXP3 (109). These genetically modified $CD4^+$ T cells (A2-CAR/FOXP3 $CD4^+$ Tregs) obtained a stable inhibitory phenotype and could suppress inflammation responses. Further, A2-CAR/FOXP3 $CD4^+$ Tregs could significantly alleviate inflammatory pathology in a GVHD mouse model (109).

Third-party and engineered Tregs raise the prospect of universal Tregs, which could be provided as off-the-shelf products for use in patients (110). Universal donor Tregs can be manufactured to reduce immunogenicity by knocking out classical HLA molecules and knocking in non-canonical HLA molecules, such as HLA-E or HLA-G, thus escaping host

immune system recognition (110). With advances in genome editing technology, induced pluripotent stem (iPS) cells may become a potential source of universal donor Tregs, with optimal immunosuppressive activity after engineering (110). Adoptive therapy of universal donor Tregs may represent a promising approach for patients with GVHD.

TREGS APPLICATION STRATEGIES IN GVHD

As mentioned above, Tregs play an essential role in maintaining immunological tolerance. Therefore, adoptive transfer of Tregs to directly reduce the incidence or severity of GVHD after allo-HSCT has been the focus in the field of transplantation. Main clinical trials have been summarized in **Table 1**. What is more, increasing the frequency and stability of Tregs *in vivo* by reagents, such as IL-2, to control excessive immune responses has also aroused interest.

Tregs to Prevent GVHD

A clinical trial showed that early adoptive transfer of Tregs freshly isolated from donors, followed by conventional T cells (Tcons) four days later, could prevent GVHD without increasing infection or relapse following haploidentical HSCT, which confirmed the prophylactic efficacy of Tregs against GVHD for the first time (82). Furthermore, Brunstein et al. also confirmed the safety of umbilical Tregs expanded *in vitro* using IL-2 and αCD3/αCD28 beads to prevent aGVHD after umbilical cord blood (UCB) transplantation without increasing the likelihood of infection, relapse, or early mortality (83). This study included 23 patients who received UCB-derived Tregs at a dose of 0.1–30 × 10^5 UCB Tregs/kg on day one after transplantation, alongside a cohort receiving a second dose of 30 × 10^5 Tregs/kg at day +15, and compared with 108 historical controls, the incidence rates of grade II–IV aGVHD were reduced by 43 and 61%, respectively (P = 0.05). A clinical trial of *in vivo* Treg expansion by injection of ultra-low dose IL-2 also reduced the incidence of GVHD (111); 16 patients were administered with ultra-low doses of IL-2 (100,000 units subcutaneously × 3 weekly for 6–12 weeks) after allo-HSCT. The percentage of Tregs increased after IL-2 therapy, with the mean of 4.8% rising to 11.1%, and no IL-2 treated patients suffered from grade II–IV aGVHD, compared with 12% (4/33) of the control group who did not receive IL-2. Besides, Pierini et al. (18) conducted a clinical trial which included 50 patients with acute myeloid leukemia (AML). An age-adapted myeloablative conditioning regimen was combined with Treg/Tcon adoptive immunotherapy, resulting in an impressive 75% moderate/severe cGVHD/relapse-free survival rate. Only two of the 50 patients relapsed, and GVL was not impaired by this therapy with Tregs, which may be related to the low levels of CXCR4 bone marrow homing receptor in Tregs.

Tregs to Treat GVHD

Notably, no large-scale trial of Treg administration to treat GVHD, particularly aGVHD, has been conducted. The first case of cGVHD patients to receive Treg infusion reported an improved outcome,

TABLE 1 | Main clinical trials with Tregs in stem cell transplantation.

Number of patients	Phase	Type of transplantation	Sources of Tregs	Dosage of Tregs	Indication	Status	Trial ID
28	II	Haplo-HSCT	donor Tregs	2×10^6/kg or 4×10^6/kg	GVHD prevention	Recruiting	(82), Di Ianni et al. NCT03977103
23	I	UCBT	UCB Tregs	$0.1–30 \times 10^5$/kg	GVHD prevention	Completed	(83), Brunstein et al. NCT00602693
43	II	Haplo-HSCT	donor Tregs	Mean 2.5×10^6/kg	GVHD prevention	Recruiting	(84), Martelli et al. NCT03977103
33	I	UCBT	UCB Tregs	$3–100 \times 10^6$/kg	GVHD prevention	Completed	(23), Brunstein et al. NCT00602693
12	I/II	MSD allo-HSCT	donor Tregs	1×10^6/kg–3×10^6/kg	GVHD prevention	Recruiting	(24), Meyer et al. NCT01660607
16	I	MSD allo-HSCT	iTregs	3×10^6/kg–10×10^8/kg	GVHD prevention	Completed	(89), MacMillan et al. NCT01634217
50	II	HLA-haploidentical HSCT	donor Tregs	2×10^6/kg	GVHD prevention	Recruiting	(18), Pierini et al. NCT03977103
35	II	allo-HSCT	donor Tregs	$\geq 0.5\times 10^6$/kg	Steroid refractory cGVHD	Recruiting	NCT01903473
20	I/II	allo-HSCT	donor Tregs	5×10^5/kg, 1×10^6/kg, 2×10^6/kg	Severe refractory cGVHD	Recruiting	NCT02749084

Allo-HSCT, allogeneic hematopoietic stem cell transplantation; Haplo-HSCT, haploidentical allogeneic hematopoietic stem cell transplantation; UCBT, umbilical cord blood transplantation; MSD, matched sibling donor; iTregs, induced regulatory T cells; GVHD, graft versus host disease; cGVHD, chronic graft versus host disease.

but this was not conclusively demonstrated in patients with aGVHD (15). In another clinical trial, Tregs from HLA-matched donors were administered to five patients with treatment-refractory cGVHD; two patients achieved symptomatic relief, while four had reduced immunosuppressive treatment with increased numbers of Tregs *in vivo*; however, two patients were diagnosed with skin cancer months after adoptive transfer of Tregs (112). Further studies are needed to evaluate the feasibility of this approach for treating GVHD. Regarding Treg infusion to treat patients with aGVHD, related reports are rare; however, the therapeutic efficacy of Tregs in established aGVHD has been investigated in a mouse model (113). The severity of tissue damage caused by aGVHD was alleviated and Tregs migrated to aGVHD targeted organs and lymphoid sites to exert immunosuppressive function, particularly in the gastrointestinal tract. Mice treated with Tregs showed relief of aGVHD symptoms and achieved prolonged survival (113). These results suggest that adoptive transfer of Tregs to treat aGVHD may be effective, although more studies are required.

Tregs Combined With DLI

Previous investigations have focused on the feasibility and safety of Tregs' transfer to control GVHD at the time of transplantation. As an effective strategy to rescue patients with relapsed hematological malignancies after allo-HSCT, donor lymphocyte infusion (DLI) can boost GVL to eliminate tumor cells, but increases the risk of severe GVHD (114). Recently, Di Ianni et al. (115) reported the case of a patient with acute promyelocytic leukemia (APL) treated with Treg-protected DLI in the early stage of recurrence after second allo-HSCT. The patient received a first infusion dose of 2.5×10^6/kg Tregs, followed by infusion of 5×10^6/kg Tcons one week later; a second infusion (2.5×10^6/kg Tregs and 2×10^6/kg Tcons) was performed two months later. The results demonstrated that complete hematological remission was achieved, with a progression-free survival of 6 months, and no apparent GVHD symptoms were observed. Whether Tregs can be administered with DLI to prevent GVHD deserves more investigation, and this is a potential new strategy to apply Tregs for GVHD control.

Tregs and Immunosuppressive Agents

Since Tregs play a vital role in maintaining peripheral immune tolerance, pharmacological immunosuppression for GVHD prophylaxis and treatment may influence the regulation of Tregs after allo-HSCT. As hypomethylating agents, azacitidine (AzaC) and decitabine (Dec) can cause $CD4^+CD25^-$ T cells to obtain suppressive properties by inducing FOXP3 expression (116). Thus, the effect of AzaC in mitigating GVHD symptoms without abrogating GVL involves the conversion of alloreactive T cells to suppress Tregs (116). Further, nTregs are essential for AzaC protection against GVHD, as depletion of nTregs *in vivo* in mice compromised the effect of AzaC (117). The GVHD prophylactic regimen based on sirolimus without calcineurin inhibitors in patients with high-risk hematological malignancies can promote Treg proliferation and accelerate immune reconstitution *in vivo* (118). Low-dose post-transplant cyclophosphamide could attenuate and prevent GVHD by increasing Treg frequency, and the effects were enhanced by combination with anti-thymocyte globulin (119). Lee et al. (120) applied metformin and tacrolimus to treat GVHD in mice and found that combined therapy with these two drugs suppressed type 1 helper T (Th1) and Th17 cell development, while it enhanced the expression of Treg-related genes. This combination therapy alleviated GVHD severity and improved mouse survival. Furthermore, combination treatment also reduced human alloreactive T cell proliferation and production of proinflammatory cytokines by balancing the Treg/Th17 cells ratio (120). Understanding the effects of pharmaceutical preparations on Tregs could help clinicians to formulate optimal regimens to generate equilibrium between relapse and GVHD, thus achieving immune homeostasis.

DISCUSSION

Treg administration for GVHD has been investigated for several years. Numerous animal studies have confirmed that Tregs have important roles in restraining excessive immune responses and can prevent GVHD, without increasing the risk of relapse and infection. Tregs may lose their immunosuppressive phenotype due to unstable expression of FOXP3 under inflammatory conditions. Notably, upstream factors that regulate Tregs' stability have not been clearly elucidated; therefore strategies to maintain FOXP3 stability warrant investigation to ensure that Tregs exert a suppressive function after adoptive transfer. The numbers of Tregs available in peripheral blood samples are far from sufficient for clinical application. In addition to exploring methods to effectively freshly isolate high purity Tregs from donors, methods for generating large numbers of functional iTregs *in vitro* deserve more attention, given the intrinsically unstable properties of Tregs cultured *ex vivo*. Notably, alloantigen-specific Tregs may possess potent inhibitory function against specific tissues, facilitating achievement of satisfactory suppressive effects using fewer cells. Furthermore, universal Tregs may broaden the utility of Treg infusion, opening a new avenue to resolution of the problem of GVHD after allo-HSCT. Many clinical trials have demonstrated that adoptive transfer of Tregs is an effective and safe way to prevent GVHD, rather than treating GVHD after it occurs. More trials are needed to verify the feasibility of cell therapy based on Treg infusion to treat GVHD, particularly aGVHD.

AUTHOR CONTRIBUTIONS

W-WG, X-HS and M-YW wrote the manuscript. X-MF, M-ZH and E-LJ revised the manuscript. All authors contributed to the article and approved the submitted version. W-WG and X-HS contributed equally to this manuscript.

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Mesenteric Lymph Node Transplantation in Mice to Study Immune Responses of the Gastrointestinal Tract

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Mesenteric lymph nodes (mLNs) are sentinel sites of enteral immunosurveillance and immune homeostasis. Immune cells from the gastrointestinal tract (GIT) are constantly recruited to the mLNs in steady-state and under inflammatory conditions resulting in the induction of tolerance and immune cells activation, respectively. Surgical dissection and transplantation of lymph nodes (LN) is a technique that has supported seminal work to study LN function and is useful to investigate resident stromal and endothelial cell biology and their cellular interactions in experimental disease models. Here, we provide a detailed protocol of syngeneic mLN transplantation and report assays to analyze effective mLN engraftment in congenic recipients. Transplanted mLNs allow to study T cell activation and proliferation in preclinical mouse models. Donor mLNs proved viable and functional after surgical transplantation and regenerated blood and lymphatic vessels. Immune cells from the host completely colonized the transplanted mLNs within 7-8 weeks after the surgical intervention. After allogeneic hematopoietic cell transplantation (allo-HCT), adoptively transferred allogeneic $CD4^+$ T cells from FVB/N ($H\text{-}2^q$) mice homed to the transplanted mLNs in C57BL/6 ($H\text{-}2^b$) recipients during the initiation phase of acute graft-versus-host disease (aGvHD). These $CD4^+$ T cells retained full proliferative capacity and upregulated effector and gut homing molecules comparable to those in mLNs from unmanipulated wild-type recipients. Wild type mLNs transplanted into MHCII deficient syngeneic hosts sufficed to activate alloreactive T cells upon allogeneic hematopoietic cell transplantation, even in the absence of $MHCII^+$ $CD11c^+$ myeloid cells. These data support that orthotopically transplanted mLNs maintain physiological functions after transplantation.

The technique of LN transplantation can be applied to study migratory and resident cell compartment interactions in mLNs as well as immune reactions from and to the gut under inflammatory and non-inflammatory conditions.

Keywords: acute graft-versus host disease, alloreactive T cells, mesenteric lymph node, lymph node transplantation, mouse models, lymph node stromal cells

INTRODUCTION

Lymph nodes (LNs), spleen and Peyer's patches (PPs) are secondary lymphoid organs that serve as sites for immune cell interaction and activation. LNs are unique in morphology and function. These filter-like structures continuously scan lymph content that moves from the afferent lymph into the subcapsular lymph node sinus and serve as spatially highly organized hubs of immune cell interactions (1). After recognition of a pathogenic antigen (Ag), an immune response is mounted, whereas in case of dietary or commensal antigens, tolerance is induced.

Surgical LN transplantation is a pre-clinical tool that has been instrumental to study tissue-resident LN stromal cells (LNSCs) in the immune microenvironment of LNs as well as their role in the modulation of cells of hematopoietic lineage under homeostatic and inflammatory conditions (2–5). In seminal work, others have utilized LN transplantation to demonstrate that LNSCs regulate peripheral tolerance (6) and that stromal cells of mLNs imprint gut-homing properties on antigen-responsive T cells to express α4β7 integrin and CC chemokine receptor 9 (CCR9) (5, 7). Furthermore, mLN stroma shapes resident dendritic cells (DCs) to attain high Treg-inducing capacity soon after birth in a Bmp2-dependent manner (8).

Worbs and colleagues elegantly showed oral tolerance induction exclusively takes place in the mLNs with antigens transported from the intestinal surface by DCs through the afferent lymphatics, whereas roles of PPs in the induction of oral tolerance were dispensable (9). Oral tolerance is in part mediated by the generation of $FoxP3^+$ Tregs (peripherally induced Tregs, pTregs) converted from conventional $FoxP3^-$ $CD4^+$ T cells (10–14).

Notably, mLNs not only serve as sites for oral tolerance to food antigens but form an important firewall blocking systemic dissemination of microbes as potential pathogens and priming of intestinal immune cells by mounting effective immune responses (15, 16). mLNs drain lymph from various sites of GIT including the small intestine (SI) and colon, however different nodes of mLNs are anatomically segregated of lymphatic drainage from the SI and colon (17–19).

As pointed out above, lymphocytes primed in mLNs are imprinted for gut tropism and accordingly the ligands specific for their homing receptors α4β7 integrin and CCR9 are found in the gastrointestinal tract (GIT) (5, 7, 20, 21). These allow for efficient homing of immune effector cells to the lamina propria of the intestinal tract *via* the blood stream to protect from intestinal infections but are also relevant in pathological inflammatory conditions such as inflammatory bowel disease (17, 22–27) and intestinal acute graft-versus-host disease (aGvHD) (28–36). Therefore, we performed surgical mLNs transplantation to study the mucosal immune system of the gut in physiology and disease conditions.

Here, we provide an in-depth methodological description of the surgical mLNs transplantation procedure in C57BL/6 mice. We show that donor mLNs are viable after surgical transplantation, retain their histologic immune architecture, topological organization, normal vascular and lymphatic function. Furthermore, by transplanting mLNs from B6.CD45.1 congenic donor mice (B6.CD45.1) revealed the kinetics of repopulation of transplanted mLNs with all lineages of host-type hematopoietic cells. Transplanted mLNs provided all the required stimuli for the effective proliferation, differentiation and expansion of $CD4^+$ T cells similar to non-transplanted mLNs, e.g., in a mouse model of aGvHD even in otherwise MHCII deficient hosts.

Hence, the described procedure is a suitable technique to study complex and dynamic immune cell interactions within the alimentary tract.

MATERIAL AND EQUIPMENT

Materials

Materials are listed in **Table 1** corresponding to the experimental procedures.

Preparation of Reagents

- Anesthetic: add 2 ml of Xylavet® (20 mg/ml, CP-Pharma) and 2 ml of Ursotamin® (100 mg/ml, Serumwerk) to 21 ml of DPBS. PromAce® (10 mg/ml, Boehringer Ingelheim) was prepared separately and used for experiments involving surgery. Inject 10 µl/g of body weight to reach desired concentration. (Xylazin 16 mg/kg, Ketamine 80 mg/kg and Acepromazine 2 mg/kg).
- Analgesic: Carprofen (5 mg/kg) to relieve pain 30 min before opening the abdominal cavity and, if needed, every 12 h up to third day after the surgery.
- Erythrocyte lysis buffer: dissolve 89.9 g of NH_4Cl, 10 g of $KHCO_3$, and 0.37 g EDTA in 1 l of autoclaved, deionized water.
- Trypan blue solution: dissolve 1 g of trypan blue in 100 ml of PBS. Dilute 1:10 in PBS to get working solution to mix at equal volume with cell suspension.
- Magnetic cell enrichment buffer: 0.375 g of EDTA and 0.5 g of BSA in 500 of DPBS, sterile-filter.

TABLE 1 | Materials used for the LN transplantation and associated techniques.

Materials	Catalog #	Company
Mesenteric lymph node transplantation operation		
Anesthetic solution		
1 ml syringe 26GA x 3/8'' (0.45 mm x 10 mm), BD Plastipak™	300015	Becton Dickinson
1 ml insulin syringe 30GA x 1/2'' (0.3 mm x 12 mm), Omnican® 100	9151141	Braun
Fibrin sealant (TISSEEL, Fibrin Sealant kit with DUPLOJECT system)	1504516	Baxter
Eye ointment (Bepanthen®)		Bayer
Analgetic (Carprofen)	53716-49-7	Midas Pharma GmbH
Hair removal cream		Müller
Sterile razors	704028	Body products, Relax Pharma u. Kosmetik GmbH
Sterile dissecting swab (Setpack® size 2)	12780	Lohmann & Rauscher
Sterile gauze swab (Gazin® 5 cm x 5 cm)	13695	Lohmann & Rauscher
Sterile cotton swab (Rotilabo®)	EH12.1	Carl Roth
Operation towel	800430	BARRIER, Mölnlycke Health Care
Pasteur pipettes	2600111	Neolab
70% ethanol	T931.3	Carl Roth
Quickpad® 70% 2-propanol		Holtsch Medizinprodukte GmbH
Povidone iodine (Bruanol® 7.5% solution)	3864065	Braun
0.9% NaCl (Aqua ad iniectabilia)	14NM32	Fresenius Kabi Deutschland
Suture, 6-0 with beveled needle	V301G	Ethicon
Cell isolation		
DPBS without Ca^{2+}/Mg^{2+}	P04-36500	Pan Biotech
Sterile scalpel blades, feather # 10	BB510	B. Braun
Disposable serological pipettes	760180, 607180, 606180	Greiner Bio-one
Pipette controller (Accu-jet® pro)	26300	Brandt
Cell counting chamber (Neubauer)	ZK03	Hartenstein
Micropipettes	042760930, 642752433, 942741768, 342733754, 042720454, 942711302	VWR
Cell strainer, 70 μm EASYstrainer™	542070	Greiner Bio-one
Tube 50 ml	227261	Greiner Bio-one
Microtubes 1.5 ml	72.706	Sarstedt
Erythrocyte lysis buffer		
DNase I	10104159001	Roche
Collagenase VIII	C2139	Sigma Aldrich
Collagenase P	11213865001	Roche
Dispase II	4942078001	Roche
Trypan blue solution	T8154-100ML	Sigma Aldrich
Magnetic cell enrichment buffer		
Antibodies		
Anti-CD3ε antibody (145-2C11) coupled to APC	100312	Biolegend
Anti-CD3 antibody (SP7)	RBG024	Zytomed
Anti-CD4 antibody (RM4-5) coupled to PE	100512	Biolegend
Anti-CD4 antibody (RM4-5) coupled to PerCP/Cy5.5	100540	Biolegend
Anti-CD8a antibody (53-6.7) coupled to PerCP/Cy5.5	100734	Biolegend
Anti-CD11c antibody (N418) coupled to AF647	117312	Biolegend
Anti-CD19 antibody (6D5) coupled to APC/Cy7	115530	Biolegend
Anti-CD19 antibody (D4V4B)	90176S	Cell Signaling
Anti-CD24 antibody (M1/69) coupled to PerCP/Cy5.5	101824	Biolegend
Anti-CD25 antibody (PC61) coupled to APC	102012	Biolegend
Anti-CD31 antibody (MEC13.3) coupled to AF488	102514	Biolegend
Anti-CD31 antibody **(**390**)** coupled to biotin	102404	Biolegend
Anti-CD31 antibody (SZ31)	DIA 310	Dianova
Anti-CD44 antibody (IM7) coupled to PE	103008	Biolegend
Anti-CD45 antibody (30-F11) coupled to PerCP/Cy5.5	103132	Biolegend
Anti-CD45.1 antibody (A20) coupled to APC-Cy7	110716	Biolegend
Anti-CD45.2 antibody **(**104**)** coupled to AF488	109816	Biolegend
Anti-CD62L (L-Selectin) antibody (MEL-14) coupled to PE-Cy7	25-0621-82	Invitrogen
Anti-CD90.1 antibody (OX-7) coupled to APC-Cy7	202519	Biolegend
Anti-LPAM-1 (Integrin α4β7) antibody (DATK32) coupled to PE	120606	Biolegend
Anti-MAdCAM-1 antibody (MECA-367) coupled to biotin	120706	Biolegend
Anti-Podoplanin antibody (8.1.1) coupled to APC	127410	Biolegend
Anti-Ter119 antibody (M1/69) coupled to PerCP/Cy5.5	116228	Biolegend
Anti-Ki67 antibody (16A8) coupled to AF647	652408	Biolegend

(Continued)

TABLE 1 | Continued

Materials	Catalog #	Company
Commercial kits		
LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit	L34955	Thermo Fisher
Foxp3/Transcription Factor Staining Buffer Set	00-5523-00	eBioscience
$CD4^+$ T cell enrichment kit (Dynabeads™ Untouched™ Mouse CD4 Cells Kit)	11415D	Thermo Fisher
T cell depletion kit (CD90.1 MicroBeads, mouse and rat)	130-121-273	Miltenyi Biotec
Streptavidin, Alexa Fluor™ 750 conjugate	S21385	Invitrogen
Streptavidin, Alexa Fluor™ 546 conjugate	S11225	Invitrogen
Other consumables		
V-bottom 96-well plate		
Normal rat serum	10710C	Thermo Fisher
Evans blue	E2129	Sigma-Aldrich
D-Lucifirin, potassium salt	7903	BioVision
Diphtheria toxin from *Corynebacterium diphtheriae*	D0564-1MG	Sigma-Aldrich
Light sheet fluorescence microscopy		
Fetal bovine serum (FBS)	10099, 10100	Gibco
Paraformaldehyde, granulated	0335.3	Carl Roth
Triton® X 100	3051.4	Carl Roth
n-hexane	139386-500 ml	Sigma Aldrich
Benzyl benzoate	B6630-1 l	Sigma Aldrich
Histology		
Target Retrieval Solution, Citrate pH 6.1 (10x)	S1699	Dako Agilent
VECTASTAIN® Elite ABC-HRP Kit, Peroxidase (Standard)	PK-6100	Vector laboratories
ImmPACT® DAB	SK-4105	Vector laboratories
Mayer´s hemalum for counter-staining	109249	Merck
Equipment		
ISMATEC Reglo analog pump	ISM795C	IDEX Health and Science LLC
Stereo microscope	SZ51	Olympus
Light source	KL1500 LCD	Schott
Isis animal shaver	GT420	Braun
X-ray irradiation source	CP-160	Faxitron
Surgery tool: 2 fine forceps, 1 pair of small scissors, 1 flexible needle holder		Karl Hammacher GmbH and mergo
2 heating mats (20 cm x 30 cm)	76085	Trixie Heimtierbedarf GmbH
Infrared lamp	BF 27	Beurer
Thermometer (dual thermo max/min)	E609790	Amarell Electronic
Centrifuge (Megafuge 40R)		Thermo Scientific
Water bath	WNB 14	Memmert
Attune NxT flow cytometer equipped with 405, 488, 561 and 638 nm lasers and an autosampler		Thermo Scientific
IVIS Spectrum	124262	Perkin-Elmer
Gas anesthesia system for IVIS imaging platform	XGI-8	Perkin-Elmer
Light sheet fluorescent microscope (LSFM)	Home build setup	(37, 38)
Software		
FlowJo	Version X	TreeStar
Imaris	Versions 7.7.2 and 8.1.1	Bitplane
Living Image®	Version 4.0	Perkin-Elmer
Matlab	Version R2016a	Mathworks
Mice		
C57BL/6 (C57BL/6NCrl)	Strain code 027	Charles River
FVB/NCrl (FVB)	Strain code 207	Charles River
FVB.L2G85		Bred in-house
C57BL/6.*Tyrc-2J* (B6 albino)		Bred in-house
C57BL/6.L2G85.CD45.1		Bred in-house
C57BL/6.L2G85.DsRed		Bred in-house
C57BL/6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J	Stock no. 003584	Bred in-house
C57BL/6.Tg(Itgax-DTR/OVA/EGFP)1Garbi	MGI no. 463655	Bred in-house

- FACS buffer: 0.375 g of EDTA and 5 ml of FBS in 500 ml of DPBS.
- FACS blocking buffer: 20% normal rat serum in DPBS.
- Luciferin: 5 g D-lucifirin in 165 ml of 0.9% NaCl (Aqua ad iniectabilia), stored at -20°C.
- 4% PFA solution: 4 g PFA in 100 ml DPBS, dissolved at 65°C, pH: 7.4
- LSFM clearing solution (BABB): 1:2 ratio of benzyl alcohol and benzyl benzoate.

- Enzyme mix for lymph node digestion: RPMI-1640, 0.8 mg/ml Dispase II, 0.3 mg/ml Collagenase P and 0.15 mg/ml DNase I.
- Enzyme mix for small intestine digestion: 10 ml solution (1.5 mg/ml Type VIII Collagenase dissolved with 40 µg/ml of DNase I in pre-warmed HBSS Ca^{2+}/Mg^{2+}, 2% FCS.

STEPWISE PROCEDURES

This protocol focuses on syngeneic mLN transplantation from a C57BL/6 mouse into another C57BL/6 mouse and the subsequent analysis of successful engraftment and the functionality of the transplanted mLN to initiate an immune response in aGvHD inflammatory settings. Therefore, these sections are in greater detail, whereas additional procedures are outlined in brief.

Animal experiments were performed according to project license number 55.2.2-2532.2-1038-9, which was approved by the Regierung von Unterfranken, Würzburg.

Mesenteric Lymph Node Transplantation

We performed mLN transplantation in 8- to 12-week-old C57BL/6 mice from syngeneic donors expressing different reporter transgenes (firefly luciferase or fluorescent proteins) or congenic markers. Preference was given to males over female animals, as they recovered faster from the operation.

Preparation

Donor and recipient were anesthetized with an intraperitoneal (i.p.) injected mixture of Ketamine (80 mg/kg body weight) and Xylazine (16 mg/kg body weight), one day prior to the operation. To allow for sterile conditions at the site of surgery, ventral fur was wetted with 70% ethanol using a dissecting swab, trimmed with a shaver and removed with hair removal cream. Alternatively, mice were anesthetized with 2% isoflurane in O_2 (XGI-8 gas anesthesia system, Perkin-Elmer) during hair removal.

On the day of operation, two sterile working places were prepared: one for the donor mLN isolation and recipient preparation while the other one for the mLN transplantation operation. For each of the spaces, a heating mat was pre-warmed and covered with a sterile OP towel, all needed surgical instruments and reagents were sterilized and laid out.

An operation cover was prepared by cutting an OP towel to a size of 15 cm x 15 cm, and an oval window was cut into the center with a diameter of 1.5 cm x 2.5 cm to be placed on the operation site.

A gauze pad was prepared by folding it to 7.5 cm x 5 cm and incising a 2 cm long central slit.

A clean recovery cage was placed under an infrared lamp, and the sensor of thermometer was placed on the bedding to monitor the local temperature and not let it rise above 30°C to avoid overheating.

Operation and Lymph Node Transplantation

Mice were anesthetized with an i.p. injected mixture of Ketamine (80 mg/kg body weight), Xylazine (16 mg/kg body weight) and Acepromazine (2 mg/kg body weight) (39) dissolved in PBS (200 – 250 µl) and placed on a sterile, heated OP towel for preparation. The animals were subcutaneously (s.c.) injected with the perioperative analgesic Carprofen (5 mg/kg) to relieve pain 30 min before opening the abdominal cavity and every 12 h up to the third day after the surgery if required. Eyes were protected from dehydration by applying eye ointment - Bepanthen® - Augen- und Nasensalbe (Bayer Vital GmbH, Leverkusen, Deutschland). The shaved abdomen was wetted with 70% ethanol using a dissection swab; the skin was disinfected with Braunol® (B. Braun, Melsungen, Germany) in an outward circling motion.

After 10 min, anesthetic depth was ensured to be stadium III.2 (surgical tolerance) by pinching the hind paw (plantar reflex). The dosage of the anesthetic used is usually very well tolerated and leads to a reliable depth of anesthesia up to stage III.2.

If in exceptional cases by checking the plantar reflex, anesthesia depth up to stage III.2 was not achieved, an i.p. additional dose of the anesthetic was provided (20% of the original dose). The volumes can easily be applied accurately using an insulin syringe. For a more precise dosage of the amount of anesthesia, the anesthetic was additionally diluted 1:1 in PBS so that the injection volume doubled.

The prepared animal was transferred onto a fresh sterile and heated OP towel and the prepared OP-cover was applied. A 1-1.5 cm midline incision was made in the skin and in the peritoneum on the *linea alba*, no bleeding should be visible. The wound was covered with the prepared gauze pad, the slit overlaid on the incision. The pad was soaked with PBS to prevent drying of externalized intestinal tissue. Next, two cotton swabs were soaked with PBS and used to handle the intestinal contents. The swabs were inserted into the peritoneum to localize and gently exteriorize the caecum, which is located on the animals' right side to the craniolateral incision. The intestinal tissue was handled with great care to prevent postoperative ileus. First, the caecum was placed on the gauze pad left to the incision, and the intestinal tissue was kept moist at all times by dripping PBS on it using a Pasteur pipette. The large intestine was then gently pulled out, starting from the caecum, until the mLN appeared attached to mesentery. The most distal part of small intestine was also gently pulled out to have visible access to the chain of mLNs and to identify the bottom node next to the caecum (**Figure 1A**).

With the help of fine surgical scissors, the mLNs of the recipient mice were excised from the bottom to the top with minimal fat and connective tissue (**Figure 1B**). Extra care was taken to avoid injuring the superior mesenteric artery lying behind the mLNs. Minor bleeding was stopped by holding the Setpack dissecting swabs (Lohmann & Rauscher, Rengsdorf, Germany) to wounded areas. The donor LN was joined to the mesenteric tissue using a two-component fibrin glue (TISSEEL (Baxter, Höchstadt, Germany): 10 µl of fibronectin (component to TISSEEL) was pipetted on the area from where the recipient mLN was excised. The donor mLNs (isolated with the same procedure as recipient mice) were dipped in thrombin solution (component to fibrin glue) and placed on the wound in the same bottom-to-top-orientation as removed recipient LN: from distal

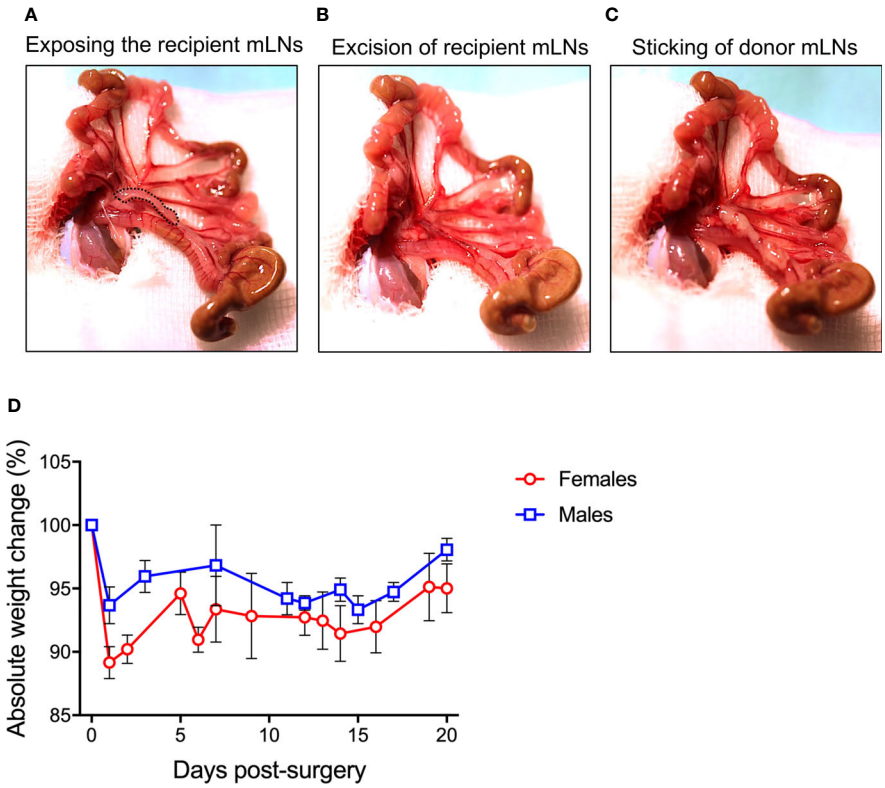

FIGURE 1 | Procedure of mesenteric lymph node (mLN) transplantation. **(A)** Peritoneal cavity of the mice was carefully opened and the bowel along with the caecum taken out and laid on DPBS-wetted gauze pad to make all the mLNs lobes visible. **(B)** All the lobes of recipient mLNs were excised out avoiding bleeding from the superior mesenteric artery lying behind the mLNs **(C)** 10 µl of fibronectin were pipetted on the scar, donor mLNs soaked in thrombin were carefully placed on the scar and incubated for 3-5 min before internalizing the complete bowel for subsequent suturing. **(D)** Absolute weight change of male and female mice undergoing surgical intervention.

(from the caecum) to the proximal region (towards the ileum) (**Figure 1C**). For firm adhesion, the glue was allowed to polymerize for 3-5 min. Subsequently, the small and large intestine along with the caecum were carefully placed back into the peritoneum. The repositioning in the correct orientation of intestinal loops was important to prevent a postoperative ileus. The peritoneum was closed with three non-consecutive stitches with coated VICRYL® (polyglactin 910) suture (Ethicon, Dülmen, Germany). Subsequently, the skin was closed with four stitches. Bepanthen® Wund- und Heilsalbe (Bayer Vital GmbH, 51368 Leverkusen, Deutschland) was applied on the closed surgical wound. The overall operation procedure took 15-25 min per animal. The mice were placed into a clean cage and held warm with an infrared lamp until recovered from anesthesia. The drinking water was supplemented with Baytril (Enrofloxacin, 0.05%) for 7 days after surgery to avoid infections.

Lymphatic Drainage Assay

Mice were anesthetized with an i.p. injected mixture of Ketamine (80 mg/kg body weight) and Xylazine (16 mg/kg body weight) and Acepromazine (2 mg/kg) (39) dissolved in PBS (200 – 250 µl) and placed on a sterile, heated OP towel for preparation. The animals were s.c. injected with the perioperative analgesic Carprofen (5 mg/kg) to relieve pain 30 min before opening the abdominal cavity. To evaluate the lymphatic drainage from the bowel into the transplanted mLNs, 5 µl Evans blue dye (2% in DPBS) was injected into PPs of the ileum using 33-gauge needle with a Hamilton syringe under a SZ51 stereo microscope (Olympus, Hamburg, Germany) by gently holding the intestine with blunt forceps. Five min after injection, mLNs were photographed for Evans blue drainage.

Preparation of Lymph Nodes for Light Sheet Fluorescent Microscopy (LSFM)

Mice were anesthetized with an i.p. injected mixture of Ketamine (80 mg/kg body weight) and Xylazine (16 mg/kg body weight) and Acepromazine (2 mg/kg) (39) dissolved in PBS (200 – 250 µl) and placed on a sterile, heated OP towel for preparation. The animals were s.c. injected of the perioperative analgesic Carprofen (5 mg/kg) to relieve pain 30 min before opening the abdominal cavity. For blood vessels staining, mice were retro-orbitally injected with 20 µg of anti-CD31 biotin antibody in 100 µl of DPBS, after 20 min mice received 10 µg of Streptavidin, Alexa Fluor™ 750 conjugate in 100 µl of DPBS.

After 20 min, anesthetic depth was ensured to be stadium III.2 (surgical tolerance) by pinching the hind paw. If anesthesia was insufficient, further anesthetic was provided retro-orbitally. Not more than 20-50 µl were injected at a time to avoid over-dosage. Prior to mLN extraction, mice were perfused for 2 min with DPBS and 8 min with paraformaldehyde (PFA) 4%. mLNs were further fixed for 2 hours in 4% PFA and washed with DPBS three times with 30 min incubations before processing at 4°C. The mLNs were blocked overnight in DPBS containing 2% FBS, 0.2% Triton X100. To stain for high endothelial venules (HEVs): Anti-MAdCAM-1 antibody (MECA-367) coupled to biotin at a dilution of 1:100 was used. Samples were incubated for 24 hours with gentle shaking at 4°C. On the next day, samples were washed with DPBS three times with 30 min incubations at 4°C and then incubated with Streptavidin, Alexa Fluor 546™ conjugate for 24 hours with gentle shaking at 4°C. After staining, mLNs were washed with DPBS three times with 30 min incubations at 4°C and dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%) for 90 min each at room temperature and in 100% overnight at 4°C. The following day, the samples were rinsed for 2 hours in 100% *n*-hexane; subsequently n-hexane was replaced stepwise by LSFM clearing solution. Special care was taken at this step to strictly avoid air exposure of the samples. mLN samples became optically transparent and suitable for LSFM imaging after incubation in the LSFM clearing solution for at least 2 hours at room temperature (30).

LSFM Setup and Data Acquisition

The LSFM modular setup is home-built providing four excitation lines of 491, 532, 642 and 730 nm as described elsewhere (37). Images acquired by LSFM were analyzed on IMARIS software v8.1.1 (Bitplane AG, CA, USA). When required, background subtraction was applied in accordance with the diameter of the cell population to eliminate unspecific background signals.

Allogeneic Hematopoietic Cell Transplantation

Donor T Cell Enrichment

Naïve T cells were enriched from the spleen of 8- to 12-week-old FVB.L2G85 (H-2^q) donor mice (28, 40), expressing firefly luciferase and congenic cell markers (CD90.1 and CD45.1). Splenocytes were enriched for T cells with (Dynabeads™ Untouched™ Mouse CD4 Cells Kit, Thermo Fisher) according to the manufacturer's protocols, counted by trypan blue exclusion, stained with Cell trace CFSE according to manufacturer protocol. Typically, T cell yields ranged between 15 to 30% of total splenocyte input with a final T cell purity of 90 to 97%.

Donor Bone Marrow Isolation and T Cell Depletion

Bone marrow cells were isolated from the hind limbs (femur and tibia) of 8- to 12-week-old wildtype donor FVB/NCrl mice. Cell numbers were determined by trypan blue exclusion and depleted of T cells by utilizing Thy 1.1 enrichment kit (Thermo Fisher), following manufacturer's protocol. Typically, bone marrow cell yields ranged between 1 – 1.5 x 10^8 cell per donor mouse, while T cell depletion purity was between 95 to 98%.

Allogeneic Hematopoietic Cell Transplantation

Untreated C57BL/6 or C57BL/6 mice transplanted with mLNs were myeloablatively irradiated (9 Gy), and 1.2 x 10^6 FVB.L2G85 donor T cells were intravenously injected *via* the retro-orbital venous plexus together with 5 x 10^6 T cell-depleted bone marrow cells in a total volume of 200 µl. The drinking water was supplemented with Baytril (Enrofloxacin, 0.05%) for 7 days after transplantation to avoid infections. aGvHD was scored clinically and body weight was assessed daily.

Diphtheria Toxin Mediated Cell Depletion

For DCs depletion in B6a.CD11c.DOG donor mice prior to the isolation of mLNs, animals were injected intraperitoneally (i.p.) with diphtheria toxin (Sigma-Aldrich, Hamburg, Germany) at doses of 20 ng/g body weight on day -5, -3, and -1 prior to the day of surgery.

Histology

Formalin-fixed, paraffin-embedded (FFPE) specimens were processed for hematoxylin and eosin staining (H&E) as previously described (41) and immunohistochemical assessment of mLN architecture. Briefly, 1 µm paraffin sections were deparaffinized and rehydrated in graded ethanol. Antigen retrieval was performed in a steam cooker (Biocarta Europe, Hamburg, Germany) at 120°C for 2.5 min using a commercially available cooking buffer: target retrieval solution, citrate pH 6.1 (Catalog #, S1699, Dako Agilent). Primary antibodies, anti-CD3 (1:200, rabbit, monoclonal, clone SP7, Catalog # RBG024, Zytomed), anti-CD19 (1:500, rabbit, monoclonal, Catalog # 90176S, Cell Signaling) and anti-CD31 (1:200, rat IgG2a, monoclonal, Catalog # 15219/01, Dianova), were added over night at room temperature. After washing biotinylated secondary antibodies were incubated for 30 minutes at room temperature (all 1:500). For detection ECTASTAIN® Elite ABC-HRP kit, peroxidase (standard), catalog # PK-6100 and ImmPACT® DAB substrate, Catalog # SK-4105, (both Vector Laboratories) were used and Mayer´s hemalum for counter-staining (Catalog # 109249, Merck). Images were taken with Zeiss Axio imager A1 at 100x original magnification.

Bioluminescence Imaging

In vivo bioluminescence imaging was performed with an IVIS Spectrum CCD-imaging system (Perkin-Elmer). Mice were anesthetized by injecting 10 µl of anesthetic solution (Ketamine and Xylazine) per gram of body weight intraperitoneally. D-Luciferin was injected in a concentration of 150 µg/g body weight and images were taken 10 min after the injection which allowed the identification of T cell proliferation and migration.

To perform *ex vivo* imaging mice were injected with the same mixture of anesthetic and D-Luciferin. 10 min after injection mice were euthanized and organs were removed within 4 min.

Ex vivo images provided higher resolution of selective organ signal distribution. Bioluminescence imaging data was analyzed on Living image® 4.5.5 software.

Isolation of Cells

Day 3- and 6- after allo-HCT mice were sacrificed, and the mLNs, and small intestine were harvested for analysis.

Cell Isolation From Lymph Nodes

Cells from the LNs were isolated using a protocol modified from Fletcher and colleagues (42). mLNs were pierced with a syringe needle 2 – 3 times and directly transferred into a centrifuge tube containing 2 ml RPMI-1640 medium on ice for further processing.

The RPMI-1640 medium was replaced with 2 ml freshly prepared enzyme mix containing 0.8 mg/ml Dispase II, 0.3 mg/ml Collagenase P and 0.15 mg/ml DNase I in RPMI-1640 medium.

Tubes were incubated at 37°C in a water bath for 20 min and gently inverted several times at 5 min intervals. To carefully disrupt the capsule after the first incubation step, the cell suspension was gently resuspended with a 1 ml pipette. Tissue pieces were allowed to settle down for 1 min and subsequently released leukocytes in the cell suspension were transferred into a new tube containing 10 ml FACS-buffer stored on ice.

2 ml of fresh enzyme-mix were added to the remaining mLNs pieces and mixed with a 1 ml pipette and again incubated for 10 min in a water bath at 37°C. After incubation, the cell suspension was again gently resuspended and released cells were transferred to the tube containing the previous supernatant in ice-cold FACS-buffer. For the last time, 2 ml of digestion-solution were added to the residual tissue fragments and this time vigorously mixed every 5 min using a 1 ml pipette until all fragments were digested. Collected supernatants, that have been stored on ice, were centrifuged at 400 g, 4°C for 5 min and counted.

Cell Isolation From Small Intestine

T cells from the small intestine were isolated as described before (43). Small intestinal tissue from the stomach to caecum was excised. Fat and mesenteric tissue was cleared from the intestine, Peyer's patches were removed, and intestine was washed with PBS. After removal of all fecal material, intestine pieces were cut into 2 cm pieces and transferred to a 50 ml centrifuge tube containing 20 ml medium 2 - HBSS medium (Ca-/Mg-, 2 mM EDTA, 5% FCS) and incubated for 20 min at 37°C with gentle rotation (100 RPM).

After incubation, gut pieces were passed through a 70 µm cell strainer over a new 50 ml centrifuge tube. The flow-through, containing the intraepithelial cells were stored on ice. Remaining gut pieces were transferred back into the tube containing medium 2 and again incubated for 20 min in a thermal incubator at 37°C with gentle rotation (100 RPM). This step disrupted the remaining intraepithelial fraction form the underlying mucosa. After incubation, gut pieces were again passed through a 70 µm cell strainer and pooled with the first fraction. This cell fraction contained the gut intraepithelial lymphocytes (IELs). IELs fraction was centrifuged at 400 g for 5 min at 4°C, resuspended in appropriate volume and counted.

To further process *lamina propria*, the remaining intestine pieces on the cell strainer were transferred onto a plastic weighing pan and reduced to pulp using fine dissection scissors. The gut-pulp was transferred to a new 50 ml centrifuge tube containing 10 ml of digestion solution: 1.5 mg/ml Type VIII Collagenase (Sigma, Hamburg, Germany) dissolved with 40 µg/ml of DNase I (Sigma, Hamburg, Germany) in pre-warmed HBSS Ca^{2+}/Mg^{2+}, 2% FCS) and incubated at 37°C for 20 min under vigorous rotation (200 RPM). Following incubation, cell suspension was vortexed for 30 secs and passed through a 100 µm cell strainer (Miltenyi Biotec, Gladbach, Germany). The cell strainer was rinsed with 30 ml of ice-cold Ca^{2+}/Mg^{2+} HBSS containing 10% FCS as washing-buffer and centrifuged at 500g for 10 min at 20°C. Cell pellet was resuspended in 10 ml PBS for counting.

Flow Cytometry

Up to 1 x 10^6 cells were stained per well in 96-well v-bottom plates. Cells were resuspended in 100 µl of blocking solution and incubated for 5 min at 4°C. 100 µl of antibody mix were added and cells were stained for 30 min at 4°C in the dark. Cells were pelleted at 400 g for 5 min 4°C and resuspended with FACS buffer for acquisition on the flow cytometer.

For intracellular staining cells were fixed, permeabilized and stained with Foxp3/Transcription Factor Staining buffer set (eBioscience) following the manufacturer's protocol.

Statistical Analysis

Data are shown as mean ± standard deviation (SD). Different groups were compared by two-tailed unpaired student's t-tests using GraphPad Prism 8 software (La Jolla, CA, USA). Level of significance was set at $p < 0.05$.

RESULTS

Donor mLNs Sustain Functionality After Surgical Transplantation

Mice undergoing surgical transplantation of mLNs lost approximately 10% of body weight post-surgery, however they recovered 5% body weight within five days (**Figure 1D**). First, we determined the viability of mLNs after surgical transplantation, in this set of experiments mLNs from B6.L2G85.CD45.1 expressing firefly luciferase were transplanted into congenic B6 albino mice. The bioluminescence signal from the transplanted cells was initially measured at day 14 post-surgery and sustained signal was acquired even until 5 weeks (35 days) post-surgery (**Figure 2A**). The decrease in bioluminescence signal can be explained by the efflux of hematopoietic cells from the LN *via* vascular and lymphatic system subsequent to successful engraftment.

To assess the functionality of the donor mLN and its connection with the vascular system of the mouse, we employed LSFM and stained the blood vessels with CD31. The vascularization of blood vessels in the transplanted mLNs as

measured by the length, diameter and density was comparable to the mLNs from wild-type mice (**Figure 2B**), suggesting adequate neovascularization and adjoining of small blood vessels to the mesenteric artery after surgery. To determine if the transplanted mLNs retained a functional post-capillary venous system, we performed 3D-LSFM imaging of mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which revealed HEVs length and diameter was not altered in the surgically transplanted mLNs however density of HEVs in the transplanted LNs was significantly reduced (**Figure 2C**). In conclusion, these findings reveal that the transplanted mLNs retain physiological vascular function after surgical transplantation.

Transplanted mLNs Retain Normal Morphology and Drain Lymph From the PPs

To evaluate the morphology and immune architecture of the transplanted mLNs, we performed H&E stainings. We observed that the lymph node architecture was preserved in transplanted mLNs similar to non-transplanted mLNs: $CD19^+$ B cells distributed regularly and accentuated in follicles in the cortex whereas $CD3^+$ T-cells predominantly located in the paracortical area. In both, transplanted and non-transplanted mLNs displayed some germinal centers (**Figure 3A**). CD31 staining revealed no obvious differences in the vasculature, moreover

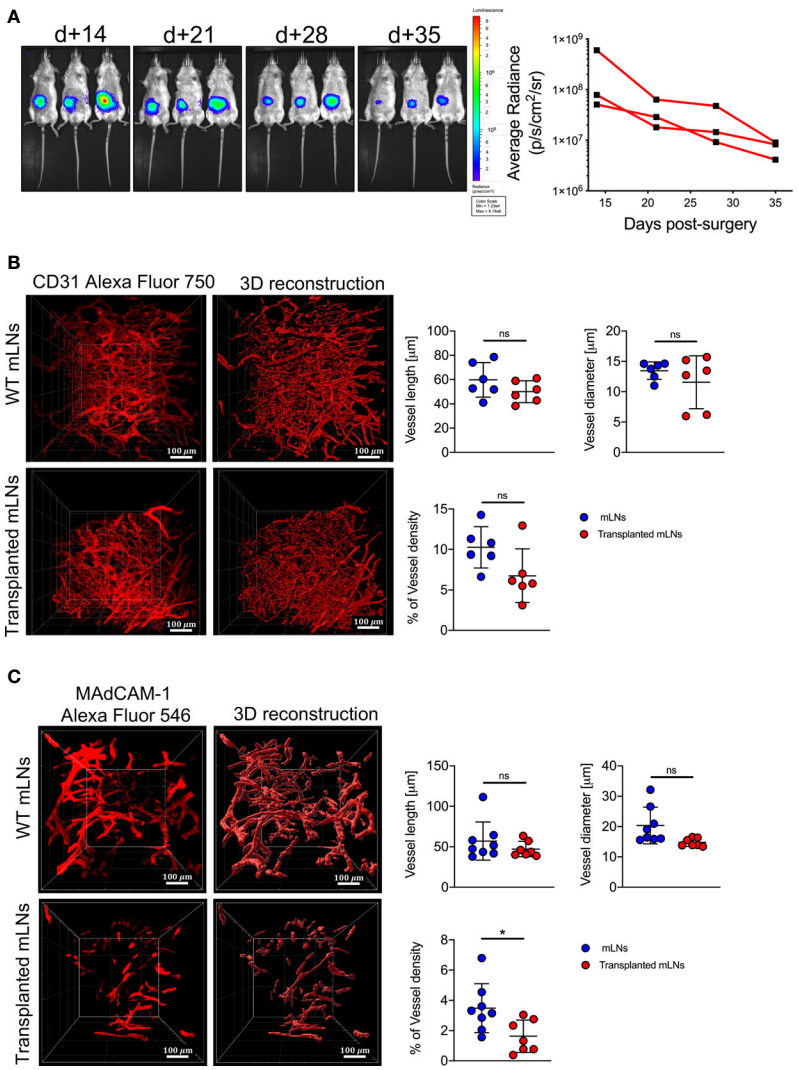

FIGURE 2 | Donor mLNs engrafted in the recipient mice after surgical transplantation. **(A)** Bioluminescence signals emitted from luc^+ mLNs from B6.L2G85 mice transplanted into a B6 albino recipient mouse and quantification (p/s/cm^2/sr) of BLI signal from donor luc^+ mLNs. **(B)** Zoomed LSFM images of blood vessels staining with anti-CD31 antibody and 3D reconstruction (20x objective, scale bar = 100 µm) and quantification of blood vessel length, diameter and density were analyzed from reconstructed images on Imaris, 5 weeks after mLN transplantation. **(C)** Zoomed LSFM images of HEVs staining with anti-MAdCAM-1 antibody and 3D reconstruction (20x objective, scale bar = 100 µm) and quantification of HEVs vessel length, diameter and density were analyzed from reconstructed images on Imaris, 5 weeks after mLN transplantation. Each data point represents one mouse, data pooled from two experiments; unpaired non-parametric Mann-Whitney test, (Mean± SD); *p < 0.05; ns, not significant.

CD31 staining on LECs in the subcapsular sinus revealed intact structure of the mLN capsule, which is necessary for the lymphatic drainage from afferent lymphatics to the medullary sinuses. As the only remarkable difference we observed signs of fibrosis of the mLN capsule in transplanted animals as opposed to untreated animals (**Figure 3A**).

To test, whether functional lymphatic anastomoses formed in transplanted mLNs, we evaluated lymphatic drainage from the small intestines. To visualize lymphatic vessels and to assess efficient lymphatic drainage we injected Evan's blue into the jejunal and iliac Peyer's patches, which drained to the transplanted mLNs (**Figure 3B**). Staining of lymph vessels and immediate drainage revealed Evans blue labeled to mLNs indicating that functional lymphatic vessel anastomoses had formed with transplanted lymph nodes.

