CUTTING-EDGE TRANSLATIONAL RESEARCH IN GRAFT-VERSUS-HOST DISEASE (GVHD) AND GRAFT-VERSUS-TUMOR (GVT) EFFECT AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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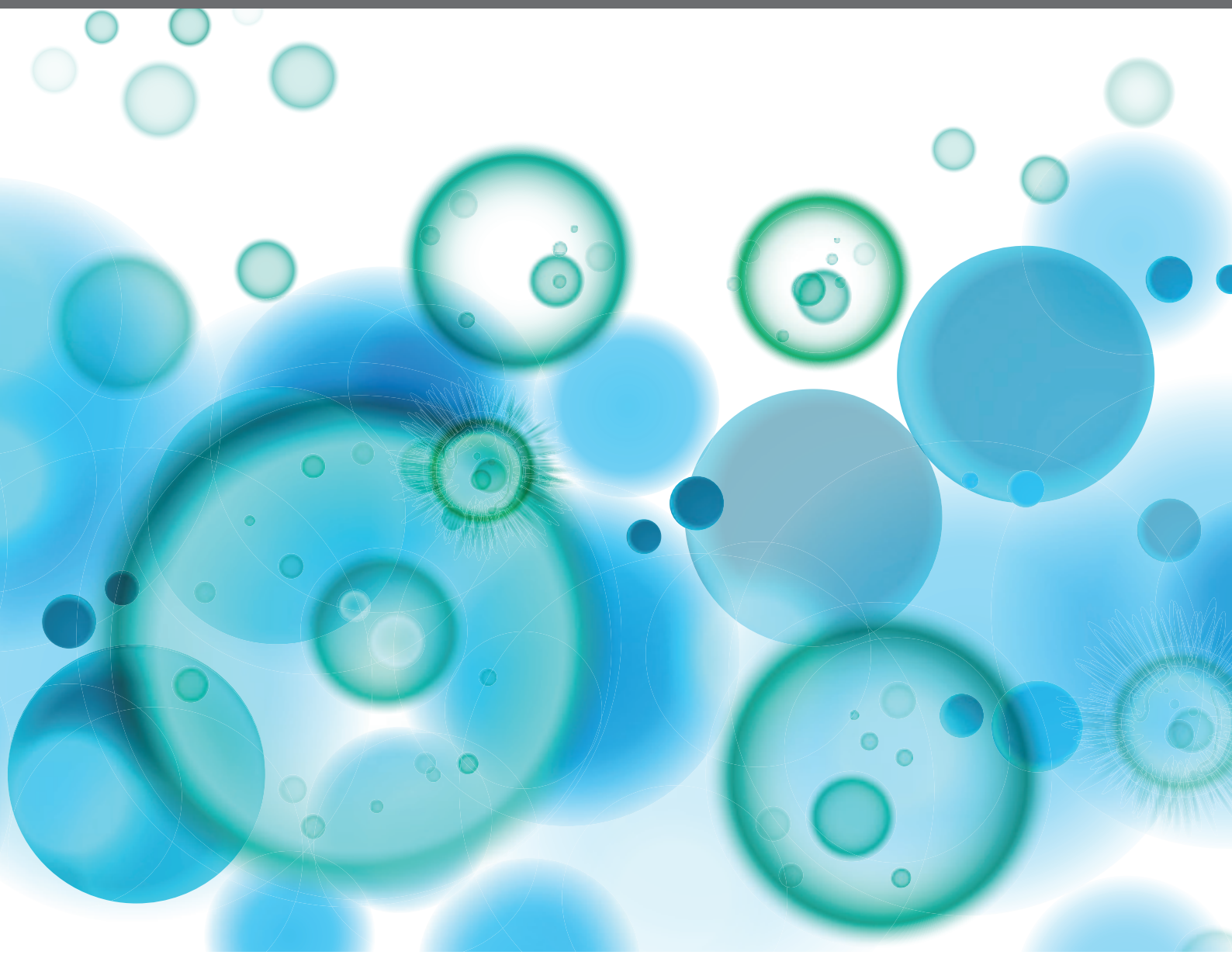

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CUTTING-EDGE TRANSLATIONAL RESEARCH IN GRAFT-VERSUS-HOST DISEASE (GVHD) AND GRAFT-VERSUS-TUMOR (GVT) EFFECT AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

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EDITORIAL
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Editorial on Translational Research in Graft-Versus-Host Disease (GVHD) and Graft-Versus-Tumor (GVT) Effect After Allogeneic Hematopoietic Cell Transplantation

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Editorial on the Research Topic

Cutting-Edge Translational Research in Graft-Versus-Host Disease (GVHD) and Graft-Versus-Tumor (GVT) Effect after Allogeneic Hematopoietic Cell Transplantation

Allogeneic hematopoietic cell transplantation (allo-HCT) is a highly effective treatment for hematological malignancies. However, the effect of allo-HCT is limited by the occurrence of acute graft-*versus*-host disease (GVHD), which is a life-threatening complication of allo-HCT that occurs in up to 50% of the patients (1).

In this Research Topic, the authors cover recent advances of pathophysiology, uncommon manifestations, prevention and treatment strategies for GVHD, as well as approaches to enhance graft-*versus*-tumor (GVT) activity. The authors also discuss the biology of GVHD in mouse models as well as aspects of clinical translation.

GVHD is mediated by alloreactive donor T cells, which recognize MHC molecules as foreign. While alloreactivity is causative for GVHD, it is also necessary to provide the beneficial GVT effect. Huang et al. describe how metabolic modulation of donor T cells could help to reduce GVHD without loss of GVT by targeting glycolysis. The beneficial effects of allo-HCT against malignant tumor cells is not restricted to hematological malignancies as Bates et al. for example explored allo-HCT as platform for the treatment of neuroblastoma. The authors assessed how combining immunocytokine treatment and *ex vivo* activated NK cell infusions could serve as intervention to provide GVT activity in a murine GD2+ neuroblastoma model. E*x vivo* expanded allogeneic T cells may also have activity in anti-viral immunity. Kim et al. describe how antigen specific T cells from donors recovered from Covid-19 can be expanded and manufactured to treat severe disease in partial HLA-matched recipients.

However, even though allo-HCT can be a life-saving intervention, the risk for developing GVHD remains an important point to consider. Research around the pathophysiology and the prevention of GVHD remains a key column for the success of allo-HCT. Due to the prominent role of alloreactive T cells in the induction of GVHD, Jiang et al. review current literature concerning the roles of different T cell subset and their respective cytokine signatures in the context of GVHD and GVT. They also outline preclinical data on the role of these subsets in both, GVHD and GVT effects, and subsequently address strategies to translate these findings to prevent GVHD in patients. Another factor that can be modified to reduce GVHD is the pre-transplant conditioning as it leads to release of danger signals (2). Based on the observation by some investigators that reduced conditioning was associated with higher relapse rates compared to full intensity conditioning (3, 4), Davis et al. elaborate on the potential benefits of repurposing ruxolitinib and venetoclax as pre-transplant medications to improve engraftment and GVT effects while reducing GVHD. Ruxolitinib was developed from the mouse model (5) into clinical application in first treatment series (6) and then in prospective phase III trials for acute and chronic GVHD (7, 8). Venetoclax, sorafenib (9) and other targeted therapies hold promise to enhance the GVT effect.

Besides the topic of strategies to reduce GVHD incidence and severity, this Research topic also covers risk factors that favor the development of GVHD. In their contribution to this series, Khuat et al. investigate how various parameters like the microbiome and high-fat diet, which are addressed using different mouse models, promote and exacerbate GVHD.

The pathophysiology of GVHD is based on a pro-inflammatory environment produced in the target organs, most prominently the skin, liver and gastrointestinal tract (GI). GI manifestations of GVHD however mostly contribute to reduced quality of life and mortality (10) and are mediated by T cells and neutrophils (11). Rayasam and Drobyski review the most foundational studies conducted in animal models that focus on preventing GI-GVHD and how these findings were translated into clinical applications. While the classical GVHD target organs are GI tract, liver and skin, increasing evidence suggests that also other organs such as the kidney, lungs or lymphatic tissues may be affected. In mouse models, T cells and microglia activation were shown to contribute to central nervous system (CNS)-GVHD (12, 13). Clinical studies on neurologic complications after allo-HCT describe the CNS as GVHD target organ (14, 15). However, also infections, vascular events, drug toxicity or other diseases may contribute to neurological symptoms like seizures or cognitive impairment (16). Vinnakota and Zeiser discuss data from mouse studies and clinical reports with a focus on how these findings increased biological understanding of underlying mechanisms and eventually may lead to novel therapy options for CNS-GVHD. Another non-classical clinical manifestation of GVHD is presenting itself as acute kidney injury (AKI). Drugs used as conditioning regimen pre allo-HCT, but also immunosuppressive drugs used to prevent GVHD are known to cause renal damage. However, renal diagnostic criteria are yet to be defined, as AKI often is the result of multiple etiologies (17). Therefore, Miyata et al. describe pathophysiology and management of kidney injury in the context of GVHD.

Mesenchymal Stromal Cell (MSC) products are a promising treatment that is under intensive investigation for GVHD. Kelly and Rasko discuss MSCs and GVHD in their contribution to this Research topic. The activity of MSCs is controversial, as different clinical studies showed responses to MSCs or failed to improve GVHD-related mortality (18, 19) which may be due to MSC preparation, transfer time point, GVHD severity or organ involvement. Murata et al. discuss two commercial MSC products and review clinical studies investigating outcome for patients.

This Research Topic presents recent advances in the field of translational research of GVHD and discuss how these advances are connected to increased mechanistic understanding of the underlying pathophysiology.

AUTHOR CONTRIBUTIONS

MC, RZ and TT performed literature research, discussed the articles and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Zeiser R, Blazar BR. Acute Graft-Versus-Host Disease - Biologic Process, Prevention, and Therapy. *N Eng J Med* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
2. Wilhelm K, Ganesan J, Müller T, Dürr C, Grimm M, Beilhack A, et al. Graft-Versus-Host Disease Enhanced by Extracellular Adenosine Triphosphate Activating P2X7R. *Nat Med* (2010) 12:1434–8. doi: 10.1038/nm.2242
3. Scott BL, Pasquini MC, Logan BR, Wu J, Devine SM, Porter DL, et al. Myeloablative Versus Reduced-Intensity Hematopoietic Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic Syndromes. *J Clin Oncol* (2017) 35(11):1154–61. doi: 10.1200/JCO.2016.70.7091
4. Wong E, Mason K, Collins J, Hockridge B, Boyd J, Gorelik A, et al. Prognostic Limitations of Donor T Cell Chimerism After Myeloablative Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic Syndromes. *Biol Blood Marrow Transplant* (2017) 23(5):840–4. doi: 10.1016/j.bbmt.2017.01.086
5. Spoerl S, Mathew NR, Bscheider M, Schmitt-Graeff A, Chen S, Mueller T, et al. Activity of Therapeutic JAK 1/2 Blockade in Graft-Versus-Host Disease. *Blood* (2014) 123:3832–42. doi: 10.1182/blood-2013-12-543736
6. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in Corticosteroid-Refractory Graft-Versus-Host Disease After Allogeneic Stem Cell Transplantation: A Multi-Center Survey. *Leukemia* (2015) 29:2062–8. doi: 10.1038/leu.2015.212

7. Zeiser R, Polverelli N, Ram R, Hashmi SK, Chakraverty R, Middeke JM, et al. Ruxolitinib for Glucocorticoid-Refractory Chronic Graft-Versus-Host Disease. *N Eng J Med* (2021) 385:228–38. doi: 10.1056/NEJMoa2033122
8. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *N Eng J Med* (2020) 382:1800–10. doi: 10.1056/NEJMoa1917635
9. Mathew NR, Baumgartner F, Braun L, O´Sullivan D, Thomas S, Waterhouse M, et al. Sorafenib Promotes Graft-Versus-Leukemia Activity in Mice and Humans Through IL-15 Production in FLT3-ITD Mutant Leukemia Cells. *Nat Med* (2018) 24:282–91. doi: 10.1038/nm.4484
10. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-Versus-Host Disease. *Lancet* (2009) 373(9674):1550–61. doi: 10.1016/S0140-6736(09)60237-3
11. Schwab L, Goroncy L, Palaniyandi S, Gautam S, Triantafyllopoulou A, Mocsai A, et al. Neutrophil Granulocytes Recruited Upon Translocation of Intestinal Bacteria Enhance GvHD *via* Tissue Damage. *Nat Med* (2014) 20:648–54. doi: 10.1038/nm.3517
12. Mathew NR, Vinnakota JM, Apostolova P, Erny D, Hamarsheh S, Andrieux G, et al. Graft-Versus-Host Disease of the CNS is Mediated by TNF Upregulation in Microglia. *J Clin Invest* (2020) 130:1315–29. doi: 10.1172/JCI130272
13. Hartrampf S, Dudakov JA, Johnson LK, Smith OM, Tsai J, Singer NV, et al. The Central Nervous System is a Target of Acute Graft Versus Host Disease in Mice. *Blood* (2013) 121:1906–10. doi: 10.1182/blood-2012-09-456590
14. Ke P, Bao X, Zhou J, Zhu Q, Zhuang J, Hu X, et al. Central Nervous System Complications After Allogeneic Hematopoietic Stem Cell Transplantation in Children. *Acta Haematol* (2019) 142(4):217–23. doi: 10.1159/000499651
15. Sheikh MA, Toledano M, Ahmed S, Gul Z, Hashmi SK. Noninfectious Neurologic Complications of Hematopoietic Cell Transplantation: A Systematic Review. *Hematol Oncol Stem Cell Ther* (2021) 14(2):87–94. doi: 10.1016/j.hemonc.2020.05.006
16. Siegal D, Keller A, Xu W, Bhuta S, Kim DH, Kuruvilla J, et al. Central Nervous System Complications After Allogeneic Hematopoietic Stem Cell Transplantation: Incidence, Manifestations, and Clinical Significance. *Biol Blood Marrow Transplant* (2007) 13(11):1369–79. doi: 10.1016/j.bbmt.2007.07.013
17. Mariotti J, Penack O, Castagna L. Acute Graft-Versus-Host-Disease Other Than Typical Targets: Between Myths and Facts. *Transplant Cell Ther* (2021) 27(2):115–24. doi: 10.1016/j.bbmt.2020.09.033
18. Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. MSC-Derived Exosomes: A Novel Tool to Treat Therapy-Refractory Graft-Versus-Host Disease. *Leukemia* (2014) 28:970–3. doi: 10.1038/leu.2014.41
19. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of Severe Acute Graft-Versus-Host Disease With Third Party Haploidentical Mesenchymal Stem Cells. *Lancet* (2004) 363:1439–41. doi: 10.1016/S0140-6736(04)16104-7

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Combining Immunocytokine and *Ex Vivo* Activated NK Cells as a Platform for Enhancing Graft-Versus-Tumor Effects Against GD2$^+$ Murine Neuroblastoma

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Management for high-risk neuroblastoma (NBL) has included autologous hematopoietic stem cell transplant (HSCT) and anti-GD2 immunotherapy, but survival remains around 50%. The aim of this study was to determine if allogeneic HSCT could serve as a platform for inducing a graft-versus-tumor (GVT) effect against NBL with combination immunocytokine and NK cells in a murine model. Lethally irradiated C57BL/6 (B6) x A/J recipients were transplanted with B6 bone marrow on Day +0. On day +10, allogeneic HSCT recipients were challenged with NXS2, a GD2$^+$ NBL. On days +14-16, mice were treated with the anti-GD2 immunocytokine hu14.18-IL2. In select groups, hu14.18-IL2 was combined with infusions of B6 NK cells activated with IL-15/IL-15Rα and CD137L *ex vivo*. Allogeneic HSCT alone was insufficient to control NXS2 tumor growth, but the addition of hu14.18-IL2 controlled tumor growth and improved survival. Adoptive transfer of *ex vivo* CD137L/IL-15/IL-15Rα activated NK cells with or without hu14.18-IL2 exacerbated lethality. CD137L/IL-15/IL-15Rα activated NK cells showed enhanced cytotoxicity and produced high levels of TNF-α *in vitro*, but induced cytokine release syndrome (CRS) *in vivo*. Infusing Perforin$^{-/-}$ CD137L/IL-15/IL-15Rα activated NK cells had no impact on GVT, whereas TNF-α$^{-/-}$ CD137L/IL-15/IL-15Rα activated NK cells improved GVT by decreasing peripheral effector cell subsets while preserving tumor-infiltrating lymphocytes. Depletion of Ly49H$^+$ NK cells also improved GVT. Using allogeneic HSCT for NBL is a viable platform for immunocytokines and *ex vivo* activated NK cell infusions,

but must be balanced with induction of CRS. Regulation of TNFα or activating NK subsets may be needed to improve GVT effects.

Keywords: immunocytokine, NK cells, neuroblastoma, graft-versus-tumor effect, cytokine release syndrome

INTRODUCTION

Neuroblastoma (NBL) is the most common extracranial solid tumor that occurs in children. For children with tumors that either have high risk biologic features or with metastatic disease, overall survival is still poor despite an aggressive treatment regimen that includes chemotherapy, surgery, autologous hematopoietic stem cell transplant (HSCT), radiation, and maintenance therapy with cis-retinoic acid (1). The addition of the monoclonal antibody (moAb) dinutuximab (which targets the NBL-associated antigen GD2), interleukin-2 (IL-2) and granulocyte-monocyte colony stimulating factor (GM-CSF) improved event-free and overall survival (2), but is not curative for the majority of patients who will ultimately relapse and die. Another treatment approach is needed that can potentially improve survival further and lead to a long-term cure.

Allogeneic HSCT was initially used in children with NBL about 30 years ago with some reports of clinical responses (3), but was never shown superior to autologous HSCT (4–6). Because there has not been convincing evidence of a graft-versus-tumor (GVT) effect against NBL (6–8), and because allogeneic HSCT introduces the life-threatening potential for graft-versus-host-disease (GVHD), autologous HSCT remains the standard of care. In fact, current protocols are incorporating tandem autologous HSCTs as consolidative therapy to improve event-free survival (9). Because of both preclinical evidence (10, 11) and case reports suggesting some clinical benefit of allogeneic HSCT in NBL, particularly in the haploidentical setting (12, 13), the objective of this preclinical study was to incorporate haploidentical HSCT as a platform for a combined immunotherapy regimen to enhance the GVT effect against NBL.

Until 2019, dinutuximab was given with GM-CSF and IL-2 in the Children's Oncology Group (COG) as separate treatments as part of upfront maintenance therapy for NBL. Due to excessive toxicity associated with systemic IL-2 administration without clear added benefit, COG eliminated usage of IL-2 and now dinutuximab and GM-CSF are used. One means by which to maintain the beneficial activation of IL-2 for antibody-dependent cellular cytotoxicity (ADCC) without systemic toxicity is to restrict its use to the immune synapse. Hu14.18-IL2 is a fusion protein consisting of human IL-2 genetically linked to the carboxyl-termini of each human IgG1 heavy chain of the GD2-specific hu14.18 moAb. This "immunocytokine" (IC) provides a local source of IL-2 at the immunological synapse between the effector cell and the NBL, activating immunity against $GD2^+$ tumors. Hu14.18-IL2 has been used in both phase I and phase II trials in children with refractory NBL and melanoma, with reversible toxicities and complete responses observed in both phase II NBL trials (14, 15). However, hu14.18-IL2 therapy is not curative when used as a single agent to treat macroscopic refractory or recurrent NBL, and has never been tested after allogeneic HSCT. The mechanism of action for hu14.18-IL2 is thought to be, at least in part, from ADCC from natural killer (NK) cells (16).

Because of the availability of clinical grade cytokines and artificial antigen presenting cells (aAPCs), infusion of high numbers of purified, *ex vivo* activated NK cells are emerging from preclinical models into clinical trials. NK cells have already been shown to have cytotoxicity *in vitro* against a variety of NBL cell lines (17) and primary patient tumors (18, 19) as well as *in vivo* with xenograft NBL models (20). In addition, the lymphopenic environment induced from the conditioning regimen for allogeneic HSCT is conducive for NK cell expansion given the presence of high levels of IL-15 (21). Lastly, NK cells produce growth factors like IL-1β, IL-6, G-CSF and GM-CSF that can support engraftment (22).

NK cells possess inhibitory receptors on their cell surface that can "turn off" the cells when they engage major histocompatibility complex (MHC) antigens (23, 24). Our current standard of administering an anti-GD2 moAb (dinutuximab) after autologous HSCT is limited in that the patient's own NK cells must engage the antibody to eliminate the tumor, and risk engaging self-MHC on the tumor that could "turn off" the NK cell. In fact, two studies in children with NBL who were treated with anti-GD2 based therapies (one with hu14.18-IL2 and one with the moAb 3F8) reported a better response to therapy in those patients that were self-killer immunoglobulin-like receptor (KIR)/KIR ligand mismatched (25, 26), something that can be easily achieved if NK cells came from an appropriately selected allogeneic donor. In this study, we explore haploidentical allogeneic HSCT in NBL-bearing mice as a means of insuring that some of the inhibitory Ly49 receptors on donor murine NK cells do not engage their cognate MHC ligand, potentially "turning on" the NK cells and maximizing anti-tumor activity after hu14.18-IL2 IC administration.