Donor mLNs Are Populated by Recipient Hematopoietic Cells After Transplantation

To address whether donor mLNs would be colonized by donor hematopoietic cells or by recipient hematopoietic cells after surgical transplantation, we transplanted mLNs from B6.L2G85.CD45.1 into congenic B6.WT (CD45.2) mice. Five weeks post-surgery the animals were euthanized and analyzed for the CD45.1$^+$ and CD45.2$^+$ population in mLNs by flow

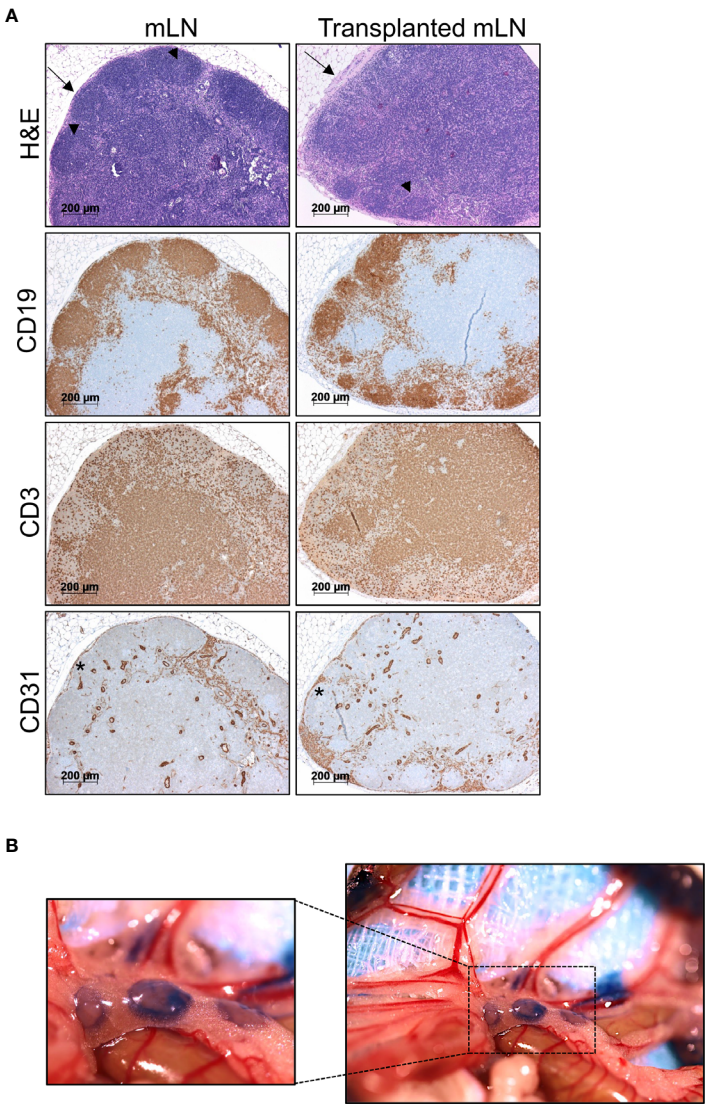

FIGURE 3 | Transplanted mLNs retain normal morphology, cell distribution and lymphatics. **(A)** H&E stainings of transplanted vs. non-transplanted mLNs reveal a regular immune architecture with B cell follicles and some germinal centers (arrowheads) in both settings in the cortex and T cell zones (top). Lymph node capsules (arrow) display signs of capsule fibrosis in transplanted mLNs. Immunohistochemical analysis of CD19$^+$ B cells confirm proper B cell follicles in the cortex and distribution of CD3$^+$ T cells dominating in the paracortical areas. CD31 staining on LECs in the subcapsular sinus (asterisk) revealed an intact structure of the mLN capsule, 5-6 weeks after mLN transplantation. **(B)** Donor mLNs lymph vessels conjoin to recipient's intestinal lymphatic vasculature after transplantation. Evans blue injected into the iliac Peyer's patches drains into the transplanted mLNs, 5 weeks after mLN transplantation.

cytometry **(Figure 4A)**. The vast majority of hematopoietic cells in the transplanted mLNs (95.4%) were of host (CD45.2) origin when compared to 0.47% of donor (CD45.1) origin. We could not detect CD45.1$^+$ myeloid cells (CD11c$^+$MHCII$^+$), however we detected very few remaining CD45.1$^+$ T cells (CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$) in the transplanted mLNs **(Figure 4B)**. The proportion of host hematopoietic cells found in the transplanted mLNs was relatively similar to the frequency in untreated C57BL/6 mice **(Figure S1)**.

To further probe the origin of non-hematopoietic LN stromal and endothelial cells we employed transgenic mice that ubiquitously expressed a red fluorescent reporter gene and transplanted mLNs from these B6.DsRed donor mice into a B6.WT recipients. After successful engraftment we could confirm fibroblastic reticular cells (FRCs), lymph endothelial cells (LECs) and blood endothelial cells (BECs) **(Figure S2A)** based on their emission of DsRed protein fluorescence supporting that they originated from the donor mLNs **(Figure 4C).**

In summary, transplanted mLNs were entirely repopulated with recipient hematopoietic cells while retaining LNSCs of donor origin within five weeks of transplantation.

Transplanted mLNs Sustain Normal T Cell Activation and Proliferation

To assess whether transplanted mLNs are fully functional and can initiate an adaptive immune response, we employed a murine model of aGvHD. After allo-HCT, we evaluated alloreactive T cell activation and proliferation in mice with and

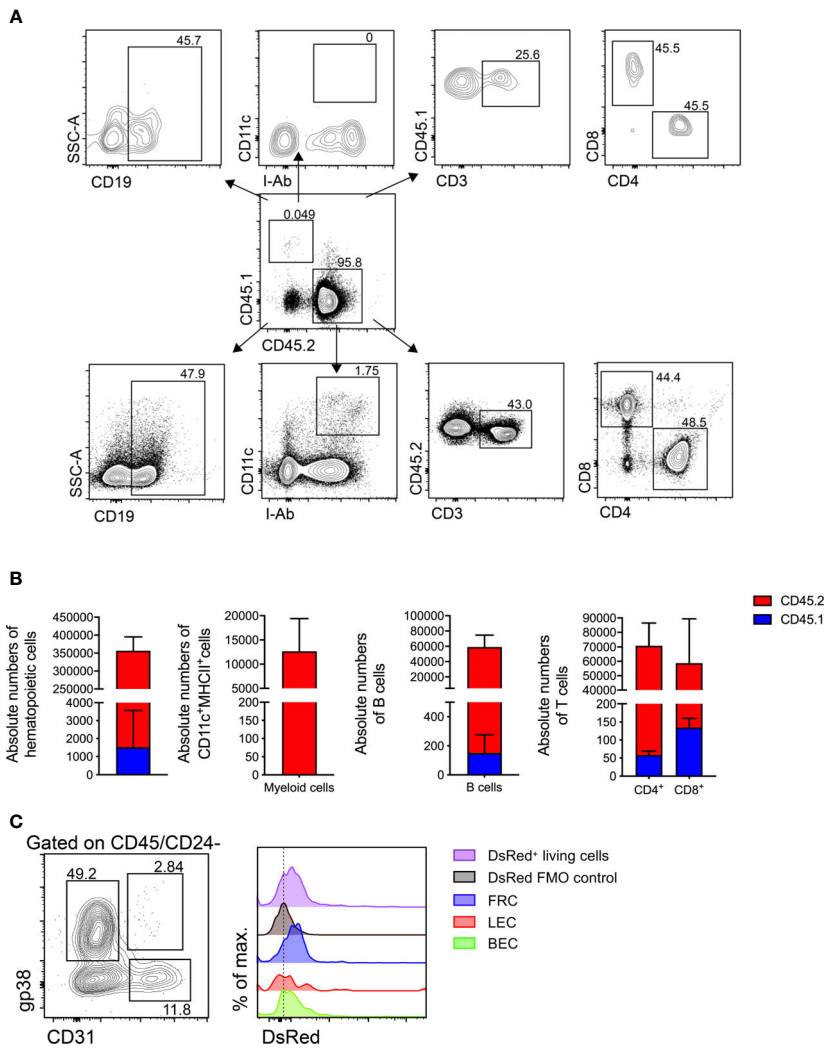

FIGURE 4 | Donor mLNs are repopulated with recipient hematopoietic cells after surgical transplantation. **(A)** Flow cytometry gating of hematopoietic cells in mLNs from B6.CD45.1 mouse transplanted in a B6.CD45.2 mouse. **(B)** Absolute numbers of hematopoietic cells, myeloid cell (dendritic cells and macrophages), B cells and CD3$^+$ pre-gated CD4$^+$ and CD8$^+$ T cells in CD45.1$^+$ and CD45.2$^+$ sub-populations, 5 weeks after mLN transplantation. Data pooled from five mice analyzed in two experiments. **(C)** Flow cytometry gating of non-hematopoietic LNSCs and DsRed fluorescence signal from B6.DsRed mLNs transplanted in a B6.WT mouse, with DsRed$^+$ living cells from a DsRed$^+$ mouse as positive and living cells from a WT mouse serving as negative controls. Analysis performed 5 weeks after mLN transplantation. Error bars represent Mean± SD.

without previously transplanted mLNs **(Figure 5A)**. In the initiation phase of aGvHD (day 3 of allo-HCT) (28), alloreactive donor $CD4^+$ T cells as gated in **(Figure S2B)** expanded in the transplanted mLNs and upregulated the expression of T cell activation markers CD44 and CD25 **(Figure 5B)**, the donor $CD4^+$ T cells in the transplanted mLNs upregulated Ki67 protein, indicating active proliferation comparable to WT group **(Figure 5C)**.

Day 6 of allo-HCT is considered as an effector phase of aGvHD, at a time-point at which the alloreactive T cells already infiltrate the aGvHD target organs (28). At this time point we detected similar BLI signal intensities derived from $luciferase^+$ donor T cells and absolute number of alloreactive $CD4^+$ T cells **(Figure S3A)**, indicating extensive alloreactive $CD4^+$ T cell expansion in transplanted mLNs **(Figure S3B)** and other aGvHD target organs, similar to allo-HCT control recipients without previous mLN transplantation. Furthermore, donor $CD4^+$ T cells upregulated T cell activation maker CD44 and intestinal homing receptor integrin $\alpha4\beta7$ **(Figure S3C)** required for the T cell migration into the intestine and functional proliferation as measured by the upregulation of Ki67 **(Figure S3D)**. Moreover, allogenic $CD4^+$ T cells differentiated into effector, effector memory and central memory T cells based on differential expression of CD44 and CD62L (L-Selectin) **(Figure S3E)**. At the same time-point we evaluated the activation status of allogeneic donor $CD4^+$ T cell infiltrating the small intestines, an organ whose immune response is especially regulated by mLNs. Likewise, here we could not detect any differences in the allogenic $CD4^+$ T cell activation and expansion that might be a result due to surgically transplanted mLNs **(Figures S4A–C)**.

Next, we asked whether non-hematopoietic cells of the mLNs can prime allogenic $CD4^+$ T cells in the absence of professional hematopoietic antigen presenting cells (APCs). To this end we isolated mLNs from CD11c.DOG mice that had been depleted of $CD11c^+$ cells by the administration of diphtheria toxin (DTx) **(Figure S5)** and surgically transplanted these mLNs into a complete MHCII deficient animal ($MHCII^{\Delta}$). Subsequently we transplanted allogeneic $MHCII^{\Delta}$ TCD BM and FVB $CD4^+$ T cells into these $MHCII^{\Delta}$ mice harboring transplanted MHC competent CD11c.DOG mLNs as well as $MHCII^{\Delta}$ recipients without transplanted mLNs **(Figure 5D)**. In $MHCII^{\Delta}$ mice harboring transplanted MHC competent mLNs we observed priming of allogeneic donor $CD4^+$ T cells that differentiated into effector/effector memory T cells ($CD44^{high}CD62L^-$). In contrast, allogeneic donor $CD4^+$ T cells mostly displayed a naïve ($CD44^-CD62L^{high}$) and central memory ($CD44^{high}CD62L^{high}$) phenotype on day +13 of allo-HCT **(Figure 5E)**.

Taken together these experiments reveal the transplanted mLNs were able to induce an effective T cell response under inflammatory conditions, even in MHCII deficient hosts.

DISCUSSION

Strategically positioned between the layers of the mesentery, mLNs locate in the center of the GIT and their dysfunction (44) or lymphadenectomy (45, 46) disrupts gut immunity. In rats, it has been demonstrated that mLNs excision protects from lethal GvHD (47, 48). Others have shown that LN transplantation can decrease pathology of lymphedema (49–51).

Here we detailed a protocol of mLN transplantation and show that this surgical procedure is a feasible method to study the immune system of the gut in steady-state or under inflammatory conditions. Transplanted mLNs are engrafted into the host mesenteric tissue within a few weeks after surgery, achieving a functional mesenteric vascular and lymphatic system.

We surgically removed all mLNs from healthy C57BL/6 mice and replaced them with mLNs from different donor mice depending on the experimental question. It is to be pointed out that here we used healthy mice as recipients, nevertheless mice already under experimental intervention can be used as recipients. However, care has to be taken as the surgical procedure is quite intensive and might result in animal mortality during or after the surgical procedure. Extended troubleshooting guideline are displayed in **Table 2**.

The transplanted mLNs in the recipient mice were viable after surgical procedure, which we confirmed with non-invasive bioluminescence imaging detecting the emission of signals from luciferase-transgenic donor mLNs. The transplanted mLNs retained physiological vascular and lymphatic function, suggesting the adjoining of blood vessels of the donor mLNs to the mesenteric artery and connection of the transplanted mLNs lymphatics vessels to the lymphatic system of the intestine within weeks after surgery. This was consistent with the report on axillary LN transplantation by Aschen and colleagues (49). However, we observed a reduction in HEVs density in transplanted mLNs when compared to endogenous non-transplanted mLNs. This could be attributed to a certain degree of vessel atrophy, as in our described procedure vessels from the host were not surgically conjoined to the donors rather, they joined spontaneously after resorption of fibrin glue during the engraftment phase. Furthermore, the transplanted mLNs maintained normal morphology with intact B cell follicles and T cell zones. However, we observed fibrosis on the capsule of transplanted mLNs, which could be explained by the deposition of fibrin glue during the surgical mLN transplantation procedure. Importantly, transplanted mLN retained their full capacity of lymphatic drainage from the intestinal tract. In line with our observations, others have shown that LN transplantation induces lymphangiogenesis, however lymphatic vessels induced by LN transplantation are abnormal in appearance but are functional and are able to transport lymph fluid and also cells (53).

Within a few weeks after surgical transplantation, the host hematopoietic cells of different lineages populated the transplanted mLNs, the efflux of donor derived hematopoietic cells resulted in the decrease of bioluminescence signal emitted by luc^+ donor cells over the course of observation. The residual bioluminescence signal that we acquired stemmed obviously from the tissue resident stromal and endothelial cells of the transplanted mLNs. Hammerschmidt and colleagues also demonstrated that donor non-hematopoietic stromal and endothelial cells survive the surgical transplantation and that

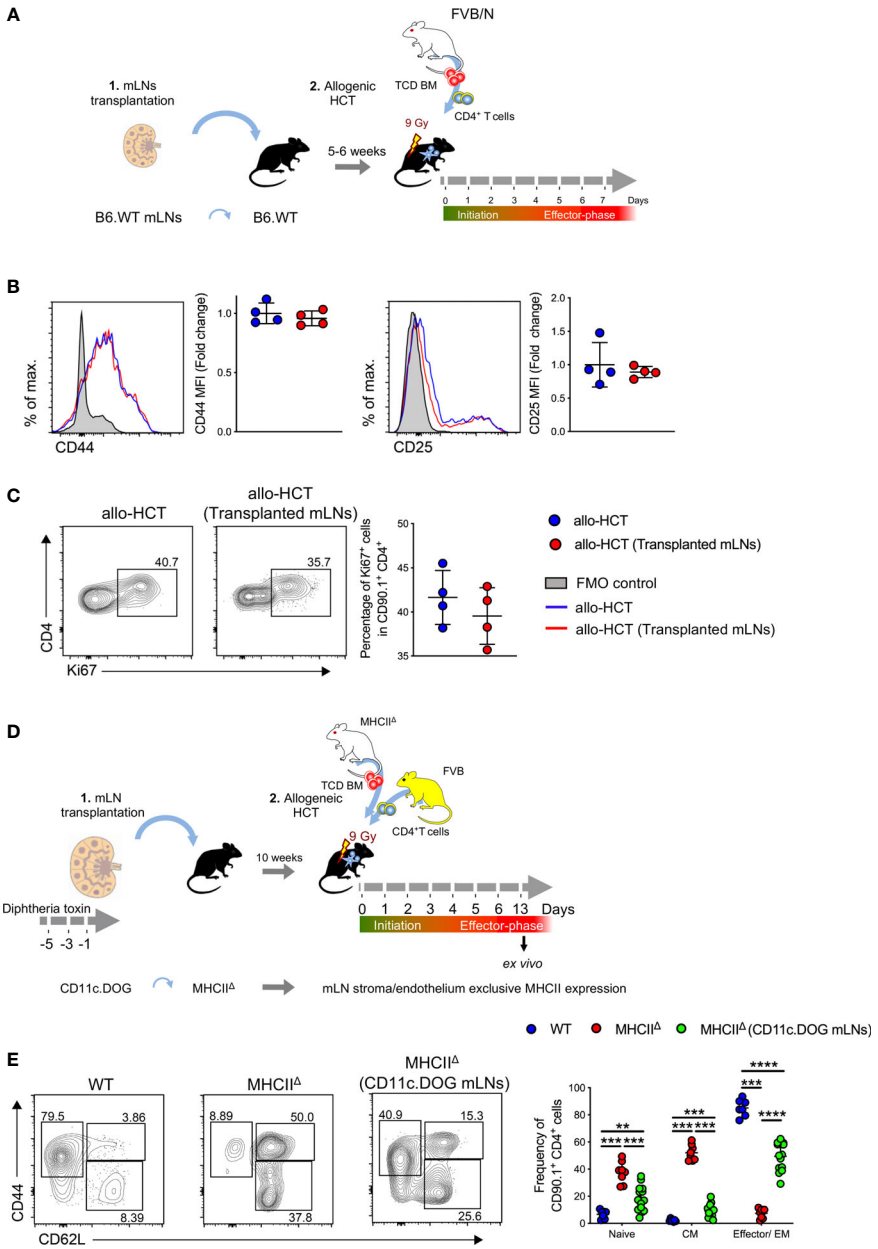

FIGURE 5 | Alloreactive CD4$^+$ T cells are robustly activated in the transplanted mLNs under aGvHD inflammatory conditions. **(A)** Experimental strategy: mLNs from B6.WT mice were transplanted into B6.WT recipient; 5-6 weeks post-surgery; MHC major mismatch aGvHD was induced by lethal irradiation (9 Gy) and allogenically transplanting with 5x10^6 TCD BM cells and 5x10^6 CD4$^+$ T cells from FVB/N mice. **(B)** Mean fluorescence intensity (MFI) of CD44 and CD25 on donor T cells d+3 of allo-HCT. **(C)** Proliferative capacity of donor CD4$^+$ T cells were analyzed by expression of Ki67 d+3 of allo-HCT. **(D)** Experimental strategy: mLNs of B6.MHCII$^\Delta$ mouse were surgically removed and transplanted with donor mLNs from B6.CD11c.DOG mice that had been depleted of CD11c$^+$ cells by i.p. administration with diphtheria toxin 20 ng/gram body weight day -5, -3, and -1 day before surgery. 10 weeks post-surgery MHCII$^\Delta$ (CD11c.DOG mLNs), untreated B6.WT and MHCII$^\Delta$ mice were myeloablatively irradiated with 9 Gy and i.v. transplanted with 5x10^6 T cell-depleted (TCD) BM and 5x10^6 CD4$^+$ T cells from MHCII$^\Delta$ and FVB mice respectively. **(E)** Analysis for T cell subsets - CD44 and CD62L (pre-gated on CD4$^+$CD90.1$^+$) on day +13 of allo-HCT from mLNs. Each data point represents one mouse, data pooled from two experiments; unpaired non-parametric Mann-Whitney test, (Mean± SD); $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

donor- and host-derived stromal cells were unevenly distributed in the transplanted LNs (7).

Whether to maintain peripheral tolerance under steady-state conditions or to mount an effective immune response under inflammatory conditions, different immune cells interact and are activated in a highly regulated microenvironment of secondary lymphoid organs. To assess the functional capacity of transplanted mLNs to induce an efficient adaptive immune cell response, we

TABLE 2 | Troubleshooting guide.

Step	Problem	Possible reason	Solution
Anesthesia	Mice reacts to footpad pinching	Anesthesia underdosed	Slowly add small doses of anesthetic i.p. until desired anesthetic depth is achieved
	Mice take longer than 1 h to recover from anesthesia	Anesthesia overdose	Do not use more anesthesia than needed to reach desired anesthetic depth
		Low body temperature	Monitor the body temperature with a rectal probe. Place the animal in its cage in front of infrared lamp (but avoid overheating)
Lethality up to 24 hr after operation despite recovery from anesthesia	Post-operative ileus	Rough handling of the bowel	Handle bowels gently and with care. Always use pre-wetted cotton swabs to handle the bowels. Refrain from pinching the bowl with forceps. Do not apply pressure to the bowel. Keep bowel lubricated at all times
	Intestinal bleeding	Aneurysm at the site of mLN removal	Care should be practiced not to rupture/cut blood vessels while removing the host mLNs. If bleeding occurs, quickly stop the bleeding by holding Setpack® size 2 at the site of bleeding until coagulation occurs. Inject 200 – 300 µl solution of 0.9% NaCl s.c. to maintain blood volume
	Peritonitis	Introduction of infection during the operation procedure	Work aseptically according to (52)
		Introduction of infection after wound closure	Suture both peritoneum and the skin sufficiently
Donor mLNs placement into recipient mesenteric tissue	Intestinal loops stuck together with fibrin glue	Excessive use of fibrin glue	Pipette minimal amount of fibronectin at the site of removed mLNs

employed a murine allo-HCT model that resulted in allogeneic T cell activation and their migration to the target tissues in aGvHD. Donor $CD4^+$ T cells effectively migrated from the blood to the transplanted mLNs in the initiation phase of aGvHD (day 3) and were activated and proliferated comparably to that in the mLNs from untreated animals. Furthermore, we observed similar $CD4^+$ T cell activation and upregulation of gut homing receptors on $CD4^+$ T cells in the transplanted mLNs indicating robust T cell priming, which was ensued by effector T cell infiltration in the *lamina propria* of the small intestine without time delay in the effector phase of aGvHD (day 6) (28–30). Taken together, these findings suggest that orthotopic syngeneic mLNs transplantation did not affect subsequent allogenic T cell activation under inflammatory conditions of aGvHD.

In recent years, a crucial role of non-hematopoietic APCs in aGvHD has emerged as it has been shown that DCs or B cells are dispensable in the initiation of aGvHD and allogenic activation of $CD4^+$ T cells (54–56). Moreover, it was recently proposed that intestinal epithelial cells have the capacity to provide allo-antigen to allogenic $CD4^+$ T cells (57). Here, we transplanted mLNs depleted of $CD11c^+$ APCs into $MHCII^{\Delta}$ mice and observed allogenic $CD4^+$ T cells priming within these mLNs. Notably, our data suggest that non-hematopoietic LNSCs in mLNs can provide priming signals to allogenic $CD4^+$ T cells under aGvHD like inflammatory conditions. However, it still remains to be determined, which subtype of LNSCs can initiate alloreactive $CD4^+$ T cell responses.

In contrast to previous LN transplantation studies on axillary LNs (49), popliteal LNs (8) and inguinal LNs (58), the use of fibrin glue served as a biological adhesive in our study and supported the engraftment of donor mLNs into the mesenteric tissue. Different nodes of mLNs drain from the jejunum, ileum and the colon (17–19) but in this study we did not elucidated if the orthotopically transplanted mLNs retained the similar lymph drainage pattern of distinct intestinal segments after surgical transplantation.

In conclusion, here we provide an elaborate protocol along with the necessary assays to perform successful mLNs transplantation in mice. This protocol can be used to study the role of resident LN stromal cells of mLNs in homeostasis (8, 12) and inflammatory conditions (6) and their interaction with the hematopoietic cells of the innate and adaptive immune system. Furthermore, mLNs being a major site of gut mucosal immunity regulation, this protocol provides an alternate tool to study gut immunology. With minor adjustments, this technique can also be applied to different disease models in mice to study e.g., the impact of microbiota and metabolites on particular stromal LN environments, modification of migrating antigen-presenting cells by distinct LN microenvironments or in cancer metastasis.

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 animal study was reviewed and approved by Regierung von Unterfranken.

AUTHOR CONTRIBUTIONS

HS designed, planned and carried out the experiments, analyzed and interpreted the data, and wrote the manuscript. ZM analyzed and interpreted the LSFM data. KJ assisted with establishing the surgery model. JV, MU, JM, EV, CG, and D-DL helped with the

experiments. AR and MB-H performed histopathologic tissue analyses. EV and KH supported LSFM imaging. JP and JH provided intellectual support. AB designed the study, interpreted the data, reviewed and co-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.2021.689896/full#supplementary-material

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REVIEW
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Experimental Models of Infectious Pulmonary Complications Following Hematopoietic Cell Transplantation

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Pulmonary infections remain a major cause of morbidity and mortality in hematopoietic cell transplantation (HCT) recipients. The prevalence and type of infection changes over time and is influenced by the course of immune reconstitution post-transplant. The interaction between pathogens and host immune responses is complex in HCT settings, since the conditioning regimens create periods of neutropenia and immunosuppressive drugs are often needed to prevent graft rejection and limit graft-*versus*-host disease (GVHD). Experimental murine models of transplantation are valuable tools for dissecting the procedure-related alterations to innate and adaptive immunity. Here we review mouse models of post-HCT infectious pulmonary complications, primarily focused on three groups of pathogens that frequently infect HCT recipients: bacteria (often *P. aeruginosa*), fungus (primarily *Aspergillus fumigatus*), and viruses (primarily herpesviruses). These mouse models have advanced our knowledge regarding how the conditioning and HCT process negatively impacts innate immunity and have provided new potential strategies of managing the infections. Studies using mouse models have also validated clinical observations suggesting that prior or occult infections are a potential etiology of noninfectious pulmonary complications post-HCT as well.

Keywords: hematopoietic cell transplantation, bone marrow transplantation, infectious pulmonary complications, herpesvirus, *Pseudomonas aeruginosa*, *Aspergillus fumigatus*

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INTRODUCTION

Hematopoietic cell transplantation (HCT) is a potentially curative treatment for high-risk hematopoietic neoplastic disorders, metabolic, genetic and immune-mediated diseases. It involves eradication or suppression of the recipient's hematopoietic cells using a conditioning regimen followed by infusion of stem cells collected from the bone marrow, placenta (cord blood) or peripheral blood (1). The source of hematopoietic cells can be either autologous (auto, recipient-derived) or allogeneic (allo, matched related or unrelated donor-derived) hematopoietic cells. HCT has been carried out increasingly over the years with 47,468 transplants in 50 European and associated countries (2), and 22,573 transplants in the United States in 2018 (3).

Unfortunately, the toxicity of conditioning regimens, alloimmune responses and immunosuppressive therapies cause severe post-transplant complications, in which the lung is

one of the most common target organs. Pulmonary complications occur in up to 60% of allo-HCT recipients (4) and 25% of auto-HCT recipients (5). The frequent pulmonary complications and their significant contribution to post-transplant morbidity and mortality limit the success of HCT (6–8). These complications are heterogeneous, and include pathologies generated by infectious agents and noninfectious disorders. Although infectious pulmonary complications after HCT have been significantly reduced due to aggressive prophylaxis and the use of broad-spectrum antimicrobial medications, these infections still remain problematic, especially among the patients with graft-*versus*-host disease (GVHD). Major noninfectious pulmonary complications include early onset idiopathic pneumonia syndrome (IPS) (7), and late onset bronchiolitis obliterans syndrome (BOS) (9). Current experimental data support alloimmunity as an underlying mechanism of these idiopathic noninfectious lung injuries (7, 9). For a review on non-infectious pulmonary complications of stem cell transplantation, please see references (10, 11).

Animal models have been extensively used for the establishment and improvement of HCT therapy (12, 13). Animal models allow manipulation of single factors during the development of complications associated with HCT, and thus are crucial for successfully improving clinical applications. Most of the current knowledge regarding defects in immune responses during infectious pulmonary complications come from studies using mouse models of HCT. In this review, we will first briefly introduce infectious pulmonary complications post-HCT, and then describe relevant mouse models and current understanding of host immune responses to lung infections post-HCT that have been acquired from studying these models.

CLINICAL PHENOTYPES OF INFECTIOUS PULMONARY COMPLICATIONS POST-HCT

The immune system of HCT recipients is eradicated or weakened by either myeloablative or less intense nonmyeloablative conditioning regimens before transplant to eradicate/reduce tumor burdens and to prevent graft rejection. Thus, it is not surprising that infections are a major complication post-HCT. Infectious complications are more frequent and severe in patients with allo-HCT due to prolonged immunosuppressive therapy and GVHD (14). The timing of reconstitution of the immune system post-HCT varies considerably among patients, depending on the type of transplant (autologous *vs* allogeneic), the intensity of conditioning regimen, the source of hematopoietic cells, the presence of GVHD and the length of immunosuppressive therapies. Nevertheless, post-HCT reconstitution can be roughly divided into three phases: severe neutropenia or pre-engraftment phase (first 2-4 weeks), early engraftment phase (second and third month) and late engraftment phase (after second or third month) (14).

The prevalence and types of infection change over time and often follow the course of immune reconstitution post-transplant in patients (14). During the pre-engraftment phase, the depletion of neutrophils and damage to the mucosal barriers caused by conditioning regimens allow opportunistic pathogens to become infectious. The predominant pathogens during this phase are *Pseudomonas, Candida* and *Aspergillus* species (15–18). During the early engraftment phase, most innate immune cell subsets such as monocytes, neutrophils, and natural killer cells repopulate at normal levels (19), but lymphocyte counts are still low. This allows the reactivation of herpesviruses, such as cytomegalovirus (CMV), Epstein–Barr virus (EBV), human herpesvirus 6 (HHV-6), and new infections with respiratory viruses (20–22). A second peak of invasive *Aspergillus* infection occurs at the end of the early engraftment phase in allo-HCT recipients due to prolonged GVHD and its immunosuppressive therapy (18). During the late posttransplant phase (about three months after transplant), innate immunity is mostly reconstituted, but the recovery of T cells takes about a year and B cells may take even longer to completely repopulate (23). Bacterial pneumonia is less common during this late phase, but allo-HCT recipients are still at risk of late CMV reactivation and fungal infection. Current preemptive therapeutic strategies have significantly reduced early onset CMV infections after allo-HCT, but the incidence of late CMV infections have increased (24, 25). CMV reactivation remains a life-threatening infectious complication that is difficult to manage following allo-HCT (26–28). Allo-HCT recipients with chronic GVHD and immunosuppressive therapy continue to be susceptible to *Aspergillus* and Gram-positive bacteria as well (29). It is thus important to understand the interplay among host immunity, pathogens and GVHD in an allo-HCT setting.

MOUSE MODELS OF INFECTIOUS PULMONARY COMPLICATIONS FOLLOWING BONE MARROW TRANSPLANTATION

There have been many functional studies on immune responses to pathogens in mouse models of HCT. These have included both syngeneic (syn) and allogeneic strain combinations to recapitulate autologous or allogeneic HCT in patients. The pneumonia pathogens studied span bacteria (mostly *Pseudomonas aeruginosa*), fungus (primarily *Aspergillus fumigatus*), and viruses (primarily herpesviruses). There are also reports of sepsis subsequent to gastrointestinal damage due to conditioning regimens in HCT mouse models (30).

Mouse Models of Post-HCT Bacterial Pneumonia

Bacterial pneumonia usually occurs early after HCT during the neutropenic period (31), but can also occur post-engraftment. *P. aeruginosa* is the most common pathogen isolated from the lower respiratory tract within 100 days post-transplant (15). *P. aeruginosa* is a ubiquitous environmental bacterium, and if inhaled into the lung airway by a immunocompetent individual, it is quickly cleared by alveolar macrophages (AMs) (32).

Because *P. aeruginosa* has become increasingly resistant to multiple antibiotics over the years (33), it can be difficult to treat multidrug resistant *Pseudomonal* pneumonia (MDRPa) (16). About 40% of the hematologic malignancy patients infected with MDRPa will die in 30 days (34) and MDRPa outbreaks are associated with a death rate as high as 80% (35).

A syn-HCT mouse model was established in our laboratory to understand why HCT recipients are susceptible to *P. aeruginosa* (36). This model is clinically relevant, as both autologous and allogeneic transplant patients are susceptible to *P. aeruginosa* infection (37). Like auto-HCT recipients, mouse syn-HCT recipients have no risk of GVHD, and thus the model can be used to explore how the transplant procedure alone impacts pulmonary immunity. Recipient C57BL/6 mice are given a split dose of 13 Gy total body irradiation (TBI) from either a ^{137}Cs or x-ray orthovoltage source with an interval of 3 hours between doses. Bone marrow is harvested from donor C57BL/6 mice, and $5x10^6$ whole bone marrow cells are infused into the recipients *via* tail vein injection. Five weeks after transplant, the percentage of donor-derived cells is approximately 95% in the spleen and the percentage of donor-derived AMs in the lung is about 83% (38). At this time point, HCT or age-matched non-HCT control mice are infected with *P. aeruginosa* PAO1 *via* intratracheal (i.t.) inoculation (36). These experiments demonstrated increased bacterial burden in the lung and dissemination to the blood at 24 h post-infection in HCT mice compared to non-transplant controls (36). See **Figure 1** for schematic illustration of the model system.

The defect in bacterial clearance in HCT mice is associated with reduced phagocytosis and killing of *P. aeruginosa* in lung AMs (39, 40) and impaired killing and defective formation of neutrophil extracellular traps (NETs) in neutrophils (41). Similar to HCT patients, the levels of immunosuppressive prostaglandin E_2 (PGE_2) are elevated in HCT mice (39, 42). Subsequent studies found that overproduction of PGE_2 impairs the functions of both AMs and neutrophils, and pharmacologic inhibition of PGE_2 production *in vivo* restores host defense of HCT mice (39, 41).

The syn-HCT model permits further dissection of the mechanisms explaining how the HCT procedure promotes AMs to overproduce PGE_2. Conditioning-associated cellular stress stimulates alveolar epithelial cells to produce TGF-β (43). TGF-β signaling stimulates AMs to transcribe microRNA (miR)-29b which suppresses the expression of DNA methyltransferases (DNMTs) (44). Under homeostatic conditions, DNMT3a and DNMT3b methylate the promoter region of cyclooxygenase (COX)-2 gene which encodes a critical enzyme for the production of PGE_2 (40). Methylation of the COX-2 promoter limits transcription and reduces COX-2 gene expression. Suppression of DNMTs by the TGF-β-miR-29b axis releases this endogenous break on COX-2 expression and thus increases the production of PGE_2 in AMs (44). Interestingly, these epigenetic changes can be long lived with human HCT

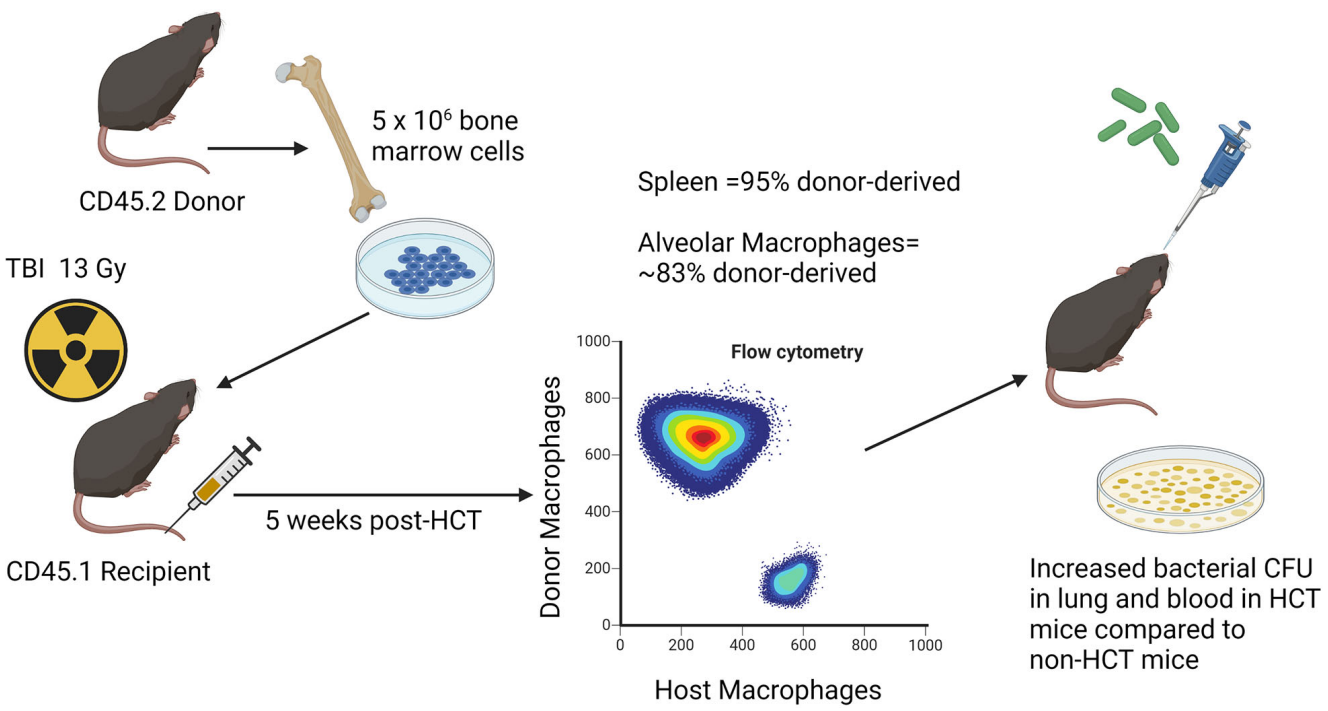

FIGURE 1 | Schematic illustration of syn HCT mouse model. The figure shows the process of syn HCT used in experiments to test impaired host defense against *Pseudomonas aeruginosa* as described in the text. The figure is created with Biorender.com.

patients (even 6 years post-HCT) showing elevated levels of PGE_2 in the lung. Thus, this mechanism likely accounts for long-lived innate immune impairment post-HCT.

The immunosuppressive function of PGE_2 is mediated by its receptors E prostanoid receptor 2 (EP2) and EP4 (44). Signaling *via* these receptors can activate a cyclic adenosine monophosphate (cAMP)-mediated signaling cascade with multiple downstream effects. One effect is downregulation of the scavenger receptor MARCO which is critical for recognition and phagocytosis of *P. aeruginosa* (45). Another effect is upregulation of IL-1 receptor associated kinase M (IRAK-M), which is an inhibitor of TLR signaling (46). Ultimately, this alteration impairs the proinflammatory cytokine response (e.g. TNF-α and IFN-γ) that could help clear bacterial infection. At the same time, PGE_2 promotes transcription of IL-1β which is a mediator of tissue damage in the lung (47). Furthermore, PGE_2 stimulates the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which negatively regulates phagocytosis and killing of *P. aeruginosa* (48). A summary figure describing some of the innate immune changes in AMs post-HCT is found in **Figure 2**.

In contrast to the impaired phagocytosis of *P. aeruginosa* post-HCT by AMs, phagocytosis of *Staphylococcus aureus* is actually enhanced (45). This increased phagocytosis of *S. aureus*, is also regulated by PGE_2, by stimulating the expression of miR-155 which upregulates scavenger receptor (SR-)AI/II (45). However, despite the enhanced uptake of *S. aureus*, AMs from HCT mice are unable to effectively kill the pathogen intracellularly as a result of the impacts on IRAK-M and PTEN described above. Additionally, the impaired innate immune function of neutrophils likely contributes to poor *S. aureus* clearance. Interestingly, a study by Zimecki et al. explored the use of bacteriophages as a therapeutic strategy for syn-HCT mice infected with *S. aureus* strain L (49). Similar to the findings reported above, HCT mice were highly susceptible to *S. aureus* infection (only 8.3% of infected mice survived whereas mice treated with phage showed 72% survival. It was also noted that the phage therapy increased the circulating leukocyte and neutrophil counts.

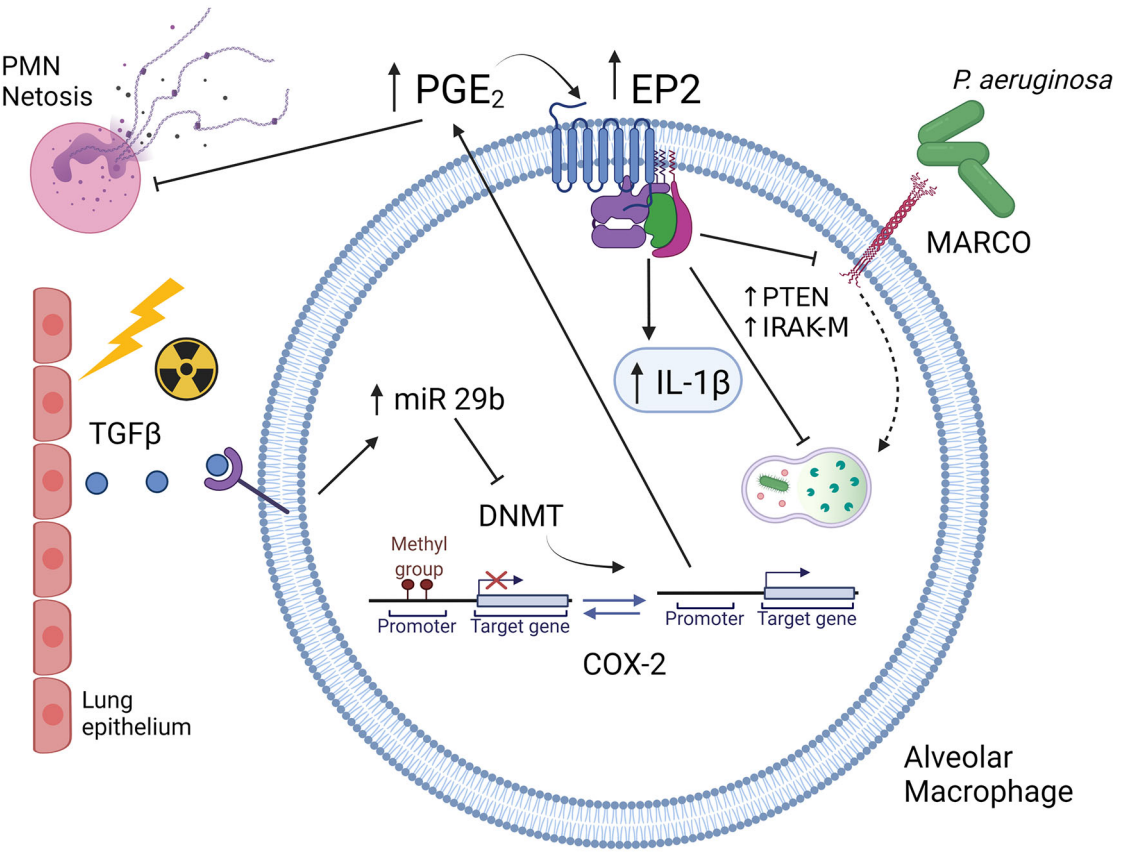

FIGURE 2 | Schematic illustration of Syn HCT induced changes in innate immunity. Conditioning with TBI causes injury to lung epithelium resulting in production of TGF-β. Binding of TGF-β to alveolar macrophages results in increased miR29b expression which then limits expression of DNA methyltransferases (DNMT). This allows for the promoter of the cyclooxygenase 2 (COX-2) gene to be unmethylated resulting in increased production of prostaglandin E2 (PGE_2). PGE_2 then binds to the E prostanoid 2 (EP2) receptor which is also upregulated post-HCT. Downstream signaling by PGE_2 results in upregulation of IL-1, elevations in phosphatase and tensin homolog on chromosome 10 (PTEN) and elevations in IL-1 receptor associated kinase (IRAK-M). These changes impair intracellular killing while downregulation of the MARCO scavenger receptor impairs phagocytosis of *P. aeruginosa*. Original references described in text. The figure is created with Biorender.com.

While the majority of the studies reviewed above show defective innate immune function, there is one study from 1989 showing that early after marrow transfer in allogeneic radiation chimeras that macrophages can be non-specifically activated by the conditioning milieu and show enhanced resistance to *Listeria monocytogenes* initially, but that this protection eventually declines with time (50).

Mouse Models of Post-HCT Fungal Pneumonia

Invasive fungi have become the leading infectious cause of morbidity and mortality in HCT recipients in the era of improved prophylaxis and treatment of bacterial and viral infections (51, 52). Invasive pulmonary aspergillosis (IPA) is the most common fungal infection in the lung of HCT recipients. IPA causes high mortality among HCT patients, ranging from 30% to 70% (53), accounting for 10% of all death among the recipients (54). IPA is highly associated with neutropenia, GVHD and its related immunosuppressive therapy, and thus the incidence of IPA peaks early in the pre-engraftment phase and then later post-engraftment in allo-HCT recipients (18). This bimodal distribution of IPA post-HCT reflects different etiologies of *Aspergillus* infection: early IPA is due to prolonged neutropenia, especially when using myeloablative conditioning regimens, while late IPA is secondary to receiving corticosteroid or other immunosuppressive therapies to treat GVHD (55). Interestingly, the pro-inflammatory status noted in allo-HCT mice without immunosuppressive treatment may enhance clearance of *Aspergillus* as noted in one study by Hildebrandt et al. (56). The significant shift to using lower intensity non-myeloablative regimens allows a shorter neutropenic period and the systematic use of antifungal prophylaxis has led to a decrease in the incidence of early IPA (57). The early and late IPA time periods present distinct immunopathology patterns in HCT recipients. Early IPA in the neutropenic phase is characterized by rapid fungal growth and low levels of inflammation, but late IPA in immunosuppressed patients usually presents with overabundant inflammation including excessive neutrophil infiltration with insufficient fungal clearance (58).

Accordingly, several animal models have been established to understand the pathogenesis of early or late aspergillosis in HCT patients (59). Neutropenic models include treating mice with chemotherapeutic agents such as cyclophosphamide (60), TBI (61, 62), and using antibody depletion of neutrophils (63). Immunomodulated models usually involve the use of corticosteroids (64). These two types of mouse models recapitulate the different pathologies of IPA that present in neutropenic and immunosuppressed patients respectively (64, 65). For the neutropenic mouse models, it is most common to administer cyclophosphamide at 150 mg/kg *via* the intraperitoneal route thrice weekly before infection. Some studies have used monoclonal antibodies to achieve neutrophil depletion. A dose of 100μg of anti-Ly6 (Gr1) rat IgG2b MAb57 (clone RB6-8C5) *via* intraperitoneal injection on the day before and 2 days after fungal intranasal inoculation dramatically reduces the number of neutrophils for up to 5 days (63). The lethal dose of irradiation varies depending on mouse strain receiving the treatment. For example, a single lethal dose of 9 Gy given to C3H/HeJ mice followed by transplants with $2x10^6$ T-cell-depleted allogeneic bone marrow cells from DBA/2 mice shows profound neutropenia 3 days after transplant (63). Immunomodulated models commonly administer cortisone at 100 to 200 mg/kg *via* subcutaneous injection thrice weekly for 1 or 2 weeks before experimental infection. In a cortisone-treated immunosuppressive model, myeloid cells such as neutrophils and macrophages, are massively recruited to the lungs upon infection, but lymphocytes fail to be recruited to the lung, indicating the requirement of lymphocytes to efficiently clear the infection (64). The most common routes of inoculation of *Aspergillus* are intranasal and intratracheal administration. A conidial suspension of *A. fumigatus* inoculated into the nares is close to natural infection, but due to upper mucociliary clearance of mice, only about 10% of the inoculum actually enters into the lungs (66). As a result, the development of IPA is highly variable in intranasally inoculated mice. Delivery of spore suspension directly into the trachea, by either tracheotomy or oropharyngeal aspiration in anesthetized mice, can more tightly control the fungal inoculum and lead to reproducible IPA (59). The most commonly used *A. fumigatus* strains are the low virulent strain AF293 (ATCC MYA4609 or CBS101355) and the high virulent strain Dal/CEA10 (ATCC MYA1163 or CBS 144.89). Depending on the strain of *A. fumigatus* and the route, the dose ranges from 1.0×10^2 to 1.0×10^9 conidia for mice infected through the intranasal route, and 1.0 to $2.0x10^7$ conidia through the intratracheal route (59).

Studies on mouse models and human patients have greatly increased our knowledge of the host immune responses to *A. fumigatus* and have aided in the development of novel therapeutic targets to treat IPA [reviewed in references (67, 68)]. Here we highlight a few advances in the field during recent years. Mouse models have confirmed or identified several important pattern recognition receptors (PRRs) on the cell surface of innate immune cells, such as AMs, which include dectin-1 (61), TLR2 (69, 70), TLR4 (70), TLR9 (71–73), NOD2 (74), soluble pentraxin-3 (75) and TREM1 (76) in recognizing *Aspergillus* components. Inflammatory cytokines such as IL-1α and IL-1β are critical for host defense against *A. fumigatus* in neutropenic mice (77, 78). The role of antigen presentation and development of antigen specific T cell subsets has been studied with respect to clearance of IPA, but with some conflicting results. For example, adoptive transfer of dendritic cells pulsed with conidia stimulates a T helper type 1 (Th1) responses and improves survival in a syn-HCT model suggesting an important role of Th responses to clear infection (79). This is consistent with several other studies in mouse HCT models that also demonstrated that Th1-mediated immunity is important in clearing *A. fumigatus* infection (80–82). Interestingly, it appears paradoxical that CCR7 deficient HCT mice whose dendritic cells cannot enter draining lymph nodes to prime T lymphocytes show improved survival in a monoclonal antibody (anti-Gr1) induced neutropenic model (83). This study suggests that retaining CD11b+ dendritic cells inside the neutropenic

lung during initial infection with *Aspergillus* is beneficial, potentially by complimenting the loss of neutrophils. The role of the Th17 response in the pathogenesis of IPA is somewhat controversial, as it can be either protective (84, 85) or pathogenic (80). Th2 responses and the production of IL-4 are detrimental to control *A. fumigatus* infection, as IL-$4^{-/-}$ mice are protected from IPA (86). Finally, regulatory T cells are producers of IL-10 which is linked to disease progression in steroid immunosuppressive experimental IPA (87, 88). **Figure 3** provides a summary of recent insights from mouse models of HCT or neutropenia with regards to Aspergillus infection.

Mouse Models of Post-HCT Viral Pneumonia

Viral infections often occur after engraftment when the reconstitution of lymphocytes is not yet complete, or when immunosuppression due to prophylaxis or treatment of GVHD in allo-HCT recipients is needed. Nearly every human being is infected with one or more herpesviruses in the first two decades of life and the viruses can establish life-long latency to escape immune surveillance and detection (20). Viral pneumonia can be caused by reactivation of latent human herpesviruses or new infection with community acquired respiratory viruses. Additionally, primary herpesvirus infections can occur in seronegative patients receiving grafts from seropositive donors (89). Besides causing direct lung injuries, such as, cytomegalovirus (CMV) pneumonia (20), occult or prior herpesvirus infections appear to trigger the development of "noninfectious" pulmonary complications at later time points after allo-HCT (21, 22, 90).

Most human herpesviruses have very strict host-species specificity, and it is thus difficult to study human herpesviruses in mice. Herpes simplex virus type 1 (HSV-1) is an exception, as it can directly cause pneumonia in HCT mice (91). To bypass this hurdle, some researchers have generated transgenic mouse models that express receptors for human herpesvirus. For example, human CD46, an HHV-6A receptor, is expressed in the brain of a mouse line to study host innate immunity against HHV-6A (92). Other researchers engrafted human CD34+ hematopoietic progenitor cells into NOD-*scid IL2Rγc*^{null} (NSG) mice which can then be directly infected with human herpesvirus (93). More often, murine homologs of their corresponding human herpesviruses are used in mouse models to study the principles of virus-host interactions that are thought to be shared among human and mouse systems. Mouse CMV (mCMV), murine gammaherpesvirus 68 (MHV-68) and murine roseolovirus (MRV) are frequently used to study human CMV, Epstein-Barr virus (EBV) and human herpesvirus (HHV)-6A/B, respectively in HCT settings.

Most murine herpesvirus models fall into two categories: pre-HCT latent infection or post-HCT infection models. Both syngeneic and allogeneic HCT have been studied. Latent infection models usually involve a primary infection in

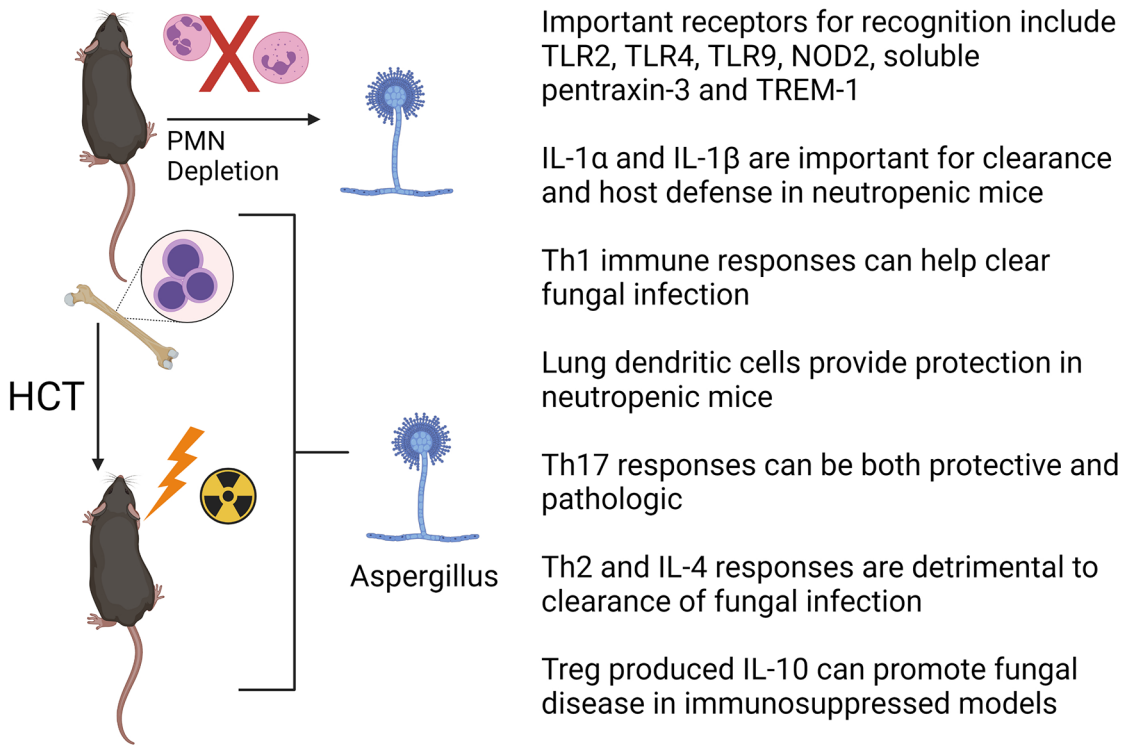

FIGURE 3 | Factors important for clearance of *Aspergillus fumigatus* in HCT or neutrophil (PMN) depleted mouse models. See text for details. The figure is created with Biorender.com.

neonates or adult mice to mimic the natural history of herpesviral infection, followed by various lengths of "waiting time" to let the virus enter latency (21, 94). After that, an allo-HCT is usually performed to stimulate viral reactivation. Latently infected mouse models are most suitable for studying the reactivation of herpesvirus, their subsequent effects and for testing novel therapeutic strategies. This model has facilitated the discovery of the critical role of humoral immunity in controlling the reactivation of mCMV (94). The half-life of preexisting antibodies in latently infected mice and the elimination of recipient plasma cells due to GVHD can lead to a loss of anti-mCMV antibodies, which eventually leads to mCMV reactivation in recipients. Importantly, the reactivation of mCMV in allo-HCT recipients can be prevented by the transfer of immune serum (94). In a similar latent infection model, two doses of leukotriene B_4 administered *via* intravenous route effectively reduced the reactivation of mCMV in allo-HCT mice through yet unknown mechanisms (95). To determine the relationship between herpesviral reactivation and noninfectious pulmonary complications, MRV, a mouse homolog of HHV-6, was given to neonatal mice and then reactivated in response to a minor histocompatibility antigen mismatched allo-HCT 8 weeks later. Indeed, the reactivation of MRV not only caused IPS-like pathology but also exacerbated histologic signs of acute GVHD in the gut (21).

Due to variable waiting times for entering latency and the heterogenous nature of post-HCT viral reactivation among latently infected mice, some researchers infect mice post-HCT or concurrent with HCT to mimic the reactivation of herpesvirus. The HCT procedure creates an immunosuppressive lung microenvironment, characterized by increased levels of PGE_2 (39), TGF-β (91, 96) and Kynurenine (97). Reduced influx or altered function of $CD8^+$ T cells, which are critical for clearance of mCMV (98, 99), HSV-1 (91) and community acquired respiratory viruses (100, 101), were observed in both syn- and allo-HCT recipients. As a result, most HCT mice experienced delayed viral clearance and persistent pneumonitis. Interestingly, the impact of HCT on T cell immunity does not seem to be mediated by the elevated levels of PGE_2 (101), but rather by TGF-β (91).

Recently, studies using MHV-68, a mouse herpesvirus genetically related to Kaposi's sarcoma-associated herpesvirus (KSHV) and EBV, in a syn-HCT model in our laboratory have advanced our understanding of host immune response to herpesvirus infection in the HCT setting. A C57BL/6 to C57BL/6 syn-HCT mouse model as described above was adopted to study MHV-68 infection. The HCT procedure not only causes an immunosuppressive environment, but also changes the structure of the lung microbiome (102). Together, these alterations in the lung microenvironment have significant impacts on the biology of conventional dendritic cells (cDCs) in HCT lungs. After exposure to MHV-68, the cDCs in syn-HCT mice increased their expression of pro-Th17 cytokines such as IL-6 IL-23 and TGF-β relative to the responses noted in untransplanted mice (103). These HCT lung cDCs also become deficient for delta like ligand 4 (DLL4), a Notch ligand, on their cell surface which further permits Th17 polarization (104). In addition, the migration of cDCs into mediastinal draining lymph nodes is impaired, significantly reducing Th1 responses, but augmenting Th17 responses which appear to be primed locally in the lung (105). Thus, the functional changes of lung cDCs post-HCT tip the balance of Th responses against MHV-68 infection from protective Th1 responses to pathogenic Th17 responses (96, 103).

Excessive IL-17A due to Th17 responses eventually causes the development of pneumonitis and pulmonary fibrosis 3 weeks after infection, when lytic MHV-68 is no longer detectable (103, 106). Administration of anti-IL-17A antibodies or using bone marrow cells isolated from IL-$17A^{-/-}$ donor mice protects HCT recipients from pneumonitis and fibrosis after infection with MHV-68 (103). **Figure 4** highlights some of the changes noted in HCT lungs post-infection with herpesvirus. Note that the pathology seen in this mouse model also resembles many histological features seen in noninfectious complications such as IPS and restrictive lung disease, suggesting a potential etiology of noninfectious pulmonary complications caused by prior or occult infections that trigger pathogenic immune responses leading to lung injury and improper repair. This hypothesis is supported by recent discoveries of occult infections in IPS patients (90), and the strong association between the infections with herpesviruses (21) or community acquired respiratory viruses (22) and noninfectious complications in HCT recipients. Furthermore, direct evidence comes from a study mentioned above showing that reactivation of MRV in allo-HCT mice causes IPS-like pathology and exacerbates acute GVHD (21).

DISCUSSION

Pulmonary infections remain a major cause of morbidity and mortality in HCT patients. This is not surprising given that the conditioning regimens create periods of cytopenia and immunosuppressive drugs are often needed to limit GVHD. The rise of antibiotic resistant strains of bacteria, challenges of vaccinating immunocompromised patients, and limited availability of vaccines for many of the common pathogens in this patient population all help explain the infectious challenges facing HCT patients. Additionally, mucosal tissue damage as a result of GVHD and bacterial colonization can significantly increase the likelihood of opportunistic infection (107–109). As discussed above, non-myeloablative conditioning and fungal and viral prophylaxis have helped reduced infections during the cytopenic/neutropenic phases; however, there is a growing appreciation that even post-engraftment, innate and adaptive immune cells display altered immune function.

Even as early as 1982, it was noted that AMs from HCT patients were defective at phagocytosis, chemotaxis towards, and killing of fungal and bacterial pathogens when studied 4 months post-HCT (110). Importantly, while phagocytic and chemotactic defects normalized, the killing defects persisted even 12 months post-HCT (110). This suggests that alterations in the lung milieu, most likely caused by conditioning regimens may interfere with

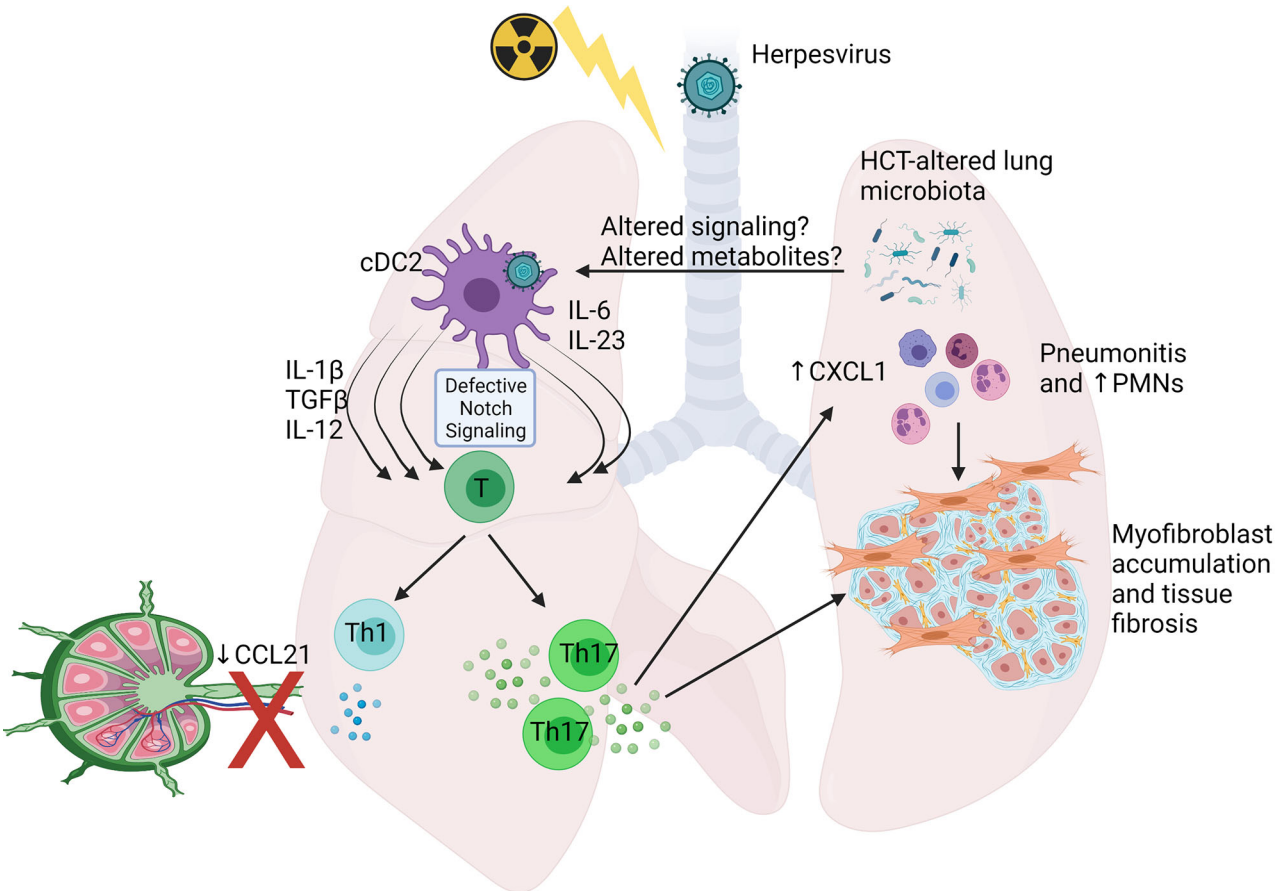

FIGURE 4 | Schematic showing alterations shown to occur in Syn HCT mouse models of murine gammaherpesvirus infection. In responses to HCT, lung conventional dendritic cells 2 (cDC2) display defective delta like ligand 4 leading to impaired notch signaling when interacting with virus-specific T cells. This leads to production of cytokines able to drive both Th1 and Th17 cell differentiation, but because of poor ability of the cDCs to migrate to the draining lymph nodes secondary to reduced CCL21 levels, the Th1 response is impaired, while the Th17 response is primed efficiently in the lungs. Elevated IL-17 levels stimulate lung epithelium to produce CXCL1 and recruit PMNs prominently, although other cell types also accumulate. The IL-17 can also directly activate myofibroblast proliferation and extracellular matrix production leading to fibrosis. Alterations to the lung and gut associated microbiome are also prominent post-HCT and may contribute to altered immune cell functions. See text for details. The figure is created with Biorender.com.

the function of innate immune cells. It also suggests that long-lived alterations may result from epigenetic alterations. A decade later, elevated levels of PGE_2 were noted in both auto and allo-HCT patients; however, this finding was not linked to impaired innate immune function (42). It was not until 2006 that murine models were able to provide a mechanistic link between these two observations and demonstrate that overproduction of PGE_2 post-HCT was responsible for impaired phagocytosis and killing by AMs (39). It took almost another decade to describe the epigenetic alterations in methylation of the COX-2 promoter that were caused by elevated levels of TGFβ1 caused by the conditioning regimen (44). Interestingly, this same mechanism that impairs innate immune function in HCT, has recently been shown to explain defective macrophage responses to wound healing and wound infection in diabetes as well (111). No doubt there are many other, yet to be discovered pathways that impair the function of innate immune cells in the transplant setting that are likely to be regulated *via* epigenetic alterations induced by the altered lung milieu.

We are also starting to learn more about how alterations of the normal microbiota in the lung and gut of HCT recipients may alter immune tone. The process of allo-HCT has been shown to reduce the diversity of the gut microbiota in humans (112). Furthermore, low diversity of gut flora at the time of neutrophil engraftment predicts mortality (113). Interestingly, when focused on the connection between gut microbiome and pulmonary complications, an observational study found that HCT patients that had low baseline gut microbiome diversity or proteobacteria domination early post-HCT had the highest incidence of pulmonary complications (114). This highlights the potential for a gut-lung axis when considering regulation of pulmonary immunity and such a concept has previously been suggested (115–118). More recently, the concept of alteration of the lung microbiome in the setting of HCT has demonstrated

that dysbiosis is associated with alterations in inflammatory cytokines and poor outcomes (102, 119). Interestingly, in the murine studies, it was found that while the HCT procedure alone altered the microbiota, there was a more profound and prolonged alteration in the setting of herpesviral infection as well (102). Similarly, HCT patients with confirmed transcriptionally active pathogens in the bronchoalveolar lavage fluid display overall lower microbiome diversity in the lung, and many of these patients had viral infections (120). Whether the alterations caused by disturbances of the lung or gut microbiota are related to altered signaling *via* pathogen recognition receptors on immune cells or altered metabolites secreted by the microbiota or both remains to be determined. Interestingly, such findings may offer new diagnostic approaches. Zinter et al. recently performed metatranscriptomic analysis of pre-HCT bronchoalveolar lavage fluid in pediatric patients and found that children with evidence of viral enrichment and innate immune activation had the highest incidence of post-HCT lung injury while patients with diverse oropharyngeal taxa and lacking inflammatory signatures rarely developed post-HCT lung injury (119). While there are known differences in the composition of human and murine microbiota, murine models should still be useful for proof-of-concept studies regarding the role of potential prebiotics, fecal microbiome transplant and other potential therapies to improve outcomes post-HCT.

Our understanding of the host-pathogen interaction in HCT recipients has been accumulated over decades from studying animal models. Mice and humans have fairly similar organs and systems, immunity and pathology. Mouse models permit tightly controlled experimental conditions and unified genetics of host and pathogens. One of the most important advantages of mice is the availability of a huge collection of gene knockout or transgenic mouse strains, and it is now relatively easy to generate such mice if they are not readily available. In practical aspects, the cost of mice is inexpensive and experiments are reproducible. However, there are a few important limitations that need to be kept in consideration. First, the immune system in mice is considerably different from that in humans (121), and thus the knowledge acquired from mice may serve as "proof-of-concept", but may not readily be translated to human clinical treatment. Second, mice are small and have short live spans. The small sizes of the body and lungs of mice may contribute to the different kinetics of immune reconstitution post-HCT and disease course in the lung compared with humans, as mouse lungs can be quickly overwhelmed by pathogens or immune cell infiltration. While there have been mouse models of BOS in mice that have provided important insights (122–128), it is not clear that the evolution of this disease in mice which have short lifespans fully recapitulate the features of disease that evolve over years in humans. Similarly, long term effects of chronic latency and reactivation of herpesviruses may be difficult to capture. Third, the host-pathogen interactions, especially with viral infections, is usually species-specific, which reduces clinical translatability. Other limitations of mouse models include a lack of parallel methodologies with the ones commonly used in clinical settings. Many of the studies have used syn-HCT models to avoid the complications of alloimmune responses, yet clinically, allo-HCT patients often have the most severe pulmonary complications and many of the current mouse models do not sufficiently explore the effects of GVHD or immunosuppressive therapy and how those factors impact immune function. Additionally, for convenience, mouse models often use TBI as the conditioning regimen, yet human HSCT is often accomplished with chemotherapy, reduced intensity irradiation or combinations. Despite these limitations however, the power of mouse genetics and the ability to genetically modify gene expression in a cell-type specific manner makes these murine models important tools that enable the dissection of fundamental mechanisms which underlie disease. Given that we do not have good anti-microbial strategies for many of the common pathogens that plague patients post-HCT, it is important to better understand how we can quickly repopulate the immune cells of the host and how we can manipulate the HCT regimens to improve the functionality of these immune cells. The power of HCT to cure inherited genetic diseases and malignancies will never be fully realized until the infectious complications, particularly in the lung can be better managed.

AUTHOR CONTRIBUTIONS

XZ and BM reviewed the literature, drafted the manuscript, created figures and edited the final document. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Pathophysiology and Treatment of Graft-*Versus*-Host Disease: Lessons Learnt From Animal Models

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Allogeneic hematopoietic cell transplantation (HCT) is a curative treatment for hematologic malignancies, bone marrow failure syndromes, and inherited immunodeficiencies and metabolic diseases. Graft-*versus*-host disease (GVHD) is the major life-threatening complication after allogeneic HCT. New insights into the pathophysiology of GVHD garnered from our understanding of the immunological pathways within animal models have been pivotal in driving new therapeutic paradigms in the clinic. Successful clinical translations include histocompatibility matching, GVHD prophylaxis using cyclosporine and methotrexate, posttransplant cyclophosphamide, and the use of broad kinase inhibitors that inhibit cytokine signaling (e.g. ruxolitinib). New approaches focus on naïve T cell depletion, targeted cytokine modulation and the inhibition of co-stimulation. This review highlights the use of animal transplantation models to guide new therapeutic principles.

Keywords: graft-*versus*-host disease, animal models, pathophysiology, history, treatment

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EARLY HISTORY

In 1956, two groups observed that mice exposed to lethal dose of total body irradiation (TBI) and administered allogeneic splenocytes survived for a shorter time than those transplanted with syngeneic splenocytes (1–3). The recipients of allogeneic cells exhibited diarrhea, weight loss, skin lesions, and died (1, 4). This syndrome was initially designated as "secondary disease", which was later renamed as graft-*versus*-host disease (GVHD). In 1963, Mathé and colleagues reported the first case of a human allogeneic bone marrow transplantation (BMT) recipient that survived beyond a year. This patient had complete engraftment and the development of a lethal "secondary disease" was described (5). Subsequently, the clinical and pathological characteristics of GVHD was described (6). The outcomes for the initial 200 patients transplanted prior to 1967 were disparaging; all patients died of either graft failure, GVHD, infection, or leukemia relapse (7). These poor outcomes reflected a limited understanding of histocompatibility matching and the requirement for immune suppression after BMT to control GVHD (8).

Although many investigators lost their enthusiasm for BMT, several groups increasingly utilized animal models to gain a better understanding of the allogeneic barrier with regard to both GVHD and graft rejection. Studies of allogeneic BMT in Seattle using dog models in the 1980s provided the scientific groundwork for the field leading to the concepts of histocompatibility matching,

conditioning regimens and pharmacological GVHD prophylaxis (9–13). These findings were soon translated to the clinic and successful clinical BMT was established (14), subsequently leading to E. Donnall Thomas being awarded the Nobel prize within Physiology or Medicine in 1990 (see **Figure 1** for a timeline).

For a detailed discussion of the various mouse models of GVHD currently available and the penetrance of disease therein, we refer the reader to some of the excellent reviews on this subject (15–17).

ACUTE GVHD

Donor T Cells

In the 1970's and 1980's, Korngold and Sprent performed extensive studies in a series of mouse models across MHC and/or minor histocompatibility antigen mismatches demonstrating the role of donor T cells in the etiology of GVHD. They showed that donor-derived T cells were causative of GVHD and identified T cell subsets (CD4 *versus* CD8) responsible for the induction of GVHD in each model (18–20). Following the observation that T cell depletion prevented GVHD in mice (18), clinical studies confirmed that GVHD also failed to develop following rigorous donor T cell depletion with CD34 positive selection of the donor inoculum and the administration of anti-thymocyte globulin (ATG); none developed GVHD even without posttransplant immunosuppression (21, 22).

Donor T cells exert graft-*versus*-leukemia (GVL) effects. In 1956, Barnes et al. reported that leukemia-bearing mice receiving allogeneic cells eventually died of GVHD without evidence of leukemia (2). Mathe et al. proposed a concept of GVL effect (23), which was soon demonstrated clinically (24–27). Importantly, pan T-cell depletion was shown to reduce GVHD at the expense of an increased risk of opportunistic infection and leukemia relapse (28). Shlomchik and colleagues refined our understanding of the subsets of mature T cells responsible for GVHD, demonstrating that naïve T cells rather than memory T cells played the major role in inducing GVHD in mice (29, 30). Early clinical trial data of naïve T cell-depleted PBSCT has shown promising results (31), but definitive randomized data is needed to confirm a role of naïve *versus* memory T cells in GVHD and GVL.

The predominant expansion of Th1/Tc1 and Th17/Tc17 cells in mice and the cytokines derived from these cells suggests that acute GVHD is primarily driven by Th1/Tc1- and Th17/Tc17-associated immune reactions (32–35). There is a crosstalk between GVHD and infection; GVHD-associated immunodeficiency, dysbiosis, and disruption of epithelial and mucosal barrier are risks for infections, while bacterial and viral infections are risks for GVHD by activating innate immunity (36). Neutrophils activated by translocation of intestinal bacteria can also accelerate GVHD early after BMT *via* tissue injury (34). Mechanistically, bacteria and virus-derived molecules behave as pathogen-associated molecular patterns (PAMPs) that accelerate allogeneic T cell responses. Candida colonization is a risk for acute GVHD and fluconazole prophylaxis is associated with reduction of severe acute GVHD (37, 38). In mouse models, fungal cell wall components such as sugar polymers, are recognized by macrophages and promote Th17 differentiation that exacerbate GVHD (39). These results highlight importance of infection prophylaxis in the control of GVHD.

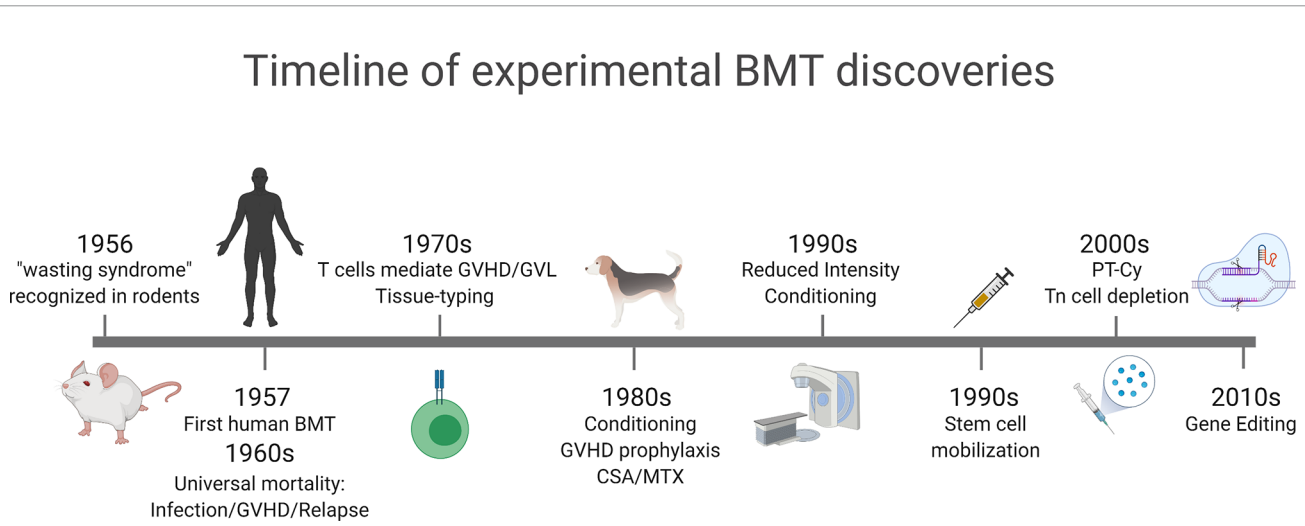

FIGURE 1 | Timeline of major experimental concepts that have translated into clinical practice. GVHD was initially described as a wasting syndrome in transplanted mice in 1956. Early clinical bone marrow transplantation was associated with high mortality due to GVHD, infection and relapse. Recognition of T cells as the mediators of GVHD and GVL and initiating rudimentary tissue-typing. Conditioning and GVHD prophylaxis regimens were developed in dog models in the 1980s that led to reduced intensity and non-myeloablative conditioning in the 1990s. Stem cell mobilization following cytokine administration was developed in the early 1990s and gained widespread clinical translation. New approaches to GVHD prophylaxis, including post-transplant cyclophosphamide (PT-Cy) and naïve T (Tn) cell depletion developed in the 2000s and are increasingly utilized in the clinic. In the last decade the widespread use of gene editing, initially in T cells, has been widely translated to modulate GVHD and GVL. Figure generated with biorender.com.

Unfortunately, it is impossible to discern cause and effect from human microbiota studies which generate associations between bacterial taxa typically derived from 16s ribosomal sequencing and transplant outcomes (40). The use of shotgun sequencing allows for the imputation of various functional properties of bacterial species (e.g. the likely ability to generate various metabolites) which provide further granularity and allows hypothesis generation (41). Recently, gnotobiotic and/or antibiotic decolonized mice have allowed true cause and effect to be ascertained whilst permitting dissection of the mechanisms by which microbiota invoke GVHD, both at initiation and amplification phases of the disease (42, 43).

Regulatory T cells (Treg) defined by the transcription factor FoxP3 are pivotal for the maintenance of self-tolerance and the induction of tolerance after allogeneic hematopoietic stem cell transplantation (HCT). Depletion of $CD25^+$ cells from the donor inoculum exacerbates acute GVHD and infusion of $CD4^+CD25^+$ Treg inhibits GVHD in mice (44–46). Mogamulizumab, anti-CCR4 antibody, eliminates Treg, in which CCR4 is highly expressed (47). Pretransplant administration of mogamulizumab is a risk for severe acute GVHD (48). Following the link between Treg and acute GVHD, early phase clinical studies of Treg infusion demonstrate safety of Treg infusion (49–54) but definitive data on efficacy is awaited. The use of epigenetic modifiers such as histone deacetylase inhibitors has been shown to attenuate acute GVHD and enhance regulatory T cell activity in preclinical systems (55). Promising activity in subsequent early phase clinical GVHD prophylaxis studies has also been seen (56). Additional immunomodulatory effects of these agents have recently been reviewed elsewhere (57).

GVHD prophylaxis using calcineurin inhibitors (cyclosporin or tacrolimus) reduce the expansion of effector T cells (Teff) by blocking IL-2 and prevent acute GVHD, but fail to reduce chronic GVHD (58–60). Calcineurin inhibitors regulate Teff at the expense of Treg inhibition and the major challenge of GVHD prophylaxis is to selectively control Teff, while preserving Treg. In addition, calcineurin inhibitors are not sufficient in isolation to control GVHD in HLA-mismatched HCT (61, 62). Alternative approaches have been explored in preclinical systems. A study in the early 1960's showed that high-dose cyclophosphamide (Cy) prolonged murine skin allograft survival only when given shortly after transplant (63). Mayumi and Nomoto then continued studies to elucidate mechanisms of tolerance induction by post-transplant Cy (PTCy) in mice (64). Tolerogenic effects of PTCy were exerted through selective elimination of alloreactive T cells, while preserving bystander T cells and Treg (65–67). Subsequently, the Johns Hopkins group translated PTCy to the clinic and confirmed a low incidence of both acute and chronic GVHD, even after haploidentical HCT (68, 69). GVHD prophylaxis using PTCy is a standard of care in haploidentical HCT and also potentially in HLA-matched related and unrelated donor transplantation, either with bone marrow or peripheral blood stem cell sources (70, 71).

Role of the Conditioning Regimen

In the setting of BMT, donor T cells are infused into recipients that have potentially experienced tissue injury by prior treatments of the underlying malignancy, infections, and more immediately, pre-transplant conditioning. The inflammatory environment invoked by these therapies predispose to a state of enhanced alloantigen-presentation. Johnson and Truitt demonstrated that delayed infusion of donor T cells induced less severe GVHD (72). The Ferrara group demonstrated that the conditioning regimen, particularly total body irradiation (TBI), induced proinflammatory cytokine secretion (e.g. IL-1 and TNF) and increased the severity of acute GVHD in animal models (73, 74). These studies demonstrated that GI tract injury and associated pathogen-derived danger signals are critical to the propagation of the "cytokine storm" characteristic of acute GVHD (75). In humans, clinical studies clearly show that myeloablative conditioning, particularly TBI is a risk for acute GVHD (76, 77). Given this link between conditioning intensity and acute GVHD, non-myeloablative and reduced intensity conditioning regimens were developed by Storb and colleagues in dog models (78). The translation of these to humans were associated with reduced incidence of acute GVHD although later-onset acute GVHD, occurring after day 100 was noted (79).

Antigen Presentation

In 1999, Shlomchik et al. demonstrated in preclinical mouse systems that recipient antigen presenting cells (APCs) were responsible for donor T cell activation and the induction of acute GVHD (80). They subsequently showed that although host APCs were much more potent, reconstituting donor hematopoietic APCs were necessary to invoke the full spectrum and severity of acute GVHD (81). They also demonstrated that these donor APCs could cross-present host antigens to induce chronic GVHD (82, 83). These hematopoietic (or professional) recipient or donor APCs were predominantly dendritic cells (DCs) (84, 85). Unexpectedly, Koyama et al. showed that non-hematopoietic recipient APCs exhibited a potent capacity to induce lethal acute GVHD (86) and consistent with this, depletion of recipient professional $CD11c^+$ or $CD11b^+$ APCs do not prevent GVHD (86, 87).

Subsequent mouse studies have demonstrated that intestine is a critical site for alloreactive T cell activation by APCs (86, 88, 89). Importantly, the pathogenic donor APCs in the colon are GM-CSF dependent, providing a potential therapeutic target [reviewed in (90)]. Intestinal epithelial cells highly express MHC class II and thereby regulate tolerance to intestinal commensals while inducing immunity against pathogens (91). Koyama et al. have demonstrated that prior to HCT, intestinal epithelial cells (IEC) express MHC class II in the ileum and can stimulate donor T cells and initiate acute GVHD, defining the lineage of the non-hematopoietic APC that initiates lethal GVHD (43). Both microbiota and conditioning invoke IL-12p40 dependent generation of interferon (IFN)-γ to mediate these effects by IEC (43). A translational clinical study has now commenced blocking IL-12p40 prior to conditioning in an attempt to prevent the initiation of MHC class II dependent GVHD within the GI tract (NCT04572815) (see also below).

Other mouse studies have been shown that intestine is a critical site for alloreactive T cell activation by APCs (86, 88). The

α4β7 integrin-MAdCAM (mucosal addressin cell adhesion molecule) -1 pathway is critical for T cell homing to the intestine (88). Such a pathway found in mice has clinical potential for translation. Maraviroc is a small-molecule drug that block CCR5. However, addition of Maraviroc to standard GVHD prophylaxis composed of tacrolimus and methotrexate failed to reduce incidence of acute GVHD (92). Vedolizumab and Natalizumab, a humanized monoclonal antibody specifically target α4β7 integrin showed potentially promising results in phase I/II studies (93, 94), and these agents are currently tested in larger studies.

Co-Stimulation

The activation, proliferation and differentiation of donor T cells requires recognition of alloantigen presented within MHC in the context of additional signals, usually a cognate costimulatory signal (characteristically CD40L – CD40 and CD28 – CD80/86 on the T cell and APC, respectively) and a differentiation signal in the form of cytokine as defined above. The recognition of the importance of cognate costimulatory signals has led to the investigation of the relative ability of inhibitory antibodies that block these pathways in preclinical models. Thus CTLA-4-Ig that inhibits CD80 and CD86, anti-CD28 and anti-CD40 have all been shown to attenuate GVHD in preclinical models (95–97). The clinical reagent abatacept (CTLA-4-Ig) has also shown promise in preventing acute GVHD in early phase clinical studies (98, 99). Additional pathways such as OX40L and ICOSL may also be clinically tractable [reviewed in (100)].

Cytokines

Cytokines play a pivotal role in the pathogenesis of GVHD. Many inflammatory cytokines (e.g. IL-1, IL-6, TNF, HMGB1) are generated in response to chemoradiotherapy during conditioning and promote the activation of APCs. Other cytokines act in a costimulatory role to promote pathogenic T cell differentiation (e.g. IL-12, IL-6). Finally pivotal T cell derived cytokines (e.g. IFNγ, GM-CSF, IL-17) can in turn invoke target tissue apoptosis and secondary myeloid cell migration to amplify GVHD [reviewed in (100)]. Initial studies in experimental GVHD models suggested that cytotoxicity mediated by cytotoxic T lymphocytes (CTLs) have a central role in GVHD target tissue injury through the Fas/Fas ligand pathway and perforin/granzyme pathways (101–104). Subsequent studies demonstrated that inhibition of inflammatory cytokines such as TNF, IL-1 and IL-6 also ameliorated GVHD (74, 105–109). In 2002, Teshima et al. formally demonstrated that cytokines alone could generate the typical acute GVHD pathology in the absence of cognate cell-to-cell dependent cytotoxic mechanisms (110). These studies facilitated clinical trials of cytokine blockade for acute GVHD. However, individual cytokine blockade (e.g. TNF-α, IL-1, and IL-2) did not demonstrate significant benefits in randomized trials, suggesting considerable redundancy in these pathways and a likely requirement to inhibit multiple cytokines to gain clinical efficacy (111, 112). With this concept in mind, Ruxolitinib inhibits the signaling of multiple cytokines involved in the pathogenesis of experimental GVHD (113, 114) and a recent randomized study has demonstrated the efficacy of this agent in the treatment corticosteroid-refractory acute GVHD (115). This represents the first successful randomized study for the treatment of acute GVHD and highlights the successful translation of our understanding of the role of cytokines in GVHD from preclinical models. Ruxolitinib also targets Jak2, which relays signals for growth and differentiation of hematopoietic cells, in addition to Jak1, which relays inflammatory cytokines. Although it remains to be determined which pathway is critical for GVHD mitigation, animal studies suggest that neutrophils recruited to GI tract in response to bacterial translocation enhance intestinal GVHD *via* tissue damage (34). α1-Antitrypsin (ATT) inactivates serine proteases produced from neutrophils and macrophages and protect tissues from proteolytic degradation. Administration of AAT ameliorates murine acute GVHD *via* multiple mechanisms (116, 117). A phase 2 study of ATT shows promising results (118) and ATT is currently tested in larger studies.

Tissue Homeostasis in GVHD

The pathophysiology of GVHD beyond donor effector T cells is now better understood. Damage to the intestine plays a central role in propagating a proinflammatory cytokine milieu and amplifying systemic GVHD. Indeed, intestinal GVHD is the major cause of non-relapse mortality after allogeneic HCT (75). Intestinal GVHD is characterized by severe villous atrophy and crypt degeneration; the latter has long been thought of as the cardinal and pathognomonic feature of intestinal GVHD (119–121). Recent data indicate that intestinal stem cells (ISCs) and their Paneth cell niche are targeted in GVHD, resulting in dysregulation of intestinal homeostasis and associated microbial ecology (122–124). Goblet cells are also significantly reduced in GVHD, resulting in disruption of inner mucus layer of the colon and increased bacterial translocation into colonic mucosa (125). In humans, reduced Paneth-cell numbers in duodenal biopsies and Goblet-cell numbers in colon biopsies correlate with the severity of GI-GVHD and transplant outcome (125, 126). Patients who undergo allogeneic HCT exhibit dysbiosis characterized by loss of diversity and expansion of potentially pathogenic bacteria (127–129). The microbiota and their metabolites shape the immune system and intestinal homeostasis (130). Lower microbial diversity and *Enterococcus* domination are associated with increased GVHD and poor survival across diverse ethnicity (40, 42). In addition, recent studies suggest an unexpected association between fungal and viral colonization and GVHD (39, 131). However, there are many open questions to be addressed in this field (132). What are the most important microbes that control transplant outcomes? Should we consider microbiota stewardship in addition to antibiotic stewardship in our transplant teams? Can we use interventions that modify the microbiome to improve transplant outcomes? What is the role of skin microbes in skin GVHD?

The sensitivity of target tissues to GVHD may be modulated by tissue-intrinsic resilience and homeostasis. Thus, integration of both immune tolerance and tissue tolerance could optimize GVHD treatment (133). In the 1990's, Ferrara's group proposed

a concept of using cytokine shields to prevent target tissue damage in the GI tract. IL-11 and keratinocyte growth factor (KGF) protect gut injury from TBI and have anti-inflammatory properties. In mice, IL-11 and KGF ameliorated GVHD (134–136). However, a clinical trial of IL-11 was halted by unexpected severe side effects (137). This trial highlighted that clinical toxicity cannot always be estimated in mice and subsequent studies have also utilized primate models to study efficacy and toxicity (138). Protection of the ISC-niche and modification of the intestinal microbiota and metabolome to restore intestinal homeostasis may also represent a novel approach to modulate GVHD and also infection. In mice, IL-22 and R-Spondin are growth factors for ISCs that ameliorate GVHD in mice (122, 123, 139, 140). Glucagon-like-peptide-2 (GLP-2) is an enteroendocrine tissue hormone. Administration of GLP-2 promoted regeneration of ISCs and Paneth cells and restored intestinal homeostasis, resulting in amelioration of GVHD (141). IL-25 protects Goblet cells and also could improve transplant outcome (125). ISCs and Paneth cells express IFN-γ receptors. IFN-γ secreted by donor T cells induces ISC and Paneth cell death *in vitro* (142, 143). Ruxolotinib inhibits IFN-γ signaling and protects ISCs and Paneth cells (142, 143), which may be an additional mechanism of ruxolitinib's action in intestinal GVHD (113, 144). Despite the promising mouse data, it remains to be elucidated whether modification of GVHD target tissue sensitivity can attenuate clinical GVHD.

Although most studies of tissue stem cell injury in GVHD have focused on the intestine, a recent study demonstrated that skin stem cells are injured in GVHD in association with impaired skin homeostasis (145). Topical corticosteroids suppressed skin inflammation but failed to protect skin stem cells and restore skin homeostasis. In contrast, topical ruxolitinib protected skin stem cells, resulting in restoration of hair regeneration and wound healing (145). These results in animals deserve further clinical scrutiny but will promote to open a new avenue for improved tissue homeostasis in GVHD beyond the GI tract.

CHRONIC GVHD

Chronic GVHD, a pleiomorphic syndrome, is the major cause of nonrelapse mortality and severely impairs the quality of life in long-term survivors of allogeneic HCT. The highly variable clinical manifestations of chronic GVHD frequently involve the skin, liver, eyes, mouth, upper respiratory tract, esophagus, and less frequently serosal surfaces, lower gastrointestinal tract, female genitalia, and fascia (146). The biological mechanisms leading to chronic GVHD are not yet as well understood as those leading to acute GVHD, complicated by the fact that chronic GVHD can present with more heterogenous phenotypes than acute GVHD. Individual mouse models have dominant disease manifestations that typically involve a limited number of organs. The B6 into B10.BR model induces chronic GVHD primarily presenting as bronchiolitis obliterans (147). The B10.D2 into BALB/c model induces scleroderma as the primary manifestation (148). G-CSF-mobilized SCT (bothB6 into B6D2F1 and Balb/c to B6 mouse models) generate scleroderma, liver disease and Sjogren's features (149). Using these preclinical models, significant progress has been made in the last decade and mouse models have demonstrated a critical role for donor Treg defects, germinal center B cell expansion and alloantibody secretion, and dysregulated Th17/Tc17 and T follicular helper (Tfh) differentiation in the development of chronic GVHD (150–157). Ibrutinib, an inhibitor of Bruton's tyrosine kinase, has showed clinical efficacy in a phase II clinical trial and was approved for chronic GVHD, representing the first such agent (158). Treg are numerically decreased and dysfunctional in patients with chronic GVHD (159, 160). Low-dose IL-2 preferentially stimulates proliferation, function, and survival of Treg; low-dose IL-2 administration to patients has been shown expands Treg and ameliorates chronic GVHD in a proportion of patients (161, 162). Efavakeukin-α, IL-2 mutein, is currently tested in a clinical trial. Ruxolitinib suppresses dysregulated inflammatory cytokine responses in chronic GVHD and is effective in patients with chronic GVHD (144); results of a prospective phase 3 trial of ruxolitinib for steroid refractory chronic GVHD are expected soon. Tfh and germinal center B cells (GCB) play a role in the development of chronic GVHD and bronchiolitis obliterans syndrome (152, 153, 163). The rho-associated coiled-coil kinase 2 (ROCK2) inhibitor, belumosudil (KD025), inhibits the differentiation of Th17 and Tfh together with GCB, and alloantibody generation (164). Syk inhibition induces apoptosis of activated B cells and ameliorates chronic GVHD (165, 166). Belumosudil and the Syk inhibitor Fostamatinib are currently being tested in clinical trials. Tissue fibrosis, the main manifestation of chronic GVHD, is characterized by increased deposition of collagen fibers secreted from activated fibroblasts in response to profibrotic cytokines such as TGF-β and PDGF-α secreted by CSF-1R-dependent macrophages (157, 167–172) [reviewed in (173)]. This pathological cascade of fibrosis defined in mice, has given rise to a number of new potential targets, including TGF-β, PDGF-α and CSF-1R; CSF-1R antibody axatilimab, which inhibits signaling through CSF-1 and IL-34, is currently undergoing assessment in clinical trials (NCT04710576).

CONCLUSIONS

Mouse models of GVHD faithfully recapitulate the pathological lesions seen in clinical disease and allow the dissection of pathogenic *versus* protective immunological mechanisms of action and tissue resistance. While the ability to tightly control MHC and minor antigen barriers is a strength, the inbred nature of these systems may overlook variables present in outbred human populations (e.g. microbiota, age, obesity, prior therapy, comorbidities, conditioning, immune suppression). Some of these limitations can be overcome by more thorough study of these variables in mice (e.g. age, obesity, conditioning, concurrent immune suppression) and/or the use of non-human

primates or dog models (especially pharmacological immune suppression). Additional limitations include the widespread use of cell lines to study graft-*versus*-leukemia effects and the lack of relevant models to study pathogen-specific immunity in the context of new therapies, at least until recently. Nevertheless, to date almost all effective therapeutic paradigms that are now established in humans have their genesis in animal models, suggesting that these systems will continue to provide valuable insights and therapeutic advances to the field. Importantly, it would seem critical that well-established preclinical systems are utilized to analyze the effects of various therapeutic interventions before they are translated into early phase clinical trials.

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REVIEW
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Novel Insights Into the Mechanism of GVHD-Induced Tissue Damage

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Prophylaxis for and treatment of graft-versus-host disease (GVHD) are essential for successful allogeneic hematopoietic stem cell transplantation (allo-SCT) and mainly consist of immunosuppressants such as calcineurin inhibitors. However, profound immunosuppression can lead to tumor relapse and infectious complications, which emphasizes the necessity of developing novel management strategies for GVHD. Emerging evidence has revealed that tissue-specific mechanisms maintaining tissue homeostasis and promoting tissue tolerance to combat GVHD are damaged after allo-SCT, resulting in exacerbation and treatment refractoriness of GVHD. In the gastrointestinal tract, epithelial regeneration derived from intestinal stem cells (ISCs), a microenvironment that maintains healthy gut microbiota, and physical and chemical mucosal barrier functions against pathogens are damaged by conditioning regimens and/or GVHD. The administration of growth factors for cells that maintain intestinal homeostasis, such as interleukin-22 (IL-22) for ISCs, R-spondin 1 (R-Spo1) for ISCs and Paneth cells, and interleukin-25 (IL-25) for goblet cells, mitigates murine GVHD. In this review, we summarize recent advances in the understanding of GVHD-induced tissue damage and emerging strategies for the management of GVHD.

Keywords: allogeneic hematopoietic stem cell transplantation, GVHD, graft-versus-host disease, intestinal stem cells, tissue stem cells, microbiota, Paneth cell, goblet cell

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INTRODUCTION

Mature epithelial cells in the gut, skin, and liver have long been recognized as the primary target of acute graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-SCT). Mature epithelial cells in the gut are composed of functionally distinct populations, including enterocytes, Paneth cells, goblet cells, tuft cells, and enteroendocrine cells. Each of these epithelial populations contributes to the maintenance of tissue homeostasis (**Table 1**). Thus, injury of these epithelial cells results in alteration of the tissue microenvironment and disruption of tissue homeostasis, potentially amplifying GVHD-induced tissue damage. Furthermore, emerging evidence indicates that adult tissue stem cells are primarily targeted by GVHD, which decreases tissue resilience in GVHD target organs (5, 7, 19). Here, we review recent advances in the understanding the cellular and molecular mechanisms of GVHD-induced tissue damage and disruption of the tissue microenvironment.

TABLE 1 | Intestinal cells that maintain intestinal homeostasis.

Cell Type	Location	Function	Mouse GVHD	Human GVHD	References
DCS cell	LI	Secrete ISC growth factors such as EGF and NOTCH ligands	Unknown	Unknown	Sasaki et al. (1), PMID: 27573849
Goblet Cell	SI/LI	Maintain the mucus layers by mucin production	↓	↓	Ara et al. (2), PMID: 32611682
ILC2	SI/LI	Secrete goblet cell growth factors such as IL-4/IL-13 in response to IL-33 and IL-25	↓ a)	↓ a),b)	Bruce et al. (3), PMID: 28375154 Munneke et al. (4), PMID: 24855210
ILC3	SI/LI	Secrete a ISC growth factor, IL-22	↓	Unknown	Hanash et al. (5), PMID: 22921121 Lindemans et al. (6), PMID: 26649819 Munneke et al. (4), PMID: 24855210
ISC	SI/LI	Differentiate into all types of intestinal epithelial cells	↓	↓	Takashima et al. (7), PMID: 21282378 Takashima et al. (8), PMID: 31811055
L Cell	SI/LI	Secrete a ISC growth factor, GLP-2	↓	↓	Norona et al. (9), PMID: 32542357
LEC	SI	Secrete a ISC growth factor, R-Spondin 3	↓	Unknown	Ogasawara et al. (10), 30013036
MRISC	LI	Secrete a ISC growth factor, R-Spondin 1 (Production of R-Spondin1 is enhanced in response to gut injury).	Unknown	Unknown	Wu et al. (11), PMID: 33658717
Paneth Cell	SI	Secrete ISC growth factors such as EGF and Wnt3 Secrete antimicrobial peptides, such as α-defensins	↓	↓	Eriguchi et al. (12), PMID: 22535662 Jenq et al. (13), PMID: 22547653 Hayase et al. (14), PMID: 29066578 Levine et al. (15), PMID: 23760615
Telocyte (at crypt base)	SI/LI	Secrete a ISC growth factor, R-Spondin 3	Unknown	Unknown	Shoshkes-Carmel et al. (16), PMID: 29720649
Tuft Cell	SI/LI	Stimulate ILC2 by production of IL-25	Unknown	Unknown	Gerbe et al. (17), PMID: 26762460 von Moltke et al. (18), PMID: 26675736

DCS cell, deep crypt secretory cell; ILC2, type 2 innate lymphoid cell; ILC3, type 3 innate lymphoid cell; ISC, intestinal stem cell; LEC, lymphatic endothelial cell; LI, large intestine; MRISC, Map3k2-regulated intestinal stromal cell; SI, small intestine.

a) Prolonged ILC2 reduction is induced by irradiation and/or chemotherapy. b) Reduction of ILC2 has been only demonstrated in the peripheral blood.

This review mainly focuses on gastrointestinal GVHD, while recent findings on the injury of tissue stem cells in the other organs are also summarized.

TISSUE DAMAGES IN GVHD

It has been recognized that bacterial and fungal pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide and α-mannan, play a critical role in initiating GVHD (20–23). PAMPs enhance production of proinflammatory cytokines, host alloantigen presentation, and infiltration of innate cells into the gastrointestinal tracts early after conditioning (21, 24, 25). Recent advances revealed the critical role of sterile damage-associated molecular patterns (DAMPs) in pathophysiology of GVHD. Tissue damage induced by conditioning chemotherapy and/or irradiation promotes release of DAMPs from damaged cells and initiates the inflammatory cascade which culminates in expansion of donor alloreactive T cells and development of acute GVHD. DAMPs are comprised of various molecules that are sequestrated in the cells in the steady state, while released into the extracellular space by cellular damages. Extracellular ATP activates host antigen presenting cells and inflammatory monocytes *via* the purinergic P2X7 and P2Y2 receptors, respectively, that exaggerates mouse GVHD (26, 27). It has been shown that lack of nucleotide-binding oligomerization domain–like (NOD) receptor protein 3 (NLRP3), a known target of ATP/P2X7 receptor signaling, in the recipient mice ameliorated GVHD, suggesting that ATP exaggerates GVHD *via* activation of NLRP3 inflammasome (28). ATP-induced NLRP3 activation in myeloid-derived suppressor cells reduces anti-GVHD effects of these cells after adoptive transfer (29). Another NLRP3 activator, uric acid is released into the extracellular space after conditioning and exaggerates GVHD (28). Interleukin-33 (IL-33) is released from epithelial cells after injury and promotes effector T-cell differentiation of donor T cells, that results in the exaggeration of GVHD (30, 31). Heparan sulfate and high-mobility group box 1 protein bind to toll like receptor 4 and induce GVHD after allo-SCT (32, 33).

DAMPs and PAMPs primarily activate myeloid inflammatory cells such as neutrophils and monocytes, and antigen presenting cells such as dendritic cells and macrophages. Conditioning-induced tissue damage promotes accumulation of host neutrophils and production of reactive oxygen species in the gastrointestinal tract, that in turn amplifies the tissue injury (25). Interestingly, neutrophils accumulated in the gastrointestinal tract early after conditioning migrate to mesenteric lymph nodes and promote activation of host antigen presenting cells and donor T cells (34). It has been shown that donor neutrophils also exaggerate GVHD (35). In patients' samples, higher density of neutrophil infiltration in the gut was associated with worse outcomes of GVHD, further emphasizing critical role of neutrophils in pathophysiology of acute GVHD (36). Monocytes and inflammatory macrophages also contribute to development of GVHD by producing proinflammatory cytokines in response to DAMPs and PAMPs and promoting activation of donor T cells (23, 27, 37). Importantly, IL-12 produced from monocytes and macrophages after irradiation enhances antigen presentation by host non-hematopoietic cells and exaggerates GVHD (24). On the other hand, host tissue resident macrophage, the ontogenetically independent population from monocytes and

inflammatory macrophages, plays a protective role against GVHD by suppressing donor T cell expansion (38–40).

TISSUE STEM CELLS AS TARGET OF GVHD

Injury of ISCs in Intestinal GVHD

Histological features of intestinal GVHD include epithelial apoptosis, crypt degeneration, and mucosal sloughing, as well as inflammatory cell infiltration (41). Early preclinical studies pointed out that proliferation of crypt cells was enhanced in less severe GVHD, while more severe GVHD abrogated crypt cell proliferation in association with villus atrophy and loss of the crypt; these findings indicated that severe GVHD targets putative tissue stem cells residing in the intestinal crypt (42). More recently, leucine-rich-repeat-containing G protein-coupled receptor 5 (LGR5) was found to be a unique marker for cycling intestinal stem cells (ISCs) residing at the crypt base of the small intestine and colon (43) (**Figure 1**). In the steady state, approximately 10 cells are produced every hour in each crypt and migrate to the villus tip in 2-3 days, and a lineage-tracing study using the LGR5-Cre reporter system revealed that LGR5$^+$ ISCs give rise to all gut epithelial lineages (43, 44). Depletion of LGR5$^+$ ISCs in the mice, in which diphtheria toxin receptor (DTR) was specifically expressed in LGR5$^+$ cells, significantly delayed epithelial regeneration after irradiation-induced intestinal damage, suggesting that LGR5$^+$ ISCs are important also for the regenerative process after gut injury (45). Adoptive transfer of eGFP-specific TCR-transgenic T cells (Jedi T cells) depleted LGR5-eGFP$^+$ ISCs and profoundly impaired the regenerative response after irradiation, suggesting that ISCs are susceptible to T cell-mediated injury (46–48). Furthermore, the crypt base region is the primary site infiltrated by donor T cells after allo-SCT; donor T cells migrate to the crypt base region in a MAdCAM-1-dependent manner as early as day 4 after murine allo-SCT, suggesting that ISCs are the primary target of gut GVHD (49).

A landmark study by Takashima et al. demonstrated that ISCs marked by another ISC-specific marker, olfactomedin-4 (Olfm4), are targeted by intestinal GVHD (7) (**Figure 2**). The reduction of LGR5$^+$ ISCs in intestinal GVHD was then confirmed using a LGR5 reporter system (5). Due to the rapid turnover of gut epithelial cells, depletion of cycling ISCs in the crypt in intestinal GVHD soon leads to villus atrophy and causes refractory colitis (44). In the small intestine, quiescent Bmi1$^+$ stem cells exist at four cell diameters above the base of the crypt and are called +4 stem cells (50). These cells are activated only after severe gut

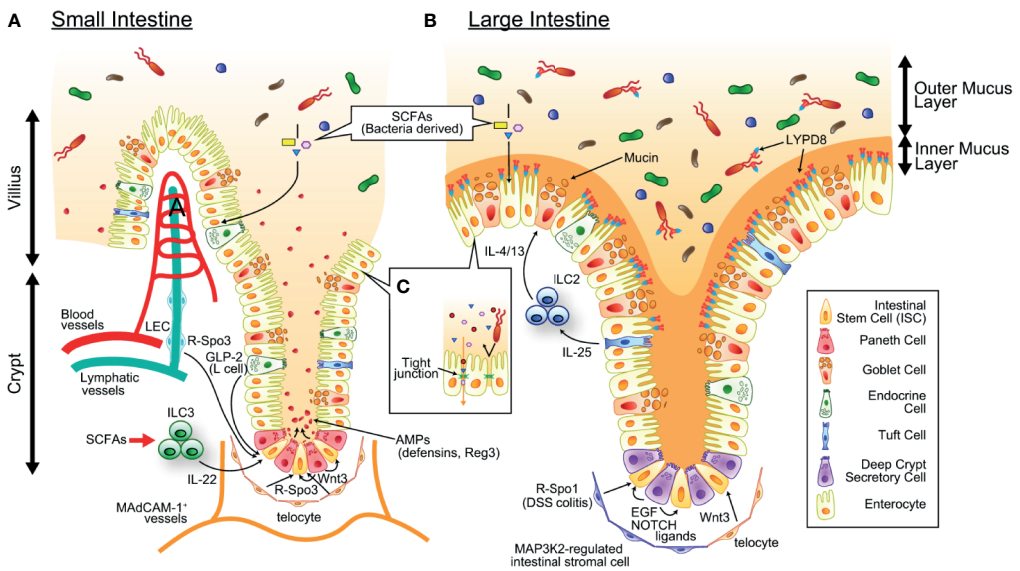

FIGURE 1 | The mechanism maintaining intestinal homeostasis. ISCs residing at the crypt base give rise to all cell lineages in the epithelium and are supported by growth factors produced by definitive and putative niche components. SCFAs produced by commensal bacteria serves as energy source of intestinal epithelial cells. **(A)** In the small intestine, Paneth cells and telocytes produce Wnt3, telocytes and LECs produce R-Spo3, L cells produce GLP2, and ILC3s (green round cells in the figure) produce IL-22. Paneth cells also produce a large amount of AMPs such as α-defensins and REG3, and maintain healthy intestinal microbiota. **(B)** In the colon, deep crypt secretory cells produce EGF and NOTCH ligands, telocytes produce WNT3, and MAP3K2-regulated intestinal stromal cells produce R-Spo1. There are tremendous numbers of bacteria in the colonic lumen, which is segregated from epithelial cells by the inner mucus layer containing mucins produced by goblet cells and antimicrobial molecules such as REG3 and LYPD8 produced by enterocytes. IL-25 produced from Tuft cells stimulates ILC2s (blue round cells in figure) to secrete goblet cell growth factors such as IL-4 and IL-13. SCFAs produced by commensal bacteria serves as energy source of intestinal epithelial cells. **(C)** The intestinal epithelial tight junctions exhibit both size and charge selectivity and regulate the selective paracellular permeability, inhibiting penetration of bacteria and bacterial components while permitting the passage of water, ions, and small molecules. AMP, antimicrobial peptide; EGF, epithelial growth factor; GLP-2, Glucagon-like peptide 2; LEC, lymphatic endothelial cell; ILC2/3, type 2/3 innate lymphoid cell; IL-4/13/22/25, interleukin-4/13/22/25; ISC, intestinal stem cell; LYPD8, Ly6/PLAUR domain-containing protein 8; R-Spo1/3, R-spondin 1/3; SCFA, short-chain fatty acid.