MATERIALS AND METHODS

Mice

C57BL/6NCr (B6, H-2^b), Balb/cAnNCr (Balb/c, H-2^d), CB6F1/Cr (CB6F1, H-$2^{b\ x\ d}$), B6Ly5.2/Cr (CD45.1^+ B6, H-2^b), A/JCr (A/J, H-2^a), and DBA/2NCr (DBA, H-2^d) mice were purchased from the National Cancer Institute (NCI) Animal Production Program and Charles River Laboratories International (Frederick, MD). B6AJF1 (H-$2^{b\ x\ a}$), C57BL/6-Prf1<tm1Sdz>/J (Perforin$^{-/-}$, H-2^b) and B6.129S-Tnf<tm1Gkl>/J (TNFα$^{-/-}$, H-2^b) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were female and used between 8 and 16 weeks of age. All animals were housed in a pathogen-free facility throughout the study. The Animal Care and

Use Committees (ACUC) at the University of Wisconsin (M005915, M01246) and National Institutes of Health (PB027) approved all protocols.

Bone Marrow Transplantation (BMT)

On BMT Day +0, bone marrow (BM) cells were harvested from donor mice and T cell depleted as previously described (27). BM recipients were lethally irradiated with a single fraction of 1100 rads (B6AJF1) or 1000 rads, (B6), 800 rads divided in two 400 rad fractions separated 4 hours apart (Balb/c) or 750 rads divided into two 375 rad fractions separated 4 hours apart (A/J). Irradiated BM recipients were then injected intravenously (i.v.) with 5 x 10^6 CD3-depleted BM cells in serum-free RPMI (Invitrogen, Carlsbad, CA). In select groups, T cells from donor mice were isolated from spleens using magnetic cell selection (Miltenyi Biotec, Auburn, CA), and injected i.v. with the BM. Mice were weighed individually biweekly, and the mean weight of each treatment group was calculated at each time point and compared with the day +0 weight. GVHD was monitored using a clinical scoring system (28). Veterinarians and veterinary technicians who were blinded to the experimental groups examined for moribund mice, and assessed the mice daily in accordance with approved institutional protocols.

Tumor Cell Lines

NXS2 is a murine $GD2^+$ NBL cell line on an $H\text{-}2^a$ background (29), and was obtained from Ralph Reisfeld (Scripps Research Institute). N18TG2 is also murine $GD2^+$ NBL cell line on an $H\text{-}2^a$ background and was obtained from Sigma-Aldrich, Inc (St. Louis, MO). Neuro-2a is a murine $GD2^-$ NBL cell line on an $H\text{-}2^a$ background, and was obtained from ATCC (Manassas, VA). 9464D is a murine $GD2^+$ NBL cell line on an $H\text{-}2^b$ background, and was obtained from Jon Wigginton (while previously at the National Cancer Institute, Frederick, MD). Yac-1 is a murine B cell lymphoma cell line on an $H\text{-}2^a$ background and was obtained from ATCC. A20 is a murine B cell lymphoma cell line on an $H\text{-}2^d$ background and was obtained from ATCC. Cell authentication was performed using short tandem repeat analysis (Idexx BioAnalytics, Westbrook, ME) and per ATCC guidelines using morphology, growth curves, and *Mycoplasma* testing within 6 months of use using the e-Myco mycoplasma PCR detection kit (iNtRON Biotechnology Inc, Boca Raton, FL). All tumor cells were maintained in culture at 37°C in 5% CO_2 in Complete Mouse Media (CMM), and used after 3-5 passages in culture after thawing.

In Vivo Tumor Challenge

Exponentially growing NBL tumor cells were prepared as a single cell suspension in serum-free RPMI and injected into the subcutaneous fat of the shaved flank at a dose of 2 × 10^6 tumor cells on day +10 after HSCT. Tumors were measured in 2 dimensions (length × width) 1-2 times a week by digital caliper. Tumor growth = length x width (mm^2). Mice were euthanized with CO_2 when tumor diameters reached 2 cm in any dimension, in accordance with animal protocols. If a mouse was found dead, the previously recorded tumor measurement was carried for the rest of the data points of the experiment for the purposes of statistical comparison. Exponentially growing A20 tumor cells were prepared as a single cell suspension in serum-free RPMI and injected as 2.5 x 10^6 cells i.v. on Day +5 into Balb/c mice.

NK Cell Isolation and Activation

NK cells were purified from single cell suspensions of spleens using magnetic cell selection (Miltenyi Biotec) and placed into CMM and 10ng/mL recombinant IL-15/IL15Rα complex (eBioscience, San Diego, CA) at 37°C in 5% CO_2. Because IL-15 is typically presented in trans by IL-15Rα, the complex was utilized to potently increase IL-15 bioactivity. Artificial antigen presenting cells (aAPCs) consisted of irradiated (10,000 rads) Yac-1 cells or Yac-1 cells transfected with CD137L (Yac1-CD137L) (30). For *in vivo* infusions, NK cells were incubated with aAPCs at a 1:1 ratio for 1 week, then washed in PBS and resuspended in serum-free RPMI prior to injection. IL-15/IL-15Rα was replaced two times per week.

Adoptive NK Cell Infusions and Immunocytokine Therapy

In select experiments, allogeneic HSCT recipients were treated on day +14 with *ex vivo* IL-15/IL-15Rα or CD137L/IL-15/IL-15Rα activated NK cells. On days 14-16, select groups were also treated with either PBS or 50mcg hu14.18-IL2 i.v. (Apeiron Biologics, Vienna, Austria) alone or in combination with CD137L/IL-15/IL-15Rα NK cells.

$Ly49H^+$ NK Depletion

$Ly49H^+$ NK cells were depleted using a purified 3D10 clone (Biolegend Cat # 144704) (31). Basically, B6AJF1 mice were transplanted as above with B6 BM and challenged with NXS2 tumors on Day +10. On Day +12, 48hrs before NK injection, 200ug of anti-Ly49H or IgG1 isotype control was given IP per mouse. On Day +14, 1 x 10^6 CD137L/IL-15/IL-15Rα NK cells were infused IV per mouse with 50ug hu14.18-IL2, and mice were followed for tumor growth.

Flow Cytometric Analysis

In brief, 1 × 10^6 freshly isolated, erythrocyte-depleted splenocytes, lymph node, BM cells, or expanded NK cells were stained at 4°C for 20 minutes with a monoclonal antibody cocktails containing either NK1.1-PerCP Cy5.5 (Cat # 108728) or NK1.1-PE (Cat # 108708) (BioLegend, San Diego, CA), Ly49C/I-FITC (Cat # 553276), Ly49H-FITC (Cat # 562536) (BD-Biosciences, San Jose, CA) or Ly49H-PE-Cy7 (Cat # 144714, BioLegend), B220-BV421 (Cat # 103251, BioLegend), CD4-eFluor 450 (Cat # 48-0048-42, Thermo Fisher Scientific), CD45.2-PerCP Cy5.5 (Cat # 109828, BioLegend) or CD45.2-FITC (Cat # 109806, BioLegend), CD8-PE (Cat # 100708, BioLegend) or CD8-APC (Cat # 100712, BioLegend), CD45.1-APC (Cat # 110714, BioLegend) or CD45.1-Pacific Blue (Cat # 110722, BioLegend), GD2 PE (Cat# 357304, BioLegend), FasL-APC (Cat # 106610, BioLegend), and TRAIL-PerCP-Cy5.5 (Cat # 109314, BioLegend) and then washed in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered salt solution with 0.2% fetal calf serum and 0.1% sodium azide). For degranulation

and intracellular cytokine analysis, expanded NK cells were incubated with or without PMA (50 ng/ml) and ionomycin (1 ug/ml) for 1 hour 37°CC in 5% CO_2. Then GolgiSTOP (monesin) and GolgiPLUG (brefeldin A) were added and the cells were incubated for an additional 4 hours. Cells were then harvested and stained with surface monoclonal antibodies. This was followed by fixation and permeabilization using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (Cat # 554714, BD) and staining with monoclonal antibody TNFα-AF647 (Cat # 506314, BioLegend). Flow cytometry data was acquired on a MACSQuant analyzer 10 (Miltenyi Biotec) and mqd files were converted to fcs files using The MACSQuantify™ Software or Attune NxT flow cytometer (Thermo Fisher). Listmode data were analyzed using FlowJo software (FlowJo, Ashland, OR).

Cytokine Production

For *in vitro* studies, NK cells were expanded with 10ng/mL IL-15/IL-15Rα alone or with Yac1-CD137L and IL-15/IL-15Rα for 1 week, then cultured at 1 x 10^6 cells/ml in CMM at 37°CC in 5% CO_2 for 3 hours. Supernatants were harvested and analyzed by enzyme-linked immunosorbent assays (ELISA) for murine TNFα (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. ELISA plates were read on a VersaMAX Microplate Reader at 450nm and analyzed using SoftMAX Pro 5 reader (Molecular Devices, Sunnyvale, CA). For *in vivo* studies, allogeneic HSCT mice had peripheral blood collected by heel stick. Serum was isolated and frozen at -20°C until used in a V-Plex Plus Pro-Inflammatory Panel 1 mouse kit according to manufacturer's directions (Meso Scale Diagnostics, Rockville, MD). Samples were run in duplicate on a MesoQuickplex SQ 120 multiplex cytokine analyzer (Meso Scale Diagnostics).

Cytotoxicity Assays

Cytotoxicity is performed using a Promega CytoTox 96 Non-Radioactive Cytotoxicity assay. Cytotoxic activity is colorimetrically measured by the amount of lactate dehydrogenase (LDH) released by the cells plated within a 96 well plate. Color formed by lysed cells is measured by wavelength absorbance (490nm). Cells plated in the assay are a ratio concentration of effector cells (NK cells) to target cells (tumor cells) diluted 2-fold starting at 20:1 to 5:1 effector:target (E:T) ratio. Effectors and target cells were co-incubated for 4 hours at 37°C before measuring wavelength absorbances on a VersaMAX Microplate Reader. Spontaneous release was determined by adding 100 µl of media to 100 µl of tumor cells. Maximum LDH release was determined by adding 100 µL of 1X-Triton X-100 detergent (Sigma-Aldrich, cat#: 9002-93-1) to tumor cells. Specific LDH release was calculated as: % lysis = 100% x (Experimental–Spontaneous)/(Maximum–Spontaneous). Additional cytotoxicity assays were performed using a calcein-AM release assay. Cytotoxic activity was measured by the amount of calcein released from lysed target cells plated within a 96 well plate. Cells were plated in the assay at a 5:1 E:T ratio. Following co-incubation for 4 hours at 37°C, supernatant calcein signal was measured using a fluorescent plate reader at 495/515 nm. Maximum calcein release was determined by adding 100 µL of 1X-Triton X-100 detergent to tumor cells and % lysis was calculated as above.

Statistical Analysis

Statistics were performed using GraphPad Prism version 9.0 for the Macintosh OS (GraphPad Software, San Diego, CA). Significant differences when comparing 2 groups were determined by the 2-tailed Mann–Whitney test or unpaired t test with Welch's correction. The Kruskal–Wallis with Dunn's multiple-comparison post-test was used to assess statistical differences among 3 or more groups. Survival analysis was plotted according to the Kaplan-Meier method, and statistical differences were determined with the log-rank test. A p value less than 0.05 was considered statistically significant.

RESULTS

Because IC have never been used after allogeneic HSCT, we established a MHC-mismatched haploidentical allogeneic HSCT model (H-2^b ➔ H-2^b x H-2^a) whereby lethally irradiated B6AJF1 recipients were transplanted with T cell depleted B6 BM and 0 – 2.5 x 10^6 B6 T cells on day +0 (**Figure 1A**). Because the donor and host cells are MHC-mismatched (in the GVH direction), the presence of T cells in the BM graft leads to weight loss (**Figure 1B**) and lethal GVHD in less than 30 days (**Figure 1C**). The addition of IC following such a transplant is safe in the absence of T cells (**Figure 1C**), but in the presence of 2.5 x 10^6 T cells there is still GVHD lethality (**Figures 1C, D**). Decreasing the amount of T cells in the donor graft reduces GVHD lethality (**Figure 1D**), suggesting there is a T cell threshold where one could safely administer IC. In fact, 2.5 x10^2 and 2.5 x 10^3 T cells are well tolerated with IC and do not induce lethal GVHD (**Figure 1D**). Analysis of immune reconstitution shows a marked decrease in $B220^+$ B cells in allogeneic HSCT recipients of 2.5 x 10^6 T cells (**Figure 1E**), a surrogate of GVHD in other murine allogeneic HSCT models (32, 33), as well as marked decreases in NK cells (**Figure 1F**) and $CD8^+$ T cells (**Figure 1G**), the cells that would typically respond to IC bound to tumor (16, 25). Both T cell depleted grafts and T cell replete grafts generate a low percentage of regulatory T cells (**Figure 1H**).

While human neuroblastomas ubiquitously express GD2, murine neuroblastomas show variable expression of GD2 (**Figure S1A**). NXS2 was selected so that allogeneic donor cells could be used from a C57BL/6 background, allowing for potential usage of knockout mice as NK donors in future experiments. Because children with solid tumors have the best outcomes when transplanted in remission, $GD2^+$ NXS2 inoculation was performed on Day +10 after HSCT to mimic tumor relapse post-HSCT. Initially we compared syngeneic and allogeneic HSCT and found that both groups developed tumors, but NXS2 tumors in allogeneic HSCT recipients were smaller (**Figure S1B**), supporting rationale for a GVT effect in this model. We next examined allogeneic HSCT recipients (with add back of a nonlethal dose of T cells in the graft to avoid lethal GVHD but maintain some residual dose to mimic clinical T cell depletion). We performed NXS2 inoculation on Day +10, followed by 3 doses of IC on Days +14-16 (**Figure 2A**) to provide

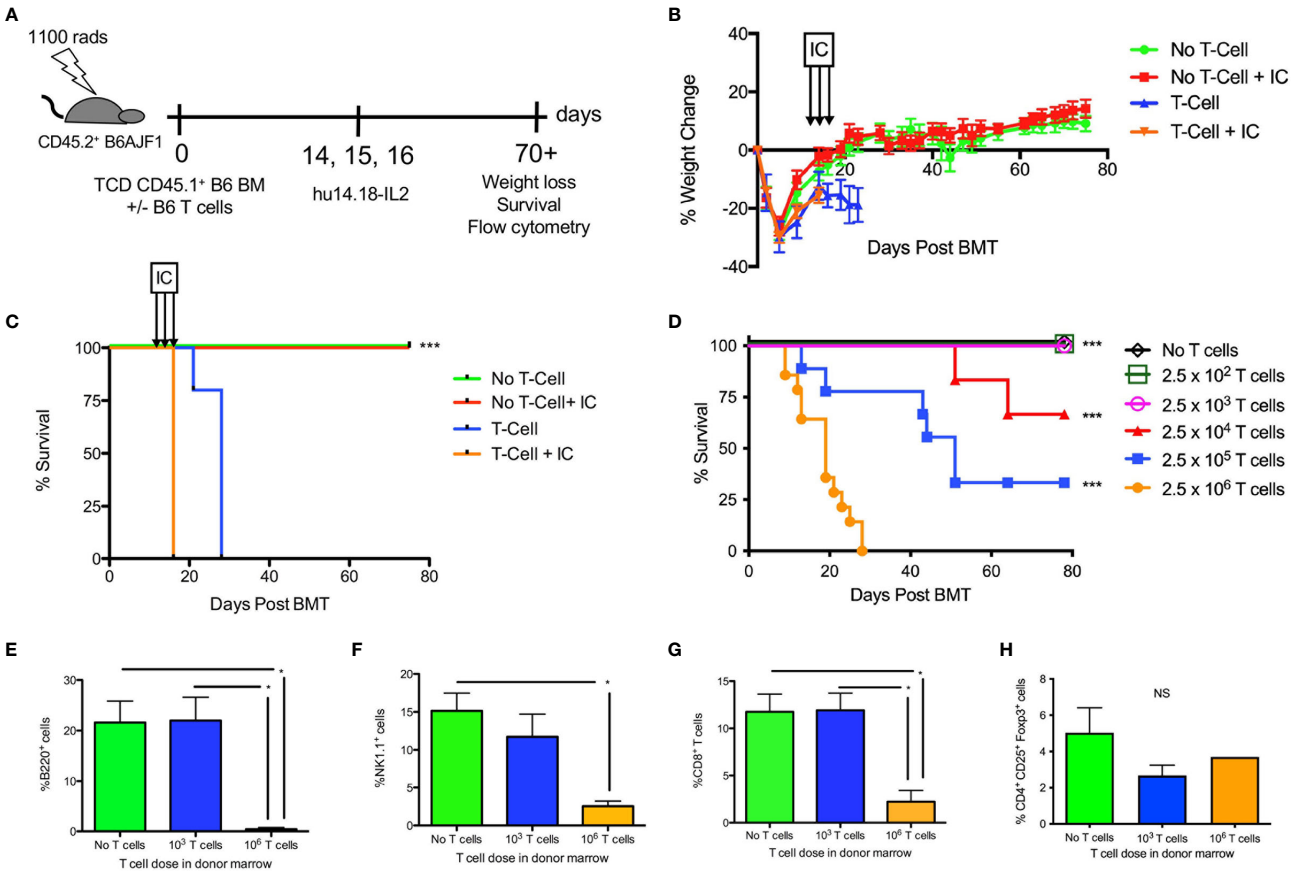

FIGURE 1 | Effect of hu14.18-IL2 after T cell deplete and replete allogeneic HSCT. **(A)** Lethally irradiated CD45.2$^+$ B6AJF1 mice (H-2$^{b\ x\ a}$) were transplanted with: either CD3e cell depleted (No T cell) or CD3e depleted BM replenished with 2.5 x 10^6 T cells (T cell) from congenic CD45.1$^+$ B6 mice (H-2^b) on Day +0. On days +14-16, PBS or hu14.18-IL-2 (IC) was administered and allogeneic HSCT recipients were followed for **(B)** GVHD-associated weight loss and **(C)** survival. N=5 mice/group. The no T cell group was compared to the corresponding T cell group. **(D)** Lethally irradiated CD45.2$^+$ B6AJF1 mice were transplanted with either no T cells or CD3e depleted BM from congenic CD45.1$^+$ B6 mice replenished with logarithmically increasing doses of T cells (2.5 x 10^2-10^6) on Day +0. On days +14-16, IC was administered and allogeneic HSCT recipients were followed for survival. Each group was compared to the 2.5 x 10^6 T cell group. Results pooled from 2 similar experiments, 5-10 mice/group. **(E)** Lethally irradiated CD45.2$^+$ B6AJF1 mice were transplanted with either no T cells or CD3e depleted BM from congenic CD45.1$^+$ B6 mice replenished with no T cells, 2.5 x 10^3 or 10^6 T cells on Day +0. On days +14-16, IC was administered and allogeneic HSCT recipients were sacrificed at Day +21 for flow cytometric analysis of B cells, **(F)** NK cells, **(G)** CD8$^+$ T cells and **(H)** CD4$^+$ regulatory T cells in the spleen. Results pooled from 2 similar experiments, 5-10 mice/group. NS, not significant *p < 0.05, ***p < 0.001.

anti-GD2 tumor targeting for donor cells from the graft. Without IC, NXS2 tumors became large (**Figure 2B**). Administering IC significantly enhances the GVT effect by reducing tumor growth after T cell replete allogeneic HSCT, but small tumors still develop (**Figure 2B**). No differences were seen after T cell depleted allogeneic HSCT (**Figure 2B**), suggesting both donor T and NK cells are needed for optimal GVT effects of the IC. Importantly the IC mediates GVT without exacerbating GVHD (**Figure 2C**) after T cell replete allogeneic HSCT. *Ex vivo* activation of additional effector cells (e.g. donor-derived NK cells) that can recognize the tumor as allogeneic and/or respond to the IC *via* ADCC may enhance the GVT effect and potentially prevent tumor growth entirely.

Human aAPCs that express the co-stimulatory molecule 4-1BB ligand (CD137L) have been shown to potently expand and activate human NK cells (34–37), however has not been explored on murine NK cells. Using a murine aAPC transfected with CD137L in the presence of IL-15/IL-15Rα expands purified murine NK cells *ex vivo* (**Figure 3A**), with the highest yields after 1 week at a 1:1 ratio of NK:aAPC (**Figure 3B**). Activating NK cells with the CD137L$^+$ aAPC without IL-15/IL-15Rα is insufficient to sustain NK cell growth (data not shown). The purity of NK cells after 7 days of *ex vivo* expansion is 90% (**Figure 3C**). While the percentage and absolute numbers of NK cells increase after *ex vivo* expansion, the percentage of NK cell subsets within that expanded population also changes. There is a mild but statistically significant increase in the percentage of Ly49C+I$^+$ NK cells after *ex vivo* activation with IL-15/IL-15Rα alone or with CD137L/IL-15/IL-15Rα compared to unexpanded NK cells (**Figure 3D**). In contrast, we did not see any differences in the percentage of Ly49H$^+$ NK cells after *ex vivo* activation (**Figure 3E**). *Ex vivo* activation also occurs, as evidenced by enhanced NK cytotoxicity as measured by potency assays *in vitro* (**Figure 3F**) and *in vivo* (**Figure S2**), and augmented TNF-α

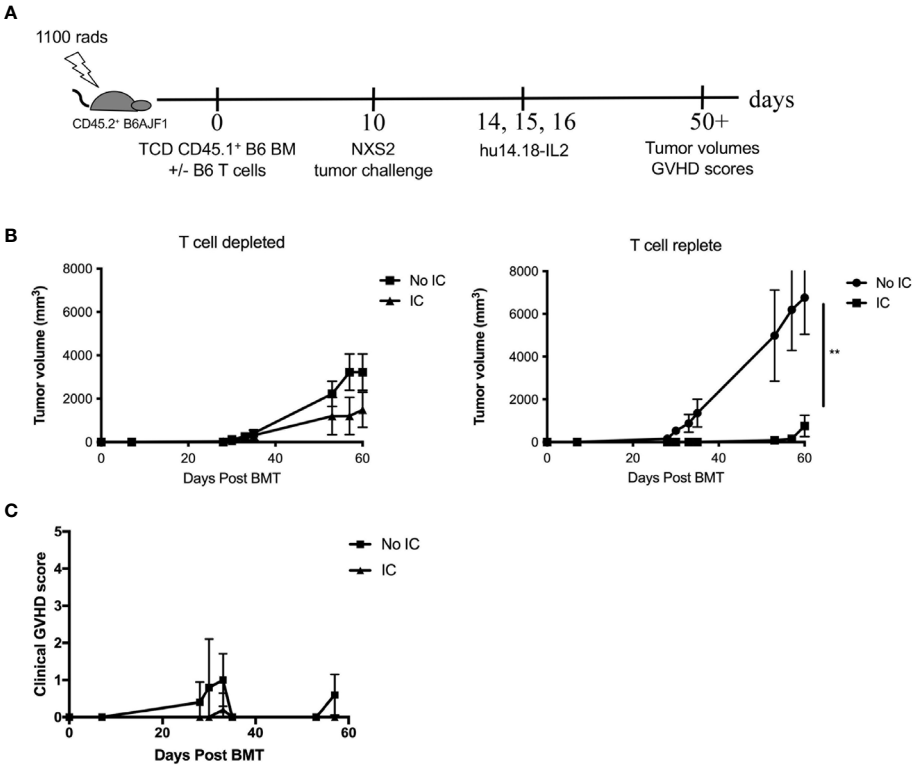

FIGURE 2 | GD2$^+$ NXS2 neuroblastoma growth after allogeneic HSCT and hu14.18-IL2. **(A)** Lethally irradiated CD45.2$^+$ B6AJF1 mice (H-2$^{b \times a}$) were transplanted with either CD3e cell depleted (T cell depleted) or CD3e depleted BM replenished with 2.5 x 10^3 T cells (T cell replete) from congenic CD45.1$^+$ B6 mice (H-2^b) on Day +0. On Day +10, 2 x 10^6 NXS2 tumor cells were inoculated into the right flank. On days +14-16, PBS (No IC) or hu14.18-IL-2 (IC) was administered and **(B)** tumor growth was monitored by using a digital caliper as well as **(C)** clinical GVHD scores. N=5 mice/group. ** p = 0.01.

production (**Figure 3G**). There are no significant changes in the percentage of cytotoxic (TRAIL$^+$, FasL$^+$ or CD107a$^+$) or TNF-α producing Ly49 NK subsets (**Figure S3**). Interestingly, *ex vivo* activated (H-2^b) NK cells demonstrate lysis of various syngeneic (H-2^b: 9464D) and allogeneic murine NBL cell lines (H-2^a: Neuro-2A, N18TG2, NXS2), however no significant improvement is seen with the addition of IC *in vitro* (**Figure 3H**). Because GVT/GVHD is a complex phenomenon that cannot be recapitulated *in vitro*, this observation led us to test if there were characteristics of the allogeneic HSCT milieu that could enhance a NK-mediated GVT effect with the addition of IC against GD2$^+$ NBL *in vivo*.

During allogeneic HSCT, the GVT effect is mediated by T cells and NK cells while GVHD is mainly mediated by α/β$^+$ T cells. To determine the contribution of NK alloreactivity to a GVT effect without contribution of donor T cells, we designed a F1 into parent allogeneic HSCT model so that (1) any residual donor T cells in the BM graft would be tolerized to host MHC and minor histocompatibility antigens in the thymus and thus not mediate GVHD (38), and (2) donor NK cells could still mediate alloreactivity since the host would lack cognate MHC ligands needed to engage donor Ly49 inhibitory receptors (39) (**Figure 4A**). When we infused F1 NK cells into one parent strain (H-2^{bxd} ➔ H-2^b), we observed that allogeneic *ex vivo* activated NK cells could mediate a mild weight loss (**Figure 4B**), but no lethality was observed (data not shown). Lethality was observed after infusion of F1 NK cells into the other parent strain (H-2^{bxd} ➔ H-2^d), with significantly more lethality observed with NK cells activated with CD137/IL-15/IL-15Rα after allogeneic HSCT than infusing NK cells activated with IL-15/IL15Rα alone (**Figure 4C**), indicating the contribution of CD137L during NK expansion and host MHC molecules in driving toxicity. More weight loss was seen with CD137/IL-15/IL-15Rα NK cells (**Figure 4D**). The infusion of *ex vivo* activated NK cells in a fully MHC-mismatched, T cell depleted, allogeneic HSCT model (H-2^b ➔ H-2^a) leads to lethality with or without hu14.18-IL2 (**Figure 4E**), suggesting IC does not contribute to lethality. Interestingly, when the allogeneic HSCT donor and recipients were MHC-matched, minor histocompatibility antigen-mismatched, no differences in weight loss (data not shown) or lethality were observed (**Figure 4F**). Histopathologic examination of classic acute GVHD target tissues (liver, gut, skin) did not show any lymphocytic infiltrate (data not shown), suggesting there was no direct attack of host tissues. Analysis of serum cytokines, however, did show cytokine release syndrome (CRS) with statistically significant increases in IL-6, IL-10 and

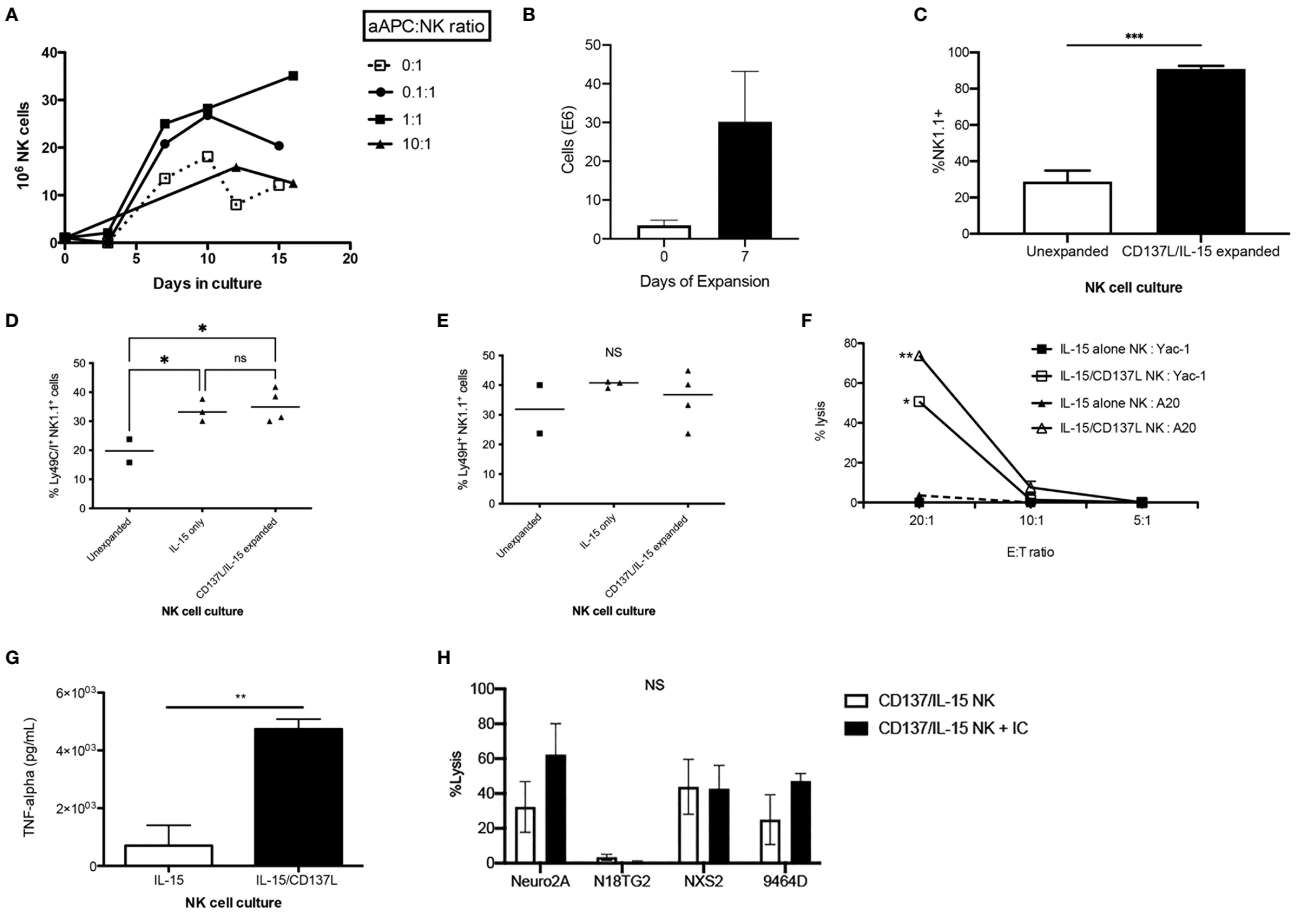

FIGURE 3 | *In vitro* characterization of *ex vivo* CD137L/IL-15/IL-15Rα expanded NK cells. Donor B6 NK cells were expanded *in vitro* with IL-15/IL-15Rα alone (0:1) or with IL-15/IL-15Rα plus an aAPC expressing 4-1BBL (CD137L) at a logarithmically increasing dose of aAPC : NK cell ratios (0.1:1-10:1). **(A)** Cell counts were enumerated twice per week. Results pooled from 4 separate cultures. **(B)** After 1 week, NK cells expanded without aAPC or IL-15/IL-15Rα (unexpanded) were compared to NK cells expanded with the aAPC at a 1:1 ratio (CD137L/IL-15/IL-15Rα expanded) for **(C)** NK1.1 purity, **(D)** inhibitory Ly49C/I expression and **(E)** activating Ly49H expression. Results pooled from 2 separate experiments, 2-7 mice/group. **(F)** Balb/c NK cells (H-2^d) expanded with IL-15/IL-15Rα or with IL-15/IL-15Rα and CD137L aAPCs at a 1:1 NK:aAPC ratio for 1 week were compared for their ability to lyse syngeneic A20 (H-2^d) or allogeneic Yac-1 (H-2^a) lymphoma cells at various E:T ratios using a 4 hour LDH release cytotoxicity assay, performed in triplicate. **(G)** NK cells expanded without aAPC (IL-15) were compared to NK cells expanded with the aAPC at a 1:1 ratio (IL-15/CD137L) and examined for TNF-α production by ELISA, performed in triplicate. **(H)** B6 (H-2^b) NK cells expanded with IL-15/IL-15Rα and CD137L aAPCs at a 1:1 NK:aAPC ratio for 1 week were tested for their ability to lyse murine neuroblastoma cell lines Neuro2a, N18TG2, NXS2, and 9464D (H-2^a) using a 4 hour calcein-AM release cytotoxicity assay, performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS, not significant.

IL-12p70 and a decrease in TNFα noted 1 and/or 2 weeks after *ex vivo* activated NK infusion as compared to recipients of allogeneic HSCT alone (**Figure 5**). No differences in IFNγ, IL-1β, IL-2, IL-4, IL-5 and CXCL1 were observed (**Figure S4**).

Immune profiling of allogeneic HSCT recipients showed mild increases in B cells and $CD8^+$ T cells after tumor inoculation, but no changes in NK cells (**Figure 6A**). While adoptive transfer of wild type NK cells did not increase the total percentage of NK cells in the host, total NK cells did increase after IC administration but without enrichment of inhibitory Ly49C/I^+ or activating Ly49H^+ NK subsets (**Figure 6A**). Because *ex vivo* activated NK cells showed superior cytotoxicity *in vitro* (**Figure 3F**) and *in vivo* (**Figure S2**), as well as high levels of TNFα production *ex vivo* (**Figure 3G**), we wanted to determine if the GVT effect was mediated by contact-dependent killing (*via* perforin) or contact-independent cytokine release (*via* TNF-α release), and whether abrogating these pathways would impact GVT. Infusion of Perforin$^{-/-}$ *ex vivo* activated NK cells with IC did lead to a slight delay in tumor growth, but ultimately tumors overtook the mice (**Figure 6B**). But when we infused *ex vivo* activated TNFα$^{-/-}$ NK cells after allogeneic HSCT with IC, we observed improved tumor control compared to *ex vivo* activated TNFα$^{+/+}$ NK cells (**Figure 6C**), suggesting TNFα may be contributing to CRS in a manner that attenuates the GVT potential of the infused NK cells. Flow cytometric analysis of splenocytes of mice treated with *ex vivo* activated TNFα$^{-/-}$ NK

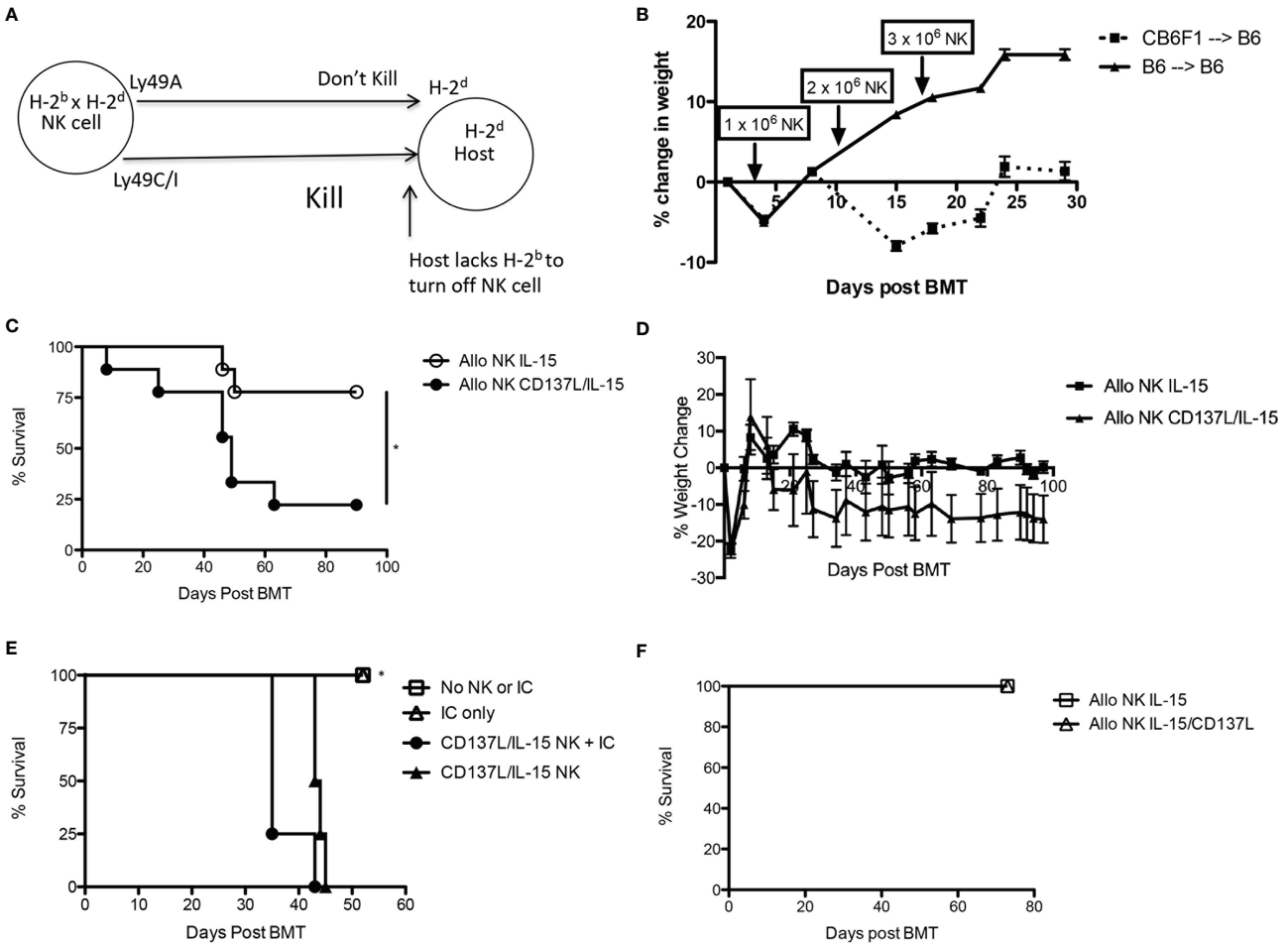

FIGURE 4 | Effects of *ex vivo* expanded NK cells after syngeneic and allogeneic MHC-matched and mismatched HSCT. **(A)** Child into parent HSCT schema showing how "missing self" in host prevents engagement of Ly49 inhibitory receptors on donor NK cells. **(B)** Lethally irradiated B6 mice (H-2^b) were transplanted with CD3e cell depleted BM from B6 (H-2^b) or CB6F1 mice (H-2^{bxd}) on Day +0. On Days +3, +10, and +17 HSCT recipients received donor-derived, CD137L/IL-15/IL-15Rα expanded NK cells in increasing dose increments of 1, 2 or 3 x 10^6 cells, and were followed for weight loss and survival. N=5 mice/group. **(C)** Lethally irradiated Balb/c mice (H-2^d) were transplanted with CD3e cell depleted BM from CB6F1 mice (H-$2^{b \times d}$) on Day +0. On Day +1 HSCT recipients received 5 x 10^6 CB6F1 NK cells (H-2bxd) cultured in IL-15 alone, or activated with CD137L/IL-15/IL-15Rα, and were followed for survival and **(D)** weight loss. Lethally irradiated Balb/c mice (H-2^d) were transplanted with CD3e cell depleted BM from CB6F1 mice (H-$2^{b \times d}$) on Day +0. On Day +1 HSCT recipients received 5 x 10^6 CB6F1 NK cells (H-2^{bxd}) cultured in IL-15 alone, or activated with CD137L/IL-15/IL-15Rα, and were followed for survival. Results pooled from 2 separate experiments, 9 mice/group. **(E)** Lethally irradiated A/J mice (H-2^a) were transplanted with CD3e cell depleted BM from B6 mice (H-2^b) on Day +0. On Days +14-16, PBS (No IC) or hu14.18-IL-2 (IC) was administered. On Day +16, select groups were infused with 2.5 x 10^6 CD137L/IL-15/IL-15Rα expanded B6 NK cells and followed for survival. N= 5 mice/group. **(F)** Lethally irradiated Balb/c mice (H-2^d) were transplanted with CD3e cell depleted BM from DBA mice (H-2^d) on Day +0. On Day +1 HSCT recipients received 5 x 10^6 CB6F1 NK cells (H-$2^{b \times d}$) cultured in IL-15/IL-15Rα alone, or activated with CD137L/IL-15/IL-15Rα, and were followed for weight loss and survival. N=5 mice/group. *$p < 0.05$.

cells showed a decrease in T cells and NK cells, with specifically less $CD69^+$, $CD107a^+$, and $TRAIL^+$ NK cells seen in the periphery (**Figure 6D**). However, there were no differences between these NK subsets within the tumor (**Figure S5**). Depletion of $Ly49H^+$ NK cells, which represent an NK subset bearing an activation receptor that can engage MHC (H-2^b) on B6AJF1 host tissues, after NK infusion also led to improved tumor control early after tumor development (**Figure 6E**), suggesting blockade of TNFα-producing or depletion of activated NK cell subsets may help regulate toxicity while preserving GVT responses against NBL.