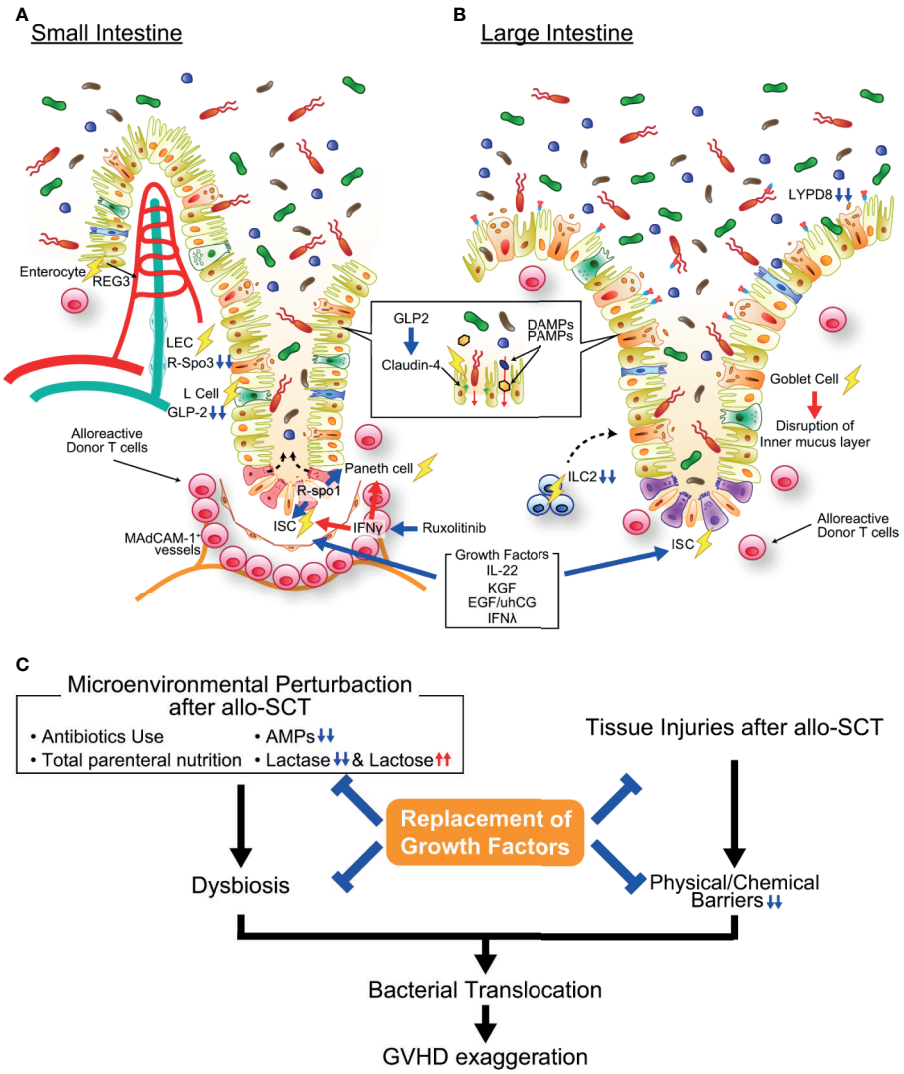

FIGURE 2 | Pathophysiology of gastrointestinal graft-versus-host disease (GVHD). **(A)** In the small intestine, activated alloreactive donor T cells (pink round cells in figure) migrate to the crypt base region early after allogeneic transplantation in a MAdCAM-1-dependent manner and damage ISCs, resulting in impairment of mature intestinal epithelial cell regeneration. Paneth cell injury causes the reduction of AMP production and loss of function as an ISC niche. IFN-γ plays an important role in both ISC and Paneth cell injury in GVHD, and ruxolitinib protects ISCs and Paneth cells against GVHD. Moreover, growth factors of ISCs such as R-Spondin 3, IL-22, and GLP-2 are reduced in the intestine due to GVHD-induced reduction of LECs, ILC3s, and L cells. The expression of tight junction molecules such as claudin-4 are also reduced in GVHD, resulting in disruption of intestinal epithelial barrier function. **(B)** In the large intestine, goblet cell injury in GVHD results in disruption of the mucus layers bleaching both chemical and mechanical barrier functions of the intestinal mucosa. ILC2s, producer of goblet cell growth factors, are profoundly depleted by conditioning radiotherapy or chemotherapy, likely inhibiting regeneration of goblet cells. **(C)** Microenvironmental perturbation after allo-SCT induced by administration of antibiotics and/or total parenteral nutrition, reduction of AMP production, and lactose malabsorption leads to intestinal dysbiosis, frequently accompanying *Enterococcus* domination. Dysbiosis and disruption of barrier function of the intestinal mucosa enhance bacterial translocation, further exaggerating GVHD. Replacement of growth factors for ISCs, Paneth cells, and goblet cells ameliorate GVHD. Allo-SCT, allogeneic hematopoietic stem cell transplantation; DAMP, damage-associated molecular pattern; EGF, epidermal growth factor; IFN-γ, interferon-γ; KGF, keratinocyte growth factor; LYPD8, Ly6/PLAUR domain-containing protein 8; PAMP, pathogen-associated molecular pattern; REG, regenerating islet-derived protein; R-Spo1, R-spondin1; uhCG, urinary-derived human chorionic gonadotropin.

injury or depletion of LGR5$^+$ ISCs and differentiate into all types of epithelial cells, including LGR5$^+$ ISCs (51, 52). However, the fate and role of this second stem cell population in GVHD remain to be clarified. The mechanisms by which GVHD causes injury of LGR5$^+$ ISCs have been studied intensively using a gut organoid culture system. Single LGR5$^+$ ISCs isolated from the intestine give rise to crypt–villus organoids containing all differentiated cell types of the intestinal epithelium without the support of niche cells (53). Coculture of intestinal organoids with activated T cells induced caspase-3/caspase-7 cleavage and apoptosis of LGR5$^+$ ISCs in the organoid, while IFN-γ blockade prevented T cell-mediated injury of the organoids, indicating that activated T cells

damage LGR5$^+$ ISCs in an IFN-γ-dependent manner (8, 54). In mouse models of allo-SCT, significantly more LGR5$^+$ ISCs persisted after transplantation with IFN-γ-deficient donor T cells than after transplantation with wild-type (WT) donor T cells (54). Furthermore, administration of IFN-γ significantly reduced LGR5$^+$ ISCs in the mice conditioned with total body irradiation, while it induced a proliferative response in the crypt in nonirradiated mice (54). Thus, IFN-γ seems to be more harmful for ISCs in the presence of genotoxic stress, such as irradiation, *in vivo*, while a high concentration of IFN-γ alone could induce apoptosis of ISCs *in vitro*. Alternatively, radiosensitive niche components could protect ISCs from IFN-γ *in vivo* to some extent.

Injury of the ISC Niche

The ISC niche, which provides survival and growth factors for ISCs, is also targeted by GVHD (**Figures 1** and **2**). Interleukin-22 (IL-22) produced by type 3 innate lymphoid cells (ILC3s) is a well-described growth factor of LGR5$^+$ ISCs (5). Total body irradiation (TBI) enhances IL-22 production from radioresistant ILC3s in an interleukin-23 (IL-23)-dependent manner, which is believed to promote regeneration of epithelial cells from radiation-induced damage. IL-22-producing ILC3s persisted after syngeneic bone marrow transplantation, while they were depleted after mouse allogeneic transplant, indicating that GVHD targets ILC3s. A reduction in IL-22 producing ILC3s in GVHD is associated with prolonged depletion of ISCs and exacerbation of gut GVHD.

Crypt bases have enriched transcription of Wnt target genes, and Paneth cells produce high levels of Wnt3, suggesting that Paneth cells are an ISC niche component (55, 56). Although the survival and proliferation of LGR5$^+$ ISCs were not affected in Paneth cell-deficient mice in the steady state, Paneth cells may protect ISCs against gut injury (57). Because Paneth cells are also susceptible to IFN-γ-induced apoptosis in GVHD, regeneration of the gut epithelium from ISCs could be further disturbed in intestinal GVHD (12, 13). On the other hand, Paneth cell-derived Wnt3 is redundant with that produced from subepithelial telocytes (16, 58–60). It remains to be clarified whether telocytes are targeted by GVHD. In the colon, which is devoid of Paneth cells, deep crypt secretory (DCS) cells residing at the crypt base act as the niche for LGR5$^+$ ISCs by producing NOTCH ligands and epidermal growth factor (EGF) (1). While DCS cells do not produce Wnt ligands, stromal tissues surrounding colonic crypts produce Wnt ligands and support colonic ISCs (58). It also remains to be clarified whether DCS cell are targeted by GVHD. R-spondins are the ligands of LGR4, LGR5, and LGR6 and enhance Wnt/β-catenin signaling by preventing ubiquitination and degradation of the Wnt receptor Frizzled (61, 62). The R-spondin family is composed of four molecules. R-Spo1-R-Spo4 share a similar structure, and each of these four molecules can bind to LGR4, LGR5 and LGR6 (63). We found that R-Spo3 is the major molecule produced in the small intestine (10). Although it has been reported that mesenchymal cells, including telocytes, produce R-Spo3, we found that CD90$^+$CD31$^+$podoplanin$^+$ lymphatic endothelial cells are the main producers of R-Spo3 in the intestine (10, 16). Importantly, both the R-Spo3 production and absolute numbers of lymphatic endothelial cells are significantly reduced in GVHD (10). On the other hand, it remains to be clarified whether R-Spo3-producing telocytes are targeted by GVHD. The importance of R-Spo3 was also demonstrated in an antibody-mediated inhibition study, in which administration of anti-R-Spo3 antibodies alone reduced LGR5$^+$ ISCs in naïve mice and suppressed the regenerative response after irradiation (64). Although this study showed that anti-R-Spo2 antibodies and anti-R-Spo3 antibodies work synergistically in the depletion of LGR5$^+$ ISCs, the cellular source of R-Spo2 in the intestine remains to be clarified. Interestingly, recent study showed that Map3k2-regulated intestinal stromal cells (MRISCs) residing around the crypt base enhance production of R-Spo1 in response to dextran sodium sulfate (DSS)-induced colitis and protect colonic ISCs (11). Map3k2-deficient mice are more susceptible to DSS-induced colitis compared with wild type controls, further emphasizing a protective role of MRISCs against inflammation of the colon (11). These findings suggest that there are distinct ISC niche systems in the small intestine and the colon, and further studies are required to assess the fate of these ISC niches in GVHD.

GVHD Prophylaxis and Treatments Targeting ISCs

Strategies that protect ISCs or induce their regeneration could be therapeutic options for GVHD that avoid strengthening immune suppression, which could lead to infection or leukemia relapse. As mentioned above, the reduction in IL-22 produced by ILC3s in GVHD leads to depletion of ISCs. IL-22 induces the proliferation and differentiation of ISCs and inhibits the apoptosis of ISCs after genotoxic stress (65). Replacement of IL-22 by administration of F-652, a recombinant fusion protein consisting of an rhIL-22 dimer and Fc fusion protein, after mouse allogeneic bone marrow transplantation enhanced the recovery of ISCs, increased epithelial regeneration, and ameliorated GVHD (6). However, the potential benefit of IL-22 could be limited because IL-22 secreted from donor T cells has been shown to aggravate GVHD by reducing Tregs and enhancing inflammatory responses (66–68). It has been suggested that IL-22 induces Th1 cell infiltration in the gastrointestinal tract *via* a host type I interferon dependent manner (69). Thus, the safety and efficacy of IL-22 replacement therapy must be evaluated in clinical studies; F-652 is now being tested for the treatment of lower gastrointestinal acute GVHD (NCT02406651). Because ILC3s produce IL-22 in response to bacterial metabolites such as short-chain fatty acids (SCFAs), probiotics that produce SCFAs could be used for GVHD prophylaxis (70).

Administration of R-spondins is also promising for GVHD prophylaxis, as this strategy protects ISCs against mouse GVHD. Recombinant human R-Spo1 (rhR-Spo1) was found to stimulate the proliferation of epithelial cells in the intestinal crypt (71). Subsequently, it was shown that rhR-Spo1 expands ISCs in naïve mice and mice undergoing allo-SCT. Importantly, rhR-Spo1

administered in the peritransplant period protects ISCs against GVHD and ameliorated GVHD after allo-SCT in TBI-conditioned mice (7). In contrast, rhR-Spo1 does not impact the severity of GVHD after allo-SCT without conditioning, potentially indicating synergistic effects of TBI and T-cell-derived IFN-γ on ILC injury (7, 54). Administration of a Robo ligand, Slit2 works synergistically with R-Spo1 in preventing ISC loss after chemoradiotherapy, suggesting that this combination could be useful for GVHD prophylaxis (72).

Type III interferon plays a protective role against gastrointestinal GVHD. Type III interferon family was discovered in 2003 and consists of four molecules, IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4 (73). Among them, IFN-λ2 and IFN-λ3 are expressed in both humans and mice, while IFN-λ1 gene is a pseudogene in mice, and IFN-λ4 gene is absent in mice. IFN-λ receptor consists of two chains, including a unique subunit, IFN-λ receptor 1 (IFNLR1) and common IL-10 receptor-β (IL-10RB) chain, which is shared with cytokines of the IL-10 family. IFNLR1 is preferentially expressed in gastrointestinal epithelium, suggesting that IFNλ is a key effector cytokine in mucosal immunity (74, 75). Recently, Henden and colleagues showed that IFNλ treatment improves the proliferative and regenerative capacity of $LGR5^+$ ISCs independently of IL-22 and ameliorates murine GVHD (76). Since, pegylated recombinant IL-29 is being developed as an adjunctive therapy for Hepatitis C, this agent may be rapidly testable for clinical GVHD (77).

Ruxolitinib, a JAK1/2 inhibitor, has been shown to ameliorate mouse and human GVHD and has been approved by the Food and Drug Administration (FDA) in the United States for the treatment of steroid-refractory acute GVHD (78, 79). Ruxolitinib profoundly suppresses T cell activation, proliferation, and differentiation toward T helper 1 (Th1), Th17 and cytotoxic T cells (79). Given the critical role of IFN-γ in ISC injury, it has been tested if ruxolitinib could protect ISCs against GVHD by inhibiting JAK1/2-STAT1 pathway, an indispensable pathway in IFN-γ receptor signaling. Organoid culture systems have demonstrated that allogeneic T cells induce apoptosis of organoids and ISCs in an IFN-γ-dependent manner (8). Ruxolitinib protected ISCs and Paneth cells in organoids from IFN-γ and allogeneic T cells (8, 54). Furthermore, ruxolitinib prevented IFN-γ-induced ISC injury after syngeneic SCT, indicating that ruxolitinib protects ISCs independent of suppression of allogeneic T cell activation (54). These ISC-targeting strategies for GVHD prophylaxis and treatment are promising and could promote regeneration of all types of intestinal epithelial cells after GVHD-mediated injury (**Figure 2**).

Tissue Stem Cells in Other Organs

Tissue stem cells in other target organs, such as the skin and liver, could be involved in GVHD pathophysiology. The fate of skin stem cells in acute cutaneous GVHD has been studied. Multiple tissue stem and/or progenitor populations of epithelial cells have been identified in the skin. The bulge of hair follicles has long been recognized to foster tissue stem cells because long-lived label-retaining cells exist in the hair bulge (80). More recently, it became possible to identify hair follicle stem cells (HFSCs) in the lower part of the bulge as $CD34^+$, cytokeratin 15 $(CK15)^+$, and $LGR5^+$ cells using flow cytometric or immunofluorescent studies (81–83). These HFSCs alone can regenerate all structures of hair follicles and hair shafts and contribute to regeneration of the epidermis after skin injury (19, 82, 84). In addition to HFSCs, $LGR6^+$ stem cells residing directly above the bulge and leucine-rich repeats and immunoglobulin-like domains 1 $(Lrig1)^+$ stem cells in the isthmus maintain the upper pilosebaceous units (85, 86). Other than stem cells in the hair follicles, there are $CK15^+$ epidermal progenitor and/or stem cells in the rete-like prominences (RLPs) of mouse tongues, a surrogate of human epidermal rete ridges of the skin.

Early studies demonstrated that donor T cells primarily migrate to stem cell-rich parts of the skin, such as mouse RLPs, human rete ridges, and the bulge of hair follicles, suggesting that skin stem cells could be targeted by GVHD (87–90). Among multiple stem cell populations, $CK15^+$ stem and/or progenitor cells in mouse RLPs have been shown to undergo cytokine-induced apoptosis in cutaneous GVHD (90–92). Recently, we found that $LGR5^+$ HFSCs were significantly reduced in mouse cutaneous GVHD, in association with reduced numbers of hair follicles, alopecia, and delayed wound healing (19). This finding was rather surprising because a previous study showed that injection of eGFP-specific Jedi T cells did not deplete LGR5-$eGFP^+$ HFSCs, suggesting that these $LGR5^+$ HFSCs are immune privileged (47). This discrepancy suggests that HFSCs are not inherently immune privileged and that the environment and/or HFSC niche protect HFSCs against immune-mediated injury. An extensive inflammatory environment or disruption of the HFSC niche could be responsible for HFSC damage in cutaneous GVHD. One of the HFSC niche components, subcutaneous fat, which acts as regulator of hair cycling and energy reservoir for HFSCs, becomes atrophic in cutaneous GVHD, which can lead to a reduction of $LGR5^+$ HFSCs (19, 93–95). Although the mechanism by which GVHD depletes $LGR5^+$ HFSCs and $CK15^+$ RLP stem cells remains to be clarified, it is worth of note that topical administration of ruxolitinib protects these stem cells from mouse GVHD (19). On the other hand, topical corticosteroids demonstrate direct toxicity that leads to depletion of HFSCs after syngeneic and allogeneic SCT, even though topical steroids dramatically reduce donor T cell infiltration to the skin in cutaneous GVHD. Protection of $LGR5^+$ HFSCs with topical ruxolitinib was associated with suppression of alopecia and enhancement of wound healing after allo-SCT, while topical corticosteroid was not (19). Based on its protective effects on both ISCs and skin stem cells, ruxolitinib could be an ideal therapeutic agent for GVHD (78, 79). The fate of other stem cell populations in the skin, such as $LGR6^+$ stem cells and $Lrig1^+$ stem cells in the hair follicles, remains to be clarified (85, 86).

The liver, another major target organ in acute GVHD, is a highly regenerative organ, and there are two main epithelial populations: hepatocytes and biliary epithelial cells (BECs). Lineage-tracing studies have shown that there are stem and/or progenitor populations of hepatocytes that maintain the hepatocyte pool in steady states, for example, studies in Axin2-

Cre/ER reporter mice (96). However, some of these lineage-tracing strains have aberrant proliferation of labeled hepatocytes, possibly due to deletion of exons of the target molecule, which potentially leads to overestimation of the contribution of labeled cells to tissue regeneration after liver injury (97). More recently, it has been shown that mid-lobular hepatocytes cycle and maintain whole hepatocytes in the liver, except glutamine synthetase (GS)-expressing hepatocytes facing the central vein, which are maintained independently from other hepatocytes (97, 98). In certain contexts of liver injury, $LGR5^+$ and $Sox9^+$ hepatocytes endowed with the potential to differentiate both into hepatocytes and BECs emerge, and BECs proliferate and contribute to the reconstitution of hepatocytes after severe liver injury (99–103). Because jaundice and biliary dysfunction are the cardinal features of liver GVHD, it should be more important to study BEC stem cells rather than stem cells of hepatocytes. Huch et al. found that single $LGR5^+$ cells isolated from the hepatic duct give rise to liver organoids that can be differentiated into both hepatocytes and BECs (99). The fate of BEC stem cells needs to be clarified in future studies.

ALTERATION OF THE MICROENVIRONMENT INDUCES INTESTINAL DYSBIOSIS IN GVHD

Intestinal dysbiosis is frequently observed after allo-SCT and is associated with exacerbation of GVHD and transplantation-related death (13, 104, 105). Multiple factors, such as antibiotics and total parenteral nutrition, can lead to dysbiosis after allo-SCT (**Figure 2** and **Table 1**). In addition, GVHD-induced tissue injury can generate a microenvironment related to dysbiosis. α-Defensins, major antimicrobial peptides (AMPs) produced from Paneth cells, exert potent bactericidal effects on pathogenic bacteria that occupy a minor proportion of the healthy microbiota but are minimally effective on nonpathogenic commensals that dominate the healthy gut microbiota (106, 107). Paneth cells are highly sensitive to GVHD, and α-defensin production is profoundly decreased in GVHD (12, 13, 108). This reduction is mediated by IFN-γ signaling, and ruxolitinib can protect Paneth cells against GVHD (54). R-Spo1, a growth factor of ISCs, is also a potent inducer of Paneth cell differentiation from ISCs, and we found that administration of rhR-Spo1 induced expansion of Paneth cells in naïve mice, leading to marked elevation of fecal levels of α-defensins such as cryptdin-1 (Crp-1) and cryptdin-4 (Crp-4) (14). In mouse GVHD, peritransplant administration of R-Spo1 protects not only ISCs but also Paneth cells, resulting in preserved α-defensin production and prevention of intestinal dysbiosis after allo-SCT (14). Short-term oral administration of Crp-4 to allogeneic recipient mice temporally mitigated intestinal dysbiosis and inflammation in the gut after allo-SCT, while dysbiosis developed after cessation of Crp-4 treatment, indicating that long-term administration of Crp-4, until Paneth cell regeneration, is required for the prevention of dysbiosis after allo-SCT (14). Paneth cell numbers in duodenal biopsies from transplanted patients are negatively related to gut GVHD severity, further emphasizing the protective role of Paneth cells against GVHD (15).

REG3, another major AMP in the intestine, is produced by intestinal epithelial cells, including Paneth cells and enterocytes, and diffuses into the inner mucus layer, segregating luminal bacteria from the gut epithelium (109, 110). In mouse models of allo-SCT, the expression levels of REG3γ, the mouse homolog of human REG3α, in the small intestine were significantly reduced in GVHD, and REG3γ leaked from the gut to the blood, leading to elevation of plasma levels of REG3γ (111, 112). In clinical allo-SCT, the plasma levels of REG3α and ST2 are now widely appreciated as diagnostic and prognostic biomarkers of acute GVHD (113–115). In mouse models of steroid-refractory GVHD, it has been shown that IL-22 produced by donor Th/Tc22 cells stimulates REG3γ production in the intestine, and excess REG3γ leads to dysbiosis and exacerbation of GVHD (67). Thus, REG3γ could be a therapeutic target for treating steroid-refractory GVHD.

Enterococcus domination, defined as a status in which 30% or more of all the bacteria in the fecal microbiota are enterococci, develops frequently after allo-SCT and is associated with blood stream infection, development and exacerbation of GVHD, and GVHD-related death after allo-SCT (105, 116–119). Because the presence of the *VanA* gene in fecal samples from allo-SCT recipients has been associated with *Enterococcus* domination, antibiotics likely contribute to the development of *Enterococcus* domination (119). However, *Enterococcus* domination is also observed after murine allo-SCT in which no antibiotics are used, indicating that antibiotics are not the only reason for *Enterococcus* domination and that GVHD may induce a microenvironment suitable for the expansion of enterococci. The growth of enterococci is strictly dependent on lactose, and the expression of lactase, a critical enzyme for the absorption of lactose from the diet, in the intestine is reduced in GVHD (119, 120). The reduction in lactase in GVHD leads to ineffective absorption and an increase in lactose availability in the gut lumen, leading to enterococcal expansion. Importantly, the lactose intolerance allele is associated with the persistence of *Enterococcus* domination after the cessation of antibiotics. These data suggest that a lactose-free diet or lactase administration could be used for prophylactic treatment of *Enterococcus* domination, which could improve the outcomes of allo-SCT. In addition to antibiotic administration and lactase reduction, reduction of α-defensins, which exert potent bactericidal effects on *Enterococcus*, could contribute to enterococcal expansion after allo-SCT (107).

THE BARRIER FUNCTION OF GUT EPITHELIAL CELLS IN GVHD

The intestinal mucosa has the complex task of acting as a semipermeable barrier that allows the absorption of nutrients and water while limiting the transport of potentially harmful microbes and microbial components. Sheets of gut epithelial cells are bound to each other *via* tight junctions, acting as a physical barrier against luminal components (**Figure 1**). Conditioning and

allogeneic T cell responses damage epithelial cells (**Table 1**), leading to the loss of the physical barrier function of the mucosal epithelium against bacteria and bacterial components , which fosters an environment prone to GVHD development (121). Thus, epithelial growth factors have been proposed as therapeutic options for acute GVHD (**Figure 2**). Keratinocyte growth factor (KGF) promotes the proliferation and differentiation of epithelial cells. It was suggested that ISCs are also supported by KGF; however, whether KGF protects ISCs against GVHD has not been explored using specific ISC markers, such as LGR5 (122, 123). Although it has been reported that KGF ameliorates murine gut GVHD (124, 125), human recombinant KGF did not demonstrate significant beneficial effects on the incidence and severity of GVHD in randomized clinical trials (126–128). Although the reason for this discrepancy between preclinical and clinical studies is not fully understood, it has been suggested that KGF could exert more potent anti-GVHD effects in recipients conditioned with TBI alone than in those conditioned with TBI in combination with cytotoxic agents; the latter strategy is used in the clinical setting (126).

Glucagon-like peptide 2 (GLP-2) is another growth factor of gut epithelial cells, and administration of a GLP-2 analog protects ISCs against irradiation-induced injury (129). GLP-2-producing enteroendocrine L cells are targeted by GVHD, and reduction of L cells in the patients' colon is associated with worse outcome after allo-SCT. Because GLP-2 is inactivated by DPP-4, a DPP-4-resistant GLP-2 analog, teduglutide, was tested for a GVHD prophylaxis. Peritransplant administration of teduglutide protected ISCs and Paneth cells against GVHD, and prolonged survival after mouse allo-SCT (9). Furthermore, GLP-2 and GLP-2 analogues enhance the expression of tight junction molecules such as claudin-4, possibly enhancing intestinal barrier function in GVHD (9, 130). A clinical trial in which teduglutide is tested for treatment of short bowel syndrome (NCT04733066) is ongoing, and future clinical studies are required to test if teduglutide could protect patients against GVHD. Interestingly, a small-scale phase II study demonstrated that peritransplant administration of high-dose sitagliptin, a DPP-4 inhibitor, prevented the onset of acute GVHD (131). Although it is most likely that DPP-4 inhibition prevents GVHD by suppressing donor T cell activation (132), DPP-4 inhibition may mitigate damage to the intestinal epithelium by inhibiting GLP-2 degradation (133). The impact of DPP-4 inhibitors on GVHD-induced damage to gut epithelial cells needs to be clarified in future studies.

In rodent models of radiation colitis, administration of EGF enhanced gut epithelial regeneration (134, 135). In a phase I clinical trial, it has been shown that administration of a urinary-derived human chorionic gonadotropin (uhCG) agent containing abundant EGF was safe and possibly effective for the treatment of high-risk or steroid-refractory acute GVHD (136). This agent may improve GVHD *via* EGF-induced protection of gut epithelial cells, while the Treg expansion observed after administration of this agent could contribute to GVHD suppression, too. This inexpensive and commercially available uhCG agent will be studied in phase II and III trials.

In mouse GVHD, the TNF-α/MLCK210 axis increases tight junction permeability to larger molecules (137). IFN-γ also regulates tight junction permeability (138). Thus, GVHD actively increases permeability through tight junctions, which promotes the absorption of bacterial components, further recruiting donor T cells and propagating GVHD (137). Prevention of increase of tight junction permeability could be another prophylactic strategy against GVHD.

Loss of commensals in intestinal microbiota after allo-SCT leads to the reduction of bacterial metabolites which contribute to maintenance of tissue homeostasis. Among these metabolites, butyrate is mainly produced by commensal anaerobes such as *Clostridia* and *Blautia*, and mitigates harmful immune reactions by promoting differentiation of regulatory T cells (139). Microbiota-derived butyrate is also taken up by intestinal epithelial cells through G-protein coupled receptor, GPR43 and serves as a major energy source of intestinal epithelial cells. Butyrate acts as a histone deacetylase (HDAC) inhibitor and promotes tricarboxylic acid cycling, improving integrity of barrier function of intestinal mucosa (140, 141). Thus, dysbiosis with the reduction of butyrogenic bacteria reduces butyrate in the intestinal epithelial cells and impairs the resilience of the gut epithelium after allo-SCT (142). Probiotics containing butyrogenic bacteria or prebiotics containing butyrogenic fibers and starch are promising therapeutic options against mouse and human GVHD (140, 143). The urinary levels of 3-indoxyl sulfate (3-IS) are positively correlated with the abundances of *Lachnospiraceae* and *Ruminococcaceae* in the gut microbiota, and higher levels of urinary 3-IS predicts better survival after allo-SCT. Although the direct role of 3-IS in GVHD remains to be clarified, 3-IS could act as a ligand for aryl hydrocarbon receptor, the critical receptor for maintenance of intestinal epithelial barrier function and production of AMPs (144–146).

THE ROLE OF THE GUT MUCUS LAYER IN GVHD

The intestinal mucus layer constitutes a critical barrier that segregates millions of microbes and environmental antigens in the gut lumen from the host immune system (**Figure 1**). The mucus layer serves as the first line of innate defense, and gel-forming mucins secreted by goblet cells form the basic scaffold of the mucus layer. Mice lacking the *Muc2* gene, encoding the major gel-forming mucin in the intestine, are devoid of mucus layers and prone to developing severe colitis, suggesting that direct contact between luminal bacteria and the intestinal mucosa triggers inflammation (147, 148). The large intestine has a system with two mucus layers; the inner mucus layer is enriched with antimicrobial molecules (AMMs), such as Ly6/Plaur domain-containing 8 (LYPD8), and devoid of bacteria, suggesting that the mucus layer also acts as a chemical barrier against luminal bacteria (149, 150).

As noted above, GVHD-induced Paneth cell injury and lactose malabsorption together with other factors, such as antibiotic administration and induction of total parenteral

nutrition, lead to intestinal dysbiosis after allo-SCT. In such a situation, the mucosal barrier must act as the final line of defense against pathogenic bacteria expanding in the gut lumen. However, GVHD leads to a reduction in intestinal goblet cells, which results in disruption of the mucus layer due to its rapid turnover (**Figure 2** and **Table 1**); the mucus layer is renewed every 1 to 2 hours by newly produced mucus from goblet cells (151–153). Recently, we studied the role of goblet cells and the inner mucus layer in the pathophysiology of acute GVHD using mouse models of acute GVHD (2). First, we confirmed that goblet cells were profoundly and persistently reduced in the colon after allo-SCT, which led to disruption of the colonic two-layered mucus system in allogeneic recipients in association with enhanced bacterial translocation, elevated plasma levels of proinflammatory cytokines, and exacerbation of GVHD. Although the mechanism by which GVHD targets goblet cells remains to be clarified, it is possible that goblet cells are reduced due to GVHD-induced depletion of ISCs considering the rapid turnover of goblet cells (3 to 7 days) (154). In the steady state and after parasite infections, ILC2s produce growth factors of goblet cells, such as interleukin 13 (IL-13), in response to interleukin-25 (IL-25) secreted from Tuft cells (17, 18). We found that pretransplant administration of IL-25 expanded goblet cells that persisted after GVHD, preventing bacterial translocation, elevation of proinflammatory cytokines, and exacerbation of GVHD (2). Conditioning TBI and chemotherapy lead to prolonged depletion of ILC2s in mice and humans (3, 4), which could further reduce goblet cells or impair regeneration of these cells. Bruce et al. showed that donor ILC2 infusion promotes IL-13 production by ILC2s and enhances the survival of donor myeloid suppressor cells, suppresses donor T cell production of proinflammatory cytokines, and reduces GVHD (3). Although this study demonstrated that transfer of donor ILC2s improves intestinal epithelial integrity, the impact on goblet cells was not addressed. Deficiency of NOD-like receptor family pyrin domain-containing 6 (NLRP6), the critical molecule for goblet cell secretion of mucus, in nonhematopoietic cells of recipients mitigates goblet cell injury after allo-SCT and ameliorates intestinal GVHD, suggesting that NLRP6 is another target molecule for protection of goblet cells after allo-SCT (155).

LYPD8 is produced by enterocytes in the colon and enriched in the inner mucus layer (149). LYPD8 binds to flagellated bacteria such as *Escherichia coli* and prevents bacterial translocation by inhibiting bacterial motility. Based on these findings, we studied the protective role of LYPD8 in murine GVHD using LYPD8-deficient mice as recipients (2). First, we found that disruption of the inner mucus layer in allogeneic recipients led to disappearance of the LYPD8-rich layer in the mucus layer. Next, we found that bacterial translocation was dramatically enhanced in LYPD8-deficient recipients compared to WT recipients after allo-SCT, in association with exacerbation of GVHD. Furthermore, goblet cell expansion using IL-25 did not ameliorate GVHD in LYPD8-deficient recipients, suggesting that the mucus layer containing LYPD8 is critical for goblet cell-mediated GVHD suppression (2).

CONCLUDING REMARKS

The discovery of specific markers for tissue stem cells has enabled us to study the fate of tissue stem cells in mouse GVHD, and we found that ISCs and HFSCs are targeted by GVHD. Furthermore, niche components that support tissue stem cells are also damaged after allo-SCT, likely inhibiting the recovery of tissue stem cells after GVHD-mediated injury. Emerging evidence also indicates that human and mouse GVHD targets specific epithelial populations, such as Paneth cells, L cells, and goblet cells, resulting in disruption of tissue homeostasis (**Figure 2** and **Table 1**). Strategies to promote recovery of tissue stem cells and maintenance of the tissue microenvironment are promising adjuncts to standard immunosuppressive GVHD prophylaxis and treatment, which may enable the separation of GVHD and graft-versus-leukemia effects.

There remain many unanswered questions in this field. Although the existence of $LGR5^+$ ISCs is also demonstrated in the human intestine, the fate of $LGR5^+$ tissue stem cells in the intestine and skin after human allo-SCT remains to be clarified (156). The role and fate of BEC stem cells need to be studied both in human and mouse liver GVHD. Furthermore, the role of tissue stem cells in pathophysiology of chronic GVHD has not been well studied, and studies about intestinal dysbiosis in chronic GVHD has only just begun (157). Although it has been shown that protection of intestinal stem cells, Paneth cells, or goblet cells represents a promising anti-GVHD treatment, these strategies have been tested only in mouse models of GVHD in a prophylactic manner. It should be tested if these strategies are also useful for treatment of established GVHD.

AUTHOR CONTRIBUTIONS

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

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Crosstalk Between Intestinal Microbiota Derived Metabolites and Tissues in Allogeneic Hematopoietic Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an evidence based-cellular immunotherapy for hematological malignancies. Immune reactions not only promote graft-versus-tumor effects that kill hematological malignant cells but also graft-versus-host disease (GVHD) that is the primary complication characterized by systemic organ damages consisting of T-cells and antigen presenting cells (APCs) activation. GVHD has long been recognized as an immunological reaction that requires an immunosuppressive treatment targeting immune cells. However immune suppression cannot always prevent GVHD or effectively treat it once it has developed. Recent studies using high-throughput sequencing technology investigated the impact of microbial flora on GVHD and provided profound insights of the mechanism of GVHD other than immune cells. Allo-HSCT affects the intestinal microbiota and microbiome-metabolome axis that can alter intestinal homeostasis and the severity of experimental GVHD. This axis can potentially be manipulated *via* dietary intervention or metabolites produced by intestinal bacteria affected post-allo-HSCT. In this review, we discuss the mechanism of experimental GVHD regulation by the complex microbial community-metabolites-host tissue axis. Furthermore, we summarize the major findings of microbiome-based immunotherapeutic approaches that protect tissues from experimental GVHD. Understanding the complex relationships between gut microbiota-metabolites-host tissues axis provides crucial insight into the pathogenesis of GVHD and advances the development of new therapeutic approaches.

Keywords: graft-versus-host disease, microbial metabolite, dysbiosis, microbiota, allogeneic stem cell transplantation

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INTRODUCTION

Hematological malignancies, such as leukemia, lead to high mortality, especially in the elderly. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an immune therapy that has become widely used for the treatment of life-threatening hematological malignancies and congenic immune deficiencies (1). However, many complications following allo-HSCT occur due to immune-related reactions, the most challenging and serious of which is graft-*versus*-host disease (GVHD) (2).

GVHD is composed of complexes of immune cells and tissues (3). After T-cells activation and migration to GVHD target organs, such as the lung, gut, liver and skin, cytotoxic T-cells and helper T-cells attack target cells in these target organs. Once GVHD develops, recipients require additional immunosuppressive therapies using steroids in addition to GVHD preventive immunosuppressants consisting of calcineurin inhibitors. However, GVHD is frequently refractory to standard steroid therapy and has a dismal prognosis, with only 5–30% overall survival (4). In steroid-refractory GVHD, novel treatment approaches include the use of anti-thymocyte globulin, extracorporeal photopheresis, mesenchymal stromal cells, mycophenolate mofetil, everolimus, sirolimus, etanercept, infliximab, and ruxolitinib (5). Intensified immunosuppressive therapies for aggravated GVHD suppress immune reactions and permit bacterial translocation from injured intestinal epithelium cells; this leads to subsequent systemic infections that increase the release of pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). This negative feedback loop can result in fatal GVHD. In addition, excessive immunosuppression increases malignancy relapse after HSCT. Thus, immunosuppressive therapies are limited.

Current focus has been shifted to the target tissues, the third player in GVHD, in addition to APCs and T-cells (3). The role of tissue-intrinsic factors that might contribute to the regulation of GVHD severity has been largely overlooked. Tissue-specific programs contribute to target tissue resilience, repair, and regeneration and mitigate the severity of GVHD without altering the load or function of alloreactive immune cells (6). In this context, the gastro-intestinal (GI) tract has close interactions with the microbiota, and recent genomic microbial analyses have revealed intricate connections between the microbiota and various diseases. Particularly, in GVHD, disturbance of microbial composition, that is, dysbiosis, is strongly associated with poor outcomes after allo-HSCT (7). However, whether a causal relationship exists remains unclear. In this review, we focus on the connection among gut microbiota, microbial metabolites and intestinal environment that affect GVHD. We also discuss the recent findings on microbiome-based immunotherapeutics that affect or mitigate GVHD and enumerate the emerging strategies for the regulation of dysbiosis and microbial metabolites in the regulation of GVHD.

Gut Microbiota and Intestinal Epithelial Cells

A close relationship between microbiota and human health has been investigated. Microbiota refers to the community comprising trillions of microorganisms, including bacteria, fungi, and viruses, that symbiotically colonize the human body, most members of which reside in the gut and are largely nonpathogenic anaerobic commensal bacteria (8). This balance is finely tuned in the gastrointestinal (GI) tract and influenced by the environment, diet, and host factors, including host physiology (9). The mammalian GI tract is a relatively hypoxic tissue and has a steep oxygen (O_2) gradient between the O_2-rich lamina propria and the gut lumen, which is dominated by anaerobic organisms (10). Aerobic and facultative anaerobic bacteria have been suggested to consume oxygen in the distal intestine and maintain hypoxia in the lumen, leading to the colonization by strict anaerobes and the production of short-chain fatty acids (SCFAs) (11). Complex dietary carbohydrates (fiber) are broken down by these bacteria into digested fermentation products, that are absorbed by the host and utilized for host nutrition, immune development, and niche protection against enteric pathogens (12–19). Members of *Clostridia* and *Bacteroidia* are the obligate anaerobic bacteria dominating in the colon and capably digest any carbohydrate complex into fermentation products (20, 21). Facultative anaerobic bacteria such as *Proteobacteria* are not specialized in consuming fiber, and rather interfere with host nutrition by changing metabolites to carbon dioxide under oxygen (22, 23). Fermented metabolite effects have been observed in a neonatal mouse model with *Clostridia* species colonization that protected neonatal mice form virulent pathogens (24). Intestinal epithelial cells (IECs) are continuously renewed from the crypts where intestinal stem cells (ISCs) are located. ISCs divide and differentiate into transit-amplifying (TA) cells, IECs, enteroendocrine cells and goblet cells (25). In contrast to TA and ISCs that utilize glucose to obtain energy through glycolysis, IECs produce energy *via* mitochondrial β-oxidation of fatty acids and oxidative phosphorylation, both of which require oxygen (26, 27). Butyrate is a favored metabolic substrate that maintains epithelial energy homeostasis (26). Despite low O_2 conditions, high butyrate levels result in the downregulation of the glycolytic pathway and the upregulation of mitochondrial respiration (28). This drastic change of energy production was triggered with the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), which is a nuclear receptor synthesized in differentiated IECs of rodents and humans (29). The energy metabolism in IECs consumes a high amount of oxygen, leading to low oxygen pressure in the lumen (30). This oxygen consumption in IECs permits epithelial hypoxia and helps to maintain anaerobic bacterial flora in the intestinal lumen (31). The hypoxic environment enables obligate anaerobic bacteria that produce carbohydrate metabolites to colonize and provide benefits to the host. Epithelial metabolism of SCFA is a primary determinant of "physiologic hypoxia" in the mucosa with O_2 consumption and hypoxia-inducible factor (HIF) stabilization that promotes barrier function (32). Therefore, IECs are uniquely adapted to this hypoxic environment, and cells programmed by "physiological hypoxia" have been shown to tonally regulate barrier function (33). These mechanisms indicated that IECs influence the shaping of beneficial microbiota, and those microbiotas bring metabolic profits to the host. In this process IECs play a central role in maintaining gut homeostasis (**Figure 1**).

The IECs and gut microbiota function in two distinct manners: protection against ingested pathogens and induction of tolerance to beneficial commensals. The intestinal immune system matured by microbiota stimulates the mucosa-producing cells (i.e., Paneth cells and goblet cells) to secrete antimicrobial

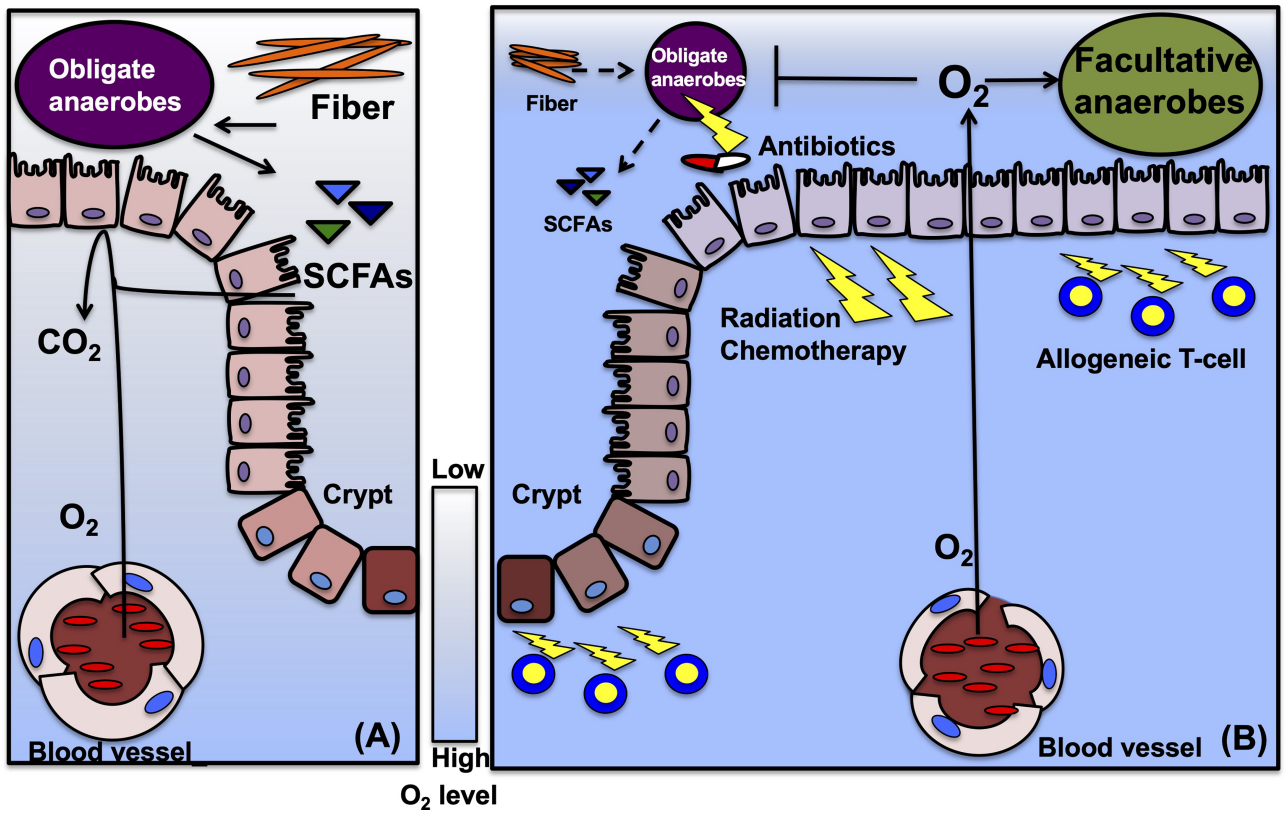

FIGURE 1 | Suggestive mechanisms of epithelial metabolism and gut microbiota in HSCT. **(A)** Before HSCT, gut microbiota, especially obligate anaerobic bacteria, ferment fiber from diet into metabolites, such as SCFAs. Intestinal epithelial cells (IECs) metabolize short chain fatty acids (SCFAs) and consume oxygen to produce energy. High oxygen consumption limits the oxygen diffusion into the lumen and maintains hypoxia in the lumen. Hypoxic condition enables obligate anaerobes to keep growth. The scale bar indicates oxygen (O_2) levels, usually between 3% to 10% in normal intestines. **(B)** During HSCT, conditioning treatments including irradiation and high-dose chemotherapy damages IECs, and injured IEC decreases oxygen consumption. Decreased dietary fiber and antibiotics treatments during HSCT disrupt microbiota composition, especially reduce obligate anaerobes, and deplete the fermentative products. Reduced SCFAs limit oxygen consumption to produce energy in IECs and permit oxygen diffusion into the gut lumen. Elevated oxygen concentration drives an expansion of facultative anaerobic bacteria and might lead to a contraction of obligate anaerobes. In GVHD, allogeneic T-cells promote IEC injuries and require antibiotics treatments, that could enhance and maintain dysbiosis in the gut.

peptides, antibodies, and mucin to form intestinal physical barriers, such as the mucus layer and tight junctions that regulates the relationship between the microbiota and the host (34). Germ-free mice have distinct gut features, such as altered intestinal morphology and slower turnover of IECs than conventional mice (35). After exposure to exogenous commensal bacteria, germ-free mice responded to intestinal bacteria. Four days post-exposure, innate immune responses and structural changes in the intestinal epithelial crypts were observed, i.e., increased TNF-α and interferon-γ (IFN-γ) expression and upregulation of MHC class I molecules (35). IECs in SPF mice also expressed MHC class II molecules that were deficient in germ free mice (36). Under the inflammatory status such as GVHD, gut microbiome stimulated IL-12 production from myeloid cells, resulting in increased expression of MHC class II on IECs through IFN-γ produced from lamina propria lymphocytes. In summary, the intestinal environment shapes and maintains the microbiota. Also, microbiota and those metabolites play important roles on both the function and metabolism in IECs.

Mechanism of Dysbiosis in Hematopoietic Cell Transplantation

Associations between the microbiota and HSCT outcomes have been extensively studied in the 1970s (37). According to these early studies, specific pathogen-free mice treated with antibiotics or germ-free mice that received HSCT developed less severe GVHD than their respective controls. These attempts were validated in HSCT patients with gut decontamination by antibiotics (38). Recently, broad-spectrum antibiotics that target anaerobic pathobionts have been revealed to increase GVHD-related mortality in mouse and human (39). Therefore, some antibiotics that perturb microbial composition were unsuitable for patients in HSCT settings. The microbial compositions of various anaerobic commensal intestinal bacterial species were robustly disturbed and reduced due to chemo/radiotherapy, antibiotics, or HSCT itself in patients (40). Decreased

diversity and expansion of specific bacteria, including *Enterococcus* spp., were reported as risk factors that resulted in poor outcomes after HSCT (41). The mono-domination of *Enterococcus* was significantly associated with severe GVHD and treatment related mortality (TRM) in mice and human studies (42). These studies indicate the causative role of *Enterococcus* in the pathogenesis of GVHD. However, the mechanism of dysbiosis post-HSCT remains unclear (7, 43). Before and after HSCT, patients receive various interventions that are likely to contribute to the microbial dysbiosis. Importantly, in a mouse model of acute GVHD exposure to a GVHD-associated gut microbiome pre- and/or post- HSCT accelerated GVHD development and severity demonstrating the complex relationship between GVHD and microbiota (44). These factors could affect the intestinal microbial composition (**Figure 1**).

Radiotherapy

As a part of allo-HSCT, systemic irradiation is frequently used to kill fast-dividing leukemia/lymphoma cells and host myeloid cells/lymphocytes. However, radiation causes intestinal damage, and the potential correlation between dysbiosis and radiation-induced damage has been revealed (45, 46). Radiation induces oxidative stress *via* the generation of reactive oxygen species (ROS), including hydroxyl radical (OH), superoxide anion (O^{2-}), and hydrogen peroxide (H_2O_2), resulting in the activation of cyclooxygenases (COX), nitric oxide synthases, lipoxygenases, and nicotinamide adenine dinucleotide phosphate oxidases. These products of oxidative stress cause DNA damage, inflammation, and apoptosis in IECs and affect the gut microbiota composition through homeostatic disturbances (45). The relationship between radiation enteritis and dysbiosis was observed in patients receiving radiotherapy. Relatively high abundances of *Proteobacteria* and *Gammaproteobacteria*, characterized by increased oxidative stress resistance in *Enterobacteriaceae, Phyllobacteriaceae,* and *Beijerinckiaceae*, and low abundance of *Bacteroides* with decreased *Bacteroidaceae* and *Ruminococcaceae* that were sensitive to oxidative stress, were detected (46). Recently, a multi-omics study reported *Lachnospiraceae* and *Enterococcaceae* as radioprotective microbes and elevated SCFA levels due to irradiation damage in mice receiving irradiation (47). *Bifidobacterium* can utilize indigestive fibers, such as fructose oligosaccharides (FOS), inulin-type fructans (ITF), and xylo-oligosaccharides (XOS), and cross-feed with lactate-converting bacteria and butyrate-producing bacteria, i.e., *Eubacterium hallii* and *Anaerostipes caccae* (48). Although *Bifidobacterium* cannot produce butyrate, it is associated with increased abundance of butyrogenic bacteria in a cross-feeding manner (49). Moreover, *Eubacterium hallii* L2-7 and *Anaerostipes caccae* L1-92 could not grow in starch but grew upon co-culture with *Bifidobacterium adolescentis* L2-32 to produce butyrate (50). Collectively, the abundance of specific bacterial species is reduced and eventually lost post irradiation before allo-HSCT.

Chemotherapy

Elaborating the purpose of repeated intensive chemotherapy before allo-HSCT reduces microbiota diversity in both human and mice and leads to the expansion of *Escherichia Coli* and *Enterococcus* spp (51). During allo-HSCT, disrupted gut microbial composition and diversity, including decreased abundances in *Bifidobacterium*, butyrate-producing *Faecalibacterium* and *Lachnospiraceae*, were highly associated with transplant-related mortality (7). Methotrexate treatments caused a significant reduction in the number of *Bifidobacterium* and *Lactobacillus* species and *Escherichia coli* in children with leukemia (52). Etoposide exhibits broad bacterial inhibitory activity that changes microbial composition *in vitro* (53). In leukemia patient data, it is suggested that chemotherapy induces dysbiosis (51). Healthy volunteers in the Human Microbiome Project (HMP) and patients with acute myeloid leukemia in the pre-induction phase showed no significant differences in terms of microbial diversity. After neutrophil recovery, microbial diversity in the leukemia group was significantly decreased and this dysbiosis was associated with an increased risk of infections and the use of broad-spectrum antibiotics. Within 3 months of anti-anaerobic antibiotic administrations including carbapenem and piperacillin-tazobactam, a significant reduction in microbial diversity was also observed before allo-HSCT in the recipients (54). Multiple intensive chemotherapies for acute leukemia patients also disrupt the microbial composition and promote the outgrowth of pathogenic bacteria such as *Enterococcus* (55). Another study demonstrated that pre-transplant microbial composition differed from the healthy controls due to decreased abundances of beneficial bacteria, *Bifidobacterium*, *Faecalibacterium*, and *Lachnospiraceae* (56). Overall, these findings suggest that pre-transplant microbe disruption increased HSCT comorbidity. However, the mechanisms of dysbiosis onset and the disruption of the microbiota by chemotherapy are still unknown. Furthermore, these studies could not separate the effects of HSCT from those of antibiotics (39).

Antibiotics

Chemotherapies for hematological malignancies cause high-grade neutropenia and require systemic antibiotic administration to prevent or treat life-threatening bacterial infections. The influence of antibiotics on the microbial composition should be accounted for. Recent microbiome studies have observed undesirable consequences in dysbiosis, including antibiotic resistance, pathogenic bacteria dominance, transient or profound loss of microbial diversity, increased susceptibility to infection, and the risk of recurrent infections, after antibiotic usage in cancer patients (57). However, immunocompromised individuals are at high risk of intestinal infections, which are difficult to treat without using broad-spectrum antibiotics (58). As reported by various studies, oxygen-tolerant species, *Enterococcus, Enterobacteriaceae, Klebsiella* spp., and *Viridans streptococci*, are most commonly translocated from damaged intestinal mucosal barrier due to chemotherapy, irradiation, and antibiotics to the bloodstream (59). Prophylactic antibiotics, such as quinolones, are usually utilized to prevent infections in patients receiving HSCT. Once they develop fever or infections, broad-spectrum antibiotics, such as piperacillin-tazobactam or meropenem, are prescribed (60). Compared to pre-transplant conditions, decreased *Firmicutes* and *Actinobacteria* and increased *Bacteroides* and

Proteobacteria have been detected at 1week post-conditioning (61). Notably, the use of rifaximin could preserve gut beneficial commensals and improve patient outcomes (62). HSCT patients receiving rifaximin showed significantly lower TRM, prolonged OS, and a tendency for low acute GI-GVHD rates than historical controls. These observations remained unknown even in patients who developed neutropenic fever and received systemic antibiotics without an increase in sepsis rates or pathogenic bacterial colonization. Efforts to investigate and identify the antibiotics that could spare microbial compositions to reduce acute GI-GVHD and adverse outcomes have been attempted (63). A study in patients from Canada also assessed the effects of prophylactic and therapeutic antibiotic administration before day 0 of HSCT (64). The antibiotic-receiving group had a significantly higher incidence of acute GVHD (aGVHD) and shorter survival. The early administration of systemic antibiotics before engraftment was also associated with low 3-IS levels and reduced Clostridia abundance in the intestines, leading to higher TRM rates than those without systemic antibiotics before engraftment (65). These studies suggest the association of antibiotic usage affecting microbial diversity and GVHD-related outcomes. Piperacillin-tazobactam and meropenem, known as broad-spectrum antibiotics that are also effective against anaerobic bacteria, caused decreased microbiome diversity during transplantation in mice and humans (39, 66). In mouse models, these antibiotics lead to microbial injury with loss of colonic mucosa and intestinal barrier function caused by mucin-degrading bacteria, *Akkermansia muciniphila* (39). The load of anti-anaerobic antibiotic usage was correlated with a significant decrease in anti-inflammatory Clostridia (AIC) abundance and aGVHD in pediatric HSCT patients (67). This decrease was recovered by the administration of AIC after clindamycin treatment and improved survival in a mouse GVHD model (67, 68). Among the broad-spectrum antibiotics administered during HSCT, meropenem showed significantly decreased microbial diversity and a higher rate of GI-HVHD onset compared to no antibiotics, but cefepime did not change the diversity with a trend of increased GI-GVHD onset rates (69). In a pediatric study, anti-anaerobic antibiotics resulted in a significant decrease of SCFA-producing bacteria, especially butyrate-producing commensals and butyrate levels in the luminal content. These patients developed aGVHD with low butyrate levels in stools compared to patients without GVHD (70). Single bacterial taxa dominated by Enterococcus and Streptococcus was observed in two-thirds of patients with bloodstream infections around the time of engraftment (71). The significant elevation of enterococcus expansion was increased by metronidazole administration, and VRE bacteremia was significantly increased (3-fold and 9-fold, respectively). Integrating these studies, although antibiotic prophylaxis and systemic administration clearly improved TRM, especially infection-related mortality, we need comprehensive choices of antibiotic usage around HSCT periods comparing weights of disease status, donor sources, history of antibiotic usage, status of microbial injuries, and other risk factors.

METABOLIC CHANGES IN COLONOCYTES

IECs might control the homeostasis that shapes the microbiota to be beneficial (72). Dysbiosis that caused increased abundance of facultative anaerobic bacteria, is observed in the population consuming a high-fat Western-style diet, patients with inflammatory bowel disease, colorectal cancer, irritable bowel syndrome or necrotizing enterocolitis (73–80). It has been proposed that shift of microbial community from obligate to facultative anaerobic bacteria that can utilize oxygen could be associated with a disruption in aerobiosis, a concept "oxygen hypothesis" in mouse inflammatory bowel disease models (81, 82). This microbial shift might be associated with the colonocyte metabolic dysfunction (31). Disruption of gut homeostasis was observed in antibiotic treatment that altered IECs metabolism by depleting microbes that produce SCFAs in a mouse colitis model (19, 32). Decreased SCFAs increases the inflammatory tone of the colonic mucosa in mice (83). In the animal model, elevation of inflammatory signals shifts the metabolism in IECs toward an aerobic glycolysis, reduced oxygen consumption and high glucose consumption and high lactate release (84). These metabolic changes result in a loss of epithelial hypoxia (32). Increased oxygen concentration elevates the amount of oxygen in mucosal surface, therefore drives an expansion of facultative anaerobic bacteria (85). These associations between metabolic condition in IECs and dysbiosis are not validated in GVHD settings and the future investigations are required.

Butyrate and Lactase Pathway

Recent evidence has suggested that dysbiosis post allo-HSCT are related to the butyrate and lactase pathways (86). Butyrate is produced from non-digestible fiber by anaerobic bacterial species, including those belonging to *Firmicutes*, *Lachnospiraceae*, *Ruminococcaceae*, *Lactobacillus* spp., and *Bifidobacterium adolescentis*. Other bacterial phyla including *Bacteroides*, *Actinobacteria, Fusobacteria*, and *Proteobacteria* are also potent butyrate producers (87). These bacteria are sensitive to oxidative stress caused by chemotherapy and irradiation administered before HSCT, leading to their reduced abundances and the subsequent decrease in butyrate volume in the lumen. At the intestinal level, butyrate is a protective molecule against inflammation, a histone deacetylase inhibitor, and an energy source for epithelial cells (68, 88). Therefore, decreased butyrate concentration led to GVHD augmentation due to the disturbance of intestinal homeostasis and the activation of inflammatory environment in the intestine. Another mechanism that causes dysbiosis post-HSCT in GVHD patients involves the domination of the lactic acid bacteria (LAB) *Enterococcus faecium* in the gut (42). In GVHD patients, metabolism of lactose and galactose is impaired by the overexpression of enzymes and lactases (89). In adults, lactase is mainly produced by the microbiota, including species belonging to *Actinobacteria, Proteobacteria*, and *Firmicutes* (90). Each LAB has distinct enzymatic characteristics and behaves differently depending on each feature. LABs are mainly gram-positive, acid-tolerant bacteria

from the genera *Lactobacillus, Enterococcus, Streptococcus, Pediococcus, Lactococcus*, and *Oenococcus* (91). They produce lactase that catalyzes the conversion of carbohydrates (such as lactose, glucose, sucrose, and galactose) into lactic acid and facilitates lactose absorption (92, 93). The reduction in the number of commensal LAB mediated by conditioning chemotherapy, radiotherapy, and antibiotics post-HSCT leads to high lactose concentrations. For example, *Enterococci* are a part of the normal intestinal microbiota and can cause clinical problems. Particularly, *Enterococcus faecium* and some *Enterococcus faecalis* encode genes related to lactose and galactose metabolism (42). *Enterococcus faecium* metabolizes citrate and lactose to produce lactate, whereas *E. faecalis* can produce superoxide and H_2O_2 that damage colonic epithelial cells (94, 95). These enterococci require lactose for growth *in vitro* (lactose auxotroph) and are associated with intestinal inflammation and damages (96). These pathways were observed following the expansion of *Enterococci* early after HSCT in patients with the increased risk of HSCT-related mortality (43). Butyrate and lactase pathway could be a target for treating dysbiosis in HSCT. The analysis of these pathways for TRM post HSCT has been reviewed in detail previously (89).

MICROBIAL METABOLITES IN GVHD

The intestinal microbiota produces various microbial metabolites that maintain intestinal homeostasis (97, 98). These metabolites affect both IECs and immune cells in the intestine to maintain intestinal barrier function and host immune responses (97). Changes in microbial composition led to poor HSCT outcomes; however, to date, there is no conclusive evidence that changes in gut microbial metabolites affect GVHD severity and TRM.

Short-Chain Fatty Acids

SCFAs, including acetate, butyrate, and propionate, are products of fermented carbohydrates by anaerobic commensal bacteria *Clostridia* spp (99). SCFAs have multipotent effects on both IECs and intestinal immune cells (**Figure 2**). Particularly, butyrate serves as an energy source for IECs as demonstrated by reduced autophagy in SPF mice compared to germ-free mice (26). In addition, SCFAs preserve the intestinal mucosal barrier by supporting goblet cells *via* upregulating mucin-related genes. The supplementation of SCFA-producing bacteria in germ-free rats showed goblet cell maturation (100–102). SCFAs also maintain the IEC barrier integrity that prevents pathogenic bacterial translocation (103). Furthermore, they play an important role in innate immunity, neutrophils, mononuclear cells, macrophages, and dendritic cells and act as histone deacetylase (HDAC) inhibitors with anti-inflammatory effects (99, 101, 102). SCFAs also affect Tregs to promote differentiation and anti-inflammatory responses in a mouse colitis model (17). Moreover, anti-inflammatory IL-10 production from Foxp3-positive Tregs *via* HDAC inhibition was observed in germ-free mice with SCFA supplementation (18, 104). A high-fiber diet and acetate activated the inflammasome and promoted IL-18 production, which modulated epithelial repair in murine colitis model (105). Butyrate also increased IL-18 expression in IECs and IL-10 expression in DCs and macrophages, thereby inducing Treg differentiation in mice (106). Recently, a metabolomic analysis of murine intestine and contents revealed a significant reduction in butyrate in the IECs from GVHD mice and that the supplementation of butyrate or butyrate-producing bacteria ameliorated acute GVHD *via* HDAC inhibition and gut integrity maintenance (68, 107). Butyrate and propionate directly protected IECs *via* inflammasome activation through SCFA-sensing receptors in murine GVHD model (108). In humans, small clinical studies have found that the high fecal butyrate and propionate concentrations are decreased in acute GVHD patients and that high circulating butyrate and propionate concentrations in the blood are associated with protection from chronic GVHD (70, 107). Other studies examined fecal SCFA concentrations after allo-HSCT and reported that the concentrations of butyrate and other SCFAs correlate with the abundance of butyrate-producing bacteria in the intestinal microbiota and are higher in patients with resistance to lower tract respiratory infections (109). However, the effect of SCFAs on GVHD needs to be examined in patients after allogeneic HSCT.

During HSCT, recipients have problems that limit oral intake due to mucositis and receive total parenteral nutrition (TPN). In both mice and humans, TPN skewed immune responses to pro-inflammatory conditions (110, 111). Positive nutritional support using sucrose improved hematopoietic recovery compared to antibiotic-treated recipients in mice (112). These studies overall suggest the potential of specific diet elements (prebiotics) during HSCT. Resistant carbohydrate supplementation can stimulate SCFA production in the intestine as the microbiota can metabolize resistant starch (RS) (113). Among various formulations of RS, that derived from potatoes was identified as a potent candidate for increasing fecal butyrate levels in healthy adults (114). Remarkably, RS administration to allo-recipients around HSCT periods was demonstrated as feasible and safe and lead to increased fecal butyrate levels concomitant with high RS degrading and butyrate-producing bacteria in a human pilot study (115). However, the clinical data on the effects of prebiotics in acute GVHD are limited. Inulin and fructo-oligosaccharides have potential as prebiotics in inflammatory bowel disease patients as they led to the expansion of intestinal microbial diversity (116, 117). To determine the exact role of nutritional modulation using RS in the microbiome and metabolites affecting GVHD severity, clinical trials are currently ongoing (www.clinicaltrials.gov: NCT02763033). A clinical trial that evaluating the safety and tolerability of fructo-oligosaccharides in HSCT patients (NCT02805075) is also underway.

Bile Acids

Bile acid is a microbial metabolite affected by intestinal microbial composition (118). Primary bile acids are generated in the liver *via* cholesterol catabolism. These primary bile acids are conjugated to glycine and taurine in the final step of synthesis and are secreted

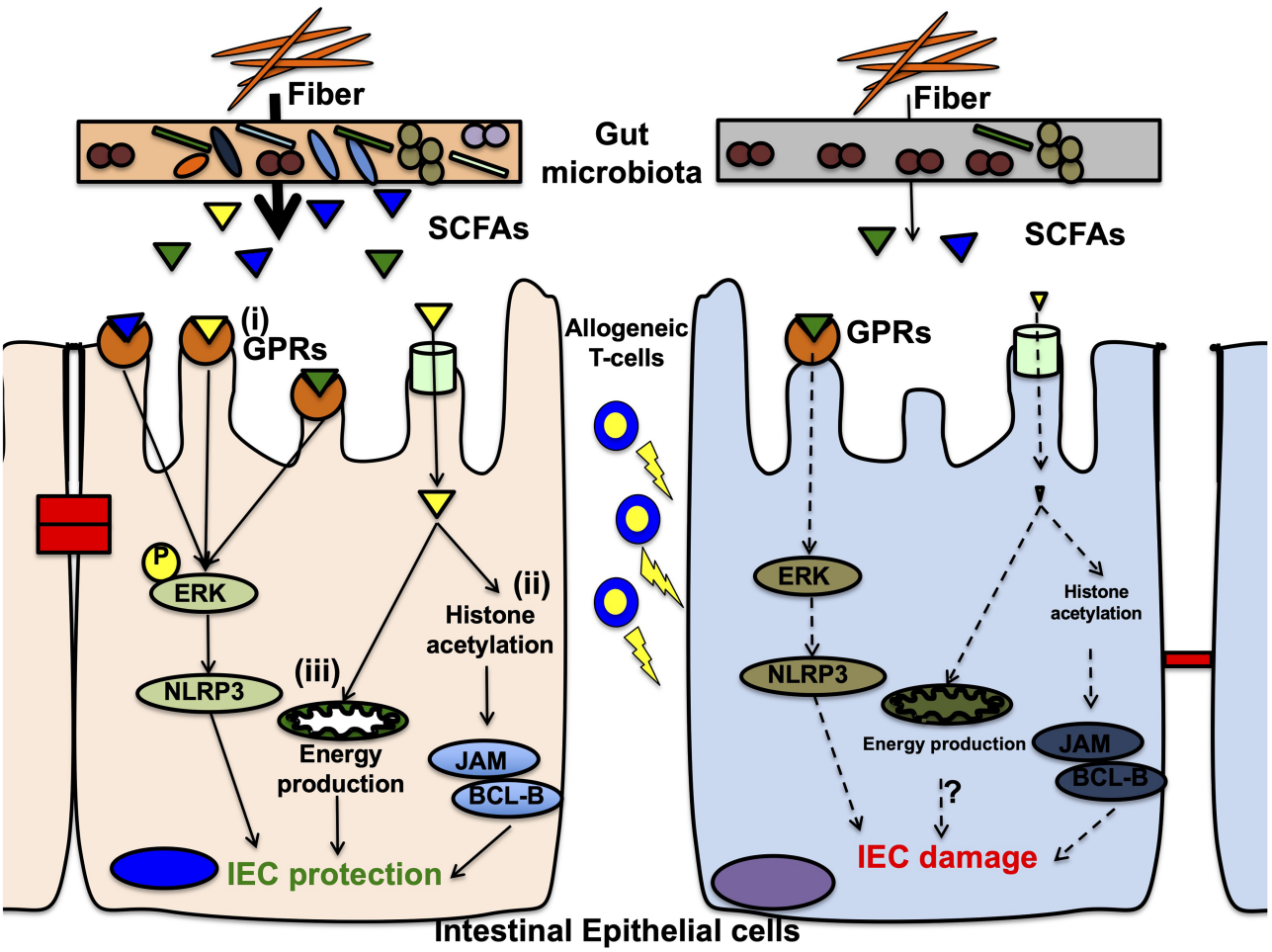

FIGURE 2 | Mechanisms of short chain fatty acids on intestinal epithelial cells in the pathophysiology of gastrointestinal GVHD. In the normal condition, short chain fatty acids (SCFAs) that are metabolites from microbial fermentation of dietary fibers, protect intestinal epithelial cells (IECs) in at least 3ways. (i) G-protein-coupled receptors (GPRs) on IECs, especially GPR43, sensor SCFAs and signal ERK phosphorylation leading to subsequent NLRP3 inflammasome activation, that promotes IEC integrity. In graft-*versus*-host disease (GVHD), decreases of GPRs on IECs and reduced SCFAs from dysbiosis diminish intracellular signaling *via* ERK-NLRP3 pathway, resulting in IEC damages. (ii) One of SCFAs, butyrate acts as a histone deacetylase inhibitor, and increases various target gene expressions, including anti-apoptotic BCL-B and the junctional protein JAM (junctional adhesion molecule). These gene expressions result in decreased IEC apoptosis and enhanced junctional integrity, leading to IEC protection. In GVHD, reduced butyrate fails to protect histones from deacetylation and decreased target gene expression, resulting in low resistance against allogeneic T-cell injuries. (iii) Butyrate activates peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling in IECs and promotes mitochondrial ß-oxidation that produce enough amount of energy to protect and maintain IECs from cellular damages. In GVHD, reduced butyrate might not activate PPAR-γ, resulting in mitochondrial dysfunction for energy production that are inappropriate for IEC protection against allogeneic T-cell injuries. This mechanism is studied in inflammatory bowel disease but not validated in GVHD. Further investigations are required.

into the intestine. Most bile acids are taken up by the IECs and transported to the liver (119). Secondary bile acids are converted by microbial enzymes from primary bile acids that were not absorbed. Two main receptors, the nuclear farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (GPBAR1; TGR5), are known. FXR-deficient mice showed abnormal IEC functions, thus causing bacterial translocation from the intestine (120). TGR5 signaling from bile acids activates macrophages and inhibits the production of pro-inflammatory cytokines TNF-α and IL-1 by inhibiting the nuclear factor-κB (121). Dietary fat-induced taurocholic acid in IL-10-deficient mice enhanced colitis in mice by altering the intestinal microbiota and pro-inflammatory Th1 responses (75). While bile acids are not well absorbed in patients with intestinal GVHD and serum sample analysis of allo-HSCT patients revealed altered bile acid concentrations, ursodeoxycholic acid, a secondary bile acid, is clinically used to reduce hepatic complications and GVHD (122–124). In a recent study, tauroursodeoxycholic acid, another bile acid, reduced the experimental murine GVHD severity (124). Tauroursodeoxycholic acid decreased antigen presentation on IECs, and the apoptosis of IECs without affecting the microbial composition and graft-*versus*-leukemia/lymphoma (GVL) effects. Taurine is a metabolite related to bile acids that stimulates the Nod-like receptor family protein domain containing 6 (NLRP6). NLRP6 stimulation leads to innate

immune cell activation, increased apoptosis/necroptosis, inflammasome-related cytokine production, and promotion of intestinal epithelial integrity (125). Taurine has been reported to stimulate NLRP6 in IECs and augment GVHD severity in mice (126). These data suggest that bile acids are promising microbial metabolites that can be used to reduce GVHD severity.

Aryl Hydrocarbon Receptor Ligands

Aryl hydrocarbon receptor (AhR) modulates mucosal immune responses *via* the ligand-activated transcription factor. AhR ligands are produced from the endogenous and exogenous pathway in the body. In an endogenous pathway, they are produced through intestinal microbial metabolism. *Lactobacilli*, *Fusobacterium*, *Bacteroides* and *Enterococcus faecalis* convert the amino acid, tryptophan, into AhR ligands, such as indole-3-aldehyde and its derivatives (127, 128). In a human GVHD cohort, preserved microbial diversity and *Clostridia spp* were correlated with 3-IS concentrations in the urine (129). In serum metabolic comparisons of HLA identical donors and recipients, indolepropionate that was derived from microbiota, was a smaller amount in recipients at the time of GVHD onset (123). 3-IS is also known as a uremic toxin associated with adverse outcomes in renal disease patients. In this setting, monocytes respond to 3-IS through AhR pathway and release TNF-α, resulting in the pro-inflammatory condition with endothelial damage leading to cardiovascular disease (130). AhR ligands maintain IEC barrier functions. AhR-deficient mice have decreased levels of anti-microbial peptides and intestinal epithelial lymphocytes and reduced IEC turnover with altered microbial composition in mice (131). AhR also regulates innate immunity in the intestine by expanding the IL-22-producing retinoic acid receptor-related orphan receptor γt (RORγt)+ group 3 innate lymphoid cells (ILC3s) (132), thereby affecting adaptive immunity (133). In murine GVHD models, a few investigations were reported. Recipient mice that exposed to lethal irradiation or chemotherapeutic conditioning regimens had lower urinary concentrations of 3-IS (134). The supplementation of indole-3 carboxaldehyde, an indole derivative, ameliorated gut epithelial damages with reduced inflammatory cytokines, leading to improved survival. In murine GVHD models, AhR-deficient T-cells ameliorated GVHD through the expansion of peripherally induced Tregs (135). AhR ligand levels were decreased in patients at the onset of GVHD (123). However, the mechanism by which the AhR pathway affects GVHD in mice and humans remains unknown.

Tyrosine Derived Metabolites

Tyrosine derived metabolites are produced from tyrosine fermentation by microbiota in the large intestine (136). Tyrosine is one of the non-essential amino acids that is involved in catecholamine synthesis (137). The function of tyrosine has been well studied in brain physiological and pathological conditions (138). In a mouse GVHD model, the metabolic profiles in recipient mice have been determined (139). The low concentration of tyrosine in the gut was observed in GVHD mice, and the tyrosine derived metabolites were decreased in the mice that has less *Blautia* and *Enterococcus*. Dietary supplementation of tyrosine ameliorated GVHD and restored the microbial diversity. In GVHD patients, the metabolite markers in the serum have been investigated (140). Alteration of tyrosine derived metabolites, such as p-cresol sulfate and 3-phenylpropionate, were identified. Also, lysine and phenylalanine were suggested as the altered metabolites. To elucidate the importance of these metabolites, further research is necessary.

Choline Derived Metabolites

Choline derived trimethylamine N-oxide (TMAO) results from oxidation by hepatic flavin monooxygenases of trimethylamine (TMA) that is a microbial metabolite of choline and other choline containing compounds in the diet (141). TMAO is well known to play a role in the initiation of atherosclerosis and thrombosis in vascular inflammation and endothelial function (142). TMAO and choline induced the GVHD progression in a murine GVHD model (143). TMAO and choline induces M1 macrophages and M1-like cytokines in tissues and bone marrow *via* NLRP3. TMAO are produced by the wide range of bacteria, *Anaerococcus hydrogenalis, Clostridium asparagiforme, Clostridium hathewayi, Clostridium sporogenes* (144).

Riboflavin (Vitamin B2) Derived Metabolites

Riboflavin derived metabolites are also known to have an important role in the GVHD pathogenesis. Various bacteria including *E. coli, Staphylococcus aureus and Pseudomonas aeruginosa*, produce these metabolites (145). These metabolites expand the numbers of mucosal associated invariant T-cells (MAIT) (146). Vitamin B2/B9 derived metabolites are presented by MR1, the MHC class I-like molecule, to MAIT cells producing IFN-γ, IL-17 and antibacterial products. In mouse and human GVHD, studies involving riboflavin derived metabolites and GVHD have been accumulating, but there is still no direct evidence that the riboflavin concentration in feces has a direct impact on HSCT complications and outcomes (147–150). In cord blood transplantation, microbial expression of enzymes in the riboflavin synthesis pathway was associated with greater MAIT reconstitution after HSCT (151).

Polyamines

Polyamines are polycationic molecules found in the gastrointestinal tract and are produced by microbial metabolism. They have various biological functions, including IEC barrier function, innate immunity, pro-inflammatory cytokines, and adaptive immunity (152). Spermine, a polyamine, preserves TGF-β and IL-10 production by inhibiting pro-inflammatory cytokine production in activated macrophages (153). Moreover, the amino acid arginine is metabolized to produce polyamines and decreases the production of pro-inflammatory cytokines when administered with *bifidobacterial LKM512* (154). The oral supplementation of spermine or spermidine promoted intraepithelial $CD8^+$ T-cell maturation and $CD4^+$ T-cell increase in the lamina propria and B cell increase in the rat spleen (155). However, the effects of polyamines on GVHD have not yet been well studied. The oral

microbiome is suggested to play a role in pathological conditions and mucositis in allo-HSCT settings (156, 157). During allo-HSCT, oral microbiota derived metabolites are altered in patients with severe oral mucositis, and reduction of N-acetylputrescine and agmatine, metabolites involved in the polyamine pathway, were reported from salivary metabolic analysis in patients (158).

Fecal Microbiota Transplant

Alterations in intestinal microbiota composition and its contribution to allo-HSCT outcomes have been studied to determine the role of the intestinal microbiota in acute GVHD severity. The strategy of manipulating or improving dysbiosis post allo-HSCT *via* fecal microbiota transplant (FMT) were introduced in the HSCT field because FMT treatment for *Clostridium difficile* infection in non-HSCT settings has been successfully reported (159). Recently, the utility of FMT in patients with refractory GVHD after allo-HSCT has also been reported (160). After FMT treatment, dysbiosis improvement was observed as evidenced by increased beneficial bacteria and resolution of clinical symptoms (161). The effects of FMT from third parties were also assessed in a pilot study, and the feasibility and effects on microbiome diversity in recipients have been reported (162). An increase of *Clostridiales* abundance was correlated with a significant increase of 3-IS concentration in the urine in this study. In a randomized trial involving autologous FMT early after allo-HSCT without broad-spectrum antibiotics, the expansion and reestablishment of gut microbiota diversity in autologous FMT recipients was reported (163). Although only a limited number of studies regarding FMT and GVHD treatment have been conducted, they have reported similar results (164). FMT products were freshly processed or frozen until subsequent use. The routes of administration mainly include oral in packed capsules, nasogastric/nasoduodenal tubes, or enema. Most FMT recipients were treated using third-line or more therapies, whereas some received second-line therapy after steroid failure. Complete response rate was high upon treatment with second-line therapy. Responses to treatment were observed within an average 14 days, with a median of two FMT administrations. Changes in the stool microbiome were analyzed using bacterial sequences. FMT recipients had increased diversity and enrichment of *Bacteroides, Lactobacillus, Bidifobacterium*, and *Faecalibacterium* compared with pre-FMTs (160). These changes were observed only when anti-anaerobic systemic antibiotics were discontinued; however, the fourth generation cephem did not affect the efficacy of FMT.

The gut microbiome comprises communities of bacteria, fungi, and viruses that affect each other. Similar to bacterial dysbiosis, alterations in the gut viral community are also associated with gut GVHD (165). For instance, human herpes virus 6 was detected in the serum, and picornavirus in the stool of acute GVHD patients (166). The intestinal fungal composition (mycobiome) after HSCT remains not well investigated. The expansion of pathogenic *Candida* species was associated with a substantial loss of bacterial diversity, especially that of anaerobes, and increased the risk of fungal bloodstream infections (167). There is a paucity of data regarding the changes in the mycobiome and virome in allo-HSCT post-FMT (168). In a GVHD patient with repeated FMT treatments, longitudinal studies observed dynamic changes in the microbiome, mycobiome, and virome in the stool. In contrast to the expansion of intestinal microbial composition post-FMT, the gut mycobiome was first expanded and decreased after multiple FMTs. The gut virome community varied substantially over time with a stable increase in diversity. Furthermore, increased microbial diversity post-FMTs was consistently reported; however, the suggested mechanisms need to be elucidated, including the changes in the microbial metabolites and the key bacterial strains.

Overall, most studies have concluded that FMTs are generally safe and effective for steroid-refractory GVHD patients. However, infectious complications and deaths have been reported, including death due to transmitted drug-resistant bacteria from the FMT donor, bacteremia not from FMT products, diarrhea due to norovirus in the FMTs, and other infections, and were attributed to the immunocompromised states of the patients. Importantly, critical complications related to the recipient death have been reported, such as the transmission of extended spectrum beta-lactamase producing *Escherichia coli* that was proven using genomic sequencing from FMT products (169). Before the clinical application of FMTs, the preparation of FMT products should be standardized, and universal stool banks are warranted (170). The precise practical aspects of FMT treatments have been reviewed in detail elsewhere (171).

FUTURE PERSPECTIVES

The effects of dysbiosis post-HSCT on GVHD have been investigated. Currently new studies on mycobiome and virome in the intestine post-HSCT are focused because intestinal environments were composed of various microorganisms other than microbiota. As viruses and fungi are also part of the gut microbiome, they are expected to provide a deeper understanding of the connection between the microbiome and GVHD. Microbial-derived metabolites, such as SCFAs and indoles, play critical roles in promoting intestinal homeostasis to overcome unwanted antibiotic influences and unfavorable outcomes after HSCT. There are various targets that can affect the results of HSCT, including microbial metabolites and specific microbial strains that potentiate as prebiotics, probiotics, or FMT in the clinical settings. All potential interventions are still under investigation or not yet determined. However, the mechanisms that cause dysbiosis post-HSCT, excluding broad-spectrum antibiotics, remain elusive. Because IECs utilize oxygen for energy, the intestinal environment has low oxygen levels (26, 89), resulting in the domination of anaerobic bacteria in the gut microbiota. As shown in an inflammatory bowel disease model, the metabolic change through PPAR-γ signaling in IECs might lead to dysbiosis in GVHD, which still require further investigation (19). The role of SCFAs in cell metabolism

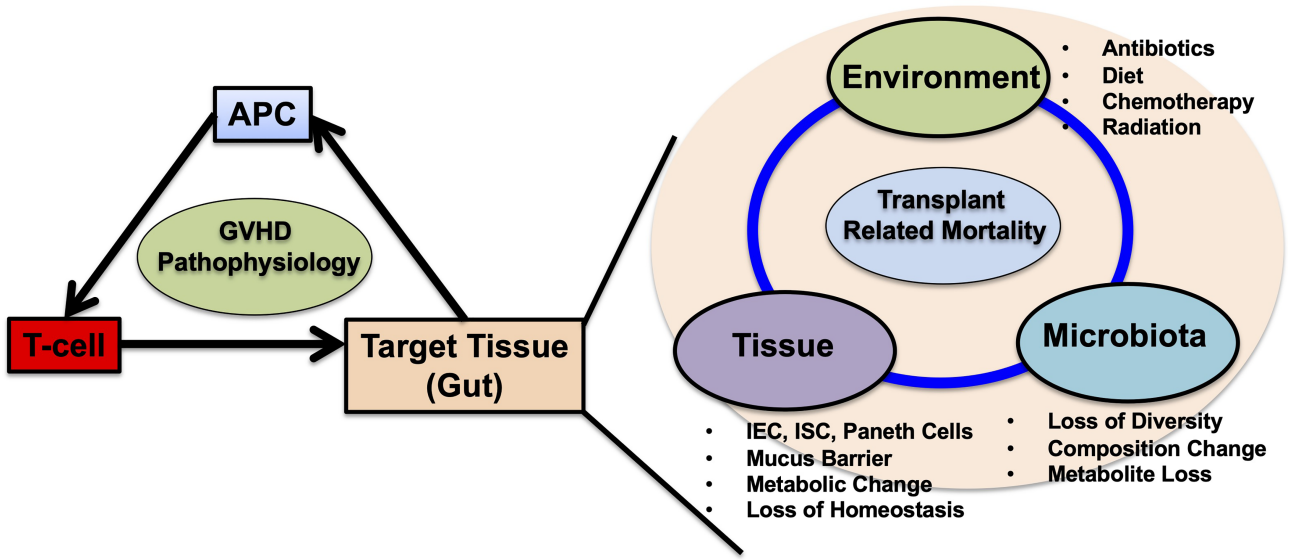

FIGURE 3 | Schematic representation of acute GVHD pathophysiology and the multifactorial interactions in the gut target tissue. Historically, antigen presenting cells (APCs), donor-T cells and target tissues are the main factors in acute GVHD pathophysiology. Among these cells, APCs and T-cells are well studied and targeted for GVHD prevention and treatment. In gut GVHD, microbial dysbiosis are highly associated with transplant related mortality. Microbial dysbiosis is one aspect of multifactorial interactions in the gut during GVHD. In HSCT, conditioning regimens including chemotherapy and irradiation cause damages to intestinal epithelial cells (IECs), intestinal stem cells (ISCs), paneth cells mucus producing goblet cells. Disruption of gut microbiota already exists before HSCT, and prophylactic and systemic antibiotic treatments worsens the microbial dysbiosis with loss of diversity, composition changes along with increase of *Enterococcus*. Dysbiosis depletes microbial derived metabolites changes the IEC metabolism skewing to less tolerant against donor T-cell damages, postpones IEC repairs and mucus barrier restorations. Expansion of Pathogenic bacteria permits blood stream infection thorough injured IECs that necessitate systemic antibiotic administration, leading to further microbial disruptions. These factors including environment, tissue and microbiota falls into the vicious cycle leading to transplant related mortality. The questions remain whether which factor could be prioritized and be targeted for treatments including fecal microbiota transplantation (FMT), prebiotics, probiotics, the attentions of the usages of anti-anaerobic antibiotics and other interventions targeting tissues. Single or combined strategies are warranted to break the vicious cycle and improve HSCT outcomes.

suggests that tissue damage can be reduced by directly intervening target cells without substantially affecting the immune system (68, 172). Tolerance mediated by tissue homeostasis reduces immunological damage from T-cells and other cells. In addition, GVHD and GVL can also be separated. Furthermore, the connection between GVL effects and dysbiosis should be explored to identify potential strategies that boost immune reactions.

Understanding the precise mechanisms and conditions in the intestinal environment can promote the development of prophylactic/therapeutic strategies targeting single or combined modalities (such as FMT or FMT + butyrate, combinations of metabolites) to reduce immunological reactions originating from the gut (**Figure 3**). Furthermore, investigating the crosstalk between local microbiota and injuries in target organs, such as the lungs, could unveil other strategies to prevent HSCT-related complications (173).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Harnessing Treg Homeostasis to Optimize Posttransplant Immunity: Current Concepts and Future Perspectives

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$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are functionally distinct subsets of mature T cells with broad suppressive activity and have been shown to play an important role in the establishment of immune tolerance after allogeneic hematopoietic stem cell transplantation (HSCT). Tregs exhibit an activated phenotype from the stage of emigration from the thymus and maintain continuous proliferation in the periphery. The distinctive feature in homeostasis enables Tregs to respond sensitively to small environmental changes and exert necessary and sufficient immune suppression; however, on the other hand, it also predisposes Tregs to be susceptible to apoptosis in the inflammatory condition post-transplant. Our studies have attempted to define the intrinsic and extrinsic factors affecting Treg homeostasis from the acute to chronic phases after allogeneic HSCT. We have found that altered cytokine environment in the prolonged post-HSCT lymphopenia or peri-transplant use of immune checkpoint inhibitors could hamper Treg reconstitution, leading to refractory graft-*versus*-host disease. Using murine models and clinical trials, we have also demonstrated that proper intervention with low-dose interleukin-2 or post-transplant cyclophosphamide could restore Treg homeostasis and further amplify the suppressive function after HSCT. The purpose of this review is to reconsider the distinctive characteristics of post-transplant Treg homeostasis and discuss how to harness Treg homeostasis to optimize posttransplant immunity for developing a safe and efficient therapeutic strategy.

Keywords: regulatory T cell, graft-*versus*-host disease, interleukin 2, immune checkpoint inhibitor, post-transplant cyclophosphamide

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment strategy for patients with various hematological disorders (1). However, graft-*versus*-host disease (GVHD) remains a cause of significant morbidity and mortality following HSCT (2, 3). The pathophysiology of acute GVHD is the response of donor-derived effector T cells to recipient tissues, including the skin, gut, and liver. During the early phase after HSCT, these immune reactions also evoke dysregulated reconstitution of T and B cell subsets, leading to the basic pathogenesis of the

development of chronic GVHD (4). Over the last decade, there has been increasing evidence showing that regulatory T cells (Tregs) play a critical role in the regulation of acute and chronic GVHD, and the robust reconstitution of Tregs is needed to reestablish a well-balanced immune system that can maintain appropriate levels of peripheral tolerance.

Tregs are a subpopulation of $CD4^+$ T cells that specifically retain their immunosuppressive functions. Tregs were initially identified as $CD4^+CD25^+$ T cells (5), and subsequent studies have shown that Forkhead box P3 (Foxp3) is a master transcription factor for this subset (6). Mice lacking Foxp3 expression were seen to develop lethal autoimmune diseases (7), and humans with a mutation in the *Foxp3* gene are known to develop immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome, which is a severe multiorgan autoimmune disease (8). Tregs comprise 5–10% of peripheral blood $CD4^+$ T cells in healthy individuals and play an essential role in maintaining peripheral self-tolerance and preventing autoimmune diseases (9, 10). In the context of allogeneic HSCT, $CD4^+CD25^+$ cells were initially found to be indispensable for immune tolerance against alloantigens using an *in vitro* mixed lymphocyte reaction assay (11). Later, using murine GVHD models, depletion of $CD25^+$ cells from the donor inoculum exacerbated the severity of GVHD, and co-infusion of *ex vivo* cultured $CD4^+CD25^+$ cells resulted in significant inhibition of rapidly lethal GVHD *in vivo* (12). In addition to murine studies, the analyses of clinical samples from patients after HSCT demonstrated that the expression of the *Foxp3* gene in peripheral blood mononuclear cells was negatively correlated with the incidence and severity of clinical acute and chronic GVHD (13, 14). High Treg contents in the donor graft were associated with the low incidence of acute GVHD (15). Further, prospective monitoring of T cell reconstitution demonstrated that the unbalanced recovery of Tregs and effector T cells contributed to the development of chronic GVHD (16, 17). Notably, studies have shown that Tregs do not abrogate the cytotoxic activity of effector T cells against leukemia cells, both *in vitro* and *in vivo*, suggesting that Tregs could separate GVHD from graft-*versus*-tumor (GVT) activity mediated by donor-derived effector T cells (18). However, in a human clinical setting, $CD25^+$ T cell-depleted donor lymphocyte infusion (DLI) showed a better disease control without increasing the incidence of acute GVHD than unmodified DLI in contemporaneous patients, indicating the possibility of Treg suppressive function against the GVT effect (19).

In general, human T cells express CD25 and Foxp3 after antigen presentation and undergo cell activation. In contrast, Tregs highly express such activated markers during migration from the thymus and maintain continuous proliferation in the periphery (**Figure 1**). The distinctive feature in homeostasis enables Tregs to respond sensitively to small environmental changes and exert necessary and sufficient immune suppression. However, on the other hand, it also predisposes Tregs to be susceptible to apoptosis. In patients after allogeneic HSCT, Tregs are exposed to a profound inflammatory

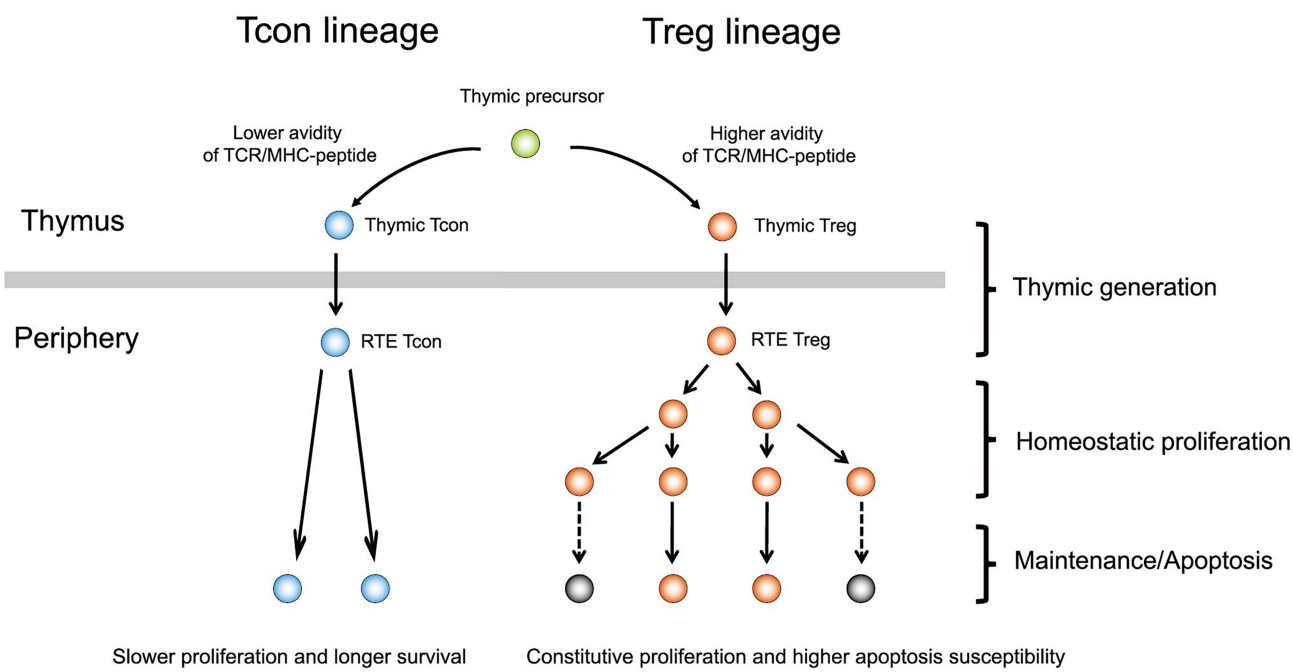

FIGURE 1 | Development and maintenance of regulatory T cells. Tregs are positively selected from a population with a higher avidity of TCR/MHC-peptide in the thymus and emigrate to the periphery. Tregs already exhibit an activated phenotype at emigration to the periphery and undergo rapid homeostatic proliferation. Constitutive proliferation predisposes Tregs to apoptosis. In contrast, Tcons generally undergo slower proliferation and, thus, stable homeostasis with lower apoptosis. Tcon, conventional T cell; Treg, regulatory T cell; RTE, recent thymic emigrant; TCR, T-cell receptor; MHC, major histocompatibility complex.

environment affected by various endogenous factors, including severe lymphopenia, allogeneic antigen stimulation, and altered cytokine milieu. In addition to such endogenous factors after HSCT, various therapeutic immune modulators have been increasingly used in the peri-transplant period in recent years (**Figure 2**). Our studies have elucidated that both endogenous and exogenous factors have major impacts, particularly on Treg homeostasis and clinical outcomes after allogeneic HSCT.

In this review, we outline the characteristics of posttransplant Treg homeostasis and discuss how to harness Treg homeostasis to optimize posttransplant immunity. In particular, we would attempt to define the intrinsic and extrinsic factors that affect Treg homeostasis from the acute to chronic phases after allogeneic HSCT.

CHARACTERISTICS OF TREG HOMEOSTASIS AFTER HSCT

The Generation and Maintenance of Tregs

Tregs are positively selected from populations with a higher avidity of T cell receptor (TCR)/major histocompatibility complex-peptide in the thymus and emigrate to the periphery (**Figure 1**). Therefore, Tregs can be considered as a physiologically preactivated population and show distinctive homeostasis in the periphery (**Figure 1**). Tregs have a high turnover and fine sensitivity to a variety of signals from the environment to regulate the cell number, localization, and function required to efficiently react to delicate changes in the immune system (20). Peripheral Tregs exhibit a high basal proliferation rate compared with conventional T cells (Tcons) in both mice (21) and humans (22); however, the high proliferation rate of Tregs is counterbalanced by a high rate of apoptosis (16). Interleukin-2 (IL-2), which is mainly produced by effector T cells, plays a pivotal role in Treg homeostasis (20). Importantly, Tregs cannot produce IL-2 independently; therefore, homeostasis critically depends on the extrinsic cytokine environment of the cell.

The Reconstitution of T Cell Subsets and Immune Tolerance

The reconstitution of T cell subsets after HSCT is a polymorphic and long-term process. In general, the initial phase of T cell reconstitution is primarily dependent on the peripheral expansion of mature T cells that are contained in the stem cell graft (23). This process is promoted by lymphopenia-induced signals and the stimulation of donor T cells by alloantigens (16). Hematopoietic stem cells (HSC) also differentiate through the thymus, and thymus-derived T cells are then exported to the periphery. However, thymus-dependent generation of donor T cells is generally delayed and incomplete in adult patients because of natural thymic involution and damage, especially in thymic epithelial cells, resulting from high-dose chemotherapy and irradiation administered as part of the conditioning regimen (24–28). Once naïve T cells are exported into the periphery from the thymus, they are subject to homeostatic signals that regulate

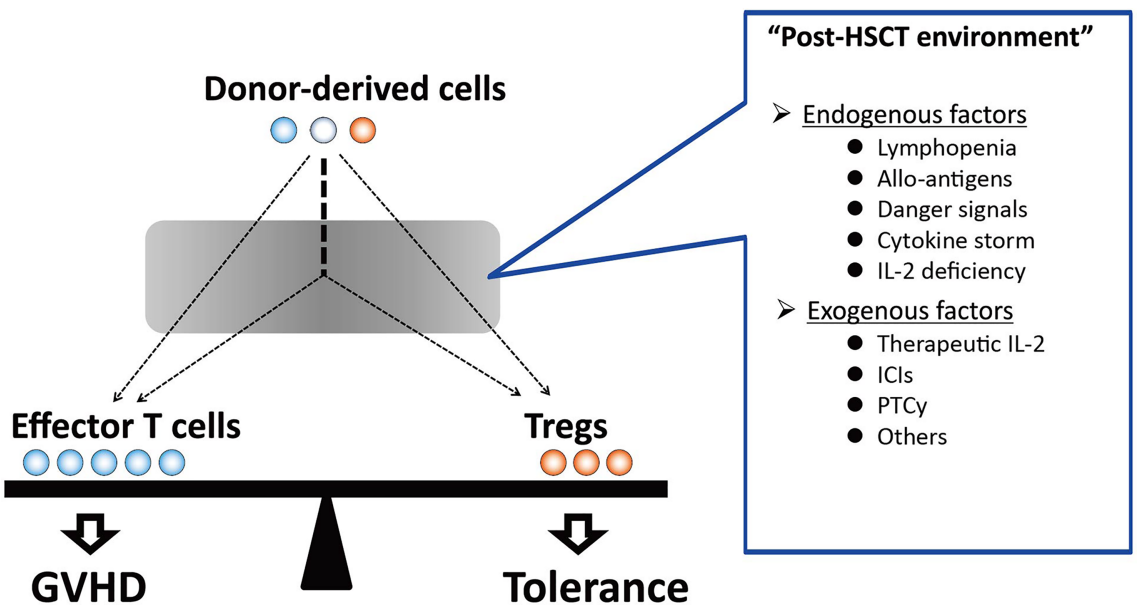

FIGURE 2 | The balance between effector T cells and Tregs in the distinct post-HSCT environment. After HSCT, donor-derived T cells are exposed to a variety of endogenous factors, including lymphopenia, alloantigens, danger signals, and cytokine storm, which affect the reconstitution of donor-derived immune cells. In addition, therapeutic interventions that have immunomodulating effects during the peri-transplant period also affect donor-derived T cell homeostasis. Unbalanced immune reconstitution may provide a basis for the pathogenesis of GVHD. In contrast, favorable Treg recovery in the post-transplant environment results in the induction of immune tolerance. GVHD, graft-*versus*-host disease; Treg, regulatory T cell; HSCT, hematopoietic stem cell transplantation; IL-2: interleukin 2, ICI, immune checkpoint inhibitor; PTCy, post-transplant cyclophosphamide.

the expansion and contraction of the T cell population to maintain the total number of T cell subsets at an appropriate level. Although the transition of peripheral T cells from mature T cells in the stem cell graft into thymic-generated naïve T cells is commonly observed in each T cell lineage after HSCT; each T cell subset is subject to distinct homeostatic controls in the periphery (29). Particularly, Treg homeostasis appears to be distinct from Tcon, and this may contribute to an imbalance between Treg and Tcon (**Figure 2**) (10, 22). We previously studied long-term Treg reconstitution after clinical HSCT and the impacts on the development of chronic GVHD (16). Our data indicated that thymic generation of Tregs was markedly impaired, while this subset maintained a significantly higher level of proliferation as compared to Tcons. Treg proliferation *in vivo* appears to be driven primarily by CD4 lymphopenia. Importantly, high levels of Treg proliferation are counterbalanced by its increased susceptibility to apoptosis. Altered Treg homeostasis in response to the homeostatic pressure under prolonged CD4 lymphopenia resulted in the selective peripheral depletion of Tregs and the subsequent development of chronic GVHD.

A recent study that analyzed $CD4^+$Treg heterogeneity with a large panel of functional markers using mass cytometry by time of flight revealed that in addition to the numerical deficiency, reduced heterogeneity of $CD4^+$Tregs was associated with the development of chronic GVHD (30). Thus, the thymic generation of *de novo* Tregs from donor HSC plays an essential role in long-term immune reconstitution. In addition to Treg heterogeneity, foxp3 stability (31) is also important for Treg survival and GVHD suppression (32–34). The post-transplant inflammatory environment may affect the genetic stability of Tregs and should be taken into consideration for the understandings of Treg homeostasis after HSCT.

In addition to donor-derived Tregs, host-derived Tregs appear to play a crucial role in immune tolerance, especially during the very early phase after HSCT. Since Tregs are relatively radioresistant (35), murine studies showed that host Tregs could survive for several weeks after HSCT and contribute to suppressing the alloimmune response in the experimental mice model in the low level of irradiation before HSCT (36).

The Basic Framework of Treg Reconstitution After Clinical HSCT

Based on the above findings, we proposed a basic framework of Treg reconstitution after clinical HSCT (**Figure 3**) (37). It comprises four phases: Treg expansion, transition, repopulation, and maintenance. Although previous studies have suggested that host-type Tregs show a temporary expansion immediately after HSCT after non-myeloablative conditioning, the kinetics of host-Tregs after HSCT is left out from this schema to simply discuss the donor-Treg engrafting processes.

Phase 1: Expansion of Graft-Derived Tregs

During the first month post-HSCT, donor stem-cell graft-derived mature Tregs promptly expand in response to lymphopenia and alloantigens. The expansion peaks around the first month after HSCT and appears to be contracted by activation-induced cell death (AICD). It remains unclear whether some part of graft-derived Tregs would survive as functional memory suppressor cells for a long time after HSCT (38).

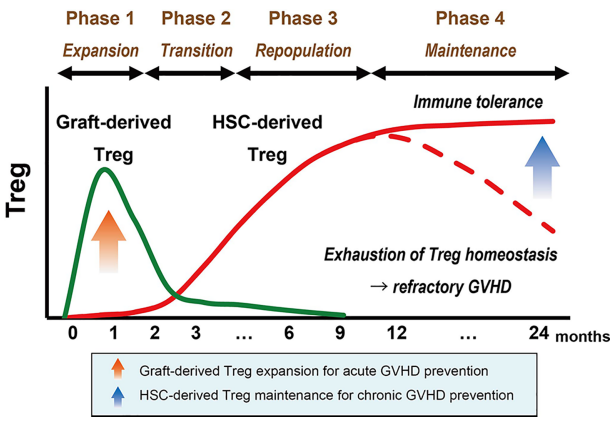

FIGURE 3 | Basic framework of Treg reconstitution after HSCT. The basic framework of Treg recovery after HSCT consists of four phases. In the first month after HSCT, lymphopenia-driven peripheral proliferation of graft-derived Tregs (shown in green) induces the initial expansion of this subset, which is important for preventing acute GVHD (Phase 1). The total number of Tregs transiently decrease between the contraction of expanded graft-derived Tregs and the emergence of HSC-derived Tregs (shown in red) (Phase 2). Prolonged lymphopenia in the first year promotes the continuous aggressive proliferation of HSC-derived Tregs, which contributes to the recovery of total Treg cells and provides an essential basis for long-term immune tolerance (red solid line) (Phase 3). However, highly proliferative Tregs can fail to maintain homeostasis due to the high susceptibility to apoptosis, and exhaustion of the Treg population could be a trigger for refractory GVHD (red dotted line) (Phase 4). GVHD, graft-*versus*-host disease; Treg, regulatory T cell; HSC, hematopoietic stem cell.

Phase 2: Transition of Peripheral Treg Source

The emergence of HSC-derived Tregs is generally delayed because of thymic dysfunction after HSCT; therefore, the total number of Tregs in the periphery decreases after the contraction of graft-derived Tregs (transitional phase). The decline in the number of Tregs in this phase may be associated with the late onset of acute GVHD or overlap syndrome.

Phase 3: Repopulation and Relocation of Thymus-Generated Tregs

After a dip in the number of Tregs in Phase 2, it takes about a year for the number of circulating Tregs to increase and approach the normal range. It is much faster than the reconstitution of Tcon subsets, which often reach a normal range within over 2 years (16). Treg repopulation in this phase is mainly constituted by HSC-derived Tregs. Although thymic generation of Tregs is markedly impaired after HSCT, newly generated Tregs maintain higher levels of proliferation during this phase. Proliferation of Tregs is relatively aggressive as compared to other Tcons, resulting in high levels of the Treg/Tcon ratio and the Treg/CD8T ratio in this phase (16, 17). The

recovery of thymus-generated Tregs is highly influenced by age-related natural thymic involution, thus Treg kinetics in Phase 3 should be carefully evaluated in the pediatric transplant cohorts.

Phase 4: Maintenance of HSC-Derived Tregs

As only naïve Tregs are capable of aggressive proliferation, the stable emergence of naïve Tregs from the thymus is thought to be critical for the maintenance of Treg homeostasis. Tregs can repopulate by compensatory homeostatic proliferation in Phase 3, even in patients with severe thymic damage; however, high levels of Treg proliferation in prolonged lymphopenia are counterbalanced by increased susceptibility to apoptosis, 6–9 months after HSCT. Importantly, peripheral depletion based on abnormal homeostasis is observed only in Tregs, but not in other Tcons, resulting in the imbalance of lymphocyte subsets, which provides a fundamental basis for the development of chronic GVHD (16, 39, 40).