DISCUSSION

While haploidentical allogeneic HSCT is effective against leukemia (40), despite the publication of preclinical data (10, 11) and clinical data from case series describing the impact of allogeneic HSCT on NBL (12, 13), significant barriers are preventing allogeneic HSCT from more widespread testing as potential therapy for children with high risk or metastatic NBL. Barriers include the absence of conclusive evidence of a GVT effect against NBL and the development of GVHD that contributes to treatment-related mortality (41). We

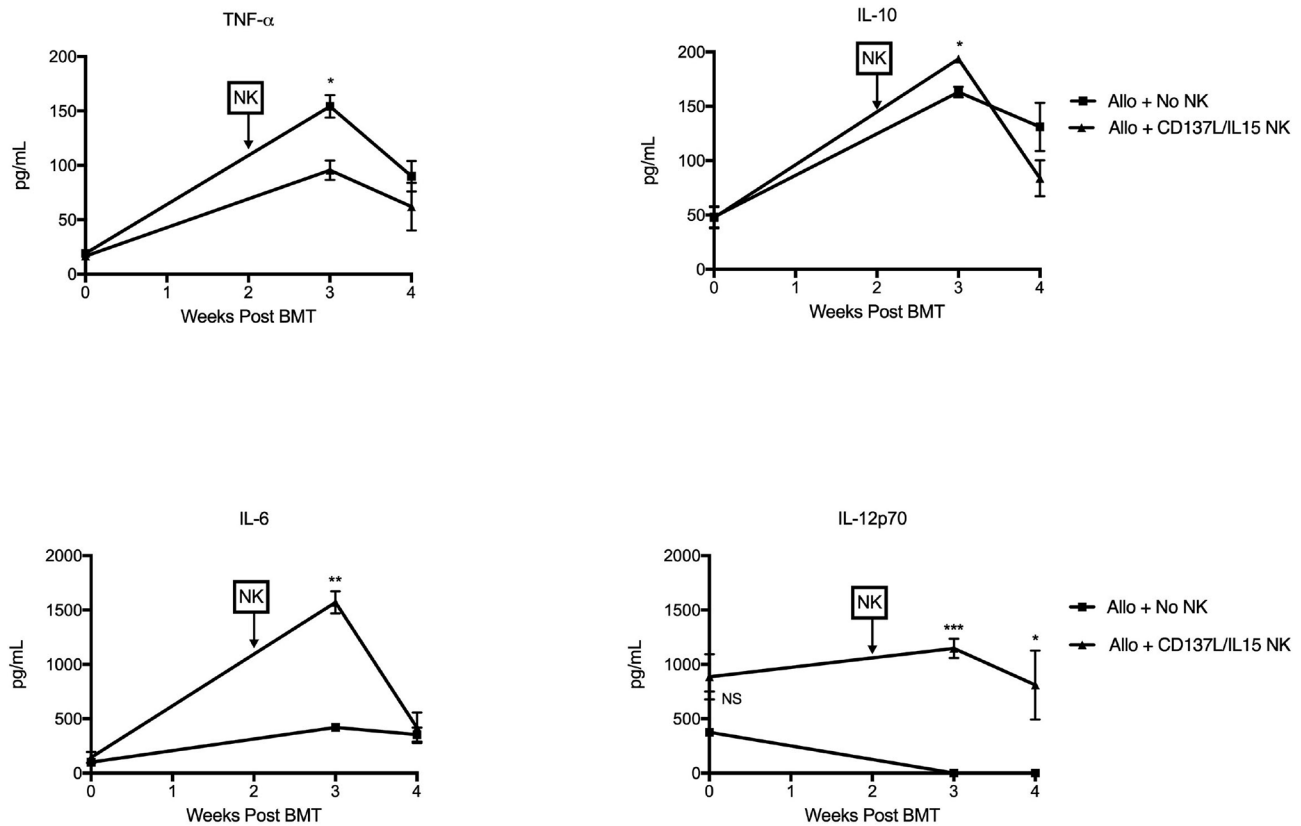

FIGURE 5 | *Ex vivo* CD137L/IL-15/IL-15Rα expanded NK cells mediate cytokine release syndrome *in vivo*. Lethally irradiated B6AJF1 mice (H-$2^{b \times a}$) were transplanted with CD3e cell depleted BM from B6 mice (H-2^{b}) on Day +0. On Day +10, 2 x 10^6 NXS2 tumor cells were inoculated into the right flank. On days +14-16, hu14.18-IL-2 (IC) was administered. On Day +16, groups were infused with PBS or 2.5 x 10^6 CD137L/IL-15/IL-15Rα expanded B6 NK cells. Peripheral blood was collected pre-alloHSCT, and then 3 and 4 weeks post-alloHSCT. Serum was isolated from each blood sample and all timepoints were frozen, batched and analyzed in duplicate by multiplex cytokine array. N= 5 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001.

hypothesized that these barriers may be overcome by: 1) using T cell-depleted haploidentical allogeneic HSCT to enhance GVT and minimize GVHD; 2) focusing the localization and activity of the GVT inducing cells in the allogeneic HSCT *via* co-administration of the anti-GD2 IC hu14.18-IL2; 3) augmenting the capability of the GVT causing cells by selecting donors with the appropriate haploidentical relationship to the patient to enable NK allo-recognition in the GVT direction; and 4) co-infusing *ex-vivo* activated NK cells.

We show for the first time that usage of an IC, in this case hu14.18-IL2, is feasible and effective after allogeneic HSCT; IC induces GVT without GVHD as long as the T cell dose is minimized in the donor bone marrow graft. Because IL-2 could activate alloreactive T cells and exacerbate GVHD, but also expand regulatory T cells and abrogate GVHD, it was not clear what the effect of infusing IC would be after allogeneic HSCT. With higher T cells doses, the IL-2 present on the IC may have unintentionally stimulated alloreactive T cells from the donor, leading to GVHD. Also, poorer immune reconstitution was observed, which could reflect immunosuppression from GVHD or reduced spleen size as allogeneic HSCT recipients were dying from GVHD. With lower T cell doses, while anti-tumor activity against $GD2^{+}$ NBL was observed as compared to allogeneic HSCT recipients without IC, tumors still developed. Because the mechanism of action of IC involves ADCC by NK cells, allogeneic HSCT recipients are lymphopenic, and the post-allogeneic HSCT milieu has high levels of IL-15 (21, 42), we hypothesized that infusions of *ex vivo* activated NK cells from the donor could enhance the GVT effect of the IC. Instead, we observed that adoptive transfer of *ex vivo* activated NK cells led to lethality in the presence or absence of IC.

NK cells have been adoptively transferred to recipients of allogeneic HSCT in preclinical models, with promising anti-tumor activity observed (43–46). Adoptive transfer of NK cells can also inhibit acute GVHD by limiting expansion and infiltration of donor T cells (47–49), producing TGF-β (45), controlling infections (50), depleting recipient dendritic cells (39), and improving lymphopenia (51). One limitation of

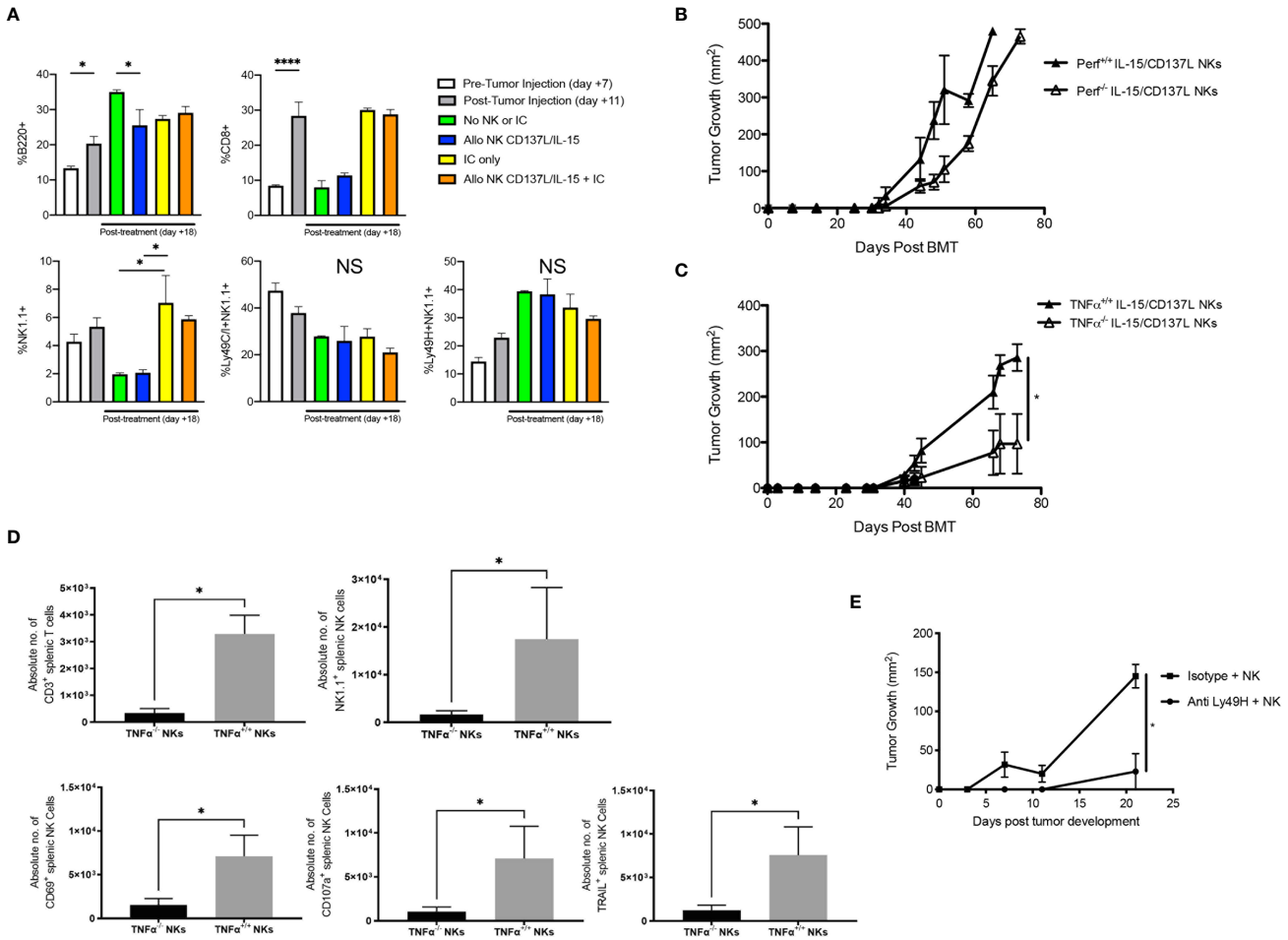

FIGURE 6 | Impact of infusing *ex vivo* CD137L/IL-15/IL-15Rα expanded NK cells on GD2$^+$ NXS2 neuroblastoma growth after allogeneic HSCT. Lethally irradiated A/J mice (H-2^a) were transplanted with CD3e cell depleted B6 BM (H-2^b) with 2.5 x 10^2 B6 T cells on Day +0. On Day +10, 2 x 10^6 NXS2 tumor cells were inoculated into the right flank. On Days +14-16, hu14.18-IL-2 (IC) was administered. **(A)** Flow cytometric analysis of the spleen was performed on Day +7 (pre-NXS2), +11 (post-NXS2, pre-IC or NK cells) or +18 (post-IC and/or NK cells). **(B)** On Day +16, select groups were infused with 2.5 x 10^6 CD137L/IL-15/IL-15Rα expanded NK cells from B6 wild type (Perf$^{+/+}$) or B6 Perforin (Perf$^{-/-}$) donors, or **(C)** B6 wild type (TNFα$^{+/+}$) or B6 TNFα$^{-/-}$ donors. **(D)** Both spleens and tumors were harvested from recipients of B6 wild type (TNFα$^{+/+}$) or B6 TNFα$^{-/-}$ donors at Day +30 and analyzed for T cells and NK cell subsets. **(E)** Mice were transplanted and challenged with NXS2 as above but on Day +16 were infused with 2.5 x 10^6 CD137L/IL-15/IL-15Rα expanded NK cells from B6 wild type and then treated with anti-Ly49H depletion or an isotype control. All mice were followed for NXS2 tumor growth. N=3-5 mice/group. *p < 0.05. ****p < 0.001. NS, not significant.

applying these murine studies to our data is that all but two of these studies infused inactivated NK cells, and none of those studies used a co-stimulatory molecule like CD137L to activate the NK cells. Using F1 into parent HSCT models, where T cells cannot cause GVHD or alloreactivity, we observed weight loss or lethality depending on the recipient strain, suggesting *ex vivo* activated NK cells can mediate toxicity independent of T cell allorecognition. While this has not been observed in prior preclinical studies of adoptively transferred NK cells, it is possible that the biology of NK cells is different *in vivo* after activation by *ex vivo* as compared to inactivated NK cells. *In vitro*, *ex vivo* activated human NK cells can overcome KIR-mediated inhibitory signals (52). In clinical studies, infusion of NK cells expanded with either IL-2 (53, 54), or IL-15 and IL-21 after HLA-mismatched allogeneic HSCT (55–57) induced low rates and/or grades of GVHD, whereas infusion of NK cells activated with CD137L/IL-15 after HLA-matched allogeneic HSCT led to higher rates and grades of GVHD (58). The exact mechanism of NK-mediated GVHD is unclear but our data suggests it could have been in part driven by CRS.

Because we used T cell depleted bone marrow and did not detect NK cells in host tissues, we hypothesized that *ex vivo* activated NK cells mediated CRS that inhibited GVT. In fact, elevated levels of IL-6, IL-10 and IL-12p70 and decreased levels of TNF-α were observed in allogeneic HSCT recipients who received *ex vivo* activated NK cells than in uninfused allogeneic

HSCT recipients. We did observe a minor population of regulatory T cells after IC administration that was not influenced by the number of T cells in the donor graft. Given the high IL-10 production observed after NK infusion, future studies should examine the contribution of IC in activating regulatory T cells and their role in GVT/GVHD/CRS in this model. In addition, increased TNF-α production by NK cells has been previously observed after haploidentical allogeneic HSCT (59), yet the decreased level noted in our model was still clinically significant. To determine if CRS was attenuating the GVT effect, adoptive transfer of purified, *ex vivo* activated TNFα $^{-/-}$ NK cells was performed and significantly attenuated tumor growth, suggesting that TNF-α production from *ex vivo* CD137L/IL-15/IL-15Rα activated NK cells may be contributing to a CRS that hinders anti-tumor effects. One potential mechanism could have been disruption of TNF-α mediated priming of regulatory T cells through TNFR2 (60, 61), reducing tolerance by/to the tumor. Future studies should examine if TNF-α may be activating regulatory T cells which in turn suppress elimination of NBL by T and NK cells. In addition, depleting *ex vivo* activated NK cells that express the activating receptor Ly49H after infusion improves early anti-tumor responses, overall suggesting that hyperactivated NK cell subsets may have to be carefully monitored after allogeneic HSCT as they may contribute to toxicities like CRS than can undo GVT effects. To our knowledge, this is the first example of CRS using adoptive transfer of *ex vivo* activated NK cells in the allogeneic HSCT setting. Because of the clinical availability of TNF-α inhibitors like infliximab, or soluble TNF-α receptor like etanercept, TNF-α blockade could be explored clinically to improve the GVT potential of *ex vivo* activated NK cells, but additional agents would likely need to be explored to better control CRS, like tocilizumab.

The preclinical data shown here provide preliminary groundwork for more mechanistic studies to enable clinical translation and evolution of existing pediatric trials using T cell depleted (e.g. α/β T cell depletion) haploidentical allogeneic HSCT for NBL by demonstrating the safety and efficacy of the combination of IC and *ex vivo* activated NK cell infusions to induce GVT effects against NBL. Clinical trials incorporating α/β T cell depletion haploidentical HSCT are underway at several pediatric centers as a means of depleting GVHD-causing α/β T cells while enriching the donor graft with GVT-promoting γ/δ T cells and NK cells (62), including for children with NBL (63, 64) (NCT02508038). A pilot trial testing the combination of IC and *ex vivo* activated haploidentical NK cell infusions in non-transplanted NBL patients is also underway (NCT03209869) (65). Further studies are warranted with these clinically available therapy platforms given the poor prognosis for high risk NBL and lack of effective salvage regimens.

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 University of Wisconsin-Madison IACUC.

AUTHOR CONTRIBUTIONS

PB, SR, JH, and MC performed research, collected, analyzed, and interpreted data, and revised the manuscript. AR performed research and revised the manuscript. RO developed and provided the aAPC and revised the manuscript. MB, TF, SG, and PS analyzed and interpreted data and revised the manuscript. CC designed and supervised research; analyzed and interpreted data, drafted and revised 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.2021.668307/full#supplementary-material

Supplementary Figure 1 | Comparison of GD2 expression on murine NBL and syngeneic versus allogeneic HSCT on NBL tumor growth. **(A)** Flow cytometric expression of GD2 on murine NBL cell lines. **(B)** A/J mice were lethally irradiated and transplanted with 5 x106 B6 BM cells and 2.5 x103 B6 T cells (allogeneic BMT) or 5 x10^6 A/J BM cells and 2.5 x10^3 A/J T cells (syngeneic BMT) on Day +0. On Day +10, mice were inoculated subcutaneously with 2 x 10^6 NXS neuroblastoma cells. Mice were followed for tumor growth. N = 5 mice/group. *p < 0.05.

Supplementary Figure 2 | *In vivo* potency of *ex vivo* activated NK cells. Balb/c mice were lethally irradiated and transplanted with 5 x 10^6 CB6F1 BM cells (allogeneic HSCT) on Day +0 as well as 2.5 x 10^6 A20 lymphoma cells on Day +5. Then PBS (No NKs) or 6 x10^6 CB6F1 IL15/IL15Rα activated or CD137L/IL15/IL15Rα activated NK cells were given on Day +13. All mice were followed for survival. N=5 mice/group. *p < 0.05 as compared to No NKs group.

Supplementary Figure 3 | Ly49 NK subset analysis. **(A)** Sample gating strategy for analyzing Ly49C/I+NK1.1+ cells pre- and post-expansion by CD137L/IL15/IL15Rα aAPCs at a 1:1 ratio for 1 week. **(B)** Flow cytometric analysis of Ly49H- and Ly49H+ NK cell subsets for cytotoxicity markers (TRAIL, FasL and CD107a) and cytokine production (TNFα) from a single expansion.

Supplementary Figure 4 | Cytokine production pre- and post-adoptive transfer of *ex vivo* activated NK cells. B6AJF1 mice were lethally irradiated on Day +0 and transplanted with 5 x10^6 B6 BM cells and 1 x10^6 B6 T cells on Day +0. Serum was collected as a baseline cytokine measurement. On Day +10, mice were inoculated with 2 x10^6 NXS2 neuroblastoma cells. On Day +14, mice wereinfused with PBS or 2.5 x 10^6 B6 CD137L/IL15/IL15Rα activated NK cells. Serum was collected 1 (Day +21) and 2 weeks (Day +28) after infusion. Serum was batched and run in duplicate per timepoint for each group. N=5 mice/group. *p < 0.05, otherwise no significant differenceswere seen.

Supplementary Figure 5 | Comparison of tumor infiltrating wild type and TNFα-deficient NK cells after allogeneic HSCT and IC treatment for NBL. Lethally irradiated A/J mice (H-2a) were transplanted with CD3e cell depleted B6 BM (H-2b) with 2.5 x 10^6 B6 T cells on Day +0. On Day +10, 2 x 10^6 NXS2 tumor cells were inoculated into the right flank. On Days +14-16, hu14.18-IL-2 (IC) was administered. On Day +16, select groups were infused with 2.5 x 10^6 CD137L/IL-15/IL-15Rα expanded NK cells from B6 wild type (TNFα+/+) or B6 TNFα-/- donors. Both spleens and tumors were harvested at Day +30 and analyzed for T cells and NK cell subsets. N=3-5 mice/group. No significant differences were seen.