Treg Migration After HSCT

Appropriate circulation and localization of Treg are crucial for systemic immune suppression. Luciferase-expressing Tregs enabled tracking the tissue migration and survival of Treg longitudinally in mice HSCT model. Transferred Tregs proliferated in secondary lymphoid organs and sequentially migrated and localized into peripheral tissue after allogeneic stimulation *in vivo* (41). The initial priming of Tregs occurring in the secondary lymph node required CD62L expression on Tregs to migrate into lymphoid organs and protect from GVHD lethality (42). The proinflammatory environment during the early period after HSCT is essential for early Treg expansion and migration to GVHD sites. These Tregs exhibited sufficient suppressive function for alloreactive effector T cell proliferation in lymphoid organ and peripheral tissue (41).

The above model of Treg reconstitution is based on the number of Tregs in peripheral blood after clinical transplantation (16, 17). Most clinical studies test Tregs in peripheral blood rather than lymph nodes or target peripheral tissues, while mouse studies often test Tregs in the spleen and lymph nodes. Therefore, interpretation of the results needs to be carefully considered. Tregs at each site after HSCT may have different properties and functions for the overall regulation of allogeneic immunity.

EFFECTS OF LOW-DOSE IL-2 ON TREG HOMEOSTASIS

IL-2 and Treg

It was initially found that IL-2 stimulates naïve T cell proliferation and generates effector and memory T cells. Later, several reports have shown that the germline knockout of IL-2 (43, 44) or blocking of IL-2 (45) or CD25 (46) results in autoimmune diseases, suggesting that IL-2 plays an essential role in immune tolerance. After identifying Tregs, IL-2 is now known as a critical homeostatic cytokine for Treg function (47), Treg differentiation in the thymus, and Treg expansion in the periphery (48, 49). The IL-2 receptor (IL-2R) is expressed as an intermediate-affinity dimer (IL-2Rβ and IL-2Rγ) or high-affinity trimers, which include IL-2Rα (CD25). Tregs constitutively express high-affinity to IL-2 receptors, which enables Tregs to respond to a small amount of IL-2, which other T cell subsets do not respond to. Initially, low-dose IL-2 was used to prevent disease recurrence owing to the GVT effect from natural killer (NK) cells, which also express CD25 in a steady state (50). In this pioneering study, low-dose IL-2 ($2–6 \times 10^5$ IU/kg/day) was administered for up to 3 months, and only one patient developed acute GVHD. Although the effect of low-dose IL-2 on lymphocyte subsets could not be fully evaluated because Treg had not been defined at the time of the study, the follow-up study revealed that the use of low-dose IL-2 induced the increasing Tregs in patients (51). The results led to the subsequent project of low-dose IL-2 therapy which aimed to reconstruct immune tolerance by increasing Tregs in patients with chronic GVHD (52).

Low-Dose IL-2 for Chronic GVHD Treatment

Based on the theory that Tregs preferentially respond to low-dose IL-2, we conducted a phase 1 trial of administering low-dose IL-2 therapy daily in patients with steroid-refractory chronic GVHD (SR-cGVHD). In this trial, a total of 29 patients were enrolled, and the maximum tolerated dose of IL-2 was 1×10^6 IU/m^2; none of the patients experienced progression of chronic GVHD or relapse of hematologic cancers. Of the evaluable 23 patients, 12 patients had a major response, and the number of $CD4^+$Tregs was preferentially increased in all patients (53). A subsequent phase 2 trial evaluated the efficacy of daily IL-2 (1×10^6 IU/m^2) administration for 12 weeks in 35 adult SR-cGVHD patients. The treatment was well tolerated, with 20 of 33 evaluable patients demonstrating clinical response at multiple chronic GVHD sites. The phase 2 trial also showed a rapid increase in the absolute number of Tregs and a rapid increase in the Tregs/Tcons ratio (54).

To elucidate the biological mechanisms of low-dose IL-2, we examined the effects of daily IL-2 therapy on the homeostasis of $CD4^+$ T cell subsets after transplantation (55). We first demonstrated that chronic GVHD is characterized by constitutive phosphorylation of Stat5 in Tcon, associated with elevated levels of IL-7 and IL-15 and relative functional deficiency of IL-2. This was promptly corrected after IL-2 therapy, which resulted in the selective increase of Stat5 phosphorylation in Tregs and a decrease in pStat5 in Tcons. This was associated with profound changes in Treg homeostasis, including increased proliferation, increased thymic export, and enhanced resistance to apoptosis (55). A single cell mass cytometry analysis confirmed that low-dose IL-2 preferentially activated p-STAT5 in $Helios^+$ naïve Tregs (56), promoted an increase in the population, and enhanced the suppressive function. In addition to increasing the number of Tregs by low-dose IL-2, it may also be important to increase the T cell

receptor (TCR) diversity of Tregs. Previous studies have already shown that the limited TCR diversity of Tregs may be associated with autoimmune diseases (57, 58) and GVHD in the experimental models (59). A recent study using samples from patients who received low-dose IL-2 demonstrated that the increased Treg diversity after IL-2 treatment was associated with the improvement of clinical symptoms of chronic GVHD (60). Taken together, low-dose IL-2 therapy restores Treg homeostasis and promotes the reestablishment of immune tolerance in patients with chronic GVHD.

To reconsider the optimum algorithm of IL-2 intervention for safe and efficient Treg expansion, we conducted a murine model study, and found that the daily administration might not be required and the intermittent administration within threshold could be sufficient for the maintenance of expanded Tregs after the initial intensive IL-2 intervention (61). Based on the idea, we have designed and started a multicenter phase I/IIa clinical trial of low-dose IL-2 for patients with SR-cGVHD (62). In the protocol, IL-2 treatment is composed of two sequential phases: the induction phase and the maintenance phase. In the induction phase, IL-2 is subcutaneously administrated once per day for 4 weeks. In the subsequent maintenance phase, IL-2 is subcutaneously administrated three times per week for following period up to 1 year. The study had been completed and showed the favorable results with the stable immunological and clinical effects (63). Data are now being analyzed for publication.

Further studies are needed to determine the optimal dosage, timing, duration, and combination of low-dose IL-2 therapy in patients with chronic GVHD. Recently, engineered Treg-selective human IL-2 was developed in humanized mice during xenogeneic GVHD (64), and Efavaleukin Alfa (AMG-592), a human IL-2 mutant designed to have greater Treg selectivity and longer half-life compared with recombinant IL-2, is now under a clinical trial for patients with SR-cGVHD (NCT03422627). Additionally, a phase 1 clinical trial focusing on the combination of donor-Treg infusion followed by low-dose IL-2 for SR-cGVHD is also in progress (NCT01937468).

EFFECTS OF IMMUNE-CHECKPOINT INHIBITORS ON TREG HOMEOSTASIS

Immune checkpoints play an important role in the activation of effector T cells and the regulation of cytotoxic activity. In addition to this, previous studies have shown that they play a pivotal role in the homeostasis and function of Tregs, which originally exhibit an activated phenotype.

PD-1 is an immune checkpoint receptor that attenuates T-cell activation by interacting with its ligands, PD-L1 and PD-L2 (65). However, the exact role of PD-1 in the immune suppressive function of Tregs remains unclear. PD-1-deficient Tregs improved the symptoms of autoimmune pancreatitis in a mouse model (66), while in human glioblastoma tissues, Tregs expressing a high level of PD-1 had an exhausted phenotype and reduced immunosuppressive function (67). Moreover, in patients with gastric cancer who developed hyper-progressive disease after PD-1 blockade, tumors possessed highly proliferative Tregs after treatment (68). These studies suggest that PD-1 may regulate the immunosuppressive function of Tregs. In contrast, in a mouse chronic infection model, PD-1 blockade attenuated immunosuppression by Tregs, mediated by direct interaction of PD-1 on Tregs with PD-L1 on $CD8^+$ T cells (69). These discrepant results regarding the role of PD-1 on the suppressive function of Tregs might be explained by the differences in the class of Tregs (70).

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an inhibitory checkpoint molecule. Tregs constitutively express a high level of CTLA-4, whereas Tcons express CTLA-4 only after activation (71). CTLA-4 competes with CD28 for CD80/CD86 ligands, resulting in the inhibition of co-stimulatory signals (72). CTLA-4 blockade was the first immune checkpoint inhibitor (ICI) approved for clinical use, and the mechanisms of the anti-CTLA-4 blockade were distinct from those of PD-1 blockade (73, 74). Regarding the effect of Tregs, several preclinical studies indicated that the anti-tumor effects of CTLA-4 blockade were dependent on the depletion of CTLA-4-expressing Tregs in the tumor microenvironment *via* antibody-dependent T cellular cytotoxicity, leading to an increase in the $CD8^+$/Treg ratio (70). However, anti-CTLA-4 immunotherapy for patients with solid cancers did not deplete Tregs (75); therefore, further analyses to address the role of CTLA-4 in Tregs in various settings are warranted.

The Effect of PD-1 on Treg Homeostasis During Low-Dose IL-2 Therapy

Low-dose IL-2 therapy increased circulating Tregs and improved clinical symptoms of chronic GVHD; however, the mechanisms that regulate Treg homeostasis during IL-2 therapy have not been well studied. To elucidate these regulatory mechanisms, we examined the role of inhibitory coreceptors on Tregs during IL-2 therapy in a murine model and in patients with chronic GVHD (76). Murine studies demonstrated that low-dose IL-2 selectively increased Tregs and simultaneously enhanced the expression of PD-1, especially on $CD44^+CD62L^+$ central-memory Tregs, whereas the expression of other inhibitory molecules, including CTLA-4, LAG-3, and TIM-3 remained stable. PD-1-deficient Tregs showed rapid Stat5 phosphorylation and proliferation soon after IL-2 initiation; however, Tregs became proapoptotic with higher Fas and lower Bcl-2 expression. As a result, the positive impact of IL-2 on Tregs was completely abolished, and Treg levels returned to baseline despite continued IL-2 administration (**Figure 4**). We also examined circulating Tregs from patients with chronic GVHD who were receiving low-dose IL-2 and found that IL-2-induced Treg proliferation was promptly followed by increased PD-1 expression on central-memory Tregs. Notably, clinical improvement of GVHD was associated with increased levels of PD-1 on Tregs, suggesting that the PD-1 pathway supports Treg-mediated tolerance. These studies indicate that PD-1 is a critical homeostatic regulator of Tregs by modulating proliferation and apoptosis during IL-2 therapy.

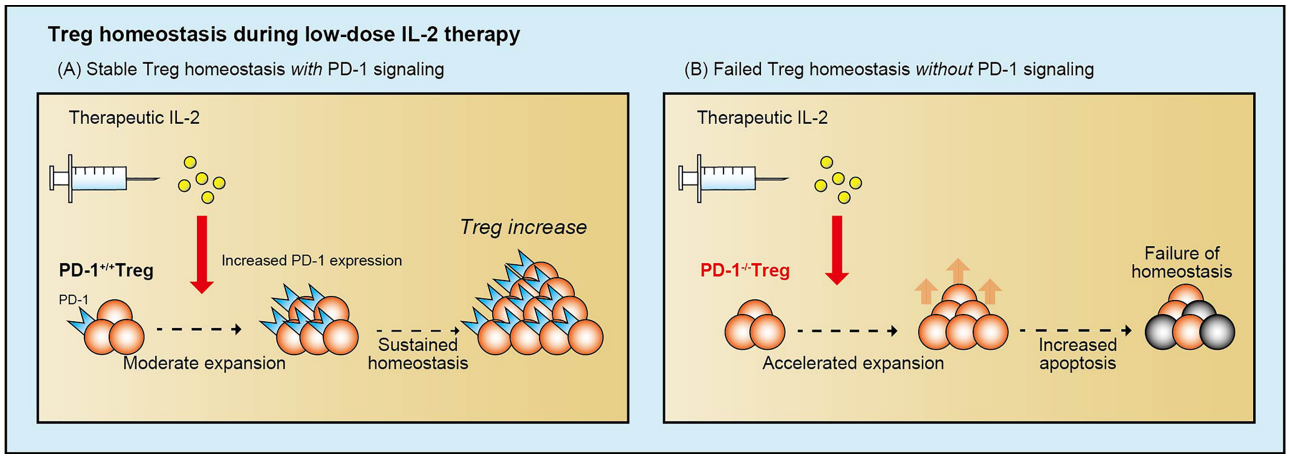

FIGURE 4 | The role of PD-1 in Treg homeostasis during low-dose IL-2 therapy. Low-dose IL-2 therapy increases PD-1 expression on Tregs, and PD-1 preserves sustainable Treg homeostasis during low-dose IL-2 therapy, resulting in the preferential expansion of Tregs and the clinical improvement of chronic GVHD symptoms **(A)**. In contrast, without sufficient PD-1 signaling, low-dose IL-2 therapy accelerates Treg expansion soon after initiation, but Tregs are predisposed to the proapoptotic status and cannot maintain the expansion **(B)**. IL-2, interleukin 2; PD-1, programmed cell death 1; Treg, regulatory T cells.

The Effect of PD-1 on Treg Homeostasis After HSCT

It has been observed that PD-1 blockade therapy is an effective strategy for hematological malignancies such as classical Hodgkin lymphoma (cHL) and it has made it possible to bridge chemotherapy-refractory patients to allogeneic HSCT (77–79). However, PD-1 blockade during the peri-transplant period theoretically enhances the donor effector T cell response, leading to an increased risk of severe GVHD. A pioneering murine model study demonstrated that PD-1 blockade potently enhanced T cell alloresponses both *in vitro* and *in vivo*, and the effect of PD-1/PD-L1 blockade was largely dependent on interferon (IFN) production (80). A subsequent study evaluating the role of PD-1 ligand for GVHD demonstrated that PD-1/PD-L1 blockade, but not PD-1/PD-L2 blockade, markedly accelerated GVHD lethality; suggesting an important differential role of host PD-L1 and PD-L2 in controlling GVHD (81). In the clinical setting, retrospective studies reported that pretransplant PD-1 blockade increased the risk of severe acute GVHD (82–84). Nieto et al. investigated clinical samples of patients treated with nivolumab before HSCT and demonstrated that nivolumab was detectable in the plasma for up to 56 days after HSCT was performed, and this residual nivolumab could bind to and block PD-1 expressed on donor effector T cells during this period. They also showed that pretransplant nivolumab resulted in a high frequency of IFN-γ-producing effector T cells, which might contribute to severe GVHD after transplantation (85).

Our group examined the effect of PD-1 blockade on Treg homeostasis after allogeneic HSCT in murine models and human clinical samples. First, we demonstrated that pre- and peri-transplant PD-1 blockade increased the severity of GVHD due to the unbalanced reconstitution of T cell subsets in recipient mice with PD-1 inhibition (86). PD-$1^{-/-}$ effector T cells aggressively increased after HSCT, whereas PD-$1^{-/-}$ Tregs could not maintain the expansion due to high susceptibility to apoptosis, leading to an unbalanced reconstitution of T cells, resulting in lethal GVHD. Interestingly, post-transplant cyclophosphamide (PTCy) restored the well-balanced reconstitution of T cell subsets and prevented tissue damage after HSCT from donor PD-$1^{-/-}$ effector T cells. Based on fundamental data from a murine study, we examined clinical samples from patients who underwent HSCT. The clinical sample analyses revealed that PTCy promoted vigorous recovery of Tregs in recipients who underwent HLA-haploidentical transplantation following nivolumab therapy (87). These results suggest that PD-1 plays a crucial role in Treg homeostasis, especially in the early phase after HSCT. Moreover, PTCy might be an optimal GVHD prophylaxis when regulatory PD-1 signaling is functionally abolished by therapeutic intervention before HSCT (**Figure 5**).

In fact, recent clinical data have clearly indicated that PTCy was associated with a low incidence of severe GVHD in recipients with pre-transplant ICIs (88–91). Furthermore, Merryman et al. recently reported the results of an international retrospective study that included 209 relapsed or refractory cHL patients who underwent HSCT after PD-1 blockade (91). They demonstrated that PTCy-based haploidentical HSCT was associated with significant improvements in the progression-free and relapse-free survival of GVHD as compared with HLA-matched HSCT without PTCy. This suggests that pretransplant ICI and subsequent PTCy-based HSCT may provide better GVHD control along with better disease control (88–91). Further investigations on the impact of peri-transplant ICIs and PTCy on GVT activity are warranted.

The Effect of PTCy on Treg Homeostasis After HSCT

PTCy is a novel GVHD prophylactic strategy for acute GVHD after HSCT from an HLA-haploidentical donor (92), and has been extended to an HLA-identical donor as a single agent GVHD prophylaxis (93–95). The mechanisms underlying the

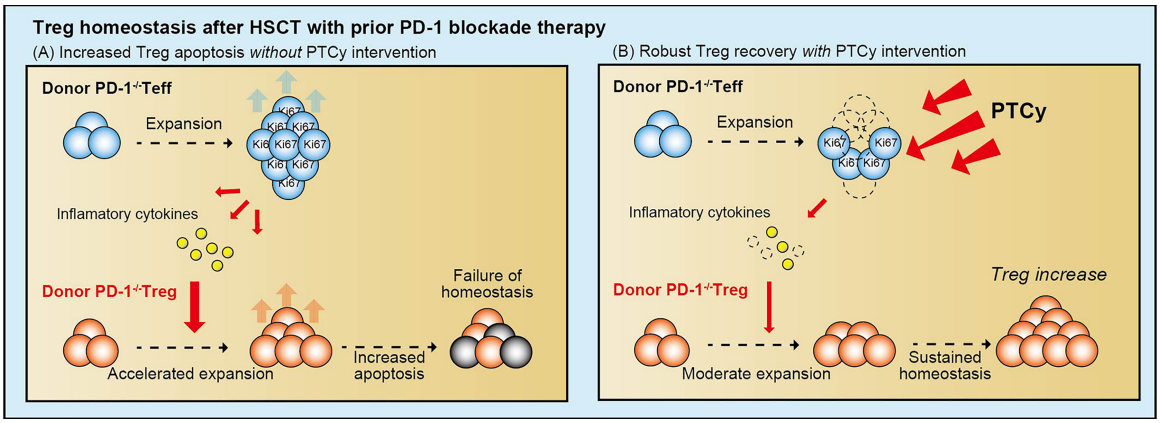

FIGURE 5 | The role of PD-1 in Treg homeostasis after allogeneic HSCT. PD-1 deficient effector T cells aggressively proliferate after HSCT and produce large amounts of inflammatory cytokines. The highly inflammatory cytokine milieu accelerates the proliferation of PD-1 deficient Tregs, but they cannot maintain proliferation due to their high susceptibility to apoptosis. Unbalanced reconstitution of T-cell subsets results in severe GVHD **(A)**. In contrast, with PTCy intervention, alloreactive proliferative effector T cells are efficiently eliminated, contributing to reduced inflammatory cytokine production. It enables Tregs to restore the appropriate levels of proliferation and maintain homeostasis, which contributes to the prevention of severe GVHD **(B)**. PD-1, programmed cell death 1; Treg, regulatory T cell; Teff, effector T cell; PTCy, post-transplant cyclophosphamide.

effect of PTCy involve impaired function of alloreactive T cells (96), preserving hematopoietic stem cells and Tregs by their high expression of aldehyde dehydrogenase (97). The indispensable role of donor-derived Tregs in preventing GVHD in PTCy was shown in a mouse model (98), which suggests that PTCy-based GVHD prophylaxis may preferentially promote the reconstitution of Tregs in a clinical setting. However, there are limited data on Treg reconstitution after HSCT with PTCy-based GVHD prophylaxis using human clinical samples (99–102). Our and other groups recently reported the reconstitution of lymphocyte subsets between HLA-haploidentical PTCy-based HSCT (PTCy-haplo) as compared to HLA-identical donor HSCT using conventional GVHD prophylaxis (101, 102). These studies demonstrated that the number of T cells was significantly lower in PTCy-haplo than that in HLA-identical HSCT in the early phase, which was due to slower $CD4^+$Tcons reconstitution. On the other hand, the recovery of Tregs after PTCy-haplo was earlier than that after HLA-identical HSCT, resulting in a significantly higher Tregs/Tcons ratio during the first 3 months after HSCT (101, 102). Although PTCy is a promising approach in terms of Treg reconstruction, further optimization of prophylaxis is required, including the optimal dose and duration of cyclophosphamide administration, the timing of calcineurin inhibitor initiation, and combining it with other immunosuppressant drugs.

EFFECTS OF OTHER IMMUNE MODULATORS ON TREG HOMEOSTASIS

Anti C-C Chemokine Receptor 4 Blockade

C-C chemokine receptor 4 (CCR4) is a chemokine receptor expressed in most patients with adult T-cell leukemia lymphoma (ATLL). Mogamulizumab (Mog) is a humanized anti-CCR4 immunoglobulin G1 monoclonal antibody that has an almost 50% efficacy in patients with relapsed or refractory ATLL (103, 104). CCR4 is also highly expressed on Tregs; therefore, pretransplant Mog may affect post-transplant Treg homeostasis and the incidence of GVHD. A multicenter, retrospective study demonstrated that pretransplant Mog was significantly associated with an increased risk of severe acute GVHD (105). Although a phase 1 study reported the half-life of Mog to be 15 to 18 days, retrospective data showed that the interval between the last administration of Mog to HSCT within 50 days was an independent prognostic factor for non-relapse mortality (105); therefore, the last treatment schedule of Mog is recommended to be ≥ 50 days prior to HSCT (106). In addition, the effect of Mog on Treg recovery after HSCT might be more profound than expected. We report a case of ATLL in which plasma exchange (PE) was conducted to eliminate residual Mog to treat severe GVHD (107). Although plasma Mog concentration was eliminated, it did not lead to the prompt elevation of Treg levels in peripheral blood, and the clinical responses of GVHD were limited to partial remission, suggesting that recovery of donor-derived Tregs in the acute phase after HSCT is multifactorial, and the single procedure of PE-based Mog depletion does not necessarily warrant the quick restoration of Treg homeostasis. As mentioned above, although Mog is a highly effective treatment for relapse and refractory ATLL, the increased risk of severe GVHD cannot be ignored. Avoiding the short interval between the last administration of Mog and HSCT and donors at high risk of GVHD should be considered to reduce the negative impact of Mog. Having said that, further clinical and basic research in this field is needed.

α-Galactosylceramide

Although low-dose IL-2 therapy induced the selective expansion of Tregs in the chronic phase after HSCT, the specificity of the

IL-2 effect may be shrunk in the acute phase after HSCT since activated effector T cells also express CD25 and may respond to the administered IL-2. In contrast, previous murine studies have demonstrated that invariant natural killer T (iNKT) stimulation mediated by α-galactosylceramide (α-GC) enables selective Treg expansion even in the very early phase following HSCT. iNKT cells are unique immunoregulatory T cell subsets that have limited TCR repertoires and recognize lipid antigens presented by CD1d on antigen-presenting cells (108). α-GC is a glycolipid originally purified from a marine sponge (109) and is also a ligand for iNKT cells in a CD1d-restricted manner. Previous studies have shown that α-GC stimulates different iNKT cell subsets depending on the method of injection (110). In the murine HSCT model, a single injection of αGC on day 0 after HSCT promoted Th2 polarization of donor T cells and the expansion of Tregs in a STAT6-dependent manner, which resulted in the reduced GVHD mortality (111, 112). Thereafter, a liposomal α-GC (lipo α-GC) was developed (113), and lipo α-GC was found to be safe and effective for acute GVHD prophylaxis in a murine model (114). Regarding the homeostasis of Tregs, a subsequent study revealed that host NKT cells induced an IL-4-dependent expansion of donor Tregs after HSCT (112). In addition, the adoptive transfer of donor iNKT cells ameliorated GVHD by expanding donor Tregs, in turn preserving the GVT effect (115). In addition to the acute GVHD model, our murine study using chronic GVHD models showed that α-GC treatment could ameliorate chronic GVHD symptoms through the early expansion of donor-derived Tregs followed by the suppression of follicular helper T cells and germinal center B cells (116). Based on the data of preclinical models, a phase 2 clinical study evaluated the efficacy of RGI-2001, a novel liposomal formulation of a synthetic derivative of α-GC in patients who underwent HSCT. A total of 29 patients received RGI-2001 on day 0 after HSCT, and 28% of recipients responded to RGI-2001 and increased the frequency and number of Tregs. These responders developed grade II to IV acute GVHD significantly less frequently than non-responders (117), suggesting that α-GC may prevent acute GVHD *via* Treg expansion in a clinical HSCT setting.

The combination of α-GC with other therapeutic modalities may be promising for further effective Treg modulation since the working mechanism of α-GC is unique and does not overlap with those of other therapies. Recently, we evaluated the effect of adding lipo α-GC after PTCy on GVHD and the GVT effect using the murine GVHD model (118). We demonstrated that a reduced dose of PTCy followed by adjuvant α-GC enhances the GVT effect without sacrificing GVHD suppression. Phenotypic analyses revealed that donor-derived B cells presented the ligand and induced preferential skewing to the NKT2 phenotype rather than the NKT1 phenotype, which was followed by the early recovery of all T cell subsets, especially Tregs. Our results propose the possibility of a novel strategy for optimizing PTCy-based transplantation.

Anti-IL-2 Antibody

As the IL-2 receptors express on the activated effector T cells, the antibody for the IL-2 receptor has been investigated for GVHD prophylaxis and treatment. However, as IL-2 is an essential cytokine for Treg survival (45), the clinical effects of IL-2 receptor blockade appear not to be straightforward in patients after HSCT. Two IL-2 receptor antagonists, basiliximab and daclizumab, were added on the standard GVHD prophylaxis and explored the prophylactic effects against GVHD. These agents showed the preventive effect against acute GVHD, and basiliximab showed a superior prophylactic effect against chronic GVHD (119). However, the subsequent prospective randomized control study showed no additional prophylactic effect of daclizumab for acute GVHD, and rather it increased the risk of chronic GVHD and decreased the risk of disease recurrence (120). In the treatment setting, daclizumab has shown efficacy for a part of patients with steroid-refractory acute GVHD in the initial phase 2 trial (121), but the subsequent randomized control study showed a significantly worse survival rate in patients receiving corticosteroid with daclizumab than corticosteroid with placebo (122).

Denileukin diftitox is a genetically engineered protein composed of human IL-2 fused to diphtheria toxin and has cytotoxicity against activated T cells based on preferential binding to the high-affinity IL-2 receptor. A phase 1 study was conducted to evaluate the safety and efficacy of denileukin diftitox for 32 patients with SR-acute GVHD. Overall, 71% of evaluable patients achieved an overall response, including 33% of complete remission and 38% partial response (123). However, animal studies suggested that it could affect Treg homeostasis, and a case study reported that fatal hyperacute GVHD following denileukin diftitox treatment (124–126)

These inconsistent results may be attributed that anti-IL-2 antibody could affect the homeostasis of both Tregs and activated Tcons those expressing the IL-2 receptor. It suggests that anti-IL-2 antibody influence the patient's immunity differently based on the balance between Tregs and activated Tcons in each patient basis, and the possible duality of the effect should be taken into account when these agents are used for patients after HSCT.

Tyrosine Kinase Inhibitors

Standard GVHD prophylaxis in recipients with HLA-matched identical donor consists of the combination of calcineurin inhibitor (CNI) and short-term methotrexate. CNI reduced IL-2 transcription and activation of effector T cells and is a critical agent to regulate post-transplant immune reaction but have been shown to be disadvantageous in maintaining Tregs reconstitution because CNI inhibits the production of IL-2 from activated Tcons (127). Recently, novel immune modulating agents targeting Janus kinase (JAK), Bruton's tyrosine kinase (BTK), or Interleukin-2 inducible tyrosine kinase (ITK) have been emerged.

Ruxolitinib (RUX) is a selective JAK1/2 inhibitor, and RUX reduced the GVHD histology score and prolonged survival in a murine GVHD model. RUX treatment suppressed the signals of inflammatory cytokines while sparing the IL-2–JAK3–STAT5 signal, which could lead to reduced $CD4^{+}IFN\text{-}\gamma^{+}$ cells and an increase of Tregs (128). In the human clinical setting, a phase 3 randomized trial compared the efficacy and safety of RUX with the investigator's choice. The overall response was significantly higher in the RUX group, and RUX treatment significantly

increased the median overall survival compared to control treatment (129).

Regarding BTK and ITK pathways, Ibrutinib, a BTK/ITK inhibitor, reduced the severity of chronic GVHD using two established chronic GVHD mice models (130). A phase 1b/2 study showed the safety and efficacy of ibrutinib treatment for patients with SR-cGVHD (131). While the significance of BTK inhibition on Treg homeostasis remains unknown, some studies showed that ITK inhibition might increase Tregs in murine models (132). Mammadli et al. reported that ITK-deficient donor T cells significantly reduced inflammatory cytokines leading to less GVHD intensity in the mice model (133). We recently demonstrated that pharmacological inhibition of ITK on donor T cells could ameliorate acute GVHD without sacrificing the GVT effect (134). In the study, we showed that ex-vivo graft manipulation with ITK inhibitor modulated donor $CD4^+$ T cell differentiation towards Th1, Th2, and Th17 with sparing Tregs, resulting in the prolonged overall survival after HSCT.

Adoptive Transfer of Tregs

In addition to the pharmacological *in vivo* modulation of Treg homeostasis described above, the GVHD-preventive effect of adoptive transferred donor-type Tregs has been evaluated in murine models and clinical studies. The adoptive transfer of Tregs enables to increase in the Treg pool at least transiently, and it may provide merit especially in patients having insufficient thymic recovery. The initial experimental study demonstrated that the adoptive transfer of Tregs to the graft suppressed the expansion of alloreactive donor T cells without impairing the GVT effect (18). In a human acute GVHD prophylaxis setting, a first in human clinical trial evaluated ex-vivo expanded umbilical cord blood (UCB)-derived Tregs for patients receiving nonmyeloablative double UCB transplantation (135). Tregs were isolated and cultured with anti-CD3/anti-CD28 monoclonal antibody-coated beads supported by IL-2. A total of 1-30 x 10^5/kg UCB-derived Tregs infused on day+1 for all participants and day+15 for an additional cohort. GVHD prophylaxis consisted of cyclosporine and mycophenolate mofetil (MMF), but later changed to sirolimus and MMF due to potential interference with Treg function and survival by cyclosporine. Grade II to IV acute GVHD and the incidence of chronic GVHD were lower than the historical control. The subsequent study from the same group demonstrated the safety and efficacy of UCB-derived Treg expanded with K562 cells. This novel approach enabled the expansion of Tregs to up to 30 times higher than the previous method. In this study, the rate of grade II to IV acute GVHD was lower than the control without increasing the infection and relapse rates (136). The Perugia group infused Tregs followed by stem cell and Tcons infusion without immunosuppression therapy after transplantation. They conducted a phase 2 study evaluating the effect of isolated donor Tregs (1 x 10^6/kg) on day-4, followed by a purified $CD34^+$ and Tcons (1 x 10^6/kg) on day0 without posttransplant immunosuppression for patients receiving HLA-haploidentical transplantation (137). In this setting, purified Tregs and Tcons were harvested from the same donor as the stem cell harvest and not cultured ex-vivo. The incidence of grade II to IV acute GVHD was 15%, and this rate of acute GVHD was similar to the historical controls. Surprisingly, although the high-risk patients' background, the relapse rate was significantly lower than the historical control group, suggesting that adoptive transfer of Tregs followed by Tcons and $CD34^+$ stem cells as acute GVHD prophylaxis strategy suppress GVHD without abrogating the GVT effect in a human setting. Recently, a subsequent study involving fifty HLA-haploidentical transplant recipients with Treg adoptive transfer showed excellent results (138). Fifteen patients developed grade II to IV acute GVHD, including 12 grade III to IV, and all recipients were treated with corticosteroids. Only 5 patients were refractory to first-line steroid therapy, and 3 of them recovered after the second-line treatment. Of note, only 2 patients relapsed, and the moderate/severe chronic GVHD/relapse-free survival was 75%.

On the contrary, the data of adoptive transfer of Tregs in the treatment setting of active ongoing acute GVHD treatment was limited. Two case series described the use of ex-vivo expanded Treg infusion for the treatment of acute GVHD and chronic GVHD (139, 140). These studies demonstrated the feasibility of the ex-vivo Treg expansion and infusion, but the effect of this approach should be evaluated by larger clinical trials.

To maintain transferred Tregs *in vivo*, low-dose IL-2 administration after adoptive transfer of Tregs is theoretically attractive. In a phase 1 study, 24 SR-cGVHD patients received freshly isolated Tregs from the original stem cell donor followed by IL-2 (1 x 10^6 U/m^2/day) treatment for 8 weeks. The response rate was 33%, and TCRβ diversity in Tregs is normalized with therapy (141). Larger clinical trials are warranted to confirm the efficacy of this combination therapy in patients with SR-cGVHD.

EMERGING PROBLEMS AND FUTURE PERSPECTIVES

Ever since the discovery, Tregs have been the focus of many studies owing to the efficient immunosuppressive function. In the allogeneic HSCT, as Tregs have a central role in the regulation of post-transplant immunity, the dynamics of Tregs have been extensively studied in murine models and clinical samples. As described in this review, Tregs originally have different characteristics in homeostasis from conventional T cell subsets, and understanding the unique homeostasis makes it possible to give Tregs selective effects *in vivo*. However, there remain many unresolved questions about Treg homeostasis in patients.

First, one of the critical problems is the localization of Tregs in a human clinical setting. Previous studies suggest that the phenotype and function of Tregs are different depending on the localization, such as lymph nodes, target tissues, and tumor micro-environments. For example, in patients with gastric cancer, Treg phenotype is different between tumor infiltrating Tregs and peripheral circulating Tregs (68). Most of human GVHD clinical studies depended on the circulating Tregs, hence, an evaluation of Treg localization and the function in each specific site would be helpful for our further understanding of Treg homeostasis in the context of GVHD.

Second, the impact of Tregs on the GVT effect has not been well characterized in human clinical transplant (142). To further elucidate the Treg effect on the GVT, further research is needed to develop clinically-relevant murine models to study anti-tumor immunity after allogeneic HSCT.

Third, the effect of novel immunotherapies on the Tregs has not been well studied. Recent developments of immunotherapies such as chimeric antigen receptor T cell (CAR-T), bispecific T-cell engagers (BiTE), and ICIs have provided significant progress for treatment. Despite improvement in the response rate, patients with refractory diseases still often require post-remission therapy with HSCT. The effects of a previous history of CAR-T or BiTE on subsequent allogeneic HSCT are largely unknown. In particular, it is important to study the effect of pretransplant novel immunotherapies on donor-derived immune cell recovery, including Tregs, after allogeneic HSCT.

Advances in the understanding of Treg homeostasis in patients after HSCT may contribute to the treatment for autoimmune diseases and solid cancers. In solid cancers, Tregs negatively influence tumor control; therefore, contrary to GVHD, reducing Tregs and activating effector T cells may promote the anti-tumor effect. Based on our previous reports regarding the role of PD-1 on Tregs after low-dose IL-2 and HSCT (76, 86), the combination treatment IL-2 and PD-1 blockade may activate effector T cell and promote Treg apoptosis in the inflammatory environment, like a tumor microenvironment.

Novel experimental methodologies may enable us to overcome the remaining issues shown here. In fact, the single-cell technique made detailed characterization of cells and their microenvironment, and to answer questions that could not be addressed by the conventional technique (143). In the context of acute GVHD, scRNA-seq has identified a novel regulator of T cell alloimmunity (144). Previous research has investigated the role of long noncoding RNA after HSCT using scRNA-seq and identified *Linc00402* as a regulator of allogeneic T cell function. In addition, the TCR sequence method provides information on the TCR diversity and clonality of these populations. Since each T cell possesses its own unique TCR, TCR-seq allows for sensitive tracking of T cells at the clonal level (145). These novel approaches may solve the limitations of previous studies by conventional approach and provide a bridge to new findings in Treg research.

AUTHOR CONTRIBUTIONS

SI wrote the paper. K-iM designed and edited the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Murine Models Provide New Insights Into Pathogenesis of Chronic Graft-*Versus*-Host Disease in Humans

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Allogeneic hematopoietic cell transplantation (allo-HCT) is a curative therapy for hematologic malignancies, but its success is complicated by graft-*versus*-host disease (GVHD). GVHD can be divided into acute and chronic types. Acute GVHD represents an acute alloimmune inflammatory response initiated by donor T cells that recognize recipient alloantigens. Chronic GVHD has a more complex pathophysiology involving donor-derived T cells that recognize recipient-specific antigens, donor-specific antigens, and antigens shared by the recipient and donor. Antibodies produced by donor B cells contribute to the pathogenesis of chronic GVHD but not acute GVHD. Acute GVHD can often be effectively controlled by treatment with corticosteroids or other immunosuppressant for a period of weeks, but successful control of chronic GVHD requires much longer treatment. Therefore, chronic GVHD remains the major cause of long-term morbidity and mortality after allo-HCT. Murine models of allo-HCT have made great contributions to our understanding pathogenesis of acute and chronic GVHD. In this review, we summarize new mechanistic findings from murine models of chronic GVHD, and we discuss the relevance of these insights to chronic GVHD pathogenesis in humans and their potential impact on clinical prevention and treatment.

Keywords: hematopoietic cell transplantation, chronic graft-*versus*-host disease, mouse models, tissue resident memory T cell, B cell

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) offers a way to eliminate residual malignant cells and prevent relapse by taking advantage of the graft-*versus*-leukemia/lymphoma (GVL) activity of alloreactive donor T cells (1–6). However, the same alloreactive T cells also mediate graft-*versus*-host disease (GVHD) (7–9). Acute GVHD is an acute alloimmune inflammatory response characterized by infiltration of donor T cells that cause apoptosis and necroptosis of epithelial cells in GVHD target tissues (10–12). Chronic GVHD is an autoimmune-like chronic inflammation variably characterized with lymphopenia, IgG autoantibodies in the serum (13), moderate donor cell infiltration, and fibrosis in certain target tissues (14). Chronic GVHD often occurs as a sequel of acute GVHD, although chronic

GVHD can occur in the absence of overt acute GVHD (15). In humans, acute and chronic GVHD can both involve the skin, liver, and gut, whereas prototypical target organs affected by chronic GVHD include salivary and lacrimal glands, oral mucosa, subcutaneous connective tissue and adipose tissue, lung, genital tract, and esophagus (15–18). Clinical manifestations of chronic GVHD typically begin between 2 and 12 months after allo-HCT (15, 19). In one retrospective study, 75% of the patients diagnosed with chronic GVHD had prior acute GVHD, and in 10% of the patients, acute GVHD evolved directly into chronic GVHD (20).

Studies of GVHD pathogenesis in humans are limited by the inaccessibility of target organ tissues other than the skin. Therefore, preclinical animal models represent important tools for elucidating the pathogenic processes leading to acute and chronic GVHD (21, 22). Murine models of allo-HCT have become the most important animal models for the GVHD mechanistic pathogenic studies, owing to the availability of genetically modified strains (21, 22), although work with canine and nonhuman primate models has produced important contributions (23, 24). Murine models of allo-HCT have demonstrated the role of recipient mismatching for major and minor histocompatibility antigens in triggering acute GVHD. These models have also elucidated the role of T-cell subsets and cytokines in acute GVHD pathogenesis (25–36). As one example, observations from murine models that NKT cells specific for nonpolymorphic CD1d suppressed acute GVHD (37) and preservation of NKT cells by conditioning regimens consisting of total lymphoid irradiation (TLI) and antithymocytes cell globulin (ATG) prevented GVHD while preserving GVL activity (38, 39) have been successfully translated into clinical application in humans (40, 41). Similarly, observations from murine models that removal of naïve T cells can ameliorate GVHD while preserving GVL activity (42, 43) have also been successfully translated into clinical application in humans (44).

Modeling chronic GVHD appeared to be more complicated, but murine models of chronic GVHD have evolved and improved during the past three decades. It was initially thought that murine models of autoimmune-like chronic GVHD required specific donor-recipient combinations that differ from those used to study acute GVHD (21, 22, 45). In this review, we will describe how we have used identical allogeneic donor and recipient strain combinations to induce acute GVHD mediated by alloreactive T cells and to induce autoimmune-like chronic GVHD. In these models, acute and chronic GVHD can occur sequentially in murine recipients (46), similar to what most often occurs in humans (15). These murine models also reflect the characteristic features of autoimmune-like chronic GVHD in humans (46). We will also summarize new insights into chronic GVHD pathogenesis through the murine models.

A MURINE MODEL CAN REFLECT CHARACTERISTIC FEATURES OF CHRONIC GVHD IN PATIENTS

We recently found that induction of acute and chronic GVHD does not require different donor and host combination (46). With the commonly used acute GVHD model of C57BL/6 donor to MHC-mismatched BALB/c recipient, acute GVHD recipients develop into chronic GVHD as long as they survive for up to 60 days after allo-HCT (46). The induction of both acute and chronic GVHD can be achieved by adjusting donor T-cell numbers in the graft, and chronic GVHD in the absence of acute GVHD can be induced by injection of purified donor $CD8^+$ T cells alone with T-cell–depleted bone marrow cells (46). Recipients with chronic GVHD induced by whole splenic T cells or by sorted donor $CD8^+$ T cells both have lymphopenia, damage in the thymus, serum autoantibodies, and damage in small intestine, liver, lung, skin, and salivary and lacrimal glands, together with collagen deposition and fibrosis in target organ tissues (46, 47). The recipients clearly showed lymphocytic bronchiolitis and interstitial collagen deposition in the lung (46, 47), although bronchial obstruction (BO) observed in a murine model conditioned with TBI plus cyclophosphamide (CY) (48) was not observed in our models. BO in murine model of chronic GVHD may require special conditioning. In addition, as summarized in **Table 1**, chronic GVHD can be induced with low-dose splenic T cells in other MHC-mismatched or MHC-matched donor–recipient combinations, including MHC-mismatched C57BL/6 ($H\text{-}2^b$) donor to B10BR ($H\text{-}2^k$) recipient and MHC-matched LP/J ($H\text{-}2^b$) donor to C57BL/6 ($H\text{-}2^b$) recipient and DBA/2 ($H\text{-}2^d$) or B10D2 ($H\text{-}2^d$) donor to BALB/c ($H\text{-}2^d$) recipient models (46, 49–55). Chronic GVHD with little acute GVHD can also be induced by naïve $CD8^+$ T cells from C3H.SW ($H\text{-}2D^b$, CD45.2) donor to MHC-matched B6/SJL ($H\text{-}2D^b$, CD45.1) recipient (46, 56) or from C57BL/6 donor to MHC-mismatched BALB/c recipient models (46).

Many characteristic features of acute and chronic GVHD in humans can be reflected by any murine model, although no single murine model captures the entire spectrum of abnormalities observed in humans, just as no single patient can represent the full spectrum of abnormalities that can be caused by the disease. Establishing murine models of acute and chronic GVHD does not require any specific donor and recipient combination. The key is to adjust the number of donor T cells in the graft to allow the recipients to survive acute phase, such that inflammation in acute GVHD can evolve into the myriad features unique to chronic GVHD.

AUTOREACTIVE PATHOGENIC $CD4^+$ T CELLS IN CHRONIC GVHD ARE DERIVED FROM BOTH MATURE $CD4^+$ T CELLS IN THE GRAFT AND *DE NOVO*-GENERATED $CD4^+$ T CELLS IN THE THYMUS

The diversity of TCRs are randomly generated by VDJ recombination during positive selection in the thymus, and autoreactive T cells in healthy thymus are depleted by negative selection in the thymic medullary mediated by medullary epithelial cells (mTECs) and dendritic cells (DCs). The mTECs express tissue-restricted antigens (TRA) in AIRE- or Fezf2-dependent manner (57–59). The $CD11c^+$ DCs in the thymic medullary include $CD11c^+B220^+PDCA\text{-}1^+$ plasmacytoid DCs

TABLE 1 | Summary of murine models of cGVHD.

Donor strain	Recipient strain	Conditioning regiment	Genetics	Main cell type contributing to phenotype	Cell type and dose	Outcome	Reference
C57BL/6 (H-2^b)	BALB/c (H-2^d)	850 cGy	Mismatched for MHCI, MHCII, and miHAs	$CD4^+$, $CD8^+$ T, and B cells	2.5×10^6 T-cell–depleted (TCD) BM cells and $0.5–1.25 \times 10^6$ unfractionated spleen cells or 0.5×10^6 $CD4^+$ or $0.5–5 \times 10^6$ $CD8^+$ T cells	Systemic disease including (1) damages in the acute and chronic GVHD overlapping targets such as thymus, gut, liver, lung, and skin, as well as chronic GVHD prototypical targets salivary and lacrimal glands; (2) increased serum autoantibodies and tissue antibody deposition; (3) collagen deposition and fibrosis in target organ tissues.	Wu et al. (46) and Kong et al. (47)
C57BL/6 (H-2^b)	B10BR (H-2^k)	Cyclophosphamide-treated (120 mg/kg/day, days –3 and –2), irradiated (8.3 Gy by radiograph, day –1)	Mismatched for MHCI, MHCII, and miHAs	$CD4^+$ and $CD8^+$ T	TCD-BM and 0.75×10^5 purified splenic T cells	Fibrosis with bronchiolitis obliterans	Katelyn Paz et al. (49)
LP/J (H-2^b)	C57BL/6 (H-2^b)	900–1,100 cGy	MHC-matched and miHA-mismatched	$CD4^+$ and $CD8^+$ T	Whole spleen (10×10^6) and TCD-BM (2.5×10^6)	Skin scleroderma	Deng et al. (50), Hamilton and Parkman (51), and DeClerck et al. (52)
DBA/2 (H-2^d)	BALB/c (H-2^d)	650 cGy	MHC-matched and miHA-mismatched	$CD4^+$ T and B cells	$2.5–10 \times 10^7$ whole spleen cells	Autoantibodies; skin scleroderma; kidney damage	Zhang et al. (53) and Zhao et al. (54)
B10D2 (H-2^d)	BALB/c (H-2^d)	850 cGy	MHC-matched and miHA-mismatched	$CD4^+$ and $CD8^+$ T	Whole spleen (10×10^6) and TCD-BM (2.5×10^6)	Skin scleroderma? Systemic disease?	Deng et al. (50), Korngold and Sprent (27), and Eyrich et al. (55)
C3H.SW (H-$2D^b$, CD45.2)	C57BL/6SJL (B6/SJL, H-$2D^b$, CD45.1)	1,000 cGy	MHC-matched and miHA-mismatched	Naïve $CD8^+$ T	TCD-BM (5×10^6) and $CD44^{low}CD8^+$ T cells (2×10^6)	Systemic disease including thymus, skin, liver, and gastrointestinal tract damage.	Zhang et al. (56)

(pDCs), $CD8^+SIRP\alpha^-$ thymus-resident DCs (tDCs), and $CD8^-SIRP\alpha^+$ migratory DCs (mDCs) (60–62). TRA from mTECs can be picked up by thymic DCs, and TRA from periphery tissues can be brought into the thymus by mDCs (59). pDCs and tDCs augment thymic negative selection with limited impact on Treg generation; in contrast, mDCs augment both negative selection and tTreg generation in the thymus (60–63). The mTEC- and DC-mediated negative selection deletes most of the autoreactive thymocytes; however, the deletion is not 100%, and a small portion of the autoreactive T cells is exported to the periphery (64). The residual autoreactive T cells in the periphery of healthy individuals are well regulated and controlled by peripheral tolerance mechanisms consisting of regulatory T cells and tolerogenic DCs (65).

Residual autoreactive T cells in the graft from healthy donors are expanded after allo-HCT due to breakdown of tolerance mechanisms. In murine models, as depicted in **Figure 1**, early after allo-HCT, donor T cells including the residual autoreactive T cells in the graft are activated by host-type APCs and differentiate into Th/Tc1 cells, and they infiltrate GVHD target tissues including gut, liver, lung, skin, thymus, and bone marrow to mediate acute GVHD. Autoreactive $CD4^+$ T cells express promiscuous TCRs that cross-react with both self-MHC-antigen complex and allo-MHC-antigen complex (66). Since autoimmune-like chronic GVHD can be induced in thymectomized and athymic recipients (53), the autoreactive $CD4^+$ T cells in those recipients are most likely derived from the residual autoreactive $CD4^+$ T cells in the graft that expanded during alloimmune responses (53, 54). The autoreactive $CD4^+$ T cells recognize both donor antigen-MHC complex and host antigen-MHC complex, such that they first act as alloreactive T cells and are activated by host-type APCs, and then they act as autoreactive T cells and are expanded by donor-type APCs, particularly by the activated donor-type B cells presenting donor- or host-type antigens (67).

The autoreactive $CD4^+$ T cells in chronic GVHD recipients are also derived from *de novo*-generated $CD4^+$ T cells from GVHD-damaged thymus (46). The thymus of allo-HCT recipients can be damaged by condition regimen and GVHD. The thymus damage by conditioning regimen alone can recover in an IL-22-dependent manner (68). Alloreactive $CD4^+$ T and $CD8^+$ T cells mediate damage of mTECs that mediate negative selection of autoreactive T cells (34, 46, 69). Although donor-type DCs augment negative selection of autoreactive antidonor and antihost T cells in non-GVHD recipients with mixed or complete chimerism (70, 71), donor-type DCs no longer augment negative selection of the autoreactive T cells in GVHD recipients due to loss or dysfunction of donor-type DCs (46, 72). Therefore, damage of thymus, especially by GVHD leads to an increased generation of autoreactive T cells.

Autoreactive $CD4^+$ T cells in chronic GVHD recipients include those derived from the mature T cells in the graft or those from *de novo*-generation in the damaged thymus. In the recipients with overt acute and chronic GVHD, majority of pathogenic $CD4^+$ T cells are from donor-type $CD4^+$ T cells from the graft (46, 47). This may result from rapid destruction of thymus by acute GVHD that ends the thymic production. However, in the recipients transplanted with sorted $CD8^+$ T cells and that developed little acute GVHD, *de novo*-generated donor-type $CD4^+$ T cells are required for induction of chronic GVHD (46). The autoreactive $CD4^+$ T cells from both

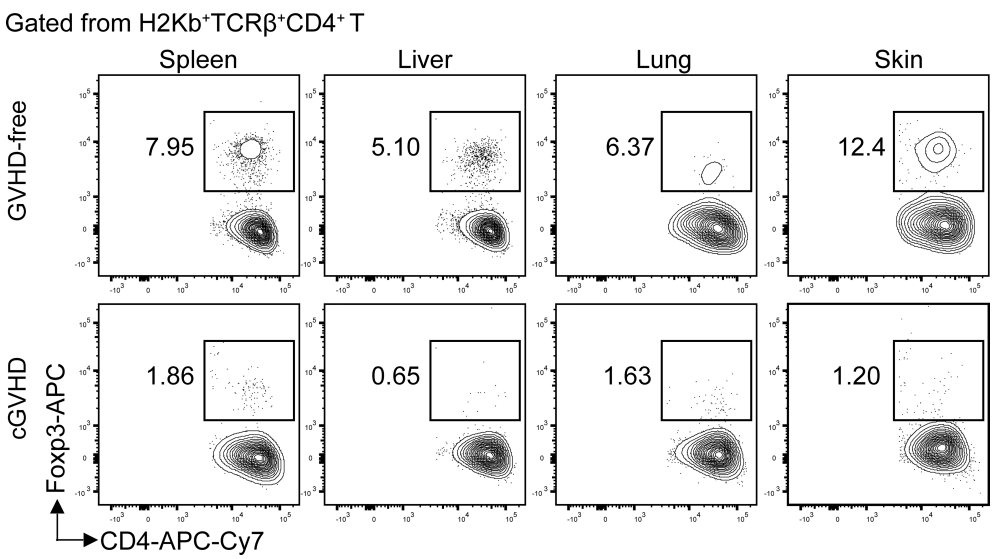

FIGURE 1 | Loss of $Foxp3^+CD4^+$ Treg cells in the target tissues of chronic GVHD recipients. Lethal TBI-conditioned BALB/c recipients were given T-cell–depleted bone marrow cells (TCD-BM, 2.5×10^6) only from C57BL/6 donors as GVHD-free control or given TCD-BM plus spleen cells (1×10^6) for induction of chronic GVHD. Sixty days after HCT, the spleen, liver, lung, and skin tissue mononuclear cells were stained with anti-H-$2K^b$, TCRb, CD4, and FoxP3. The gated donor-type H-$2K^{b+}CD4^+TCR\beta^+$ T cells are shown in CD4 *versus* Foxp3. The $FoxP3^+$ Treg cells are boxed, and the percentage of the Treg cells among the $CD4^+$ T cells is shown beside the box. One representative is shown of four recipients in each group.

sources recognize donor antigen-MHC complex and host antigen-MHC complex, and they interact with autoreactive B cells to produce autoantibodies that further damaged the thymus and causes lymphopenia in chronic GVHD recipients (46, 50, 67, 73). Therefore, the autoreactive $CD4^+$ T cells derived from the preexisting autoreactive $CD4^+$ T cells in the graft play a major role in mediating chronic GVHD pathogenesis in recipients with overt acute and chronic GVHD, and the *de novo*-generated autoreactive $CD4^+$ T cells from damaged thymus play a major role in chronic GVHD pathogenesis in recipients with little prior acute GVHD.

CHRONIC GVHD PATHOGENESIS DOES NOT REQUIRE GERMINAL CENTERS AND ITS ONSET IS ASSOCIATED WITH DESTRUCTION OF LYMPHO-FOLLICLES AND GERMINAL CENTERS

Patients with active chronic GVHD have marked reduction of $PD\text{-}1^{hi}CXCR5^+CD4^+$ follicular T helper cells (Tfh) among peripheral blood mononuclear cells (PBMC), but high serum concentrations of IgG autoantibodies and CXCL13, the ligand of CXCR5 (13, 74), suggesting intense T helper activity for B cells. The results were interpreted to indicate that Tfh had been recruited into germinal centers of lymphoid follicles in secondary lymphoid organs, consistent with previous preclinical studies showing that chronic GVHD onset was associated with enlarged germinal centers in some murine models of chronic GVHD (74, 75). However, this interpretation conflict with observations that patients with chronic GVHD usually have lymphopenia (76–79), and that somatic hypermutation (SHM) in the memory B cells is low at 1 year after HCT (80, 81).