REFERENCES

1. Morgenstern DA, Baruchel S, Irwin MS. Current and Future Strategies for Relapsed Neuroblastoma: Challenges on the Road to Precision Therapy. *J Pediatr Hematol Oncol* (2013) 35(5):337–47. doi: 10.1097/MPH.0b013e318299d637
2. Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, et al. Anti-GD2 Antibody With GM-CSF, Interleukin-2, and Isotretinoin for Neuroblastoma. *N Engl J Med* (2010) 363(14):1324–34. doi: 10.1056/NEJMoa0911123
3. August CS, Serota FT, Koch PA, Burkey E, Schlesinger H, Elkins WL, et al. Treatment of Advanced Neuroblastoma With Supralethal Chemotherapy, Radiation, and Allogeneic or Autologous Marrow Reconstitution. *J Clin Oncol* (1984) 2(6):609–16. doi: 10.1200/JCO.1984.2.6.609
4. Matthay KK, Seeger RC, Reynolds CP, Stram DO, O'Leary MC, Harris RE, et al. Allogeneic Versus Autologous Purged Bone Marrow Transplantation for Neuroblastoma: A Report From the Childrens Cancer Group. *J Clin Oncol* (1994) 12(11):2382–9. doi: 10.1200/JCO.1994.12.11.2382
5. Philip T, Ladenstein R, Lasset C, Hartmann O, Zucker JM, Pinkerton R, et al. 1070 Myeloablative Megatherapy Procedures Followed by Stem Cell Rescue for Neuroblastoma: 17 Years of European Experience and Conclusions. European Group for Blood and Marrow Transplant Registry Solid Tumour Working Party. *Eur J Cancer* (1997) 33(12):2130–5. doi: 10.1016/s0959-8049(97)00324-9
6. Wawrzyniak-Dzierzek E, Gajek K, Rybka B, Ryczan-Krawczyk R, Weclawek-Tompol J, Raciborska A, et al. Feasibility and Safety of Treosulfan, Melphalan, and Thiotepa-Based Megachemotherapy With Autologous or Allogeneic Stem Cell Transplantation in Heavily Pretreated Children With Relapsed or Refractory Neuroblastoma. *Biol Blood Marrow Transpl* (2019) 25(9):1792–7. doi: 10.1016/j.bbmt.2019.05.006
7. Illhardt T, Toporski J, Feuchtinger T, Turkiewicz D, Teltschik HM, Ebinger M, et al. Haploidentical Stem Cell Transplantation for Refractory/Relapsed Neuroblastoma. *Biol Blood Marrow Transpl* (2018) 24(5):1005–12. doi: 10.1016/j.bbmt.2017.12.805
8. Sung KW, Park JE, Chueh HW, Lee SH, Yoo KH, Koo HH, et al. Reduced-Intensity Allogeneic Stem Cell Transplantation for Children With Neuroblastoma Who Failed Tandem Autologous Stem Cell Transplantation. *Pediatr Blood Cancer* (2011) 57(4):660–5. doi: 10.1002/pbc.23035
9. Park JR, Kreissman SG, London WB, Naranjo A, Cohn SL, Hogarty MD, et al. Effect of Tandem Autologous Stem Cell Transplant vs Single Transplant on Event-Free Survival in Patients With High-Risk Neuroblastoma: A Randomized Clinical Trial. *JAMA* (2019) 322(8):746–55. doi: 10.1001/jama.2019.11642
10. Ash S, Gigi V, Askenasy N, Fabian I, Stein J, Yaniv I. Graft Versus Neuroblastoma Reaction is Efficiently Elicited by Allogeneic Bone Marrow Transplantation Through Cytolytic Activity in the Absence of GVHD. *Cancer Immunol Immunother* (2009) 58(12):2073–84. doi: 10.1007/s00262-009-0715-6
11. Willems L, Fevery S, Sprangers B, Rutgeerts O, Lenaerts C, Ibrahimi A, et al. Recipient Leukocyte Infusion Enhances the Local and Systemic Graft-Versus-Neuroblastoma Effect of Allogeneic Bone Marrow Transplantation in Mice. *Cancer Immunol Immunother* (2013) 62(11):1733–44. doi: 10.1007/s00262-013-1479-6
12. Lang P, Pfeiffer M, Muller I, Schumm M, Ebinger M, Koscielniak E, et al. Haploidentical Stem Cell Transplantation in Patients With Pediatric Solid Tumors: Preliminary Results of a Pilot Study and Analysis of Graft Versus Tumor Effects. *Klin Padiatr* (2006) 218(6):321–6. doi: 10.1055/s-2006-942256
13. Kanold J, Paillard C, Tchirkov A, Merlin E, Marabelle A, Lutz P, et al. Allogeneic or Haploidentical HSCT for Refractory or Relapsed Solid Tumors in Children: Toward a Neuroblastoma Model. *Bone Marrow Transpl* (2008) 42 Suppl 2:S25–30. doi: 10.1038/bmt.2008.279
14. Osenga KL, Hank JA, Albertini MR, Gan J, Sternberg AG, Eickhoff J, et al. A Phase I Clinical Trial of the Hu14.18-IL2 (EMD 273063) as a Treatment for Children With Refractory or Recurrent Neuroblastoma and Melanoma: A Study of the Children's Oncology Group. *Clin Cancer Res* (2006) 12(6):1750–9. doi: 10.1158/1078-0432.CCR-05-2000
15. Shusterman S, London WB, Gillies SD, Hank JA, Voss SD, Seeger RC, et al. Antitumor Activity of Hu14.18-IL2 in Patients With Relapsed/Refractory Neuroblastoma: A Children's Oncology Group (COG) Phase II Study. *J Clin Oncol* (2010) 28(33):4969–75. doi: 10.1200/JCO.2009.27.8861
16. Neal ZC, Yang JC, Rakhmilevich AL, Buhtoiarov IN, Lum HE, Imboden M, et al. Enhanced Activity of Hu14.18-IL2 Immunocytokine Against Murine NXS2 Neuroblastoma When Combined With Interleukin 2 Therapy. *Clin Cancer Res* (2004) 10(14):4839–47. doi: 10.1158/1078-0432.CCR-03-0799
17. Klob S, Bochennek K, Huenecke S, Zimmermann S-Y, KuÁi S,M, ller T, et al. A Novel Five-Colour Flow Cytometric Assay to Determine NK Cell Cytotoxicity Against Neuroblastoma and Other Adherent Tumour Cells. *J Immunol Methods* (2007) 325(1-2):140–7. doi: 10.1016/j.jim.2007.06.013
18. Zobel MJ, Zamora AK, Wu HW, Sun J, Lascano D, Malvar J, et al. Initiation of Immunotherapy With Activated Natural Killer Cells and Anti-GD2 Antibody Dinutuximab Prior to Resection of Primary Neuroblastoma Prolongs Survival in Mice. *J Immunother Cancer* (2020) 8(2):1–10. doi: 10.1136/jitc-2020-001560
19. Nguyen R, Moustaki A, Norrie JL, Brown S, Akers WJ, Shirinifard A, et al. Interleukin-15 Enhances Anti-GD2 Antibody-Mediated Cytotoxicity in an Orthotopic PDX Model of Neuroblastoma. *Clin Cancer Res* (2019) 25(24):7554–64. doi: 10.1158/1078-0432.CCR-19-1045
20. Leung W, Iyengar R, Leimig T, Holladay MS, Houston J, Handgretinger R. Phenotype and Function of Human Natural Killer Cells Purified by Using a Clinical-Scale Immunomagnetic Method. *Cancer Immunol Immunother* (2005) 54(4):389–94. doi: 10.1007/s00262-004-0609-6
21. Boyiadzis M, Memon S, Carson J, Allen K, Szczepanski MJ, Vance BA, et al. Up-Regulation of NK Cell Activating Receptors Following Allogeneic

Hematopoietic Stem Cell Transplantation Under a Lymphodepleting Reduced Intensity Regimen is Associated With Elevated IL-15 Levels. *Biol Blood Marrow Transpl* (2008) 14(3):290–300. doi: 10.1016/j.bbmt.2007.12.490
22. Murphy WJ, Keller JR, Harrison CL, Young HA, Longo DL. Interleukin-2-Activated Natural Killer Cells can Support Hematopoiesis *In Vitro* and Promote Marrow Engraftment *In Vivo*. *Blood* (1992) 80(3):670–7. doi: 10.1182/blood.V80.3.670.670
23. Murphy WJ, Parham P, Miller JS. NK Cells–From Bench to Clinic. *Biol Blood Marrow Transpl* (2012) 18(1 Suppl):S2–7. doi: 10.1016/j.bbmt.2011.10.033
24. McDowell KA, Hank JA, DeSantes KB, Capitini CM, Otto M, Sondel PM. NK Cell-Based Immunotherapies in Pediatric Oncology. *J Pediatr Hematol Oncol* (2015) 37(2):79–93. doi: 10.1097/MPH.0000000000000303
25. Delgado DC, Hank JA, Kolesar J, Lorentzen D, Gan J, Seo S, et al. Genotypes of NK Cell KIR Receptors, Their Ligands, and Fcgamma Receptors in the Response of Neuroblastoma Patients to Hu14.18-IL2 Immunotherapy. *Cancer Res* (2010) 70(23):9554–61. doi: 10.1158/0008-5472.CAN-10-2211
26. Tarek N, Le Luduec JB, Gallagher MM, Zheng J, Venstrom JM, Chamberlain E, et al. Unlicensed NK Cells Target Neuroblastoma Following Anti-GD2 Antibody Treatment. *J Clin Invest* (2012) 122(9):3260–70. doi: 10.1172/JCI62749
27. Capitini CM, Nasholm NM, Chien CD, Larabee SM, Qin H, Song YK, et al. Absence of STAT1 in Donor-Derived Plasmacytoid Dendritic Cells Results in Increased STAT3 and Attenuates Murine GVHD. *Blood* (2014) 124(12):1976–86. doi: 10.1182/blood-2013-05-500876
28. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte JJr., Crawford JM, et al. An Experimental Model of Idiopathic Pneumonia Syndrome After Bone Marrow Transplantation: I. The Roles of Minor H Antigens and Endotoxin. *Blood* (1996) 88(8):3230–9. doi: 10.1182/blood.V88.8.3230.bloodjournal8883230
29. Lode HN, Xiang R, Varki NM, Dolman CS, Gillies SD, Reisfeld RA. Targeted Interleukin-2 Therapy for Spontaneous Neuroblastoma Metastases to Bone Marrow. *J Natl Cancer Inst* (1997) 89(21):1586–94. doi: 10.1093/jnci/89.21.1586
30. Yan X, Johnson BD, Orentas RJ. Murine CD8 Lymphocyte Expansion *In Vitro* by Artificial Antigen-Presenting Cells Expressing CD137L (4-1BBL) is Superior to CD28, and CD137L Expressed on Neuroblastoma Expands CD8 Tumour-Reactive Effector Cells *In Vivo*. *Immunology* (2004) 112(1):105–16. doi: 10.1111/j.1365-2567.2004.01853.x
31. Brown MG, Dokun AO, Heusel JW, Smith HR, Beckman DL, Blattenberger EA, et al. Vital Involvement of a Natural Killer Cell Activation Receptor in Resistance to Viral Infection. *Science* (2001) 292(5518):934–7. doi: 10.1126/science.1060042
32. Capitini CM, Herby S, Milliron M, Anver MR, Mackall CL, Fry TJ. Bone Marrow Deficient in IFN-{Gamma} Signaling Selectively Reverses GVHD-Associated Immunosuppression and Enhances a Tumor-Specific GVT Effect. *Blood* (2009) 113(20):5002–9. doi: 10.1182/blood-2008-11-187385
33. Puliaev R, Nguyen P, Finkelman FD, Via CS. Differential Requirement for IFN-Gamma in CTL Maturation in Acute Murine Graft-Versus-Host Disease. *J Immunol* (2004) 173(2):910–9. doi: 10.4049/jimmunol.173.2.910
34. Mimura K, Kamiya T, Shiraishi K, Kua LF, Shabbir A, So J, et al. Therapeutic Potential of Highly Cytotoxic Natural Killer Cells for Gastric Cancer. *Int J Cancer* (2014) 135(6):1390–8. doi: 10.1002/ijc.28780
35. Lapteva N, Durett AG, Sun J, Rollins LA, Huye LL, Fang J, et al. Large-Scale *Ex Vivo* Expansion and Characterization of Natural Killer Cells for Clinical Applications. *Cytotherapy* (2012) 14(9):1131–43. doi: 10.3109/14653249.2012.700767
36. Cho D, Shook DR, Shimasaki N, Chang YH, Fujisaki H, Campana D. Cytotoxicity of Activated Natural Killer Cells Against Pediatric Solid Tumors. *Clin Cancer Res* (2010) 16(15):3901–9. doi: 10.1158/1078-0432.CCR-10-0735
37. Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, et al. Expansion of Highly Cytotoxic Human Natural Killer Cells for Cancer Cell Therapy. *Cancer Res* (2009) 69(9):4010–7. doi: 10.1158/0008-5472.CAN-08-3712
38. Sykes M, Sheard M, Sachs DH. Effects of T Cell Depletion in Radiation Bone Marrow Chimeras. I. Evidence for a Donor Cell Population Which Increases Allogeneic Chimerism But Which Lacks the Potential to Produce GVHD. *J Immunol* (1988) 141(7):2282–8.
39. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants. *Science* (2002) 295(5562):2097–100. doi: 10.1126/science.1068440
40. Chang YJ, Wang Y, Xu LP, Zhang XH, Chen H, Chen YH, et al. Haploidentical Donor is Preferred Over Matched Sibling Donor for Pre-Transplantation MRD Positive ALL: A Phase 3 Genetically Randomized Study. *J Hematol Oncol* (2020) 13(1):27. doi: 10.1186/s13045-020-00860-y
41. Hale GA, Arora M, Ahn KW, He W, Camitta B, Bishop MR, et al. Allogeneic Hematopoietic Cell Transplantation for Neuroblastoma: The CIBMTR Experience. *Bone Marrow Transpl* (2013) 48(8):1056–64. doi: 10.1038/bmt.2012.284
42. Dulphy N, Haas P, Busson M, Belhadj S, Peffault de Latour R, Robin M, et al. An Unusual CD56(bright) CD16(low) NK Cell Subset Dominates the Early Posttransplant Period Following HLA-Matched Hematopoietic Stem Cell Transplantation. *J Immunol* (2008) 181(3):2227–37. doi: 10.4049/jimmunol.181.3.2227
43. Olson JA, Zeiser R, Beilhack A, Goldman JJ, Negrin RS. Tissue-Specific Homing and Expansion of Donor NK Cells in Allogeneic Bone Marrow Transplantation. *J Immunol* (2009) 183(5):3219–28. doi: 10.4049/jimmunol.0804268
44. Lundqvist A, McCoy JP, Samsel L, Childs R. Reduction of GVHD and Enhanced Antitumor Effects After Adoptive Infusion of Alloreactive Ly49-Mismatched NK Cells From MHC-Matched Donors. *Blood* (2007) 109(8):3603–6. doi: 10.1182/blood-2006-05-024315
45. Asai O, Longo DL, Tian ZG, Hornung RL, Taub DD, Ruscetti FW, et al. Suppression of Graft-Versus-Host Disease and Amplification of Graft-Versus-Tumor Effects by Activated Natural Killer Cells After Allogeneic Bone Marrow Transplantation. *J Clin Invest* (1998) 101(9):1835–42. doi: 10.1172/JCI1268
46. Zeis M, Uharek L, Glass B, Steinmann J, Dreger P, Gassmann W, et al. Allogeneic MHC-Mismatched Activated Natural Killer Cells Administered After Bone Marrow Transplantation Provide a Strong Graft-Versus-Leukaemia Effect in Mice. *Br J Haematol* (1997) 96(4):757–61. doi: 10.1046/j.1365-2141.1997.d01-2101.x
47. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK Cells Mediate Reduction of GVHD by Inhibiting Activated, Alloreactive T Cells While Retaining GVT Effects. *Blood* (2010) 115(21):4293–301. doi: 10.1182/blood-2009-05-222190
48. Meinhardt K, Kroeger I, Bauer R, Ganss F, Ovsiy I, Rothamer J, et al. Identification and Characterization of the Specific Murine NK Cell Subset Supporting Graft–Leukemia- and Reducing Graft–Host-Effects. *Oncoimmunology* (2015) 4(1):e981483. doi: 10.4161/2162402X.2014.981483
49. Huber CM, Doisne JM, Colucci F. IL-12/15/18-Preactivated NK Cells Suppress GvHD in a Mouse Model of Mismatched Hematopoietic Cell Transplantation. *Eur J Immunol* (2015) 45(6):1727–35. doi: 10.1002/eji.201445200
50. Ghadially H, Ohana M, Elboim M, Gazit R, Gur C, Nagler A, et al. NK Cell Receptor NKp46 Regulates Graft-Versus-Host Disease. *Cell Rep* (2014) 7(6):1809–14. doi: 10.1016/j.celrep.2014.05.011
51. Chen G, Wu D, Wang Y, Cen J, Feng Y, Sun A, et al. Expanded Donor Natural Killer Cell and IL-2, IL-15 Treatment Efficacy in Allogeneic Hematopoietic Stem Cell Transplantation. *Eur J Haematol* (2008) 81(3):226–35. doi: 10.1111/j.1600-0609.2008.01108.x
52. Zhang H, Cui Y, Voong N, Sabatino M, Stroncek DF, Morisot S, et al. Activating Signals Dominate Inhibitory Signals in CD137L/IL-15 Activated Natural Killer Cells. *J Immunother* (2011) 34(2):187–95. doi: 10.1097/CJI.0b013e31820d2a21
53. Koehl U, Sorensen J, Esser R, Zimmermann S, Gruttner HP, Tonn T, et al. IL-2 Activated NK Cell Immunotherapy of Three Children After Haploidentical Stem Cell Transplantation. *Blood Cells Mol Dis* (2004) 33(3):261–6. doi: 10.1016/j.bcmd.2004.08.013
54. Choi YB, Son MH, Cho HW, Ma Y, Lee JW, Kang ES, et al. Safety and Immune Cell Kinetics After Donor Natural Killer Cell Infusion Following Haploidentical Stem Cell Transplantation in Children With Recurrent Neuroblastoma. *PLoS One* (2019) 14(12):e0225998. doi: 10.1371/journal.pone.0225998
55. Choi I, Yoon SR, Park SY, Kim H, Jung SJ, Jang YJ, et al. Donor-Derived Natural Killer Cells Infused After Human Leukocyte Antigen-Haploidentical Hematopoietic Cell Transplantation: A Dose-Escalation Study. *Biol Blood Marrow Transpl* (2014) 20(5):696–704. doi: 10.1016/j.bbmt.2014.01.031

56. Yoon SR, Lee YS, Yang SH, Ahn KH, Lee JH, Lee JH, et al. Generation of Donor Natural Killer Cells From CD34(+) Progenitor Cells and Subsequent Infusion After HLA-Mismatched Allogeneic Hematopoietic Cell Transplantation: A Feasibility Study. *Bone Marrow Transpl* (2010) 45 (6):1038–46. doi: 10.1038/bmt.2009.304
57. Ciurea SO, Schafer JR, Bassett R, Denman CJ, Cao K, Willis D, et al. Phase 1 Clinical Trial Using mbIL21 *Ex Vivo*-Expanded Donor-Derived NK Cells After Haploidentical Transplantation. *Blood* (2017) 130(16):1857–68. doi: 10.1182/blood-2017-05-785659
58. Shah NN, Baird K, Delbrook CP, Fleisher TA, Kohler ME, Rampertaap S, et al. Acute GVHD in Patients Receiving IL-15/4-1BBL Activated NK Cells Following T-Cell-Depleted Stem Cell Transplantation. *Blood* (2015) 125 (5):784–92. doi: 10.1182/blood-2014-07-592881
59. Guo H, Chang YJ, Hong Y, Xu LP, Wang Y, Zhang XH, et al. Dynamic Immune Profiling Identifies the Stronger Graft-Versus-Leukemia (GVL) Effects With Haploidentical Allografts Compared to HLA-Matched Stem Cell Transplantation. *Cell Mol Immunol* (2021) 18(5):1172–85. doi: 10.1038/s41423-020-00597-1
60. Pierini A, Strober W, Moffett C, Baker J, Nishikii H, Alvarez M, et al. TNF-Alpha Priming Enhances CD4+FoxP3+ Regulatory T-Cell Suppressive Function in Murine GVHD Prevention and Treatment. *Blood* (2016) 128 (6):866–71. doi: 10.1182/blood-2016-04-711275
61. Leclerc M, Naserian S, Pilon C, Thiolat A, Martin GH, Pouchy C, et al. Control of GVHD by Regulatory T Cells Depends on TNF Produced by T Cells and TNFR2 Expressed by Regulatory T Cells. *Blood* (2016) 128 (12):1651–9. doi: 10.1182/blood-2016-02-700849
62. Chaleff S, Otto M, Barfield RC, Leimig T, Iyengar R, Martin J, et al. A Large-Scale Method for the Selective Depletion of Alphabeta T Lymphocytes From PBSC for Allogeneic Transplantation. *Cytotherapy* (2007) 9(8):746–54. doi: 10.1080/14653240701644000
63. Liu APY, Lee PPW, Kwok JSY, Leung RYY, Chiang AKS, Ha SY, et al. Selective T Cell-Depleted Haploidentical Hematopoietic Stem Cell Transplantation for Relapsed/Refractory Neuroblastoma. *Pediatr Transpl* (2018) 22(6):e13240. doi: 10.1111/petr.13240
64. Koh KN, Im HJ, Kim H, Kim N, Choi ES, Park CJ, et al. Alphabeta T-Cell-Depleted Haploidentical Hematopoietic Cell Transplantation and Zoledronate/Interleukin-2 Therapy in Children With Relapsed, High-Risk Neuroblastoma. *Bone Marrow Transpl* (2019) 54(2):348–52. doi: 10.1038/s41409-018-0305-3
65. Damodharan SN, Walker KL, Forsberg MH, McDowell KA, Bouchlaka MN, Drier DA, et al. Analysis of *Ex Vivo* Expanded and Activated Clinical-Grade Human NK Cells After Cryopreservation. *Cytotherapy* (2020) 22(8):450–7. doi: 10.1016/j.jcyt.2020.05.001

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Treatment of Steroid-Refractory Acute Graft-*Versus*-Host Disease Using Commercial Mesenchymal Stem Cell Products

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Acute graft-*versus*-host disease (GVHD) is a life-threatening complication that can develop after allogeneic hematopoietic stem cell transplantation. In particular, the prognosis of patients with steroid-refractory acute GVHD is extremely poor. Ryoncil™ (remestemcel-L), a human bone marrow-derived mesenchymal stem cell (MSC) product, failed to show superiority over placebo in patients with steroid-refractory acute GVHD, but it was approved for use in pediatric patients in Canada and New Zealand based on the results of a subgroup analysis. Temcell®, an equivalent manufactured MSC product to remestemcel-L, was approved in Japan based on small single-arm studies by using a regulation for regenerative medicine in 2016. The efficacy of Temcell was evaluated in 381 consecutive patients treated with Temcell during the initial 3 years after its approval. Interestingly, its real-world efficacy was found to be equivalent to that observed in a prospective study of remestemcel-L with strict eligibility criteria. In this article, the potential of MSC therapy in the treatment of acute GVHD is discussed. A meticulous comparison of studies of remestemcel-L and Temcell, remestemcel-L/Temcell and ruxolitinib, and remestemcel-L/Temcell and thymoglobulin showed that the precise position of remestemcel-L/Temcell therapy in the treatment of acute GVHD remains to be determined.

Keywords: graft-*versus*-host disease (GVHD), mesenchymal stem cell (MSC), steroid, ruxolitinib, thymoglobulin

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INTRODUCTION

Acute graft-*versus*-host disease (GVHD) is a life-threatening complication that can develop after allogeneic hematopoietic stem cell transplantation (HSCT) (1, 2). Systemic corticosteroid is a standard first-line treatment, but the response rate ranges from 40% to 60% (3, 4). Many agents have been evaluated as second-line treatment for acute GVHD (5, 6). However, no consensus has been reached regarding the optimal approach for the management of steroid-refractory acute GVHD (SR-aGVHD) (7). A recent, randomized, phase 3 study comparing ruxolitinib and control (nine treatment options) for SR-aGVHD demonstrated a higher overall response (OR), defined as complete response (CR) and partial response (PR), in the ruxolitinib group (8). However, the study failed to demonstrate a significant advantage of using ruxolitinib in terms of overall survival

(OS) or non-relapse mortality (NRM). Another recent, randomized, phase 3 trial comparing inolimomab and control (antithymocyte globulin) also demonstrated no significant advantage using inolimomab in terms of OS (9). Thus, no second-line treatment has been proven to improve survival in patients with SR-aGVHD.

Mesenchymal stem cells (MSCs) have been extensively studied as a treatment for SR-aGVHD (10). Efficacy of the commercial MSC product remestemcel-L (Ryoncil™, Mesoblast, Ltd, Melbourne, Australia; formerly Prochymal®, Osiris Therapeutics, Columbia, MD, USA) was evaluated in a randomized, phase 3 trial comparing the administration of remestemcel-L and of placebo in conjunction with another second-line therapy (11). Unfortunately, the study failed to meet its primary endpoint of durable CR and the secondary endpoint of the OR rate. Thus, despite a number of reports of positive outcomes of MSC therapy, unambiguous evidence of efficacy from randomized studies is still lacking (12).

However, the *post hoc* analyses of the randomized trial demonstrated that the pediatric patients, as well as the patients with liver involvement, in the remestemcel-L group had a significantly higher OR rate than those in the placebo group (11). In Japan, Temcell® (JCR Pharmaceuticals Co. Ltd, Ashiya, Japan) was approved based on the results of small single-arm studies as "regenerative medicine" *via* a new Japanese initiative on stem cell therapies, which requires the results of additional clinical trials to confirm safety and to predict likely efficacy, in 2016. Temcell, which has no generic name, is the equivalent manufactured MSC product to remestemcel-L, derived from unrelated adult bone marrow. We recently reported the outcomes of 381 consecutive patients who were treated with Temcell during the initial 3 years after its approval (13). Interestingly, the treatment outcomes of Temcell in the real-world setting achieved an efficacy equivalent to that obtained in a prospective study of remestemcel-L. The multivariate analyses identified some factors to predict a higher OR rate and lower NRM after Temcell therapy in patients with SR-aGVHD.

This Mini Review article will discuss the potential of MSC therapy in the treatment of acute GVHD based mainly on data from the studies of a large number of patients receiving remestemcel-L or Temcell.

A BRIEF REVIEW OF MSC THERAPY FOR ACUTE GVHD

MSCs, which are alternatively defined as mesenchymal "stromal" cells, can be isolated and expanded from various tissues including bone marrow (14), umbilical cord (15), placenta (16), adipose tissue (17), and dental pulp (18). In the bone marrow, MSCs at different stages of maturation form the hematopoietic stem cell (HSC) niche, which play an important role in the maintenance and renewal of HSCs (19). These properties may contribute to facilitating the engraftment of transplanted HSCs, and therefore co-transplantation of MSCs with HSCs has been widely studied to promote engraftment in autologous and allogeneic HSCT (20–34). On the other hand, MSCs interact with the innate and adaptive immune systems *via* the direct cell-to-cell contact and the release of soluble factors, resulting in the regulation of immune activities (19). These properties may contribute to treating immune-mediated diseases, and therefore administration of MSCs has been widely studied to treat acute and chronic GVHD (11, 13, 35–64). A recent mouse study suggested that MSCs promoted the proliferation of innate lymphoid cells and their production of interleukin-22 (65), which stimulate proliferation and differentiation of intestinal stem cells to regenerate the damaged tissue (66). However, this remains to be proved clinically. Another recent study raised an alternative hypothesis that an apoptosis of infused MSCs by recipient cytotoxic cells may contribute to MSC-induced immunosuppression (67).

MSCs were initially manufactured at each transplant hospital or factory by using various cell sources and culture methods, resulting in heterogeneity of MSCs (68). Thereafter, a commercial MSC product, remestemcel-L (Prochymal, currently Ryoncil), was developed in the United States (38). In Europe, a clinical-grade MSC product, called MSC-Frankfurt am Main, can now be used in clinical practice across several European countries (46, 52). According to the literature, more than 1400 patients have received MSCs as treatment of acute GVHD in the world (11, 13, 35–59). The numbers of acute GVHD patients treated with MSCs by year of publication are shown in **Figure 1**. Interestingly, there is a trend that OR rates were higher in previous studies than in recent larger studies. For all of these reported cases, the overall OR rate was 63%. Overall, MSC therapy is well tolerated. Infusion-related reactions were observed in 1.8% of patients who received remestemcel-L, less than that in patients who received placebo (2.5%) (11). No ectopic tissue formation has been reported. Although one retrospective study found a significant increase of pneumonia-related death with MSC therapy than without MSC therapy (59), another retrospective study demonstrated no difference in the risks of infections and relapse between MSC and non-MSC therapies (58). There were no differences in the rates of infection and relapse between the remestemcel-L and placebo groups in a randomized study (11).

In this review, we particularly focus on three major studies of commercial MSC products, including large number of patients (11, 13, 56), in order to avoid heterogeneity of MSC product.

MSC THERAPY WITH REMESTEMCEL-L FOR ACUTE GVHD

A randomized, phase 3 trial of remestemcel-L *vs.* placebo added to another second-line therapy according to institutional standards in patients with SR-aGVHD was conducted (11). The main results of the study are summarized in **Table 1**. Of the 260 randomized patients, 163 received at least one infusion of remestemcel-L, and 81 received at least one infusion of placebo. Remestemcel-L therapy did not meet the primary endpoint of greater durable CR for at least 28 days within the first 100 days

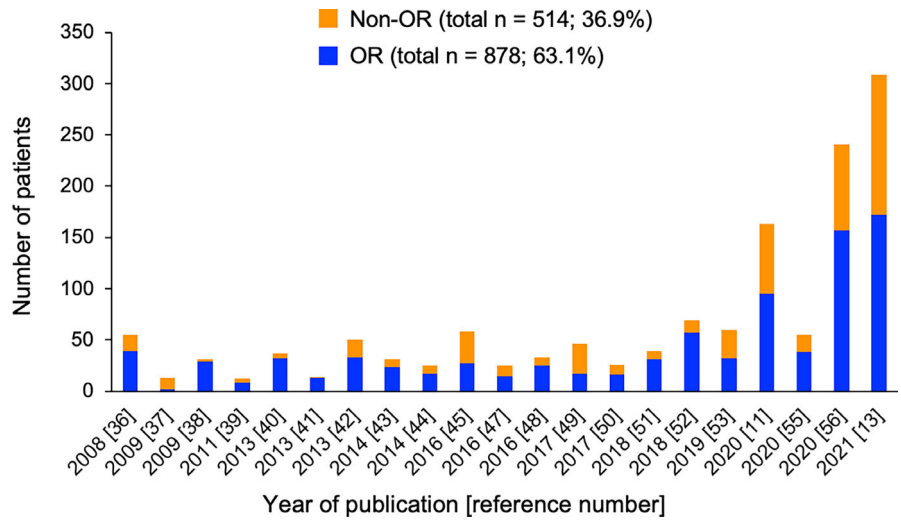

FIGURE 1 | Number of patients with (blue) and without (orange) overall response (OR) in each study of MSC therapy for acute GVHD. Total numbers and percentages of patients with or without OR as the sum of all reported cases are provided.

after enrollment (35% *vs.* 30%; $P = 0.42$). The proportions of patients achieving OR on day 28 (58% *vs.* 54%; $P = 0.59$) and OS on day 180 (34% *vs.* 42%; $P = 0.60$) were not different between the remestemcel-L and placebo groups.

A single-arm expanded access treatment with remestemcel-L in 241 patients under the age of 18 years with acute GVHD resistant to multiple immunosuppressive therapies was conducted (56). The main results of the study are summarized in **Table 1**. Despite the fact that 50% of patients had grade D acute GVHD (70), and 79% were classified as high-risk acute GVHD (71), 65% of patients achieved OR on day 28. The achievement of OR on day 28 was associated with higher OS on day 100: 82% in patients with OR and 39% without OR ($P < 0.0001$, log-rank test).

MSC THERAPY WITH TEMCELL FOR ACUTE GVHD

More recently, real-world outcomes for 381 patients who received Temcell as a health insurance-covered treatment for acute GVHD were reported from Japan (13). The main results of the study are summarized in **Table 1**. Of the 309 patients, 56% achieved OR on day 28. Of the 153 patients who received Temcell as a second-line therapy following first-line steroid therapy for classic acute GVHD, 61% achieved OR on day 28. Thus, the treatment of acute GVHD with Temcell covered by health insurance in Japan achieved an efficacy equivalent to that obtained in prospective studies of remestemcel-L with strict eligibility criteria.

On multivariate analysis, liver involvement of acute GVHD and longer duration from first-line steroid therapy to second-line MSC therapy (≥14 days *vs.* <14 days) were associated with a lower OR rate. Older patient (18 to 49 years and ≥50 years *vs.* <18 years), higher grade of GVHD (III and IV *vs.* ≤II), higher number of GVHD therapies before MSC therapy (≥2 *vs.* ≤1), and non-achievement of OR on day 28 were associated with a higher NRM. OS was significantly higher in patients with an OR on day 28 than in those without an OR.

IS THE EFFICACY OF REMESTEMCEL-L/TEMCELL GREATER IN PEDIATRIC PATIENTS?

A *post hoc* analysis of a randomized study demonstrated that the OR rate was significantly higher in the pediatric patients receiving remestemcel-L than in the pediatric patients receiving placebo (64% *vs.* 23%; $P = 0.05$) (11). However, it should be noted that only 13 pediatric patients were allocated to the placebo group, and only three (23%) of them achieved OR, in sharp contrast to the OR rate of 60% in 68 adult patients allocated to the placebo group (11). In the retrospective study of Temcell, there was no significant difference in OR rates among three age groups (< 18, 18 to 49, and ≥ 50 years) (13). Thus, there is not enough evidence to prove greater efficacy of remestemcel-L/Temcell in pediatric patients compared with adult patients.

IS REMESTEMCEL-L/TEMCELL EFFECTIVE FOR LIVER ACUTE GVHD?

A *post hoc* analysis of a randomized study demonstrated that the OR rate in patients with liver acute GVHD was significantly higher in the remestemcel-L group than in the placebo group (55% *vs.* 26%; $P = 0.05$) (11). OR rates in the remestemcel-L group were almost equal among the patients with liver, skin, and gut acute GVHD (55%, 58%, and 57%, respectively) (11).

TABLE 1 | Comparison of outcomes among selected studies of therapy for acute graft-*versus*-host disease.

Report [reference number]	Kebriaei et al. (11)		Kurtzberg et al. (56)	Murata et al. (13)		Zeiser et al. (8)		Murata et al. (69)
Study design	Randomized, double-blinded		Single-arm, prospective	Retrospective		Randomized, open-label		Retrospective
Study therapy	Remestemcel-L+ another therapy	Placebo+ another therapy	Remestemcel-L	Temcell	Temcell	Ruxolitinib	Unspecified MSC	Thymoglobulin
Treatment(s) prior to study therapy	Steroid therapy only	Steroid therapy only	Multiple therapies	Multiple therapies	Steroid therapy only	Steroid therapy only	Steroid therapy only	Steroid therapy only
Number of patients	163	81	241	309	153	154	15	99
Median age (range), y	44 (0 to 70)	40 (0 to 70)	9 (0 to 18)	49 (0 to 72)	49 (0 to 70)	53 (12 to 73)	No data	39 (2 to 69)
Grade III-IV or C-D acute GVHD, %	77	74	80	80	79	64	No data	75
OR rate on day 28, %	58	54	65	56	61	62	60	60
NRM, %	No data	No data	No data	67 at 1y	59 at 1y	43 at 1y	No data	71 at 1y
OS, %	34 on day 180	42 on day 180	67 at day 100	27 at 1y	33 at 1y	49 at 1y	No data	27 at 1y
Infection								
Evaluation period	Within 180 days	Within 180 days	No data	Within 52 weeks	Within 52 weeks	Up to the data cut off*	No data	Within 100 days
At least one infection, %	88	82	No data	47	45	80	No data	59
Bacterial infection, %	No data	No data	No data	30	24	48	No data	28
Fungal infection, %	No data	No data	No data	9	6	17	No data	11
CMV antigenemia, %	No data	No data	No data	20	22	No data	No data	25
Viral infection, %	No data	No data	No data	42	40	57	No data	35

OR indicates overall response; NRM, non-relapse mortality; OS, overall survival; CMV, cytomegalovirus.
**The follow-up period ranged 0.03 to 23.62 months.*

Similar results were obtained in a single-arm prospective study of remestemcel-L for pediatric patients; OR rates in the pediatric patients with liver, skin, and gut acute GVHD were 62%, 68%, and 65%, respectively (56).

On the other hand, a significantly lower OR rate in the patients with liver involvement of acute GVHD was reported in a retrospective study of Temcell; the OR rate in patients with liver acute GVHD was 36%, whereas OR rates in patients with skin or gut acute GVHD were 64% and 57%, respectively (13). Other immunosuppressants, such as ruxolitinib, antithymocyte globulin, and infliximab, are also reported to be less effective for liver acute GVHD than for skin or gut acute GVHD (69, 72–74). In the current situation where there is no fully effective treatment for liver acute GVHD, remestemcel-L/Temcell is an option for liver acute GVHD, but the efficacy of MSC therapy for liver acute GVHD remains unclear.

WHEN IS THE BEST TIME TO INITIATE REMESTEMCEL-L/TEMCELL THERAPY?

In a retrospective study of Temcell, multivariate analysis of all evaluable patients demonstrated a lower NRM in patients with no or one GVHD therapy before Temcell therapy compared with two or more GVHD therapies (odds ratio, 0.65; 95% confidence interval, 0.47 to 0.91) (13). Multivariate analysis of patients who received second-line Temcell therapy following first-line steroid therapy demonstrated a higher OR rate in patients with < 14 days than ≥ 14 days between first-line steroid therapy and second-line Temcell therapy (odds ratio, 2.27; 95% confidence interval, 1.09 to 4.76) (13).

In contrast, a single-arm prospective study of remestemcel-L for pediatric patients demonstrated no significant difference in the OR rate among the three groups of durations between first-line therapy and remestemcel-L therapy (74% for ≤ 14 days, 56% for 15 to 28 days, and 67% for ≥ 28 days). Thus, although it is possible that early initiation of remestemcel-L/Temcell therapy may have an advantage in terms of a higher response rate and/or lower NRM, further analysis of a larger cohort is required to provide an accurate answer to this question.

HOW MANY INFUSIONS OF REMESTEMCEL-L/TEMCELL ARE REQUIRED TO OBTAIN A RESPONSE?

Remestemcel-L/Temcell at a dose of 2×10^6 cells/kg was infused twice weekly for 4 consecutive weeks with additional infusion once weekly for a further 4 weeks in prospective studies (11, 41, 47, 55, 56). Analysis of real-world data of Temcell in Japan demonstrated that 61% of patients received ≤ 8 infusions, and 39% received 9 to 12 infusions (13). In patients who achieved OR by Temcell therapy, 50% and 90% achieved it by day 15 and day 28, respectively. These data suggest that less than 8 infusions may be sufficient in most patients, whereas more than 8 infusions are required only in a small population of patients.

On the other hand, 10% of the patients who achieved OR by day 28 with 8 infusions of Temcell experienced GVHD relapse as of day 90. Taken together, the administration schedule of remestemcel-L/Temcell therapy has not been optimized, or it has to be individually optimized.

WHICH IS MORE EFFECTIVE FOR ACUTE GVHD, RUXOLITINIB OR REMESTEMCEL-L/TEMCELL?

It must be stated again that ruxolitinib is the only drug that has been proven to be significantly more effective than control (nine therapies) in a randomized, open-label trial (8). Ruxolitinib inhibits Janus kinase 1/2 signaling, resulting in the blockade of multiple cytokines, dendritic cell activation, and neutrophil activation (75). The main results of this study (8) are summarized in **Table 1**. The patients assigned to the ruxolitinib therapy (n = 154) achieved an OR rate of 62% on day 28, which was significantly higher than that (39%) in 155 patients receiving control therapy ($P < 0.001$). MSC was one of nine options for patients assigned to the control therapy; 15 patients received MSCs and 9 (60%) of them achieved OR on day 28 (**Table 1**). Although this study was not designed to compare ruxolitinib and each control treatment, OR rates in the patients receiving ruxolitinib and MSCs were similar (8). This has been the only prospective comparison of efficacy between ruxolitinib and MSC therapies for treatment of SR-aGVHD.

Unfortunately, NRM in the patients receiving MSCs as a control therapy was not analyzed in the randomized trial (8). Although NRM cannot be directly compared between prospective and retrospective studies, NRM at 1 year in patients with ruxolitinib therapy in a prospective study (8) was much lower than that in patients with Temcell therapy in a retrospective study (13) (43% *vs.* 59%). Future comparative studies between ruxolitinib and MSC therapies, in which the primary endpoint is defined as NRM or OS, but not the response rate, are of interest.

The most common adverse events of ruxolitinib therapy have been reported to be thrombocytopenia, anemia, and cytomegalovirus infection (8). The incidences of each infection after Temcell therapy (13) might be lower than those after ruxolitinib therapy (8) (**Table 1**). However, due to a difference in the evaluation period and a lack of detailed information about clinical course, it is not possible to conclude that Temcell therapy is less likely to cause infection compared with ruxolitinib therapy.

WHICH IS MORE EFFECTIVE FOR ACUTE GVHD, ANTITHYMOCYTE GLOBULIN OR REMESTEMCEL-L/TEMCELL?

Antithymocyte globulin affects not only T cells, but also B cells, dendritic cells, regulatory T cells, and natural killer T cells, resulting in its diverse effects on the immune system (76). The major adverse events of antithymocyte globulin therapy are infusion reaction and viral and fungal infections (77). There has been no prospective study comparing antithymocyte globulin and MSC therapies for acute GVHD treatment. However, the outcome of 99 patients who received thymoglobulin as a second-line treatment for SR-aGVHD (69) was comparable to that of 153 patients who received Temcell as a second-line treatment for SR-aGVHD (13). Both retrospective studies included consecutive patients during the initial three or four years after their health insurance approval in Japan.