SHM takes place in the B cells during differentiation in the GCs (82–84). With variety murine models, we have demonstrated that chronic GVHD onset is associated with destruction of lymphoid follicles and GCs in the spleen. In addition, we showed that GC formation is not required for induction of chronic GVHD, because recipients with an absence of BCL6 in donor B cells that could not form GCs nonetheless developed chronic GVHD (50, 73). Recipients with overt chronic GVHD had no detectable GCs, Tfh cells, or GC B cells, although recipients with mild chronic GVHD had remnants of GCs, residual Tfh, and GC B cells (47, 50, 73).

EXTRAFOLLICULAR $PSGL1^{lo}CD4^+$ T AND B CELL INTERACTIONS AUGMENT AUTOIMMUNE DEVELOPMENT AND CHRONIC GVHD PATHOGENESIS

P-selectin glycoprotein ligand 1 (PSGL1, also known as CD162) is an adhesion and coinhibitory receptor; PSGL1 are widely expressed in almost all T cells in the blood and binds to E-selectin and P-selectin (85, 86). A subset of activated $CD4^+$ T cells in the spleen of SLE mice downregulate expression of PSGL1 and become $CD44^{hi}CD62L^-PSGL1^{lo}CD4^+$ T ($PSGL1^{lo}CD4^+$ T) cells (87). $PSGL1^{lo}CD4^+$ T cells localize at the extrafollicular sites of systemic lupus mice and express high levels of CXCR4, ICOS, and CD40L without expression of CXCR5 (88). We served that chronic GVHD onset is associated with expansion of $PSGL1^{lo}CD4^+$ T helpers in the GVHD target tissues (47, 50). Extrafollicular $PSGL1^{lo}CD4^+$ T helpers for autoreactive B cells were first identified as $CD4^+$ T helpers in the spleen of systemic lupus mice (87). The differentiation of the $PSGL1^{lo}CD4^+$ T helpers in chronic GVHD recipients depends on the IL-6R-Stat3-BCL6 pathway, and Stat3 or BCL6 deficiency in donor $CD4^+$ T cells prevented expansion of the $PSGL1^{lo}CD4^+$ T cells in GVHD target tissues (47, 50). The $PSGL1^{lo}CD4^+$ T cell interaction with B cells results in autoantibody production and augmented thymus damage early after HCT (50). Prevention of $PSGL1^{lo}CD4^+$ T expansion by BCL6 or Stat3 deficiency and by blockade of ICOS or PD-1 interaction with ICOSL or PD-L2 on B cells markedly reduced serum concentrations of autoantibodies and decreased the severity of chronic GVHD (47, 50). In addition, we observed that chronic GVHD tissues had high levels of CXCL13 as measured with liver tissue homogenates, and $PSGL1^{lo}CD4^+$ T cells expressed high levels of CXCL13 mRNA (Kong, unpublished data). Taken together, these results suggest that the low number of Tfh cells in the PBMC of active chronic GVHD patients is unlikely due to redistribution of the Tfh cells into GCs in the lymphoid follicles, and it is more likely due to the destruction of GCs and lymphoid follicles. The high concentrations of CXCL13 and IgG autoantibodies in the serum of the patients may result from expansion of extrafollicular $CD4^+$ T and B cells in GVHD target tissues.

EXTRAFOLLICULAR $PSGL1^{lo}CD4^+$ T HELPER CELLS ARE TISSUE RESIDENT MEMORY T CELLS THAT INTERACT WITH MEMORY B CELLS IN THE GVHD TARGET TISSUES DURING CHRONIC GVHD PATHOGENESIS

As mentioned above, extrafollicular $PSGL1^{lo}CD4^+$ T cells were identified in the spleen of systemic lupus more than a decade ago (87), but their role in human systemic lupus pathogenesis remains unknown. We have recently found that $PSGL1^{lo}CD4^+$ T cells were not detectable in the peripheral blood of murine or human chronic GVHD recipients (47). Instead, they were $CD4^+$ tissue-resident memory T (Trm) cells with upregulated expression of CD69, CXCR6, P2RX7, and PD-1 and downregulated expression of Klf2, S1PR1, and CCR7 (47), consistent with Trm cell phenotype reported by others in infection and autoimmune colitis models (89). These observations explain why extrafollicular $PSGL1^{lo}CD4^+$ T cells are not detectable in the peripheral blood of mice or patients with chronic GVHD. This may also explain why their role

in the pathogenesis of systemic lupus has not been investigated in humans.

The PSGL1^{lo}CD4$^+$ Trm cells interact with memory B cells in the GVHD target tissues in murine recipients, humanized murine recipients, and in the liver of cGVHD patients (47). The humanized murine model was established by injection of HLA-A2$^-$DR4$^-$ human PBMC into MHC$^{-/-}$HLA-A2$^+$DR4$^+$ NSG mice (47). The PSGL1^{lo}CD4$^+$ T cells were juxtaposed to memory B cells in the liver of murine recipients, humanized murine recipients, and patients with chronic GVHD, as indicated by immunofluorescent and immunohistochemistry staining of the tissue-infiltrating cells (47). Sorted PSGL1^{lo}CD4$^+$ T cells from GVHD target tissues (liver and lung) of murine and humanized murine recipients augmented *in vitro* differentiation of syngeneic or autologous memory B cells but not naïve B cells into IgG-producing plasma cells in a manner that depended on PD-1/PD-L2 interaction and IL-21 (47).

On the other hand, the expansion of human memory B and plasma B cells in the GVHD target tissue liver and lung of humanized murine recipients was associated with expansion of PSGL1^{lo}CD4$^+$ T cells, but little B cell activation and expansion were observed in the MHC$^{-/-}$ control recipients (47). We also observed that PD-1 deficiency in donor T cells and PD-L2 deficiency in donor B cells were associated with reduction of serum anti-dsDNA, reduction of tissue IgG deposition, reduction of tissue fibrosis, and reduction of chronic GVHD severity (47). Finally, sorted PD-1$^{+/+}$ or PD-1$^{-/-}$ PSGL1^{lo}CD4$^+$ T and PSGL1^{hi}CD4$^+$ T cells from liver and lung GVHD target tissues were adoptively transferred into GVHD-free adoptive recipients with PD-L2$^{+/+}$ or PD-L2$^{-/-}$ B cells. The results showed that PSGL1^{lo} and PSGL1^{hi} CD4$^+$ memory T cells preferentially migrated back to the original GVHD target tissues in the adoptive recipients, but only PSGL1^{lo}CD4$^+$ T cells augmented expansion of plasma cells in the GVHD target tissues and increased serum concentration of total IgG and anti-dsDNA-IgG in a manner that required PD-1 interaction with PD-L2 (47). Taken together, these results show that PSGL1^{lo}CD4$^+$ Trm cell interaction with memory B cells in GVHD target tissues contributes to perpetuation of chronic GVHD pathogenesis.

EXTRAFOLLICULAR PSGL1^{lo}CD4$^+$ T HELPERS ARE DERIVED FROM PSGL1^{hi}CD4$^+$ T CELLS IN THE GRAFT IN AN IL-6-STAT3-BCL6-DEPENDENT MANNER

We observed that all peripheral blood CD4$^+$ T cells from healthy murine and human donors were PSGL1^{hi} (47). After transplantation into murine and humanized murine recipients, a portion (5%–20%) of PSGL1^{hi}CD4$^+$ T cells differentiated into PSGL1^{lo}CD4$^+$ Trm cells in an IL-6-Stat3-dependent manner (47) because Stat3 deficiency in the CD4$^+$ T cells and administration of blocking anti-IL-6R mAb markedly reduced the expansion of PSGL1^{lo}CD4$^+$ T cells in the GVHD target tissues of murine recipients (47). We have also observed expansion of *de novo*-generated PSGL1^{lo}CD4$^+$ T cells in chronic GVHD recipients induced by sorted donor CD8$^+$ T cells (Kong, unpublished data). These results indicate that PSGL1^{lo}CD4$^+$ T differentiation is similar to prefollicular CD4$^+$ T differentiation that is controlled by IL-6-Stat3-BCL6 pathways (50, 84).

CIRCULATING ANTIBODIES AUGMENT SCLERODERMATOUS CUTANEOUS CHRONIC GVHD

In humans, autoantibodies such as PDGF-1 have been associated with increased severity of cutaneous chronic GVHD (90). We found that high serum concentrations of autoantibody were associated increased IgG deposition and fibrosis in the skin tissues of murine and humanized murine recipients (47). Donor-derived IgG antibodies were required to perpetuate cutaneous chronic GVHD (73). Unexpectedly, we found no PSGL1^{lo}CD4$^+$ T or B cells in the skin tissues of murine or humanized murine recipients with chronic GVHD, although PSGL1^{lo}CD4$^+$ T and memory B cells were present in the liver and lung (47). Studies are in progress to determine whether B cells or PSGL1^{lo}CD4$^+$ T cells are present in the skin of patients with cutaneous chronic GVHD.

Taken together, the preclinical results indicate that circulating autoantibodies contribute to pathogenesis of cutaneous chronic GVHD. We also observed that circulating IgG antibodies augmented DC secretion of IL-23 and expansion of Th17 cells in the skin of chronic GVHD mice (73). MacDonald et al. showed that in an IL-17-dependent cutaneous chronic GVHD model, donor-type F4/80$^+$CSF-1R$^+$ type 2 macrophages augmented cutaneous chronic GVHD in a G-CSF but not GM-CSF-dependent manner, in which the macrophages mediate fibrosis *via* their production of TGF-β (91). Whether circulating IgG autoantibodies regulate the differentiation and expansion of type 2 macrophages during cutaneous GVHD remains to be studied.

LOSS OF FUNCTIONAL THYMIC DCS AND PERIPHERAL PD-L1^{hi} PLASMACYTOID DCS MAY CONTRIBUTE TO LOSS OF FOXP3$^+$CD4$^+$ TREG CELLS IN CHRONIC GVHD TARGET TISSUES

Chronic GVHD patients had markedly low percentages of Foxp3$^+$CD4$^+$ regulatory T (Treg) cells in the blood (92). Low-dose IL-2 preferentially expanded CD4$^+$ Treg cells by binding to high affinity IL-2Rα (CD25) and ameliorated clinical manifestation of chronic GVHD (93–95). Consistently, in a chronic GVHD model with DBA/2 donors and BALB/c recipients, loss of CD4$^+$ Treg cells was associated with chronic GVHD onset, and infusion of donor-type Treg cells prevented the disease onset or ameliorated the progression of chronic GVHD (96, 97). Importantly, we observed that percentages of Treg cells were high among CD4$^+$ T cells in the spleen, liver,

lung, and skin of healthy donor or GVHD-free recipients, but few Treg cells were found among $CD4^+$ T cells in the same tissues from mice with chronic GVHD (**Figure 1**).

The low number of $CD4^+$ Treg cells may result from reduced thymic Treg (tTreg) output, reduced differentiation of conventional $CD4^+$ T (Tcon) cells into peripheral Treg (pTreg) cells, and reduced Treg expansion and survival in the periphery. Thymic damage or engraftment with $MHCII^{-/-}$ donor DCs resulted in reduced generation of tTreg cells in GVHD recipients (98, 99), while engraftment of donor-type DCs increased donor- and host-type thymic tTreg generation in GVHD-free MHC-mismatched or haploidentical mixed chimeras (71, 100, 101). Plasmacytoid DCs that express high levels of PD-L1 (PD-$L1^{hi}$ pDCs) augment Tcon differentiation into pTreg cells in a PD-L1/PD-1 interaction-dependent manner (102–104). DC PD-L1 interaction with CD80 on Treg cells also augments Treg survival and expansion (96). GVHD in bone marrow reduced the production of PD-$L1^+$ pDCs, leading to reduced generation and expansion of Treg cells (105). Therefore, loss of functional DCs in the thymus and loss of bone marrow generation of PD-$L1^{hi}$ pDCs may contribute to the marked reduction of Treg cells in the chronic GVHD recipients, and prevention of thymus and bone marrow GVHD as well as restoration of bone marrow production of pDCs might reverse chronic GVHD.

CONCLUSIONS

In summary, with murine models of chronic GVHD, we have found that extrafollicular $CD4^+$ T and B interactions and $CD4^+$ Trm cells in the GVHD target tissues play critical roles in chronic GVHD pathogenesis, and these findings have been linked to chronic GVHD pathogenesis in humans through studies with humanized $MHC^{-/-}$HLA-$A2^+DR4^+$ NSG mice and patient GVHD target tissues (47). These studies have provided new insights into chronic GVHD pathogenesis in humans.

As depicted in the diagram (**Figure 2**), we propose how donor $CD4^+$ T cells mediate autoimmune-like chronic GVHD pathogenesis. Step 1, early after allo-HCT, in the lymphoid

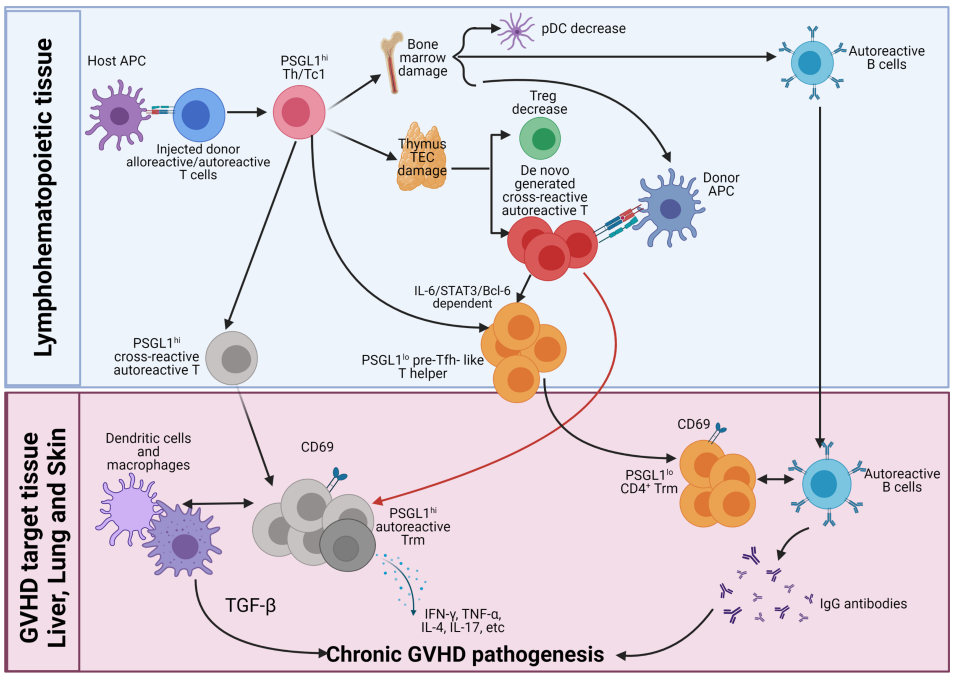

FIGURE 2 | Pathogenesis of chronic GVHD. Early after allo-HCT, donor-type $CD4^+$, and $CD8^+$ T cells including autoreactive $CD4^+$ T cells are activated by host APCs in the lymphoid tissues. The majority of the injected alloreactive T cells differentiate into PSGL1^{hi} Th1/Tc1 cells to cause acute GVHD. At the same time, some of the autoreactive $CD4^+$ T cells differentiate into PSGL$1^{lo}CD4^+$ pre-Tfh-like cells *via* IL-6-Stat3-BCL6 pathway, and they interact with activated donor B cells to augment antibody production, and some of them remain PSGL1^{hi}. The Th1/Tc1 cells infiltrate GVHD target tissues including thymus and bone marrow. Damage of thymic medullary epithelial cells (mTECs) leads to decreased generation of thymic Tregs (tTreg) cells and increased release of autoreactive T cells that are cross-reactive with donor antigen-MHC complex and host antigen-MHC complex. Damage of bone marrow microenvironment results in increased production of autoreactive B cells and reduced production of tolerogenic plasmacytoid dendritic cells (pDCs). Acute GVHD destroys lymphoid tissues. As acute GVHD subside into chronic GVHD, alloreactive pathogenic memory T, especially $CD4^+$ memory T cells, that can cross-react with donor APCs become autoreactive $CD4^+$ T cells and gather in the GVHD target tissue. The *de novo*-generated autoreactive $CD4^+$ T cells from damaged thymus also infiltrate the GVHD target tissues. The autoreactive $CD4^+$ T cells from both sources interact with donor-type APCs and become $CD69^+$ tissue resident memory T (Trm) cells in the tissues. The PSGL1^{hi} autoreactive Trm cells interact with DCs and macrophages to mediate pathogenesis *via* their production of cytokines such as TGF-β, IFN-γ, TNF-α, IL-4, IL-17, and IL-22. The pre-Tfh-like PSGL$1^{lo}CD4^+$ helper T cells interact with B cells to augment memory B-cell differentiation into plasma cells that produce IgG autoantibodies. IgG autoantibodies enter circulation and deposit in the GVHD target tissues such as skin to augment GVHD pathogenesis.

tissues, donor $CD4^+$ and $CD8^+$ T cells including cross-reactive residual autoreactive $CD4^+$ T cells in the graft act as alloreactive $CD4^+$ and $CD8^+$ T cells; they are activated by interaction with host-type APCs. The alloreactive T cells differentiate into $PSGL1^{hi}$ Th1/Tc1 cells and infiltrating GVHD target tissues. The small portion of autoreactive $CD4^+$ T cells differentiate into $PSGL1^{hi}CD4^+$ memory T cells and $PSGL1^{lo}CD4^+$ pre-Tfh-like cells *via* IL-6-Stat3-BCL6 pathway. The alloreactive Th1/Tc1 cells migrate into and cause damage in the thymus and bone marrow, among other GVHD target tissues such as the liver, lung, and skin. Those alloreactive Th1/Tc1 cells also destroy secondary lymphoid tissues as time goes on. Due to GVHD damage of thymic medullary epithelial cells and defective negative selection, the thymus increases production of autoreactive $CD4^+$ T cells that recognize both donor antigen-MHC complex and host antigen-MHC complex, as well as reduces production of $CD4^+$ tTreg cells. Those cross-reactive autoreactive $CD4^+$ T cells are activated in the periphery and infiltrate GVHD target tissues. The GVHD-damaged bone marrow has low production of tolerogenic PD-$L1^{hi}$ pDCs, defective negative selection of autoreactive B cells, and markedly reduced output of B cells and myeloid cells, leading to lymphopenia with relative expansion of autoreactive B cells in the periphery.

Step 2, the cross-reactive autoreactive $CD4^+$ T cells derived from the residual autoreactive $CD4^+$ T cells in the graft and from *de novo*-generation in the damaged thymus interact with donor-type DCs/macrophages or B cells, leading to their survival and expansion after acute GVHD subsides. The cross-reactive autoreactive $CD4^+$ T cells infiltrate GVHD target tissues and become $CD69^+$ Trm cells. The $PSGL1^{hi}$ Th1, Th2, and Th17 cross-reactive autoreactive Trm cells interact with DCs and macrophages to mediate chronic GVHD pathogenesis *via* their production of cytokines such as TGF-β, IFN-γ, TNF-α, IL-4, IL-17, and IL-22. The pre-Tfh-like autoreactive $PSGL1^{lo}CD4^+$ T cells become extrafollicular $PSGL1^{lo}CD4^+$ helper T cells in the GVHD target tissues (i.e., liver and lung). They attract and interact with autoreactive B cells in the tissues and become Trm cells. Their interaction with B cells augments memory B-cell differentiation into plasma cells that produce IgG autoantibodies that augment local inflammation and fibrosis or enter circulation. The circulating IgG antibodies deposit in the tissues such as skin to augment GVHD pathogenesis.

Finally, lack of tolerogenic pDCs and Treg cells allow the cross-reactive autoreactive $CD4^+$ Trm cells that recognize both donor antigen-MHC complex and host antigen-MHC complex to continuously interact with DCs, macrophages, and B cells to perpetuate chronic GVHD pathogenesis. Therefore, $PSGL1^{hi}$ and $PSGL1^{lo}$ $CD4^+$ Trm cells, macrophage, dendritic cells, B cells, and circulating IgG autoantibodies, all contribute to the pathogenesis of chronic GVHD, but $CD4^+$ Trm cells play the essential role. Targeting autoreactive $CD4^+$ Trm cells in the GVHD target tissues for treatment of chronic GVHD is under investigation.

We would like to point out that the proposed model of cGVHD pathogenesis is more relevant to chronic GVHD pathogenesis in recipients with obvious acute GVHD. However, in the clinic, some chronic GVHD patients did not have a clear phase of acute GVHD. The origin of the pathogenic T cells in those patients remains unclear. They may derive from *de novo* thymus-generated T cells because our murine model showed that sorted donor $CD8^+$ T cells induced thymus damage and chronic GVHD in the absence of acute GVHD (46). The roles of Tfh and extrafollicular $PSGL1^{lo}CD4^+$ T-cell interaction with B cells in the pathogenesis of chronic GVHD without obvious acute GVHD remain unclear and are under investigation.

AUTHOR CONTRIBUTIONS

QS, XK, and DZ wrote the review manuscript. PM critically reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Mouse Models of Antigen Presentation in Hematopoietic Stem Cell Transplantation

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Allogeneic stem cell transplantation (alloSCT) is a curative therapy for hematopoietic malignancies. The therapeutic effect relies on donor T cells and NK cells to recognize and eliminate malignant cells, known as the graft-versus-leukemia (GVL) effect. However, off target immune pathology, known as graft-versus-host disease (GVHD) remains a major complication of alloSCT that limits the broad application of this therapy. The presentation of recipient-origin alloantigen to donor T cells is the primary process initiating GVHD and GVL. Therefore, the understanding of spatial and temporal characteristics of alloantigen presentation is pivotal to attempts to separate beneficial GVL effects from detrimental GVHD. In this review, we discuss mouse models and the tools therein, that permit the quantification of alloantigen presentation after alloSCT.

Keywords: transplantation, antigen presentation, allogeneic stem cell transplantation, graft-*versus*-host disease, graft-*versus*-leukemia effects

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INTRODUCTION

Allogeneic stem cell transplantation (alloSCT) remains a curative therapy for a broad range of hematopoietic malignancies including acute myeloid leukemia (AML) and myelodysplastic syndrome. The therapeutic effect largely resides in graft-versus-leukemia (GVL) effects where graft-derived donor T cells and NK cells recognize allogeneic, hematopoietic or tumor-associated antigens. Unfortunately, this process is closely related to adverse immune effects, namely graft-versus-host disease (GVHD), where donor T cells attack normal recipient tissue. To date, the separation of beneficial GVL from detrimental GVHD remains the greatest unmet need in alloSCT.

The immunological pathways of both GVL and GVHD are initiated by the presentation of allogeneic antigens to donor T cells: Autologous or syngeneic (from an identical twin donor) transplants do not induce classical GVL or GVHD due to the lack of alloreactivity (1). AlloSCT using rigorous T cell-depletion (TCD) prevents severe GVHD but increases leukemia relapse (2–4), indicating that the recognition of alloantigens by the donor T cell is essential in disease pathophysiology. Therefore, studies to elucidate potential spatial and temporal differences in antigen presentation within GVL and GVHD have been undertaken (i.e. what types of antigen presenting cells (APC) and donor T cell subsets are involved, in which organs, and at what time after transplant?).

When choosing mouse models to study GVHD, at least three factors should be considered. Firstly, who are the cellular mediators of disease? As we previously reviewed (5, 6), both donor CD4 and CD8 T cells which recognize alloantigens presented by MHC class II (MHC-II) and class I (MHC-I) respectively, can mediate distinct patterns of GVL and GVHD. Host APC initiate both GVHD and GVL, in contrast, donor APC predominantly invoke GVHD but not GVL (7–9). To define the specific pathways of MHC-I *vs* MHC-II dependent T cell GVHD and the donor *vs*. host APC involved, alloantigen-specific T cell receptor (TCR) transgenic T cells are useful tools, particularly when combined with mutant mice bearing defined genetic defects in antigen presentation. Secondly, donor T cell responses can generally be initiated by one of two types of alloantigen presentation. One is the presentation of host minor histocompatibility antigens (miHAs) derived from polymorphic proteins which are present in recipients but not donors (10, 11). A miHA is presented by MHC which is shared by the donor and the host, and donor T cells recognize miHAs in the same way as pathogen-derived antigens are seen. This process includes a process known as epitope or antigen spreading whereby T cells recognize a family of antigens that have diversified from the original parental epitope (12). This explains why HLA-matched unrelated donors are at a higher risk for GVHD than HLA-matched sibling donors (13), since the frequency of genetic disparity for individual miHA is generally two-fold higher (10, 14). The other type of antigen recognition by the donor T cell is within the complex of mismatched MHC and a non-polymorphic peptide. Naive T cells have been educated in the thymus to react a peptide loaded on self MHC. Therefore, donor T cells are not designed to react to antigen presented by a mismatched MHC. However, a scenario can occur whereby the molecular complex of a peptide and mismatched MHC is structurally sufficiently similar to that of another peptide and matched MHC to activate a donor T cell. This type of donor T cell antigen recognition happens in MHC-mismatched transplantation and is known as molecular mimicry (15). This process explains why increased numbers of mismatched MHC loci (6/8, 7/8 *vs* 8/8 HLA match) significantly increases GVHD and decreases overall survival regardless of the underlying type of malignant disease (16). Thirdly, the pathophysiology and manifestations of GVHD seen after transplant should recapitulate those in clinical GVHD. The pathways of antigen presentation leading to GVHD are highly promiscuous in xenograft systems where immune deficient mice (and their APC) stimulate a human T cell response, such that their usefulness in studying the mechanisms of GVHD is somewhat limited.

Many non-transgenic donor and host combinations have been well established for the study of GVHD. These include MHC mismatched or MHC-matched but miHA-mismatched models which are typically dominated by MHC-I or II dependent GVHD that is largely strain dependent. We direct the reader to excellent reviews on the subject of these non-transgenic models (17). Here we focus on antigen-specific models of GVHD.

ALLOANTIGEN PRESENTATION IN MODELS OF GVHD TARGETING MINOR HISTOCOMPATIBILITY ANTIGENS

In MHC matched systems, the cognate recognition of antigen by a mature, thymically educated, donor T cell requires the TCR to interact with host polymorphic peptide (miHA) presented by a HLA molecule common to donor and host. Defined human miHAs have been the subject of recent reviews (10, 11, 14). While the most common molecular mechanism generating miHAs is single nucleotide polymorphisms (SNPs) within gene exons that modify peptide binding to MHC or TCR, other mechanisms such as altered protein transport/processing or transcription can also cause the generation of dramatically new epitopes (10, 11). The expression of ovalbumin (Ova) in BMT recipients under control of ubiquitous (e.g. β-actin) promoters may mimic the latter setting. Since ova is not expressed by normal mice, transgenic ova production by recipient mice can be a dominant antigen to $CD4^+$ and $CD8^+$ T cells (18, 19). When ova expression is limited to specific cell types such as hematopoietic cells (20, 21) or leukemic cells (22), it may mimic hematopoiesis-specific or leukemia-specific miHA. Given that these hematopoietic- or leukemia-restricted miHAs have attracted attention as targets for clinical TCR transgenic T cell therapy (23, 24), these antigen model systems can be useful in understanding immunity within these contexts. In regard to ova, extensive tool reagents are available. Ova peptide-specific $CD4^+$ and $CD8^+$ TCR transgenic mice (OT-II, DO11.10 and OT-I mice) can be utilized as a source of donor T cells, whereby short term T cell activation and expansion can be used to quantify antigen presentation (18, 19, 25). Ova peptide-MHC tetramers can be utilized to detect peptide-specific T cells within polyclonal T cells (26) and the monoclonal antibody (25-D1.16) can also quantify ova-peptide loaded within MHC -I to quantify direct antigen presentation (27). Many foreign peptide/proteins other than ova, such as virus-derived proteins, can also be exploited in a similar fashion (**Table 1**).

Similarly, strain-specific models of endogenous antigen also exist. H60 protein, a ligand for NKG2D, is expressed by hematopoietic cells but not parenchyma cells in a strain-specific manner. H60 is expressed by BALB.B mice but not C57Bl6 (B6) nor C3H.SW mice, and these three stains are all MHC-matched ($H\text{-}2^b$) but miHA disparate. Recently, H60 transduction has been undertaken into B6-background leukemia or recipient mice and H60 peptide-$H2K^b$ tetramers used to detect responding $CD8^+$ T cells in combination with MHC-I deficient mice ($H\text{-}2K^{b\text{-}/\text{-}}$) to demonstrate how defects in leukemia antigen presentation promote exhaustion of donor T cells and ineffective GVL (42) (**Table 1**).

HLA molecules are highly polymorphic (43). Since HLA-mismatched transplants (including haplo-identical transplantation) are all semi HLA-matched, mismatched HLA-derived peptides can be presented by another shared HLA. To mimic this scenario, TEa transgenic TCR (Vα2/Vβ6) T cells and YAe antibody recognize the same complex of mismatched MHC-derived peptide presented within MHC-II (Eα52-68 peptide and

TABLE 1 | Minor antigens within MHC matched systems and antigen-specific TCR transgenic T cells (top), peptide-MHC tetramer to detect antigen-specific T cells (middle) and antibodies to quantify antigen-MHC complexes (bottom).

Ag protein	Ag peptide	MHC-restriction	TCR-Transgenic mouse	Reference
Ova albumin	OVA257-264 (SIINFEKL)	H-2K^b	OT-I	(19)
H60	LTFNYRNL	H-2K^b	J15	(28, 29)
H-Y, *Uty* gene	WMHHNMDLI	H-2D^b	MataHari	(30)
H-Y	unknown	H-2D^b	HY-TCR	(31)
H-Y	unknown	I-A^b	Rachel	(32)
H-Y, *Dby* gene	NAGFNSNRANSSRSS	I-A^b	Marilyn	(33, 34)
I-E	Eα52-68	I-A^b	TEa	(35)
Ova albumin	OVA323-339	I-A^b	OT-II	(18)
Ova albumin	OVA323-339, 327-333, 328-338	I-A^d	DO11.10	(25)

Ag protein	Ag peptide	MHC-restriction	TCR Detection tetramer	Reference
H60	LTFNYRNL	H-2K^b	H6/H-2K^b tetramer	(36, 37)
H-Y, *Uty* gene	WMHHNMDLI	H-2D^b	HY-Uty/H-2D^b tetramer	(37)
Ova albumin	OVA323-339	I-A^b	OVA323-339/I-A^b tetramer	(38)
Ova albumin	OVA323-339	I-A^d	OVA323-339/I-A^d tetramer	(26)

Ag protein	Ag peptide	MHC-restriction	Antibody reactivity	Clone	Reference
Ova albumin	OVA257-264 (SIINFEKL)	H-2K^b	Against SIINFEKL bound to H-2K^b	25-D1.16	(27)
I-Ea chain	Eα52-68	I-A^b	Against Eα52-68 peptide bound to I-A^b	Y-Ae	(39, 40)
hen egg lysozyme (HEL)	HEL-derived peptide (HEL48-62)	I-A^k	Against HEL peptide (residue 48-62) bound to I-A^k	AW3.18	(41)

Superscript letters indicate MHC types.

I-A^b respectively) (35, 39, 40). B6 mice lack I-Eα chain, hence, do not express I-E, a MHC-II locus, whereas many other strains express the I-Eα chain. Thus the Eα52-68 peptide derived from the I-Eα chain of relevant recipient strains can stimulate TEa T cells and bind to the YAe antibody in an I-A^b-restricted manner. To our knowledge, YAe, the aforementioned 25-D1.16 and AW3.18 which binds to hen egg lysozyme (HEL) peptide loaded on I-A^k, are the only antibodies that bind to specific peptide-MHC complexes that are commercially available (**Table 1**). They are highly useful tools for the quantification of antigen presentation. Thus while antigen-specific T cell expansion detected by tetramer or as TCR transgenic T cell expansion reflects overall antigen presentation, these antibodies allow direct quantification of antigen presentation within individual APC subsets that are distinguishable by flow cytometry (e.g. donor cell *vs*. host cells, dendritic cell subsets *vs* macrophages, and within different organs) (44).

The transplantation of a female-derived graft into a male recipient is a known risk factor for GVHD (13). Multiple H-Y antigens encoded by Y-chromosome genes have been identified (e.g. SMCY, UTY, DBY, DEFRY) and reactive T cell clones have been isolated from female transplant recipients rejecting male grafts and male recipients transplanted with female grafts (45–48). Multiple murine TCR clones and TCR transgenic lines reactive to H-Y antigens (e.g. UTY, DBY) have been generated on a B6 background (**Table 1**) (30–34). In addition to their clear clinical relevance, these systems allow the use of male B6 mice from most transgenic and mutant strains (e.g. MHC-deficient recipients) to delineate mechanistic pathways of antigen presentation. As such, these systems provide powerful tools for the study of GVHD. The incorporation of reporter constructs such as luciferase into these TCR transgenic systems allows detailed and tissue specific compartmentalization of antigen presentation (20, 44, 49, 50).

ALLOANTIGEN PRESENTATION IN MODELS CHARACTERIZED BY MOLECULAR MIMICRY

It is well established in studies some 50 years ago that 1 - 10% peripheral T cells are reactive to non-self (mismatched) MHC, although the frequency of T cells that can respond to self (matched) MHC-expressing cell loaded with foreign Ag is likely at least 100-fold lower (51, 52). However, the mechanism underlying the high degree of clonal T cell alloreactivity to MHC-mismatched antigen has only recently been elucidated (53, 54). For decades there was a controversy over whether a T cell reacts to peptide-alloMHC (mismatched MHC) complexes in a peptide-centric or MHC-centric manner. In the former, TCR primarily interacts with the peptide rather than mismatched MHC, whereas the latter anticipates that a TCR primarily recognizes structural determinants on the (mismatched) MHC structure (15, 54, 55). The dispute has now been settled in favor of reactivity against the hybrid of peptide- and MHC-centric hypothesis. A TCR can thus recognize peptide-loaded allogeneic MHC 1) in docking modes disparate to those that are germline-encoded following thymic education (55) and 2) in the germline-encoded mode *via* molecular mimicry whereby the TCR binds to very similar structure formed by a foreign peptide presented on self-MHC and an endogenous peptide presented on allogeneic MHC (15). Both theories potentially explain allogeneic MHC reactivity. The former scenario of disparate docking modes has been demonstrated for 2C TCR (H-2K^b) T cells which react to a

self-peptide (dEV8, also known as Ndufa4$_{54–61}$) derived from enzyme NADH-ubiquinone oxidoreductase, loaded on H-2K^b (56, 57) and indeed this clone has been used to study positive selection in the thymus (57, 58). This scenario is of questionable relevance to transplant immunology where non-self-reactive mature T cells recognize MHC-mismatched cells. In the latter setting, what is striking is the demonstration that a TCR clone (LC13) recognizing Epstein-Barr virus (EBV)-derived peptide on self-MHC (HLA-B*0801) can recognize self-peptide on some allogeneic MHCs (HLA-B*4402 and B*4405, but not B*4403) due to a similar conformation (molecular mimicry) after TCR ligation (15). HLA-B*4402 or B*4405 transfected HLA class I-deficient (C1R) cell lines (C1R-B*4402 and C1R-B*4405) but not a B*0801 transfected one (C1R-B*0801) can activate LC13, indicating that endogenous antigens (e.g. ATP-binding cassette protein) can stimulate TCR clones when they are presented by some but not all allogeneic HLA molecules. Vice versa, an EBV-peptide can stimulate LC13 when it is presented by self-HLA (B*0801) but not allogeneic HLA (B*4405). Indeed, healthy individuals that are heterozygous for HLA-B*0801 and B*4402 do not possess this dominant LC13 TCR clonotype, demonstrating this clonotype has been clonally deleted due to potential self-reactiveness, and instead, they generate alternative clonotypes reactive to the same viral epitope (59). This suggests a phenomenon whereby one TCR clone reactive to a foreign peptide also responds to endogenous peptides presented by other MHC molecules within one individual. In the MHC-mismatched allogeneic transplant a donor TCR repertoire will encounter new MHC molecules, and be activated by host mismatched MHC molecules loaded with endogenous (non-polymorphic) peptides [a schematic illustration depicting the different modes of alloantigen presentation has been published previously (5)].

To study alloantigen presentation by a mismatched MHC molecule, MHC-mismatched models can be chosen [e.g. B6 (H-2^b) → BALB/c (H-2^d)]. MHC-partial mismatched or haplo-mismatched models include the possibility that matched MHC molecules present miHAs derived from mismatched MHC molecules. In this context, B6-background Bm1 (MHC-I mutation resulting in amino acid substitution) and Bm12 (MHC-II mutation resulting in amino acid substitution) mice are useful (**Table 2**). When either CD4$^+$ or CD8$^+$ T cells and BM cells from wild-type B6 were injected into lethally irradiated Bm1 and Bm12 recipients, donor CD4$^+$ T cells induced lethal GVHD in only Bm12 recipients, and donor CD8$^+$ T cells did so only in Bm1 recipients (67). When Bm12 T cells were transplanted in MHC-II deficient or wild-type B6 mice, serum IFN-γ was elevated in wild-type recipients but not in MHC-II deficient recipients (68). Similarly, *in-vitro* culture (mixed lymphocyte reaction) demonstrated that B6 CD4$^+$ T cells proliferate in response to Bm12 cells but not Bm1 or B6 (self) cells, and B6 CD8$^+$ T cells proliferate to Bm1 cells but not Bm12 or B6 cells (67). Despite the potential possibility that Bm1 and Bm12 mutation themselves serve as miHAs on conserved MHC-I or II molecules, this scenario would generate both CD4$^+$ and CD8$^+$ T cell responses, and so can be discounted. Instead, they suggest that both mutated MHC-I and II, H2-K^{bm1} and H2-Ab1^{bm12}, are loaded with endogenous peptides that bind B6 CD8$^+$ and CD4$^+$ TCR repertoires, respectively. There are other many similar MHC-I-mutated mice, most of which have mutation in the H-2K locus (e.g. bm3 and bm8) (69, 70), while MHC-II-mutated strains are limited to Bm12 (**Table 2**).

In contrast to studies utilizing specific mutations within MHC class I or II, TCR transgenic T cells which react to specific MHC disparities have also been exploited. In addition to the previously described 2C TCR transgenic CD8$^+$ T cells, 4C TCR transgenic CD4$^+$ T cells from B6 mice respond to an endogenous and ubiquitously expressed mouse non-polymorphic peptide presented on I-A^d (66).

ANTIGEN PRESENTATION IN XENOGRAFT TRANSPLANT MODELS

There has been a controversy in regard to how faithfully inbred murine allogeneic transplant models recapitulate GVHD in outbred humans. A number of studies have thus been conducted in xenogeneic transplant systems whereby human hematopoietic cells [most commonly peripheral blood mononuclear cells (PBMC)] are transplanted into severely immunodeficient mice (e.g. NSG, NRG, NOG mice) (71). PBMC is predominantly composed of lymphocytes, although APC including monocytes and dendritic cells are included. However, there is no hematopoietic progenitor or stem cell components, hence, the differentiation of human APC is not sustained. There is also a question as to whether human T cells can appropriately recognize murine MHC and if not whether these systems are indeed clinically relevant. In addition, there are three other major constraints to the interpretation of xenogeneic transplant systems. Firstly, it does not phenocopy clinical GVHD. While clinical acute GVHD typically targets the skin, liver and gastrointestinal (GI) tract and the intestinal disease usually determines lethality, the skin and GI tract display only very mild changes after xenogeneic transplant (72, 73). The major pathogenic manifestations of GVHD in xenogeneic transplant models are predominantly observed in the liver and lung and give rise to lethality. Second, since human cell engraftment is limited and predominantly of T cells after PBMC are transplanted, the GVHD induced is unlikely to recapitulate the spectrum seen following full T and myeloid cell engraftment seen in species-specific systems. Finally, it is unclear the role that mouse anti-human graft rejection (e.g. by myeloid cells) plays in the spectrum of GVHD seen in these systems (72, 74, 75).

It has been demonstrated that murine MHC-I and II molecules stimulate human T cells after human PBMC injection into NSG mice (73, 75, 76). When recipient NSG mice lack murine MHC-I expression, disease lethality and the frequency of human CD3$^+$ T cells in the recipient are reduced. The presence or absence of murine MHC-II expression is less important in isolation since its deficiency does not attenuate lethality (75). Although these data suggest that human T cells can react to murine MHC, human T cells primarily respond to

TABLE 2 | Antigen presentation within mismatched MHC. miHA matched.

Mismatched MHC	MHC mutation	Known peptide/MHC complex	T cell's self-MHC	Reactive TCR Detection by	T cell from	Reference
H-2K^{bm1}	7 nucleotides		H-2K^{b}		Multiple clones from C57BL6	(60)
H2-Ab1^{bm12}	3 nucleotides		H2-Ab1^{b}		Multiple clones from C57BL6	(61, 62)
H-2L^{d}		dEV8 (self-peptide)/H-2K^{b}, dEV8/H-2K^{bm3}, SIYR (foreign peptide)/H-2K^{b}, p2Ca/H-2L^{d}, QL9/H-2L^{d}	H-2^{b}	1B2 (anti-2C TCR mAb)	2C TCR transgenic mice	Originally BALB.B CD8^{+} T cells when immunized P815 (DBA/2 mastocytoma line) and BALB/c splenocytes (63–65).
H2-IA^{d} (I-A^{d})		unknown non-polymorphic mouse peptide/I-A^{d}	H2-Ab1 (I-A^{b})		4C TCR transgenic mice	(66)

Superscript letters indicate MHC types.

human MHC (HLA) molecules rather than murine MHC *in vitro* (77, 78). Therefore, multiple immunodeficient mouse strains expressing HLA class I (e.g. HLA-A2) and class II (e.g. HLA-DR1 or DR4) have been developed (73, 79, 80). These mice have been demonstrated to develop HLA-restricted anti-virus human T cell clones after human HSC transplantation (79, 80), suggesting the transgenic human HLA indeed preferentially invoke human TCR responses. However, physiological upregulation of MHC and antigen presentation therein are not assured. While multiple cytokines (i.e. interferon (IFN)-γ, interleukin (IL)-4, IL-6, IL-10, IFN-α/β and tissue necrosis factor) and glucocorticoids modulate MHC-I and II expression (81, 82) and many are secreted by human T cells after xenogeneic transplantation, the majority are not cross-reactive with the relevant murine receptors.

The presence of both murine and human MHC in these humanized transgenic systems likely creates promiscuous antigen recognition. NSG mice with intact murine MHC (H-2) and transgenic HLA-A*0201 expression develop accelerated lethality after transplantation with human PBMC, but equivalent histopathology (relative to HLA-A*0201-negative NSG mice) (83). *In vitro* assays demonstrate that multiple human CD4^{+} and CD8^{+} T cell clones are reactive to both murine MHC-I and II (84). In addition, highly aberrant CD4^{+}CD8^{+} T cell expansion within tissue has been seen after xenogeneic but not clinical transplantation and this likely reflects non-physiological antigen presentation (85). Thus while the mechanism by which human TCR can respond to a murine peptide-MHC complex is an intriguing question, the reality is that disordered antigen presentation is a serious confounding factor.

Another possible approach of *in vivo* model is to utilize the mice which have already been reconstituted by human hematopoiesis. To achieve human hematopoietic APC engraftment, the transplant of human bone marrow or cord blood (CB) derived CD34^{+} HSC into immune deficient mice is promising (74, 86, 87). These methods achieved stable and high level of human cell engraftment in the BM and spleen (> 50%) but with a low frequency of CD33^{+} or CD14^{+} human myeloid cells. Human CB-derived HSC injection into newborn NSG mice demonstrated the presence of human HLA-DR^{+}CD11c^{+} cell in the spleen three months after transplant (86). NSG-SGM3 (NSGS) mice which express additional transgenic genes for human IL-3, GM-CSF and SCF and MISTRG mice which express human IL-3, GM-CSF, M-CSF, thrombopoietin and SIRPα, significantly improve human myeloid cell reconstitution (88–90). Nevertheless, the issue of concurrent murine MHC expression in these systems remains a confounding variable.

CONCLUSIONS

The advantage of fully murine models that permit delineation of antigen-specific responses includes the ability to spatially and temporally track antigen presentation and resultant T cell responses *in vivo*, coupled with extensive availability of mutant and transgenic strains to delineate mechanisms of disease. Nevertheless, it remains important to validate these results with polyclonal T cells in MHC-mismatched or miHA-mismatched transplant models. The use of xenograft models are increasingly important for the examination of immune independent therapeutic effects (e.g. the effect of a drug on a human leukemia *in vivo*) or human-human cellular interactions *in vivo* (e.g. a human CAR T cell or TCR transgenic T cell response against a human leukemia). In contrast, the species mismatch inherent in these systems at the APC-T cell interface makes them more problematic as a robust preclinical transplant platform. Hence, the use of xenogeneic models for GVHD/GVL studies ought to be used cautiously, sparingly, and ideally as an adjunct to appropriate allogeneic models.

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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XBP-1s Promotes B Cell Pathogenicity in Chronic GVHD by Restraining the Activity of Regulated IRE-1α-Dependent Decay

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Allogeneic hematopoietic cell transplantation (allo-HCT) is an effective therapeutic procedure to treat hematological malignancies. However, the benefit of allo-HCT is limited by a major complication, chronic graft-versus-host disease (cGVHD). Since transmembrane and secretory proteins are generated and modified in the endoplasmic reticulum (ER), the ER stress response is of great importance to secretory cells including B cells. By using conditional knock-out (KO) of XBP-1, IRE-1α or both specifically on B cells, we demonstrated that the IRE-1α/XBP-1 pathway, one of the major ER stress response mediators, plays a critical role in B cell pathogenicity on the induction of cGVHD in murine models of allo-HCT. Endoribonuclease activity of IRE-1α activates XBP-1 signaling by converting unspliced XBP-1 (XBP-1u) mRNA into spliced XBP-1 (XBP-1s) mRNA but also cleaves other ER-associated mRNAs through regulated IRE-1α-dependent decay (RIDD). Further, ablation of XBP-1s production leads to unleashed activation of RIDD. Therefore, we hypothesized that RIDD plays an important role in B cells during cGVHD development. In this study, we found that the reduced pathogenicity of XBP-1 deficient B cells in cGVHD was reversed by RIDD restriction in IRE-1α kinase domain KO mice. Restraining RIDD activity per se in B cells resulted in an increased severity of cGVHD. Besides, inhibition of RIDD activity compromised B cell differentiation and led to dysregulated expression of MHC II and costimulatory molecules such as CD86, CD40, and ICOSL in B cells. Furthermore, restraining the RIDD activity without affecting XBP-1 splicing increased B cell ability to induce cGVHD after allo-HCT. These results suggest that RIDD is an important mediator for reducing cGVHD pathogenesis through targeting XBP-1s.

Keywords: allo-HCT, chronic GVHD, IRE-1α, XBP-1, RIDD, ER stress, UPR

INTRODUCTION

Although it is an effective therapy to treat hematological disease, the benefit of allogeneic hematopoietic stem cell transplantation (allo-HCT) is limited by the induction of complications such as acute and chronic graft-versus-host disease (GVHD) (1, 2). Chronic GVHD (cGVHD) is still the main cause for the morbidity and mortality of long-term survivors after allo-HCT (3). Despite the continuous effort to understand and reduce the cGVHD pathogenicity, the practical option for treatment is still very limited except for steroids. Therefore, new strategies to both prevent and treat cGVHD are urgently required.

It is well established that T cells play a major role in GVHD development, but emerging evidence from pre-clinical studies and clinical trials emphasize the importance of B cell involvement in the pathology of cGVHD (4–6). B cells from cGVHD patients showed increased B cell receptor response and resistance to apoptosis resulting in the constant activation of B cells (5, 7, 8). The presence of auto-antibody secreting B cells promoted by alloreactive donor CD4 T cells is an important mediator of autoimmune and fibrotic features of cGVHD (4, 9, 10). Very recently, the US Food and Drug Administration approved ibrutinib, a Bruton's tyrosine kinase inhibitor, which is important for B cell receptor signaling, as second-line therapy of steroid-refractory cGVHD (11). It shows that B cell regulation can be one of the essential targets for cGVHD treatment. Thus, we focus on the role of the unfolded protein response (UPR) mediators in regulating B cell activity after allo-HCT.

The UPR consists of highly conserved signaling pathways that allow the cells to manage ER stress in response to the accumulation of unfolded or misfolded protein (12). There are three primary UPR mediators including IRE-1α, PERK, and ATF6 (13). Endoribonuclease activity of IRE-1α activates XBP-1 signaling by converting unspliced XBP-1 to spliced XBP-1 (XBP-1s) and also mediates regulated IRE1α-dependent decay (RIDD) of ER-associated mRNAs harboring structures and sequences similar to XBP-1 mRNA stem loops (14–16). It has been demonstrated that IRE-1α/XBP-1s signaling is required for normal B cell development and also pivotal for plasma cell differentiation, which can secrete large amounts of immunoglobulin (Ig) (17–21). While XBP-1s has been previously shown to promote immunoglobulin production and secretion by plasma cells, deletion of IRE-1α kinase or RNase activity, that results in impaired XBP-1s production but also blocked RIDD activity, does not significantly compromise the capability of plasma cells in producing and secreting immunoglobulin (22, 23). Since deletion of XBP-1s can enhance RIDD of immunoglobulin mRNAs through upregulating the expression levels and kinase/ribonuclease activity of IRE-1α leading to scarcity of immunoglobulin proteins, further targeting IRE-1α kinase/RNase activity by deleting IRE-1α or mutating serine 729 to alanine (S729A) in IRE-1α kinase activation loop indeed results in the recovery of immunoglobulin production and secretion by XBP-1s-deficient plasma cells (20, 22, 23). We identified a critical role of IRE-1α/XBP-1s signaling in B cells for the development of cGVHD through genetic and pharmacological inhibition of XBP-1s in mouse allo-HCT models (24). However, it is unclear whether RIDD plays an important role in reducing the severity of cGVHD.

Here, we identified the role of RIDD in B cell-mediated cGVHD pathogenicity. Deletion of XBP-1s reduced B cell activity and ability to stimulate allogeneic CD4 T cells in an RIDD-dependent manner which reduced the severity of cGVHD in the murine allo-HCT model. Our results showed that activating RIDD by targeting XBP-1s is a useful strategy to reduce cGVHD.

MATERIALS AND METHODS

Mice

BALB/c (H-2^d), and FVB (H-2^q) mice were purchased from the National Cancer Institute (Frederick, MD). B cell conditional XBP-1^{KO} (XBP-$1^{flox/flox}$CD19-Cre^+), IRE-$1\alpha^{KO}$ (kinase domain (aa652-751) flanked by LoxP site (IRE-$1\alpha^{flox/flox}$CD19-Cre^+), XBP-1/IRE-1α double KO (DKO) (XBP-$1^{flox/flox}$IRE-$1\alpha^{flox/flox}$CD19-Cre^+), and littermate wild-type control (XBP-$1^{flox/flox}$CD19$^-$) mice on a C57BL/6(B6, H-2^b) background were generated as described before (20, 23, 25, 26). The S729A knock-in mouse model was generated as previously described (23). Mice were maintained at pathogen-free facilities in the American Association for Laboratory Animal Care–accredited Animal Resource Center at Medical University of South Carolina (MUSC) and the animal facility at the Houston Methodist Research Institute (HMRI). All animal experiments were approved by the Institutional Animal Care and Use Committees at MUSC and HMRI.

Antibodies and Reagents

Antibodies were purchased as followed: Anti-CD4-V450, anti-CD8α-APCcy7, anti-B220-V450, anti-CD138-Pe-cy7, anti-CD86-Pe-cy5, anti-FAS-PE, anti-GL-7-APC, anti-CD40-APC, anti-MHCII-FITC, anti-IL-4-PE, anti-IL-5-PE, anti-IgM-Pe-cy7, anti-IgG1-APC, anti-IFN-r-Percp5.5, anti-TNF-α-PE, and anti-IL-17-Pe-cy7 were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-PDL-1-biotin (eBioscience, San Diego, CA), anti-XBP-1s (Cell Signaling Technology, Danvers, MA), and anti-Rabbit IgG-FITC (Thermo Fisher Scientific, Waltham, MA) antibodies were purchased from commercial sources. Recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ) and LPS (Sigma-Aldrich, St. Louis, MO) were purchased from the commercial companies. Goat F(ab')2 Anti-Mouse IgM (1022-01, SouthernBiotech, Birmingham, AL) and anti-mouse CD40 (BE0016-2, BioXCell, Lebanon, NH) were commercially purchased from companies.

Allogeneic Bone Marrow Transplantation

Recipient BALB/c mice (8-10 weeks old) were lethally irradiated at 650 – 700 cGy using X-RAD 320 irradiator (Precision X-ray Inc., North Brandford, CT). 5×10^6 T cell depleted bone marrow (TCD-BM) cells were transplanted into recipient mice with or without $0.3 - 0.5 \times 10^6$ splenocytes. Survival, body weight, and clinical scores of cGVHD from recipient mice were monitored as described previously (27). Clinical scores were calculated with the combination of 7 parameters established in the previous

report (28) including weight loss, posture, activity, fur texture, skin integrity, diarrhea, and eye inflammation or conjunctivitis. Individual mice were scored 0 to 2 for each criterion and 0 to 12 overall. We arbitrarily considered mice falling under the score categories 0.5–3 as mild, 4–7 as moderate, and 8–12 as showing severe symptoms where scores ≥ 8 required euthanasia as a humane endpoint.

In Vitro Mixed Lymphocyte Reaction

B cells purified from WT, XBP-1^{KO}, IRE-1α^{KO}, and DKO B6 mice were stimulated with LPS (1μg/ml) and IL-4 (10ng/ml) for 24 h. LPS and IL-4 were removed from culture plate wells. T cells from FVB mice were stained with Carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Molecular Probes, Inc., Eugene, OR) and co-cultured with pre-stimulated B cells for 3-4 days. T cell proliferation and cytokine expression were determined with flow cytometry analysis.