The main results of the thymoglobulin study are summarized in **Table 1**. The OR rate on day 28 in the thymoglobulin study (60%) was equal to that of Temcell therapy (61%). However, NRM at 1 year was higher with thymoglobulin therapy than with Temcell therapy (71% *vs.* 59%). This difference resulted from neither patient age nor severity of GVHD, because the median age was not higher, but rather lower in the thymoglobulin study than in the Temcell study (39 *vs.* 49 years, respectively), and the proportion of grade III to IV acute GVHD was slightly lower in the thymoglobulin study than in the Temcell study (75% *vs.* 79%, respectively). Of note, the incidence of any additional infection within the first 100 days after the start of thymoglobulin therapy was 59%, whereas that within 52 weeks after Temcell therapy was 45% (**Table 1**), suggesting that Temcell therapy may have an advantage of a lower NRM associated with infectious complications compared with thymoglobulin therapy. Further analysis with detailed information, such as severity and therapeutic response of each infection, is required.

HAS THERE BEEN A STUDY TO COMPARE MSCs AND OTHER THERAPIES FOR ACUTE GVHD?

There have been no other prospective or retrospective studies to compare the efficacy of MSCs and other immunosuppressants in the treatment of acute GVHD.

CONCLUSION

It is known that the incidence of severe acute GVHD is lower in Japanese than Caucasian patients (78), but the outcome of SR-aGVHD seems to be equally poor (4). Thus, effective second-line treatments for SR-aGVHD are an unmet need. Ruxolitinib is widely used as an acute GVHD treatment in the United States and Europe, but the use of MSCs has not been approved as a health insurance treatment in those countries. In contrast, Temcell is widely used in Japan, but ruxolitinib remains under review. Thus, it is currently impossible to compare the efficacy of remestemcel-L/Temcell and ruxolitinib in a real-world setting. As described in an earlier section, there has been a randomized, prospective study of remestemcel-L and placebo (11). The correct interpretation of the study is that the addition of

remestemcel-L to another second-line therapy was not superior to a second-line therapy without remestemcel-L. In other words, the efficacy of remestemcel-L alone has not been prospectively compared with other immunosuppressive drugs in the treatment of acute GVHD.

In conclusion, the appropriate use of remestemcel-L/Temcell for acute GVHD remains to be determined. Future study is needed to establish more precisely the position of remestemcel-L/Temcell in the treatment of acute GVHD.

AUTHOR CONTRIBUTIONS

MM designed the review and wrote the manuscript, and TT wrote the manuscript and supervised the process. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Zeiser R, Blazar BR. Acute Graft-*Versus*-Host Disease - Biologic Process, Prevention, and Therapy. *N Engl J Med* (2017) 377(22):2167–79. doi: 10.1056/NEJMra1609337
2. Toubai T, Magenau J. Immunopathology and Biology-Based Treatment of Steroid-Refractory Graft-*Versus*-Host Disease. *Blood* (2020) 136(4):429–40. doi: 10.1182/blood.2019000953
3. MacMillan ML, Weisdorf DJ, Wagner JE, DeFor TE, Burns LJ, Ramsay NK, et al. Response of 443 Patients to Steroids as Primary Therapy for Acute Graft-*Versus*-Host Disease: Comparison of Grading Systems. *Biol Blood Marrow Transplant* (2002) 8(7):387–94. doi: 10.1053/bbmt.2002.v8.pm12171485
4. Murata M, Nakasone H, Kanda J, Nakane T, Furukawa T, Fukuda T, et al. Clinical Factors Predicting the Response of Acute Graft-*Versus*-Host Disease to Corticosteroid Therapy: An Analysis From the GVHD Working Group of the Japan Society for Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant* (2013) 19(8):1183–9. doi: 10.1016/j.bbmt.2013.05.003
5. Martin PJ, Rizzo JD, Wingard JR, Ballen K, Curtin PT, Cutler C, et al. First- and Second-Line Systemic Treatment of Acute Graft-*Versus*-Host Disease: Recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant* (2012) 18(8):1150–63. doi: 10.1016/j.bbmt.2012.04.005
6. Murata M. Prophylactic and Therapeutic Treatment of Graft-*Versus*-Host Disease in Japan. *Int J Hematol* (2015) 101(5):467–86. doi: 10.1007/s12185-015-1784-2
7. Martin PJ. How I Treat Steroid-Refractory Acute Graft-*Versus*-Host Disease. *Blood* (2020) 135(19):1630–8. doi: 10.1182/blood.2019000960
8. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-*Versus*-Host Disease. *N Engl J Med* (2020) 382(19):1800–10. doi: 10.1056/NEJMoa1917635
9. Socié G, Vigouroux S, Yakoub-Agha I, Bay JO, Fürst S, Bilger K, et al. A Phase 3 Randomized Trial Comparing Inolimomab *vs* Usual Care in Steroid-Resistant Acute GVHD. *Blood* (2017) 129:643–9. doi: 10.1182/blood-2016-09-738625
10. Burnham AJ, Daley-Bauer LP, Horwitz EM. Mesenchymal Stromal Cells in Hematopoietic Cell Transplantation. *Blood Adv* (2020) 4(22):5877–87. doi: 10.1182/bloodadvances.2020002646
11. Kebriaei P, Hayes J, Daly A, Uberti J, Marks DI, Soiffer R, et al. A Phase 3 Randomized Study of Remestemcel-L *Versus* Placebo Added to Second-Line Therapy in Patients With Steroid-Refractory Acute Graft-*Versus*-Host Disease. *Biol Blood Marrow Transplant* (2020) 26(5):835–44. doi: 10.1016/j.bbmt.2019.08.029
12. Fisher SA, Cutler A, Doree C, Brunskill SJ, Stanworth SJ, Navarrete C, et al. Mesenchymal Stromal Cells as Treatment or Prophylaxis for Acute or Chronic Graft-*Versus*-Host Disease in Haematopoietic Stem Cell Transplant (HSCT) Recipients With a Haematological Condition. *Cochrane Database Syst Rev* (2019) 1(1):CD009768. doi: 10.1002/14651858.CD009768.pub2
13. Murata M, Terakura S, Wake A, Miyao K, Ikegame K, Uchida N, et al. Off-The-Shelf Bone Marrow-Derived Mesenchymal Stem Cell Treatment for Acute Graft-*Versus*-Host Disease: Real-World Evidence. *Bone Marrow Transplant* (2021). doi: 10.1038/s41409-021-01304-y
14. Kopen GC, Prockop DJ, Phinney DG. Marrow Stromal Cells Migrate Throughout Forebrain and Cerebellum, and They Differentiate Into Astrocytes After Injection Into Neonatal Mouse Brains. *Proc Natl Acad Sci USA* (1999) 96(19):10711–6. doi: 10.1073/pnas.96.19.10711
15. Erices A, Conget P, Minguell JJ. Mesenchymal Progenitor Cells in Human Umbilical Cord Blood. *Br J Haematol* (2000) 109(1):235–42. doi: 10.1046/j.1365-2141.2000.01986.x
16. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, et al. Isolation of Mesenchymal Stem Cells of Fetal or Maternal Origin From Human Placenta. *Stem Cells* (2004) 22(7):1338–45. doi: 10.1634/stemcells.2004-0058
17. Zannettino AC, Paton S, Arthur A, Khor F, Itescu S, Gimble JM, et al. Multipotential Human Adipose-Derived Stromal Stem Cells Exhibit a Perivascular Phenotype *In Vitro* and *In Vivo*. *J Cell Physiol* (2008) 214 (2):413–21. doi: 10.1002/jcp.21210
18. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal Human Dental Pulp Stem Cells (DPSCs) *In Vitro* and *In Vivo*. *Proc Natl Acad Sci USA* (2000) 97(25):13625–30. doi: 10.1073/pnas.240309797
19. Uccelli A, Moretta L, Pistoia V. Mesenchymal Stem Cells in Health and Disease. *Nat Rev Immunol* (2008) 8(9):726–36. doi: 10.1038/nri2395
20. Koç ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, et al. Rapid Hematopoietic Recovery After Coinfusion of Autologous-Blood Stem Cells and Culture-Expanded Marrow Mesenchymal Stem Cells in Advanced Breast Cancer Patients Receiving High-Dose Chemotherapy. *J Clin Oncol* (2000) 18(2):307–16. doi: 10.1200/JCO.2000.18.2.307
21. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, et al. Cotransplantation of *Ex Vivo* Expanded Mesenchymal Stem Cells Accelerates Lymphocyte Recovery and may Reduce the Risk of Graft Failure in Haploidentical Hematopoietic Stem-Cell Transplantation. *Blood* (2007) 110 (7):2764–7. doi: 10.1182/blood-2007-04-087056
22. Macmillan ML, Blazar BR, DeFor TE, Wagner JE. Transplantation of *Ex-Vivo* Culture-Expanded Parental Haploidentical Mesenchymal Stem Cells to Promote Engraftment in Pediatric Recipients of Unrelated Donor Umbilical Cord Blood: Results of a Phase I-II Clinical Trial. *Bone Marrow Transplant* (2009) 43(6):447–54. doi: 10.1038/bmt.2008.348
23. Guo M, Sun Z, Sun QY, Han Q, Yu CL, Wang DH, et al. A Modified Haploidentical Nonmyeloablative Transplantation Without T Cell Depletion for High-Risk Acute Leukemia: Successful Engraftment and Mild GVHD. *Biol Blood Marrow Transplant* (2009) 15(8):930–7. doi: 10.1016/j.bbmt.2009.04.006

24. Gonzalo-Daganzo R, Regidor C, Martín-Donaire T, Rico MA, Bautista G, Krsnik I, et al. Results of a Pilot Study on the Use of Third-Party Donor Mesenchymal Stromal Cells in Cord Blood Transplantation in Adults. *Cytotherapy* (2009) 11(3):278–88. doi: 10.1080/14653240902807018
25. Baron F, Lechanteur C, Willems E, Bruck F, Baudoux E, Seidel L, et al. Cotransplantation of Mesenchymal Stem Cells Might Prevent Death From Graft-*Versus*-Host Disease (GVHD) Without Abrogating Graft-*Versus*-Tumor Effects After HLA-Mismatched Allogeneic Transplantation Following Nonmyeloablative Conditioning. *Biol Blood Marrow Transplant* (2010) 16(6):838–47. doi: 10.1016/j.bbmt.2010.01.011
26. Bernardo ME, Ball LM, Cometa AM, Roelofs H, Zecca M, Avanzini MA, et al. Co-Infusion of *Ex Vivo*-Expanded, Parental MSCs Prevents Life-Threatening Acute GVHD, But Does Not Reduce the Risk of Graft Failure in Pediatric Patients Undergoing Allogeneic Umbilical Cord Blood Transplantation. *Bone Marrow Transplant* (2011) 46(2):200–7. doi: 10.1038/bmt.2010.87
27. Liu K, Chen Y, Zeng Y, Xu L, Liu D, Chen H, et al. Coinfusion of Mesenchymal Stromal Cells Facilitates Platelet Recovery Without Increasing Leukemia Recurrence in Haploidentical Hematopoietic Stem Cell Transplantation: A Randomized, Controlled Clinical Study. *Stem Cells Dev* (2011) 20(10):1679–85. doi: 10.1089/scd.2010.0447
28. Lee SH, Lee MW, Yoo KH, Kim DS, Son MH, Sung KW, et al. Co-Transplantation of Third-Party Umbilical Cord Blood-Derived MSCs Promotes Engraftment in Children Undergoing Unrelated Umbilical Cord Blood Transplantation. *Bone Marrow Transplant* (2013) 48(8):1040–5. doi: 10.1038/bmt.2013.7
29. Wu Y, Wang Z, Cao Y, Xu L, Li X, Liu P, et al. Cotransplantation of Haploidentical Hematopoietic and Umbilical Cord Mesenchymal Stem Cells With a Myeloablative Regimen for Refractory/Relapsed Hematologic Malignancy. *Ann Hematol* (2013) 92(12):1675–84. doi: 10.1007/s00277-013-1831-0
30. Wu KH, Sheu JN, Wu HP, Tsai C, Sieber M, Peng CT, et al. Cotransplantation of Umbilical Cord-Derived Mesenchymal Stem Cells Promote Hematopoietic Engraftment in Cord Blood Transplantation: A Pilot Study. *Transplantation* (2013) 95(5):773–7. doi: 10.1097/TP.0b013e31827a93dd
31. Wu Y, Cao Y, Li X, Xu L, Wang Z, Liu P, et al. Cotransplantation of Haploidentical Hematopoietic and Umbilical Cord Mesenchymal Stem Cells for Severe Aplastic Anemia: Successful Engraftment and Mild GVHD. *Stem Cell Res* (2014) 12(1):132–8. doi: 10.1016/j.scr.2013.10.001
32. Li XH, Gao CJ, Da WM, Cao YB, Wang ZH, Xu LX, et al. Reduced Intensity Conditioning, Combined Transplantation of Haploidentical Hematopoietic Stem Cells and Mesenchymal Stem Cells in Patients With Severe Aplastic Anemia. *PLoS One* (2014) 9(3):e89666. doi: 10.1371/journal.pone.0089666
33. Xiong YY, Fan Q, Huang F, Zhang Y, Wang Y, Chen XY, et al. Mesenchymal Stem Cells *Versus* Mesenchymal Stem Cells Combined With Cord Blood for Engraftment Failure After Autologous Hematopoietic Stem Cell Transplantation: A Pilot Prospective, Open-Label, Randomized Trial. *Biol Blood Marrow Transplant* (2014) 20(2):236–42. doi: 10.1016/j.bbmt.2013.11.002
34. Goto T, Murata M, Nishida T, Terakura S, Kamoshita S, Ishikawa Y, et al. Phase I Clinical Trial of Intra-Bone Marrow Cotransplantation of Mesenchymal Stem Cells in Cord Blood Transplantation. *Stem Cells Transl Med* (2021) 10(4):542–53. doi: 10.1002/sctm.20-0381
35. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of Severe Acute Graft-*Versus*-Host Disease With Third Party Haploidentical Mesenchymal Stem Cells. *Lancet* (2004) 363(9419):1439–41. doi: 10.1016/S0140-6736(04)16104-7
36. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal Stem Cells for Treatment of Steroid-Resistant, Severe, Acute Graft-*Versus*-Host Disease: A Phase II Study. *Lancet* (2008) 371(9624):1579–86. doi: 10.1016/S0140-6736(08)60690-X
37. von Bonin M, Stölzel F, Goedecke A, Richter K, Wuschek N, Hölig K, et al. Treatment of Refractory Acute GVHD With Third-Party MSC Expanded in Platelet Lysate-Containing Medium. *Bone Marrow Transplant* (2009) 43 (3):245–51. doi: 10.1038/bmt.2008.316
38. Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, et al. Adult Human Mesenchymal Stem Cells Added to Corticosteroid Therapy for the Treatment of Acute Graft-*Versus*-Host Disease. *Biol Blood Marrow Transplant* (2009) 15(7):804–11. doi: 10.1016/j.bbmt.2008.03.012
39. Prasad VK, Lucas KG, Kleiner GI, Talano JA, Jacobsohn D, Broadwater G, et al. Efficacy and Safety of *Ex Vivo* Cultured Adult Human Mesenchymal Stem Cells (Prochymal™) in Pediatric Patients With Severe Refractory Acute Graft-*Versus*-Host Disease in a Compassionate Use Study. *Biol Blood Marrow Transplant* (2011) 17(4):534–41. doi: 10.1016/j.bbmt.2010.04.014
40. Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, et al. Multiple Infusions of Mesenchymal Stromal Cells Induce Sustained Remission in Children With Steroid-Refractory, Grade III-IV Acute Graft-*Versus*-Host Disease. *Br J Haematol* (2013) 163(4):501–9. doi: 10.1111/bjh.12545
41. Muroi K, Miyamura K, Ohashi K, Murata M, Eto T, Kobayashi N, et al. Unrelated Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells for Steroid-Refractory Acute Graft-*Versus*-Host Disease: A Phase I/II Study. *Int J Hematol* (2013) 98(2):206–13. doi: 10.1007/s12185-013-1399-4
42. Resnick IB, Barkats C, Shapira MY, Stepensky P, Bloom AI, Shimoni A, et al. Treatment of Severe Steroid Resistant Acute GVHD With Mesenchymal Stromal Cells (MSC). *Am J Blood Res* (2013) 3(3):225–38.
43. Introna M, Lucchini G, Dander E, Galimberti S, Rovelli A, Balduzzi A, et al. Treatment of Graft *Versus* Host Disease With Mesenchymal Stromal Cells: A Phase I Study on 40 Adult and Pediatric Patients. *Biol Blood Marrow Transplant* (2014) 20(3):375–81. doi: 10.1016/j.bbmt.2013.11.033
44. Sánchez-Guijo F, Caballero-Velázquez T, López-Villar O, Redondo A, Parody R, Martínez C, et al. Sequential Third-Party Mesenchymal Stromal Cell Therapy for Refractory Acute Graft-*Versus*-Host Disease. *Biol Blood Marrow Transplant* (2014) 20(10):1580–5. doi: 10.1016/j.bbmt.2014.06.015
45. von Dalowski F, Kramer M, Wermke M, Wehner R, Röllig C, Alakel N, et al. Mesenchymal Stromal Cells for Treatment of Acute Steroid-Refractory Graft *Versus* Host Disease: Clinical Responses and Long-Term Outcome. *Stem Cells* (2016) 34(2):357–66. doi: 10.1002/stem.2224
46. Kuçi Z, Bönig H, Kreyenberg H, Bunos M, Jauch A, Janssen JW, et al. Mesenchymal Stromal Cells From Pooled Mononuclear Cells of Multiple Bone Marrow Donors as Rescue Therapy in Pediatric Severe Steroid-Refractory Graft-*Versus*-Host Disease: A Multicenter Survey. *Haematologica* (2016) 101(8):985–94. doi: 10.3324/haematol.2015.140368
47. Muroi K, Miyamura K, Okada M, Yamashita T, Murata M, Ishikawa T, et al. Bone Marrow-Derived Mesenchymal Stem Cells (JR-031) for Steroid-Refractory Grade III or IV Acute Graft-*Versus*-Host Disease: A Phase II/III Study. *Int J Hematol* (2016) 103(2):243–50. doi: 10.1007/s12185-015-1915-9
48. Erbey F, Atay D, Akcay A, Ovali E, Ozturk G. Mesenchymal Stem Cell Treatment for Steroid Refractory Graft-*Versus*-Host Disease in Children: A Pilot and First Study From Turkey. *Stem Cells Int* (2016) 2016:1641402. doi: 10.1155/2016/1641402
49. Dotoli GM, De Santis GC, Orellana MD, de Lima Prata K, Caruso SR, Fernandes TR, et al. Mesenchymal Stromal Cell Infusion to Treat Steroid-Refractory Acute GvHD III/IV After Hematopoietic Stem Cell Transplantation. *Bone Marrow Transplant* (2017) 52(6):859–62. doi: 10.1038/bmt.2017.35
50. Salmenniemi U, Itälä-Remes M, Nystedt J, Putkonen M, Niittyvuopio R, Vettenranta K, et al. Good Responses But High TRM in Adult Patients After MSC Therapy for GvHD. *Bone Marrow Transplant* (2017) 52(4):606–8. doi: 10.1038/bmt.2016.317
51. Ringden O, Baygan A, Remberger M, Gustafsson B, Winiarski J, Khoein B, et al. Placenta-Derived Decidua Stromal Cells for Treatment of Severe Acute Graft-*Versus*-Host Disease. *Stem Cells Transl Med* (2018) 7(4):325–31. doi: 10.1002/sctm.17-0167
52. Bader P, Kuçi Z, Bakhtiar S, Basu O, Bug G, Dennis M, et al. Effective Treatment of Steroid and Therapy-Refractory Acute Graft-*Versus*-Host Disease With a Novel Mesenchymal Stromal Cell Product (MSC-FFM). *Bone Marrow Transplant* (2018) 53(7):852–62. doi: 10.1038/s41409-018-0102-z
53. Galleu A, Milojkovic D, Deplano S, Szydlo R, Loaiza S, Wynn R, et al. Mesenchymal Stromal Cells for Acute Graft-*Versus*-Host Disease: Response at 1 Week Predicts Probability of Survival. *Br J Haematol* (2019) 185(1):89–92. doi: 10.1111/bjh.15749
54. Bonig H, Kuçi Z, Kuçi S, Bakhtiar S, Basu O, Bug G, et al. Children and Adults With Refractory Acute Graft-*Versus*-Host Disease Respond to Treatment With the Mesenchymal Stromal Cell Preparation "MSC-FFM"-Outcome Report of 92 Patients. *Cells* (2019) 8(12):1577. doi: 10.3390/cells8121577