Serum Immunoglobulin Detection

Using DNA from calf thymus (Sigma-Aldrich), we made double-strained DNA (dsDNA) (27). ELISA plates were coated with a 5μg/ml dsDNA overnight at 37°C. The plates were blocked with 1% BSA solution in PBS for 30 min. After blocking, serum or cell supernatant was added at a 1:10 to 1:100 ratio in PBS containing 0.05% Tween and 1% BSA. Plates were incubated at room temperature (RT) for 45 minutes and then washed. Biotin-conjugated IgM or IgG1 antibody (BD Bioscience) was added at a 1:4000 ratio and incubated for another 45 min in RT. Plates were then washed and added with streptavidin-HRP antibody (Invitrogen) at a 1:250 ratio and incubated for 45 min in RT. After washing the plates, TMB Substrate (eBioscience) was added to the plates. The reaction was stopped after 15 min using 1M phosphoric acid and the plates were read at 450nm.

Statistics

Data were presented by means ± standard deviation (SD) or means ± standard error of the mean (SEM) and statistical analyses were performed by GraphPad Prism software, version 9. Statistics for GVHD scoring and mice weight were performed using two-way ANOVA with Tukey's multiple comparison test. Comparison of the survival distributions of any given groups were done using log-rank test. One way analysis of variance (ANOVA) with the Tukey's multiple comparison test was used for multiple groups comparisons unless otherwise stated. $p < 0.05$ is considered statistically significant.

RESULTS

Inhibition of IRE-1α/XBP-1 Signaling Reduces B Cell Activation, Differentiation, and IgM Secretion Through RIDD-Dependent Manner

We hypothesized that enhanced RIDD resulting from XBP-1s deletion is an important factor to reduce B cell pathogenicity in cGVHD development. To test this hypothesis, we compared the activation, differentiation, and immunoglobulin (Ig) production of B cells from WT or genetically modified mice that have XBP-1s, IRE-1α, or XBP-1s/IRE-1α deficiency specifically on their B cells. Since IRE-1α/XBP-1 signaling is known to be activated by LPS and IL-4 stimulation, which are also commonly presented in BMT recipients (29), we stimulated B cells with them. First, we measured the expression of XBP-1s, and confirmed that it was eliminated in XBP-1^{KO}, IRE-1α^{KO}, and DKO B cells compared to WT B cells (**Supplementary Figure 1**) (23). Activated B cells increase the expression of MHCII and costimulatory molecules including CD86, CD40, and ICOS. The expression of CD86 and ICOSL was reduced on XBP-1^{KO} B cells compared with WT counterparts, but it was increased on IRE-1α^{KO} and DKO B cells where RIDD was also impeded (**Figures 1A, B**). The expression levels of MHCII and CD40 were significantly increased in IRE-1α^{KO} and DKO B cells compared to WT B cells (**Figures 1C, D**). Germinal center (GC) B cell development was also significantly lower in XBP-1^{KO} B cells, but it was reversed in IRE-1α^{KO} and DKO groups (**Figures 1E, F**). IgM is the first antibody produced by plasma cells and its mRNA is the representative substrate of RIDD in B cells (22). B cells from XBP-1^{KO} mice expressed a significantly lower level of IgM compared to WT B cells, which is related to the reduced GC B cell population by XBP-1s deletion (**Figures 1E, F**) and also confirms that RIDD was increased in XBP-1s-deficient B cells (**Figures 1G, H**). Therefore, ablation of RIDD by deleting IRE-1α in IRE-1α^{KO} or DKO B cells restored the IgM expression compared to XBP-1^{KO} B cells (**Figures 1G, H**). On the other hand, intracellular IgG1 expression was not dramatically affected in XBP-1^{KO}, IRE-1α^{KO} or DKO B cells (**Supplementary Figures 2A, B**). XBP-1^{KO} B cells showed reduced IL-4/IL-5 expression but such a reduction was not restored by IRE-1α deletion suggesting these cytokines are not regulated by RIDD (**Supplementary Figures 2C, D**).

XBP-1s is also required for the proper signaling through the B cell receptor (BCR) (20). BCR is composed of a membrane-bound immunoglobulin (IgM or IgD) and the disulfide-linked Igα/Igβ heterodimer. To induce BCR signaling, we stimulated B cells with anti-IgM and anti-CD40 for stable activation. Similar to stimulation with LPS and IL-4, XBP-1s deficiency repressed the B cell activation, GC cell differentiation and sIgM expression but these phenotypes were reversed by RIDD inhibition in IRE-1^{KO} and DKO B cells after BCR stimulation (**Supplementary Figure 3**). Taken together, these data indicate that inhibition of XBP-1s in B cells reduces B cell activation and differentiation through both RIDD-dependent and -independent manners.

RIDD Is Essential for the Ability of XBP-1s-Deficient B Cells to Activate T Cells

Activated B cells are important APCs in alloantigen-rich immunologic microenvironment, which can prime allogenic T cells in ongoing cGVHD (30). Therefore, we tested whether and how RIDD may affect B cell activity to stimulate allogeneic T cells *in vitro*. WT T cells from FVB mice were stimulated with allogeneic B cells from different B6 mice (WT, XBP-1^{KO}, IRE-1α^{KO}, or DKO). T cells, particularly CD4 T cells, had a reduced proliferation reflected by a decreased CFSE dilution when

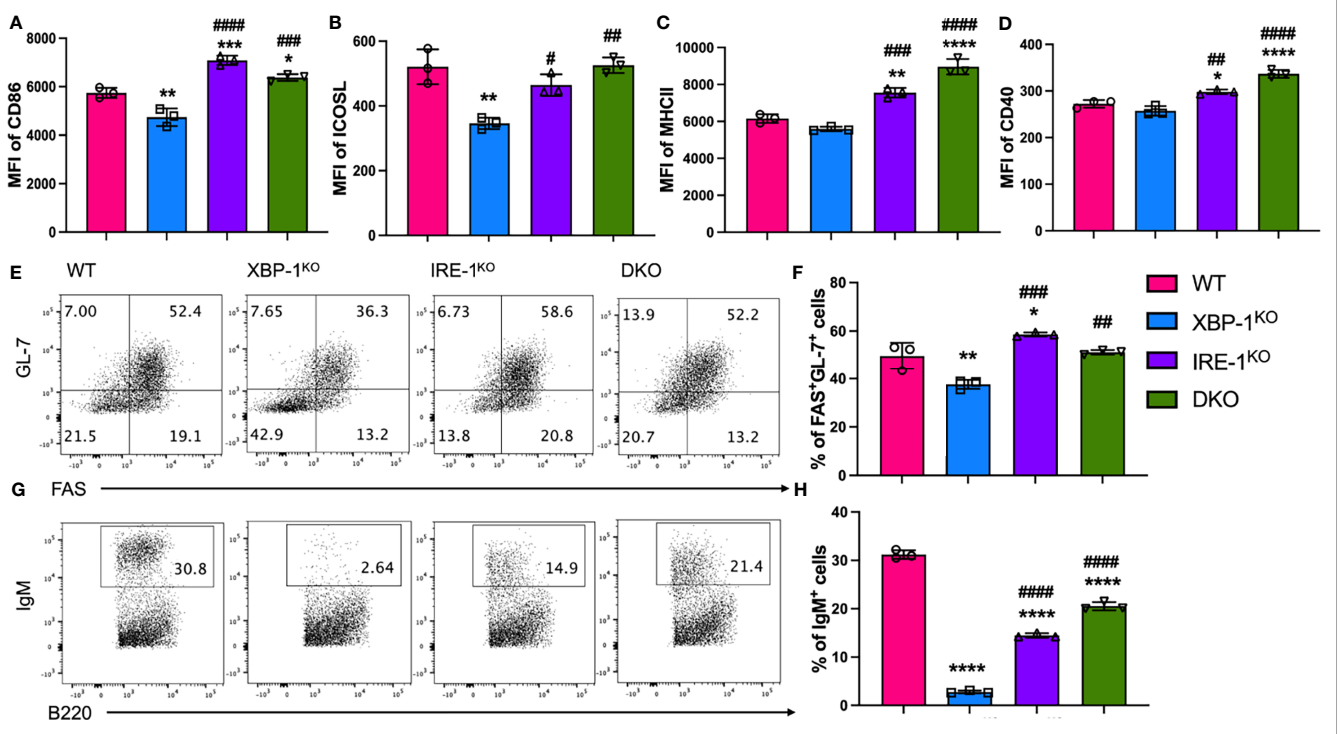

FIGURE 1 | Roles of XBP-1s and IRE-1α in B cell activation, differentiation and IgM production *in vitro*. B cells were isolated from WT, XBP-1^{KO}, IRE-1α^{KO}, and IRE-1α^{KO}/XBP-1^{KO} (double KO; DKO) mice and stimulated with 1 mu;g/ml LPS and 10 ng/ml IL-4 for 4 days. The cell surface expression levels of CD86 **(A)**, ICOSL **(B)**, MHCII **(C)**, and CD40 **(D)** were measured by flow cytometry analysis. Germinal Center B cells (FAS^{+}GL-7^{+}) were detected by flow cytometry **(E, F)**. B cells were stimulated with PMA and Ionomycin for another 4 hrs. B cells were intracellularly stained and analyzed for IgM production **(G, H)**. Data are representative graphs and flow cytometry plots of three independently repeated experiments. Data are shown as means ± SD. MFI, mean fluorescence intensity. Statistics were performed using ordinary one-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001 when compared to WT. $^{\#}$p < 0.05, $^{\#\#}$p < 0.005, $^{\#\#\#}$p < 0.0005, and $^{\#\#\#\#}$p < 0.0001 when compared to XBP-1^{KO} group.

stimulated with XBP-1^{KO} B cells, but such a reduction was not observed in those stimulated with IRE-1α^{KO} or DKO B cells (**Figures 2A, B**). The proliferation of CD8 T cells was slightly increased by IRE-1α^{KO} or DKO B cell stimulation (**Figures 2C, D**). Moreover, CD4 T cells, but not CD8 T cells, expressed significantly less inflammatory cytokines including IFN-γ (**Figures 2E–G**) and TNF-α (**Figures 2H–J**) when stimulated by XBP-1^{KO} B cells. Both CD4 and CD8 T cells produced decreased levels of IL-17A when stimulated with XBP-1s-deficient B cells (**Figures 2K–M**). However, similar or even higher levels of cytokines were observed when CD4 T cells were stimulated with IRE-1α^{KO} and DKO B cells in which not only XBP-1s was not expressed but also RIDD was ablated. Collectively, our results indicate that activated RIDD in XBP-1^{KO} B cells is essential for optimal activation of allogeneic CD4 and potentially CD8 T cells.

XBP-1s Deficiency on B Cells Decreases Severity of cGVHD Through RIDD

We showed that B cell activation and differentiation can be regulated by RIDD activation resulting in altered alloreactive T cell activation. Here, we investigated the role of XBP-1s deficiency-mediated activation of RIDD in the prevention of cGVHD using an MHC-mismatched murine BMT (B6 to BALB/c) model. The recipients transplanted with XBP-1s-deficient donor-graft showed significantly reduced cGVHD severity (**Figures 3A–C**). Induction of follicular helper T cell (T_{FH}) is required for cGVHD development by supporting GC formation and maintenance (31). We found that T_{FH} phenotype was notably increased in the recipients of IRE-1α^{KO} or DKO graft (**Supplementary Figures 4A, B**). Besides, donor CD4 T cells in the recipients of XBP-1^{KO} graft produced a significantly lower level of IFN-γ and IL-17, but T cells from those of RIDD-ablated IRE-1α^{KO} or DKO grafts expressed similar, or even higher levels of cytokines compared to WT (**Supplementary Figures 4C–F**). These data support that XBP-1s deficiency-mediated activation of RIDD in B cells affects the T cell pathogenicity and consequently regulates the cGVHD severity in the long-term period.

RIDD Regulates B Cell Activation and cGVHD Pathogenesis

To determine the cellular mechanisms associated with cGVHD development, we used B6 to BALB/c cGVHD model and euthanized recipient mice at 4 weeks after allo-BMT for analyses of donor B and T cell responses. Recipients transplanted with allogeneic grafts from B cell-specific XBP-1^{KO} donors showed attenuated severity of GVHD represented by reduced GVHD scoring and improved weight loss (**Figures 4A, B**). However, the

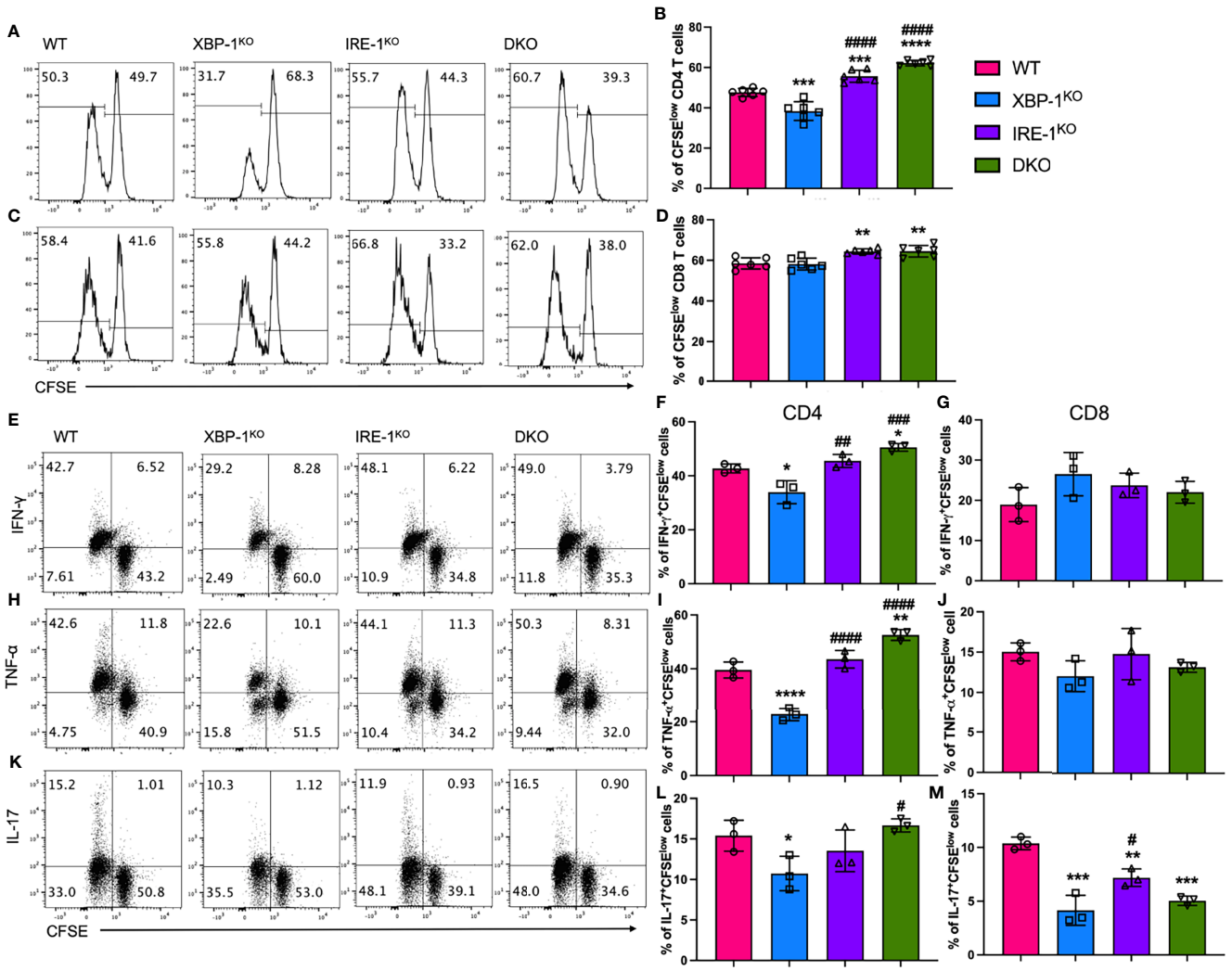

FIGURE 2 | Effects of XBP-1s and IRE-1α on B cell ability to stimulate allogeneic T cells *in vitro*. B cells isolated from WT, XBP-1^{KO}, IRE-1α^{KO}, and DKO on B6 background mice are stimulated with 1 μg/ml LPS and 10 ng/ml IL-4 for 24 hrs. T cells were isolated from FVB mice and labeled with CFSE and then incubated with activated B cells for another 4 days. The representative flow panels of CFSE dilution and percentages of CFSE diluted cells on CD4 **(A, B)** and CD8 **(C, D)** T cells. T cells were stimulated with PMA and Ionomycin for 4 h before intracellular cytokine staining for cytokine detection. The representative flow panels and the percentages of IFN-γ **(E–G)**, TNF-α **(H–J)** or IL-17 **(K–M)** among proliferated (CFSE^{low}) CD4 and CD8 T cells were shown. Data show representative flow cytometry plots and graphs from three independently repeated experiments. Statistics were performed using ordinary one-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001 when compared to WT. #p < 0.05, ##p < 0.005, ###p < 0.0005, and ####p < 0.0001 when compared to XBP-1^{KO} group.

recipients with IRE-1α^{KO} or DKO donor grafts showed similar levels of GVHD development compared to WT counterparts (**Figures 4A, B**). The recipients of XBP-1^{KO} donor-grafts had higher frequencies of donor-derived B cells in their spleens, but this was not the case in the recipients transplanted with IRE-1α^{KO} or DKO donor-grafts where RIDD was also ablated in donor B cells (**Figures 4C, D**). B cells from the recipients with XBP-1^{KO} donor-grafts showed significantly decreased expression levels of MHCII, CD86, ICOSL, and CD40, which were restored in B cells from those with IRE-1α^{KO} or DKO donor-grafts (**Figures 4E–H**). In the recipients with XBP-1^{KO} donor-grafts, B cell showed significantly decreased expression of IgM (**Figure 4I**). Ablation of RIDD in B cells in IRE-1α^{KO} or DKO donor-grafts enhanced IgM production compared to those in XBP-1^{KO} donor-grafts (**Figure 4I**). Consistent with *in vitro* data, IgG1, IL-4 and IL-5 expression levels were reduced in XBP-1s-deficient B cells, but such reductions could not be restored by further deleting IRE-1α (**Supplementary Figure 5**). These data indicate that RIDD is a key mediator, which can reduce cGVHD in the context of targeting XBP-1s.

RIDD Regulates the Capabilities of B Cells in Activating T Cells During cGVHD

We observed that MHCII expression and costimulatory activity of B cells were regulated by RIDD, which can affect T cell pathogenicity in the induction of cGVHD. Therefore, we tested donor T cell activation in the recipients transplanted with

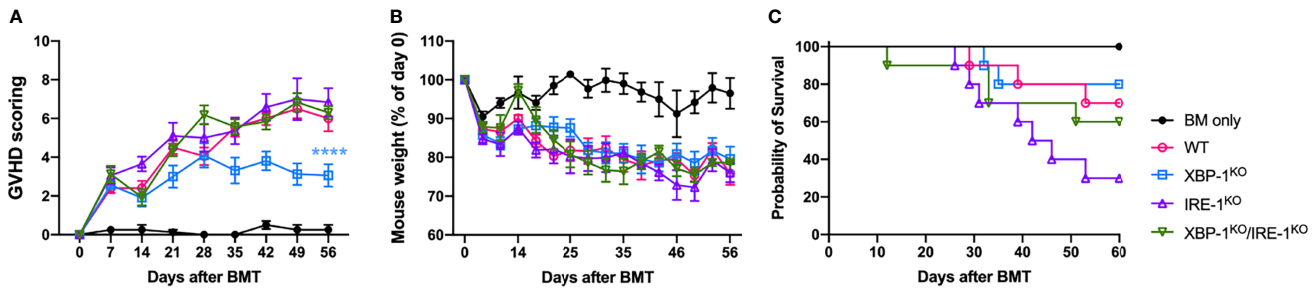

FIGURE 3 | Long-term impact of XBP-1s and IRE-1α on B cell pathogenicity in the induction of cGVHD. BALB/c mice were lethally irradiated and transplanted with 5 x 10^6 T cell-depleted bone marrow (TCD-BM) cells from WT (n = 4) or TCD-BM from WT, XBP-1^{KO}, IRE-$1\alpha^{KO}$, and DKO mice on a B6 background with 0.35 - 0.5 x 10^6 splenocytes (n = 40). Recipient mice were monitored for the GVHD scoring **(A)**, weight loss **(B)**, and mortality **(C)** until 60 days after BMT. Data show results from two out of three independently repeated experiments. 22 recipient mice were used for each experiment. Statistics for scoring and weight were performed using two-way ANOVA with Tukey's multiple comparison test. Comparisons of the survival distributions of any given groups were done using log-rank test. ****$p < 0.0001$ when compared to WT.

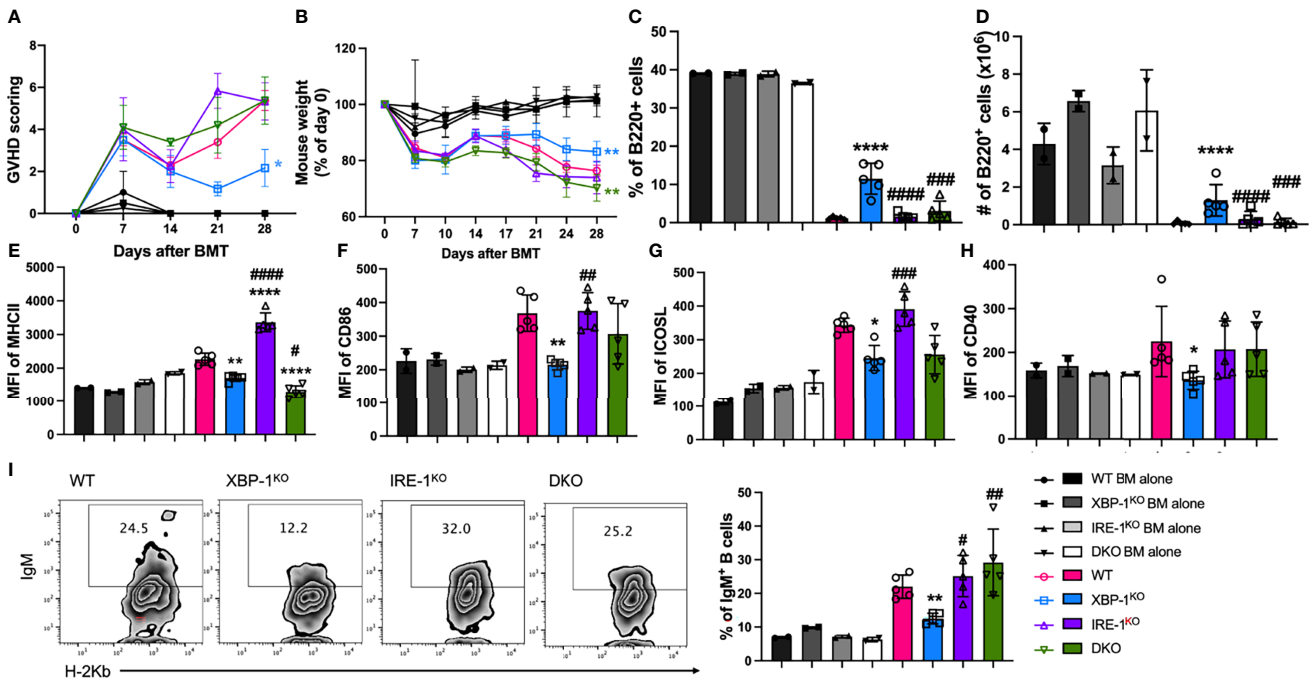

FIGURE 4 | Effect of XBP-1s and IRE-1α on B cell activation and cGVHD pathogenicity. BALB/c mice were lethally irradiated and transplanted with 5 x 10^6 TCD-BM cells from WT, XBP-1^{KO}, IRE-$1\alpha^{KO}$, and DKO mice on a B6 background with (n = 20) or without (n = 8) 0.35 - 0.5 x 10^6 splenocytes. GVHD scoring **(A)** and mouse weight **(B)** were monitored during the experiment. Subsets of recipient mice were euthanized on day 28, and spleens were dissected and processed into single-cell suspension. The percentages of $B220^+$ B cells **(C)** and the expression levels of MHCII **(D)**, CD86 **(E)**, ICOSL **(F)**, and CD40 **(G)** were detected by flow cytometry analysis. Intracellular expression of IgM **(H, I)** was also determined using flow cytometry. Data show the representative result from three independently repeated experiments. 28 recipient mice were used for each experiment. Statistics were performed using two-way ANOVA with Tukey's multiple comparison test for **(A, B)** One-way ANOVA with Tukey's multiple comparison test was used for others. *$p < 0.05$, **$p < 0.005$, and ****$p < 0.0001$ when compared to WT. #$p < 0.05$, ##$p < 0.005$, ###$p < 0.0005$, and ####$p < 0.0001$ when compared to XBP-1^{KO} group.

different donor grafts. We found that Treg differentiation was increased in T cells in the recipients of XBP-1^{KO} donor-grafts (**Figures 5A, B**). Furthermore, Treg frequency was reversed in T cell populations from the recipients transplanted with IRE-$1\alpha^{KO}$ or DKO donor-grafts, consistent with CD86 expression on corresponding types of donor B cells (**Figure 4F**). Opposite to Tregs with a suppressive role, T helper (Th)1, Th2, and Th17 cells play a pathogenic role in GVHD development (32, 33). We measured the presence of Th2 (IL-4/IL-5+), Th1 (IFN-γ+), and Th17 (IL-17+) T helpers in donor T cell populations from the

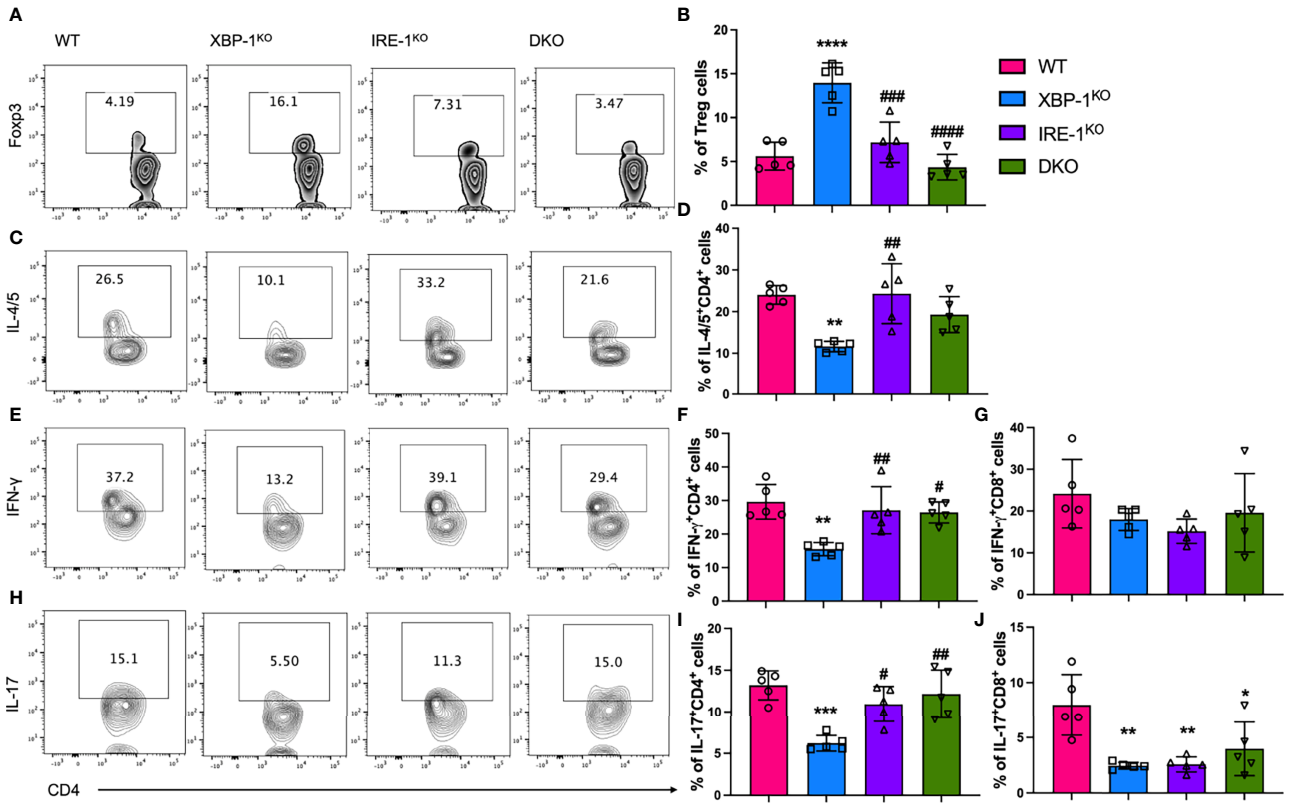

FIGURE 5 | Effect of XBP-1s and IRE-1α on B cell ability to stimulate allogeneic T cells during cGVHD. Lethally irradiated BALB/c mice were transplanted with 5 x 10^6 TCD-BM cells from WT, XBP-1^{KO}, IRE-$1\alpha^{KO}$, and DKO mice on a B6 background with 0.35 - 0.5 x 10^6 splenocytes (n=20). Recipient mice were euthanized on day 28 after BMT and single cells were isolated from recipient spleens. The expression of Foxp3 transcription factor **(A, B)** was measured by flow cytometry analysis. The cytokine levels in CD4 and CD8 T cells including IL-4/IL-5 **(C, D)**, IFN-γ **(E–G)**, and IL-17 **(H–J)** were determined after intracellular staining by flow cytometry analysis. Data show the representative result from three independently repeated experiments. 20 recipient mice were used for each experiment. Statistics were performed using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001 when compared to WT. $^{\#}$p < 0.05, $^{\#\#}$p < 0.005, $^{\#\#\#}$p < 0.0005, and $^{\#\#\#\#}$p < 0.0001 when compared to XBP-1^{KO} group.

recipients transferred with various donor-grafts. In XBP-1^{KO} donor-graft transplanted mice, T cells showed decreased Th1, Th2, and Th17 differentiation, which was reversed in the recipients transplanted with IRE-$1\alpha^{KO}$ or DKO donor grafts (**Figures 5C–F, H, I**). On the other hand, there was no significant difference in IFN-γ secreting type 1 CD8 T cell (Tc1) differentiation among the four groups (**Figure 5G**). T cells from the recipients transplanted with XBP-1^{KO} or IRE-$1\alpha^{KO}$ donor grafts were significantly less capable of differentiating into IL-17-producing CD8 T cells (Tc17) (**Figure 5J**). Collectively, these results suggest that activated RIDD as a result of XBP-1s deficiency specifically in B cells can change CD4 T cell differentiation during cGVHD.

RIDD Affects Activation and Differentiation of B Cells, and Their Ability to Activate T Cells

The kinase domain of IRE-1α is decisive for the ribonuclease activity of IRE-1α, and especially phosphorylation of the S729 residue is important for RIDD, because IRE-1α carrying the S729A mutation shows ablated RIDD activity but unabated activity in splicing XBP-1 mRNA (23). First, we confirmed that XBP-1s signaling is not affected by S729A mutation in B cells (**Supplementary Figure 6**). To evaluate the role of RIDD in B cells, we investigated activation and differentiation of B cells from S729A and XBP-1^{KO}/S729A mice and compared them with B cells from WT and XBP-1^{KO} mice, after B cells were treated with F(ab')2 and anti-CD40. As we expected, the reduced expression levels of MHCII and CD86 resulted from XBP-1s deficiency were reversed in XBP-1^{KO}/S729A B cells (**Figures 6A, B**). The formation of GC B cells was also decreased when XBP-1s is deleted in B cells, but it was restored by further introducing S729A into IRE-1α in XBP-1^{KO} B cells (**Figure 6C**). Decreased IgM-positive B cells and reduced IgM levels secreted into culture media resulted from XBP-1s deficiency were also reversed by further introducing the S729A mutation into IRE-1α (**Figures 6D, F**). However, reduced IgG1 expression levels resulted from XBP-1s deficiency were not reversed by the S729A mutation of IRE-1α (**Figures 6E, G**), similar to our

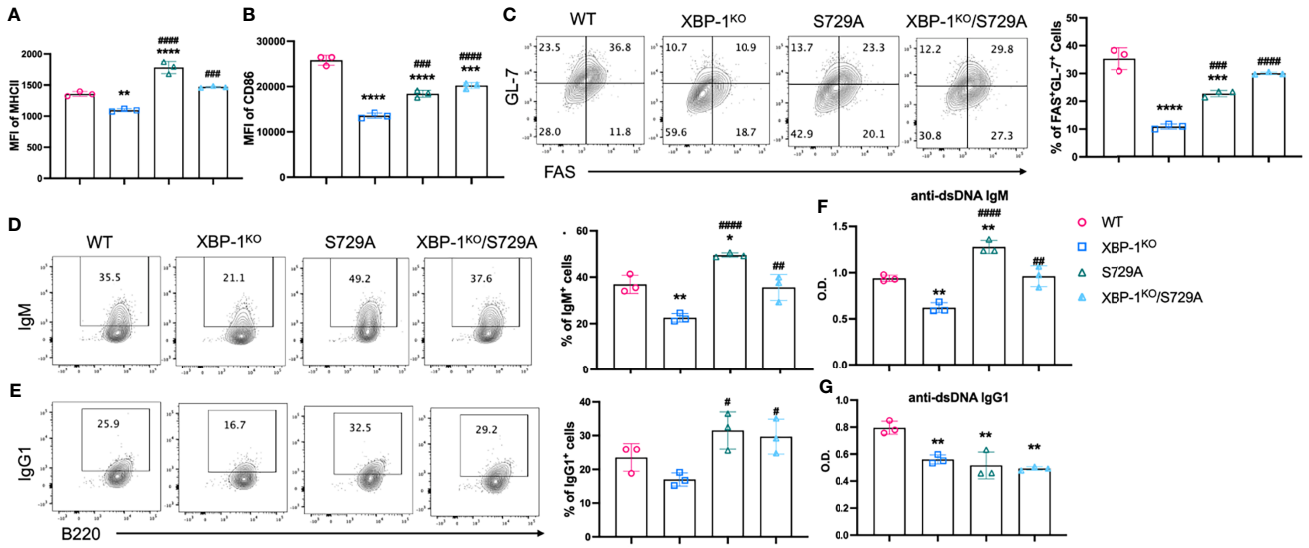

FIGURE 6 | Roles of XBP-1s and the S729A mutation of IRE-1α in B cell activation, differentiation, and IgM production *in vitro*. B cells were isolated from WT, XBP-1^{KO}, S729A, and XBP-1^{KO}/S729A mice and stimulated with anti-IgM F(ab')2 (10 mu;g/ml) and anti-CD40 (10 mu;g/ml) for 48 hrs. The expression of MHCII **(A)** and CD86 **(B)** were detected by flow cytometry. The percentages of germinal center B cells ($FAS^+GL\text{-}7^+$) were presented **(C)**. **(D, E)** B cells were stimulated with PMA and Ionomycin for 4 hrs on day 4. Intracellular levels of IgM **(D)** and IgG1 **(E)** were detected by flow cytometry analysis. Cell culture media were also collected, and anti-dsDNA autoantibodies were detected using ELISA **(F, G)**. Statistics were performed using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001 when compared to WT. #p < 0.05, ##p <0.005, ###p < 0.0005, and ####p < 0.0001 when compared to XBP-1^{KO} group.

IRE-1α kinase deletion results (**Supplementary Figures 5A, B**). B cells with S729A mutation showed even higher MHCII expression and IgM secretion compared to WT B cells after stimulation with F(ab')2 and anti-CD40 (**Figures 6A, F**). However, B cells from S729A mice have already shown curtailed activity and reduced GC B cell formation compared to WT B cells when we stimulate B cells with LPS and IL-4 (**Supplementary Figures 7A–E**) while IgM expression is still increased in S729A B cells (**Supplementary Figure 7F**). These data showed that S729A B cells may respond differently to TLR4 signaling compared to BCR signaling. Taken together, these results suggest that RIDD is essential for reduced activation and differentiation of XBP-1s-deficient B cells.

We next tested whether RIDD inhibition by introducing the S729A mutation to IRE-1α regulates B cell ability to stimulate allogeneic T cells. CFSE-labeled allogeneic T cells were stimulated with B cells from WT, XBP-1^{KO}, S729A, or XBP-1^{KO}/S729A B6 mice *in vitro*. Similar to the IRE-$1\alpha^{KO}$ and DKO (**Figures 2A, B**), B cells from XBP-1^{KO} mice carrying the S729A mutation did not reduce CD4 T cell proliferation (**Supplementary Figures 8A, B**). On the other hand, there was no significant difference in CD8 T cell proliferation among all four groups (**Supplementary Figures 8C, D**). CD4 T cells stimulated with XBP-1s-deficient B cells produced significantly less IFN-γ, IL-4 and IL-5, but the levels of these cytokines were restored by further introduction of the S729A mutation of IRE-1α into XBP-1^{KO} B cells (**Supplementary Figures 8E, F, H, I**). S729A knock-in B cells showed reduced expression of MHCII and costimulatory molecules when stimulated with LPS and IL-4 (**Supplementary Figures 7A–D**). Consequently, coculture of T cells with LPS and IL-4 stimulated allogeneic S729A B cells exhibited reduced proliferation and cytokine production in CD4 but not CD8 T cell populations (**Supplementary Figures 8 E–J**). Taken together, these data support inhibition of XBP-1s in B cells can reduce the allogeneic T cell activity through activating RIDD.

RIDD Affects the Severity of cGVHD

We next wanted to directly test the role of RIDD activity in B cell pathogenicity in the induction of cGVHD by taking advantage of the S729A mutation that specifically inhibits RIDD activity of IRE-1α while preserving its ability in splicing XBP-1 (**Supplementary Figure 6**). Because S729A is germline mutation, we isolated B cells from IRE-1α S729A mutant or control mice and compared their ability to induce cGVHD. The recipients transferred with S729A mutant B cells developed more severe cGVHD as reflected by decreased survival, increased GVHD scores, and weight loss (**Figures 7A–C**). The recipients of S729A mutant B cells demonstrated significantly increased thymus damage (**Figure 7D**) and reduced frequencies of the donor-derived B cells in the spleens (**Figure 7E**). These recipients also displayed a significantly increased percentage of GC B cells, exhibited higher expression levels of MHCII, CD86, ICOSL, and CD40 (**Figures 7F–J**), and produced significantly higher levels of anti-dsDNA IgM (**Figure 7K**) as compared with WT B cells. Taken together, these data indicate that RIDD activity of B cells contributes to a diverse repertoire of B cell function including activation, differentiation, and immunoglobulin production that resulted in reduced cGVHD (**Figure 8**).

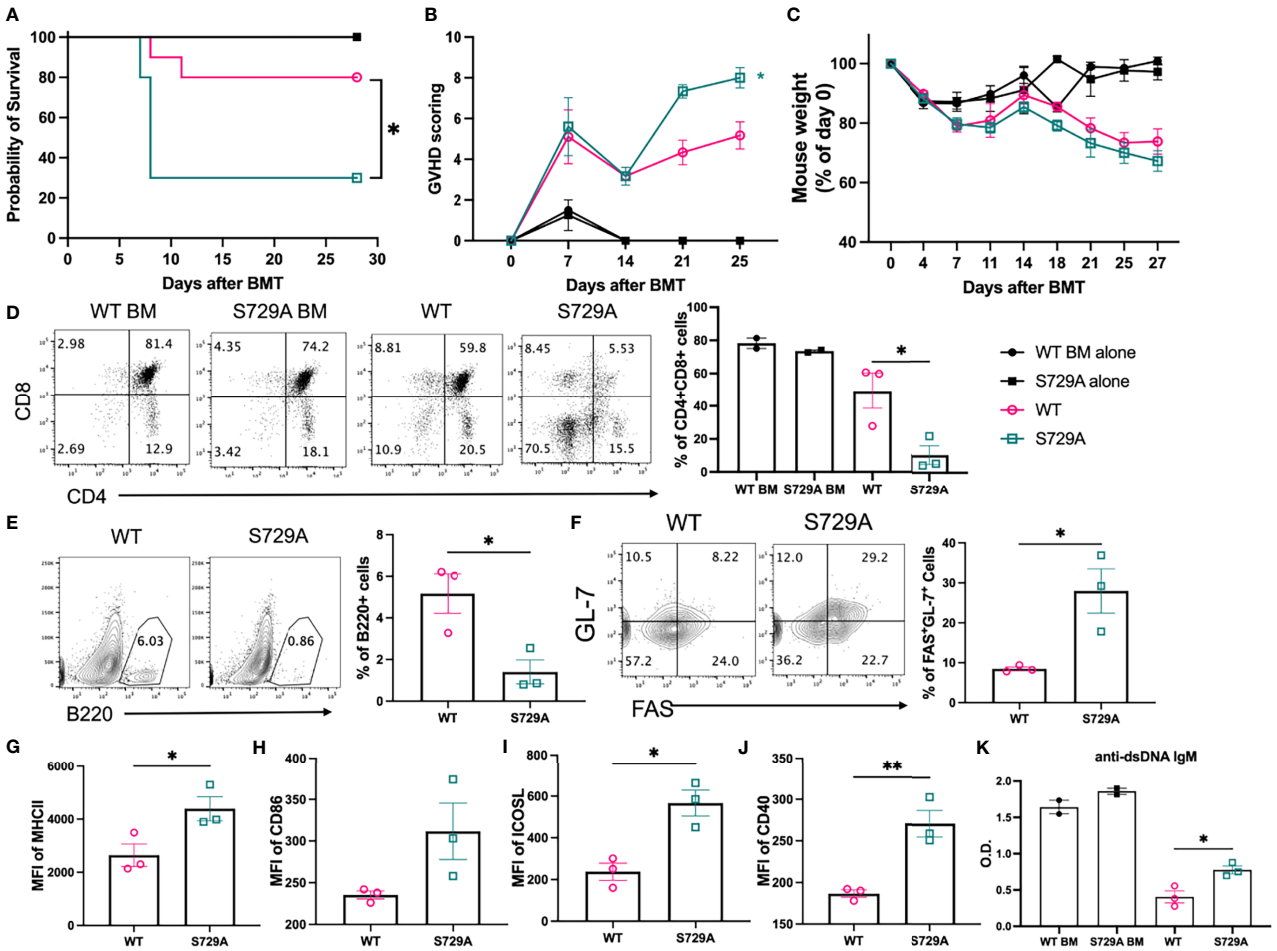

FIGURE 7 | Effect of S729A mutation on B cell activation and cGVHD pathogenicity. BALB/c mice were lethally irradiated and transplanted with 5 x 10^6 TCD-BM cells from WT and S729A mice on a B6 background with (n = 10) or without (n = 4) 0.12 x 10^6 B cells from WT or S729A mice and 0.18 x 10^6 B cell deleted WT splenocyte. Survival **(A)**, GVHD score **(B)** and mouse weight **(C)** were monitored during the experiment. Subsets of recipient mice were euthanized on day 28, and thymi and spleens were dissected and processed into single-cell suspension. CD4 and CD8 double positive cell percentages in thymus were determined using flow cytometry **(D)**. The percentages of $B220^+$ B cells **(E)**, germinal center B cells ($FAS^+GL\text{-}7^+$) **(F)**, the expression of MHCII **(G)**, CD86 **(H)**, ICOSL **(I)**, and CD40 **(J)** were detected by flow cytometry analysis. Serum isolated from recipients was collected on day 28 and assayed for anti-dsDNA autoantibodies using ELISA **(K)**. **(A–C)** shows combined data from two independently repeated experiments. **(D–K)** shows representative data from two independently repeated experiments. 14 recipient mice were used for each experiment. Comparison of the survival distributions in **(A)** was done using log-rank test. Statistics were performed using two-way ANOVA with Tukey's multiple comparison test for **(B, C)** One-way ANOVA with Tukey's multiple comparison test was used for comparing multiple groups and 2-tailed Student *t* test was used for comparing between two groups. *p < 0.05, **p < 0.005, and ****p < 0.0001 when compared to WT. #p < 0.05, ##p < 0.005, ###p < 0.0005, and ####p < 0.0001 when compared to $XBP\text{-}1^{KO}$ group.

DISCUSSION

We previously demonstrated that targeting XBP-1s in B cells efficiently prevented cGVHD by reducing activation and differentiation of B cells (24). However, how XBP-1s deficiency regulates B cell response in cGVHD was not fully defined. Since the absence of XBP-1s in B cells has been reported to confer the upregulation of IRE-1α expression accompanied by increased kinase and ribonuclease activity, we hypothesized that RIDD can be a key mediator in response to targeting XBP-1s for cGVHD prevention. IRE-1α has kinase and RNase domains in the cytoplasmic region, and under ER stress, autophosphorylation of the kinase domain results in RNase activity (34). In this study, we used B cell specific IRE-1α kinase domain deleted mice to determine how RIDD impacts the role of XBP-1s deficiency in B cells and found that deletion of IRE-1α kinase activity attenuated the effect of targeting XBP-1s in the prevention of cGVHD. *In vitro* assay revealed that the expression of MHCII and costimulatory molecules are regulated by RIDD in B cells and consequently alloreactive stimulation of T cells is attenuated by activated RIDD in XBP-1s-deficient B cells.

We confirmed that RIDD was significantly increased in XBP-1s-deficient B cells through IgM expression (**Figure 1**). However, the absence of IRE-1α did not fully restore IgM production to the

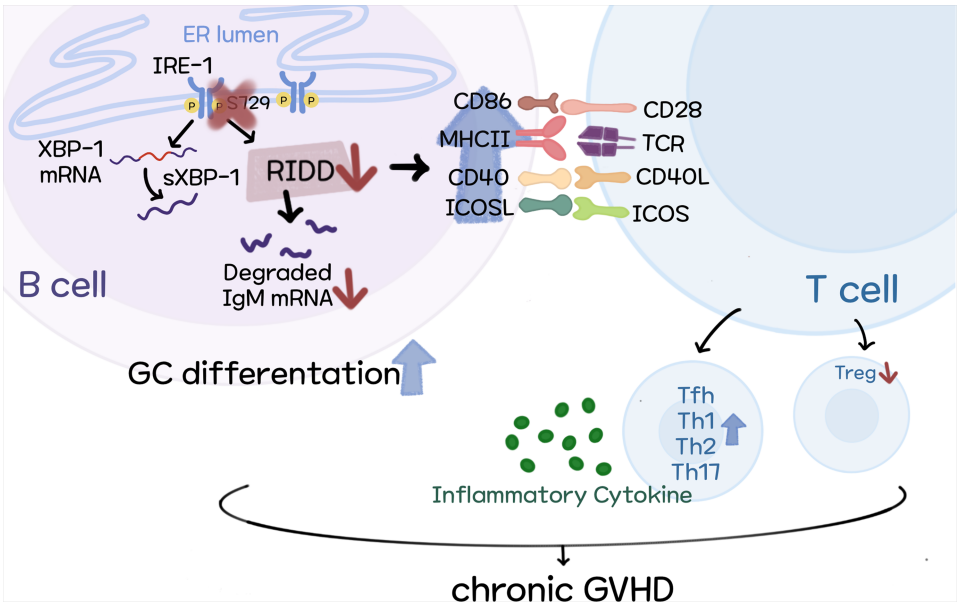

FIGURE 8 | Schematic diagram of our proposed model.

levels of WT B cells (**Figure 1**). We reason that IRE-1α-XBP-1s signaling is also needed to promote plasma cell differentiation in an RIDD-independent manner, thus insufficient differentiation lowers the IgM expression in IRE-1α KO or DKO B cells compared to WT counterparts. Increased splenic germinal centers that significantly enhance Tfh and GC B cell differentiation have been reported to be closely correlated with cGVHD development (31, 35). Moreover, inducible ICOS/ICOSL and CD40L/CD40 signaling between Tfh cells and B cells are essential to initiate a germinal center reaction and promote cGVHD (36, 37). We found that the expression of ICOSL and CD40 were repressed by XBP-1s deletion but restored in IRE-1α^{KO} or DKO B cells (**Figure 1**), suggesting that RIDD activation can suppress ICOSL and CD40 expression on B cells likely through an indirect mechanism. Accordingly, GC B cell and Tfh differentiation were also modulated by RIDD activation (**Figure 1** and **Supplementary Figure 4**).

Donor B cell-derived antibodies have been directly implicated in cGVHD progress by augmenting the fibrosis of target organs and inflammatory T cell infiltration (6, 38). RIDD has been shown to regulate IgM and IgG2b expression while IgG1 response relied on a different downstream element of IRE-1α/XBP-1s signaling (22). Besides, S729A mice with repressed RIDD activity have also been reported to produce increased serum IgM and IgG2b levels in response to immunization (23). Likewise, *in vitro* assay revealed that IgM expression was partially restored in IRE-1α^{KO} and DKO B cells (**Figures 1G–H**). In murine GVHD model, IgM production was more obviously restored in IRE-1α^{KO} and DKO B cells in transplanted recipients (**Figure 4I**) while IgG1 was curtailed to a level similar to the XBP-1^{KO} group (**Supplementary Figures 5A, B**). These data implicate that RIDD plays a critical role for plasma cells to reduce the production of IgM and certain subclasses of IgG in GVHD development. It has been suggested that deposition of IgG can lead to infiltration and activation of T cells and macrophages resulting in the cGVHD progress (6, 38). The role of secretory IgM on cGVHD has not been defined yet, however, it was reported that both IgG and IgM were frequently found in the basal epidermis in both acute and chronic GVHD patient biopsies, and their number was positively related to the degree of epidermal necrosis observed in histologic section (39). It has also been reported that patients with hyper IgM syndrome are prone to exposure the autoimmune disease (40, 41). Although one report suggests that inhibition of secretory IgM can induce more autoreactive IgG resulting in the more severe autoimmune disease in lupus-prone lymphoproliferative mice (42), it is possible that that sIgM may play a different role in cGVHD. In addition, the previous paper suggested sIgM suppressed the development of IgG, but we consider that increased IgG development may be because of the increased Ig class switching by restricting IgM secretion. In our mouse GVHD model, the recipients transferred with XBP-1s deficient B cells showed reduced sIgM expression, but they didn't show increased IgG1 expression (**Figure 4I** and **Supplementary Figure 5**). Since IgM is the main target of RIDD in B cells and their expression in serum positively correlated with the severity of the disease, we reason deposition of IgM may play an essential role in the development of cGVHD. Further studies are needed to determine this hypothesis.

Alloreactive T cells need to be primed by APCs to initiate GVHD, and specifically, CD86 and CD40 mediated-costimulation from APCs has been demonstrated to play an essential role in eliciting cGHVD (43, 44). In light of our *in vivo* and *in vitro* data on costimulatory molecules, RIDD

inhibition by deleting IRE-1α restored the expression of CD86 and CD40 in XBP-1s-deficient B cells (**Figure 1** and **4**). Besides, the expression of MHCII on B cells that present antigens to activate CD4 T cells was also modulated by RIDD (**Figures 1A**, **4E**). As a result, we demonstrated that alloreactivity of T cells, especially CD4 T cells, can be recovered by suppressing RIDD in XBP-1s-deficient B cells (**Figure 2**). It has been assumed that Th1 cells play a dominant role in acute GVHD, whereas Th2 cells are important for cGVHD. However, recent studies have reported that cytokines from Th1 (IFN-γ) and Th17 (IL-17) cells also play important roles in cGVHD progression (45, 46). Similar to previous reports, our data demonstrated that the differentiation of Th1, Th2, and Th17 was reduced upon interaction with XBP-1s-deficient B cells but restored by suppressing RIDD *via* further deleting IRE-1α, which in turn contributed to the severity of cGVHD (**Figure 5**). Besides, poor Treg reconstitution after allo-HCT has been suggested to result in the expansion of Th1 and Th17 cells that released proinflammatory cytokines and increased the risk of cGVHD (47, 48). We determined that CD28 mediated-Lck signaling suppresses the generation of iTreg (49). Based on these reports, we reason altered CD86 expression by RIDD increased CD28 costimulatory signaling in T cells, affected Treg differentiation during cGVHD development, and consequently influenced Th1 and Th17 differentiation (**Figure 5A**).

It has been reported that phosphorylation of the S729 residue in IRE-1α contributes to the upregulation of RIDD in XBP-1s-deficient B cells (23). We demonstrate that RIDD triggered by XBP-1s deletion could also be diminished by introducing the S729A mutation (**Figure 6D** and **Supplementary Figure 7F**). As a result, activation, differentiation, and alloreactivity of B cells were also restored in S729A/XBP-1^{KO} B cells (**Figure 6** and **Supplementary Figure 8**). RIDD inhibition with intact XBP-1s expression in S729A B cells resulted in much higher IgM and MHCII expression compared to WT, XBP-1^{KO} and S729A/XBP-1^{KO} B cells (**Figure 6** and **Supplementary Figure 7**), highlighting the role of RIDD in suppressing GVHD. Unexpectedly, S729A B cells showed reduced costimulatory factor expression and GC formation (**Figures 6B,C** and **Supplementary Figures 7A–E**). Since RIDD is important in maintaining basal ER homeostasis in B cells, we surmise that the increased accumulation of RIDD target molecules including IgM μ chain mRNA may compete for ribosomes and limit the expression of costimulatory factors in S729A B cells. Besides, since S729A B cells still produced XBP-1s, we interpret that these B cells may upregulate some chaperon genes under RIDD inhibition. Although S729 is located in the activation loop of IRE-1α, phosphorylation of IRE-1α is not essential for splicing the XBP-1 mRNA (23). Consistently, we also found that mutating the S729 phosphorylation site of IRE-1α did not limit B cells to produce XBP-1s. Altogether, we provide direct evidence showing that selective inhibition of RIDD activity of IRE-1α increases B cell pathogenicity in cGVHD induction (**Figure 7**).

To sum up, we demonstrate the mechanisms by which targeting XBP-1s alleviates cGVHD development. Our results indicate that activated RIDD resulted from XBP-1s deficiency may be responsible for reduced pathogenicity of B cells in the development of cGVHD, possibly through reducing IgM secretion and limiting B cell activation and differentiation (**Figure 8**).

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 The Institutional Animal Care & Use Committee.

AUTHOR CONTRIBUTIONS

Contribution: H-JC participated in research design, execution of experiments, statistical analysis and interpretation of data, and manuscript writing. C-HT generated mouse models, prepared mouse spleens and bone marrow for transplantation, interpreted data, and manuscript editing. LT, YW, MS, TT, and SS participated in conducting experiment and acquiring data. C-CH and X-ZY designed research, interpreted data, and edited 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.2021.705484/full#supplementary-material

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