55. Kurtzberg J, Abdel-Azim H, Carpenter P, Chaudhury S, Horn B, Mahadeo K, et al. A Phase 3, Single-Arm, Prospective Study of Remestemcel-L, *Ex Vivo* Culture-Expanded Adult Human Mesenchymal Stromal Cells for the Treatment of Pediatric Patients Who Failed to Respond to Steroid Treatment for Acute Graft-*Versus*-Host Disease. *Biol Blood Marrow Transplant* (2020) 26(5):845–54. doi: 10.1016/j.bbmt.2020.01.018
56. Kurtzberg J, Prockop S, Chaudhury S, Horn B, Nemecek E, Prasad V, et al. Study 275: Updated Expanded Access Program for Remestemcel-L in Steroid-Refractory Acute Graft-*Versus*-Host Disease in Children. *Biol Blood Marrow Transplant* (2020) 26(5):855–64. doi: 10.1016/j.bbmt.2020.01.026
57. van der Wagen LE, Miranda-Bedate A, Janssen A, Fernando F, Appukudige N, van Dooremalen S, et al. Efficacy of MSC for Steroid-Refractory Acute GVHD Associates With MSC Donor Age and a Defined Molecular Profile. *Bone Marrow Transplant* (2020) 55(11):2188–92. doi: 10.1038/s41409-020-0910-9
58. Zhao K, Lou R, Huang F, Peng Y, Jiang Z, Huang K, et al. Immunomodulation Effects of Mesenchymal Stromal Cells on Acute Graft-*Versus*-Host Disease After Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2015) 21(1):97–104. doi: 10.1016/j.bbmt.2014.09.030
59. Forslöw U, Blennow O, LeBlanc K, Ringdén O, Gustafsson B, Mattsson J, et al. Treatment With Mesenchymal Stromal Cells Is a Risk Factor for Pneumonia-Related Death After Allogeneic Hematopoietic Stem Cell Transplantation. *Eur J Haematol* (2012) 89(3):220–7. doi: 10.1111/j.1600-0609.2012.01824.x
60. Zhou H, Guo M, Bian C, Sun Z, Yang Z, Zeng Y, et al. Efficacy of Bone Marrow-Derived Mesenchymal Stem Cells in the Treatment of Sclerodermatous Chronic Graft-*Versus*-Host Disease: Clinical Report. *Biol Blood Marrow Transplant* (2010) 16(3):403–12. doi: 10.1016/j.bbmt.2009.11.006
61. Weng JY, Du X, Geng SX, Peng YW, Wang Z, Lu ZS, et al. Mesenchymal Stem Cell as Salvage Treatment for Refractory Chronic GVHD. *Bone Marrow Transplant* (2010) 45(12):1732–40. doi: 10.1038/bmt.2010.195
62. Pérez-Simon JA, López-Villar O, Andreu EJ, Rifón J, Muntion S, Diez Campelo M, et al. Mesenchymal Stem Cells Expanded *In Vitro* With Human Serum for the Treatment of Acute and Chronic Graft-*Versus*-Host Disease: Results of a Phase I/II Clinical Trial. *Haematologica* (2011) 96 (7):1072–6. doi: 10.3324/haematol.2010.038356
63. Peng Y, Chen X, Liu Q, Zhang X, Huang K, Liu L, et al. Mesenchymal Stromal Cells Infusions Improve Refractory Chronic Graft *Versus* Host Disease Through an Increase of CD5+ Regulatory B Cells Producing Interleukin 10. *Leukemia* (2015) 29(3):636–46. doi: 10.1038/leu.2014.225
64. Boberg E, von Bahr L, Afram G, Lindström C, Ljungman P, Heldring N, et al. Treatment of Chronic GvHD With Mesenchymal Stromal Cells Induces Durable Responses: A Phase II Study. *Stem Cells Transl Med* (2020) 9 (10):1190–202. doi: 10.1002/sctm.20-0099
65. van Hoeven V, Munneke JM, Cornelissen AS, Omar SZ, Spruit MJ, Kleijer M, et al. Mesenchymal Stromal Cells Stimulate the Proliferation and IL-22 Production of Group 3 Innate Lymphoid Cells. *J Immunol* (2018) 201 (4):1165–73. doi: 10.4049/jimmunol.1700901
66. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR, et al. Interleukin-22 Promotes Intestinal-Stem-Cell-Mediated Epithelial Regeneration. *Nature* (2015) 528(7583):560–4. doi: 10.1038/nature16460
67. Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, et al. Apoptosis in Mesenchymal Stromal Cells Induces *In Vivo* Recipient-Mediated Immunomodulation. *Sci Transl Med* (2017) 9(416):eaam7828. doi: 10.1126/scitranslmed.aam7828
68. Rizk M, Monaghan M, Shorr R, Kekre N, Bredeson CN, Allan DS. Heterogeneity in Studies of Mesenchymal Stromal Cells to Treat or Prevent Graft-*Versus*-Host Disease: A Scoping Review of the Evidence. *Biol Blood Marrow Transplant* (2016) 22(8):1416–23. doi: 10.1016/j.bbmt.2016.04.010
69. Murata M, Ikegame K, Morishita Y, Ogawa H, Kaida K, Nakamae H, et al. Low-Dose Thymoglobulin as Second-Line Treatment for Steroid-Resistant Acute GvHD: An Analysis of the JSHCT. *Bone Marrow Transplant* (2017) 52 (2):252–7. doi: 10.1038/bmt.2016.247
70. Rowlings PA, Przepiorka D, Klein JP, Gale RP, Passweg JR, Henslee-Downey PJ, et al. IBMTR Severity Index for Grading Acute Graft-*Versus*-Host Disease: Retrospective Comparison With Glucksberg Grade. *Br J Haematol* (1997) 97 (4):855–64. doi: 10.1046/j.1365-2141.1997.1112925.x
71. MacMillan ML, Robin M, Harris AC, DeFor TE, Martin PJ, Alousi A, et al. A Refined Risk Score for Acute Graft-*Versus*-Host Disease That Predicts Response to Initial Therapy, Survival, and Transplant-Related Mortality. *Biol Blood Marrow Transplant* (2015) 21(4):761–7. doi: 10.1016/j.bbmt.2015.01.001
72. Jagasia M, Perales MA, Schroeder MA, Ali H, Shah NN, Chen YB, et al. Ruxolitinib for the Treatment of Steroid-Refractory Acute GVHD (REACH1): A Multicenter, Open-Label Phase 2 Trial. *Blood* (2020) 135(20):1739–49. doi: 10.1182/blood.2020004823
73. Khoury H, Kashyap A, Adkins DR, Brown RA, Miller G, Vij R, et al. Treatment of Steroid-Resistant Acute Graft-*Versus*-Host Disease With Anti-Thymocyte Globulin. *Bone Marrow Transplant* (2001) 27(10):1059–64. doi: 10.1038/sj.bmt.1703032
74. Couriel D, Saliba R, Hicks K, Ippoliti C, de Lima M, Hosing C, et al. Tumor Necrosis Factor-Alpha Blockade for the Treatment of Acute GVHD. *Blood* (2004) 104(3):649–54. doi: 10.1182/blood-2003-12-4241
75. Teshima T, Reddy P, Zeiser R. Acute Graft-*Versus*-Host Disease: Novel Biological Insights. *Biol Blood Marrow Transplant* (2016) 22(1):11–6. doi: 10.1016/j.bbmt.2015.10.001
76. Mohty M. Mechanisms of Action of Antithymocyte Globulin: T-Cell Depletion and Beyond. *Leukemia* (2007) 21(7):1387–94. doi: 10.1038/sj.leu.2404683
77. Bacigalupo A. Antilymphocyte/thymocyte Globulin for Graft *Versus* Host Disease Prophylaxis: Efficacy and Side Effects. *Bone Marrow Transplant* (2005) 35(3):225–31. doi: 10.1038/sj.bmt.1704758
78. Kanda J, Brazauskas R, Hu ZH, Kuwatsuka Y, Nagafuji K, Kanamori H, et al. Graft-*Versus*-Host Disease After HLA-Matched Sibling Bone Marrow or Peripheral Blood Stem Cell Transplantation: Comparison of North American Caucasian and Japanese Populations. *Biol Blood Marrow Transplant* (2016) 22(4):744–51. doi: 10.1016/j.bbmt.2015.12.027

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Venetoclax or Ruxolitinib in Pre-Transplant Conditioning Lowers the Engraftment Barrier by Different Mechanisms in Allogeneic Stem Cell Transplant Recipients

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Allogeneic stem cell transplantation (alloSCT) is utilised to cure haematological malignancies through a combination of conditioning regimen intensity and immunological disease control *via* the graft versus tumour (GVT) effect. Currently, conventional myeloablative chemotherapeutic or chemoradiation conditioning regimens are associated with significant side effects including graft versus host disease (GVHD), infection, and organ toxicity. Conversely, more tolerable reduced intensity conditioning (RIC) regimens are associated with unacceptably higher rates of disease relapse, partly through an excess incidence of mixed chimerism. Improvement in post-alloSCT outcomes therefore depends on promotion of the GVT effect whilst simultaneously reducing conditioning-related toxicity. We have previously shown that this could be achieved through BCL-2 inhibition, and in this study, we explored the modulation of JAK1/2 as a strategy to lower the barrier to donor engraftment in the setting of RIC. We investigated the impact of short-term treatment of BCL2 (venetoclax) or JAK1/2 (ruxolitinib) inhibition on recipient natural killer and T cell immunity and the subsequent effect on donor engraftment. We identified striking differences in mechanism of action of these two drugs on immune cell subsets in the bone marrow of recipients, and in the regulation of MHC class-II and interferon-inducible gene expression, leading to different rates of GVHD. This study demonstrates that the repurposed use of ruxolitinib or venetoclax can be utilised as pre-transplant immune-modulators to promote the efficacy of alloSCT, whilst reducing its toxicity.

Keywords: venetoclax, ruxolitinib, reduced intensity conditioning, graft versus tumour effect, MHC class-II, graft versus host disease, allogeneic stem cell transplantation

INTRODUCTION

Allogeneic stem cell transplantation (alloSCT) is used to cure a range of haematological malignancies in part through the induction of the graft versus tumour (GVT) response mediated by engrafted donor immunity (1). Myeloablative conditioning (MAC) regimens have been the mainstay of allogeneic transplantation and produce reliable donor T cell engraftment but are associated with transplant-related toxicity and graft-versus host disease (GVHD), which collectively contribute to a transplant-related mortality of 20% in most series (2). In order to mitigate these toxicities in older patients or in those with comorbidities, a range of reduced-intensity conditioning (RIC) regimens have been employed over the last 20 years of alloSCT practice and now accounts for nearly two thirds of all transplant conditioning regimens (3, 4). However, RIC is often associated with mixed donor cell chimerism and a concurrent reduction in the GVT effect (5–7). Therefore, novel approaches to conditioning are required to enhance and maintain donor engraftment and GVT effect following RIC, whilst avoiding the toxicity and mortality rates associated with MAC.

We have previously shown that following RIC, residual recipient immunity acts as a barrier to donor engraftment that can be overcome by the addition of targeted therapy to RIC regimens (8, 9). Importantly, the brief pharmacological inhibition of BCL2 using venetoclax prior to RIC in mice resulted in depletion of residual recipient immunity and subsequent rapid donor cell engraftment in most recipients. Additionally, an absence of inflammatory cytokine production and avoidance of GVHD onset was observed, whilst the GVT effect against acute myeloid leukaemia (AML) was maintained (8). The Janus Kinase (JAK) 1/2 inhibitor ruxolitinib first showed its ability to profoundly decrease inflammatory cytokines in the treatment of myelofibrosis (10), and was the first drug to be approved by the FDA for the treatment of steroid-refractory GVHD (11, 12) *via* reduction of inflammatory cytokine production by T, NK and dendritic cells [reviewed by (13)]. Ruxolitinib has been established as an important and safe component of salvage therapy for the treatment of steroid-refractory acute GVHD (14, 15).

Based on our observations that donor engraftment and anti-tumour efficacy of alloSCT following RIC can be enhanced through venetoclax-induced depletion of residual recipient immunity, we hypothesised that suppression of inflammatory cytokines using ruxolitinib may also lower the engraftment barrier in RIC and result in similar post-alloSCT outcomes. In this paper we explored the effects of ruxolitinib in a RIC alloSCT model and compared the mechanisms to those observed in a venetoclax-containing RIC regimen.

MATERIALS AND METHODS

Experimental Mice

Experimental mice were specific-pathogen-free (SPF) and all animal work was conducted with standard operating procedures approved by institutional animal ethics committees. The alloSCT experiments were performed either at the Biological Research Facility of the Victorian Comprehensive Cancer Centre (VCCC) or the Bioservice Department of the Walter and Eliza Hall Institute of Medical Research (WEHI). IL-15 KO (16) mice with C57BL/6 background were bred and used at WEHI. All mice used as recipients for transplantation were 6-14 weeks of age when the experiments were set up. BALB/c donors were purchased at 6-8 weeks of age, and sex-matched to the recipients.

The MHC-mismatched allogeneic SCT (alloSCT) model used mice with C57BL/6 background (H-2Kb) as recipients and BALB/c (H-2Kd) allogeneic donors. Recipients (n=6/group) received split-dose total body irradiation (TBI) by a cobalt-60 irradiator, of either myeloablative (MAC) (2 × 550 rad) or reduced intensity conditioning (RIC) dose (2 × 400 rad) delivered two hours apart. 7.5 $x10^6$ bone marrow (BM) cells and 1 $x10^6$ T cells (splenic CD4+ T cells and CD8+ T cells mixed in a 2:1 ratio) from BALB/c donors were intravenously injected into recipients at least two hours after irradiation. AlloSCT recipients were monitored regularly for body weight and clinical scores based on posture, activity, and eye appearance [scores of 3 for each, adapted from (17)], and were humanely killed once 20% of initial body weight loss or clinical scores of 4 were reached. Donor haematopoietic cell engraftment examined the donor:recipient (H-2Kd/H-2Kb) ratio within peripheral blood. Donor cell engraftment and cell profiles within organs were also analysed at the experimental endpoint.

Chemical Compounds

The BCL2 inhibitor venetoclax and JAK1/2 inhibitor ruxolitinib (SelleckChem, Houston, TX) were used to treat C57BL/6 WT mice for two days prior to alloSCT. Venetoclax (100 mg/kg) and its vehicle (60% phosal R 50 PG (Merck, Germany), 30% polyethylene glycol (PEG) 400 (Merck, Germany), 10% ethanol) were administered by oral gavage once daily for two days, with a cumulative total dose of 4 mg. Ruxolitinib (180 mg/kg) and its vehicle (2% DMSO, 30% PEG 300 (Merck, Germany), ddH2O) were administered twice a day by oral gavage for two days, with a cumulative total dose of 14.4 mg.

GVHD Histology

Recipient rectum and colon tissue without stool were preserved in 10% neutral buffered formalin (Merck, Germany), and Haemotoxylin and Eosin (H&E) staining and digital images *via* a 20x slide scanning were processed by the Histology Department of WEHI. Histology scores were given to the gut tissues according to the number of apoptotic cells, mucosal integrity, and lymphocyte infiltration (each scored out of 3), by an independent, blinded pathologist.

Graft Versus Tumour Model

Wild type C57BL/6 mice were inoculated with 0.8 × 10^6 (mixed lineage leukaemia) GFP+ MLL-AF9 acute myeloid leukaemia (AML) cells. After 8 days, mice were treated with ruxolitinib (180 mg/kg) by oral gavage twice daily for two days. The following day mice were irradiated with RIC and injected with 7.5 $x10^6$ BM cells and 1 $x10^6$ T cells from BALB/c donors. Mice were

monitored regularly for body weight, clinical scores, donor cell engraftment and AML burden in the blood, and were killed after 21 days post alloSCT.

Flow Cytometric Analysis

Peripheral blood samples were collected with or without EDTA to separate blood cells and plasma/serum, which were stored at -20°C for cytokine analysis. Single cell suspension of splenocytes, peripheral blood, BM and liver cells (purified using a 33.75% Percoll R Density Gradient (GE Healthcare, Sweden), were resuspended in FACS buffer (PBS + 2% FCS) after red blood cell lysis. Cells were mixed with FACS buffer containing 1/100 mouse Fc blocking antibody (purified Rat anti-Mouse CD16/CD32, 2.4G2, BD Biosciences, San Jose, CA) and specific antibody cocktail on ice for 30 minutes. After unbound antibodies were washed away, cells were fixed in 2% paraformaldehyde and analysed on a BD LSRFortessaII (BD Biosciences). The following antibodies were used to identify donor cells (H-2Kd; e450, SF1-1.1.1), recipient cells (H-2Kb; PE, AF6-88.5), leukocytes (CD45; BV611, 30-F11), T cells (CD3; BV785, 17A2), CD4+ T cells (CD4; BUV805/APCe780, GK1.5), CD8+ T cells (CD8a; PerCP-Cy5.5/PE-Cy7/BUV395, 53-6.7), memory T cells (CD44 and CD62L; APC-Cy7, IM7; PE-Cy7, MEL-14), B cells (CD19; BV711, ID3), myeloid cells (CD11b and Ly6C/G; BUV395, M1/70; APC, RB6-8C5), NK cells (NK1.1, NKp46 and CD49b; BV650, PK136; PECy7, 29A1.4; BB700, HMα2), mature NK cells (CD11b and CD27; BV605, M1/70; APC-e780; LG.7F9), ILC1s (NK1.1, NKp46 and CD49a; BV650, PK136; PECy7, 29A1.4; BV711, Ha31/8). All mAbs were from BD Biosciences, except for CD27, H2Kd and NKp46 (Thermo Fisher, Waltham, MA).

FlowJo (BD Biosciences, San Jose, CA) analysis was used to identify NK (NK1.1+CD3-), cNK (NKp46+CD49b+), ILC1s (NKp46+CD49a+), CD4 (CD3+CD4+) and CD8 (CD3+CD8+) T cells, B cells (CD19+), and granulocytes (CD11b+Ly6G+). Phenotypic subsets were characterised by the expression of the following cell surface markers: M1 Mature (CD11b+CD27+), M2 mature (CD11b+CD27-) and immature (CD11b-CD27+) NK cells, naive (N; CD44-CD62L+); central memory (CM; CD44+CD62L+); effector memory (EM; CD44+CD62L-) CD4 and CD8 T cells; and virtual memory (VM; CD8+CD44+CD62L+CD49d+) T cells.

Cytometric Bead Array

Plasma/serum samples from specific timepoints post-alloSCT were tested using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Jose, CA) as per manufacturer's instructions. The CBA was analysed using FCAP Array v3.0 Analysis Software (BD Biosciences, San Jose, CA).

Gene Expression Analysis

Total BM RNA was extracted from cohorts of mice (n=3-4) days 1, 3 and 7 post-drug treatment or from untreated controls using the Qiagen RNeasy Kit (Qiagen, Venlo, The Netherlands). Gene expression was determined using the NanoString Mouse PanCancer Immune Profiling Panel (NanoString Technologies, Seattle, WA) as per manufacturer's instructions. All raw data was reviewed, and all samples in downstream analysis had no quality control flags and detection of at least 20% of probes. All experiments were normalised and analysed using nCounter Advanced Analysis (version 2.0.115; NanoString Technologies).

Statistical Analysis

Statistical analysis was conducted using, unpaired T test, Mann-Whitney unpaired T test, Ordinary One-Way Anova Holm-Sidak's multiple comparisons test, and Pearson's Correlation coefficient as indicated, using GraphPad Prism V9.2.0 (San Diego, CA). Significance is indicated as follows: $p<0.05$(*), $p<0.01$(**), $p<0.001$(***), $p<0.0001$(****).

RESULTS

Recipient NK and CD8+ T Cells Regulate Donor Cell Engraftment and Onset of Acute GVHD

NK cells present an engraftment barrier in RIC treated mice (9), and NK cell survival is dependent on IL-15 signalling (18, 19). To explore the outcome of alloSCT in recipients in which the engraftment barrier was absent, we compared C57BL/6 WT mice transplanted using MAC compared with IL-15 KO mice transplanted using RIC. IL-15 KO mice lack mature NK cells and also have 10-fold fewer immature NK cells compared to WT mice (**Supplementary Figures 1A, B**). Within 3 days post alloSCT, IL-15 KO recipients developed rapid weight loss (**Figure 1A**) and high clinical GVHD scores (**Figure 1B**), and had to be killed by day 6 due to hyperacute GVHD. Donor cell engraftment was greater than 80% by day 6 post-transplant, and was accompanied by elevated IFNγ and IL-6 levels, and high GVHD histology scores in the gut (**Figures 1C–E**). Therefore, while IL-15 KO successfully removed the recipient-derived engraftment barrier, it was at the cost of unmitigated donor T cell expansion, cytokine production and onset of severe, fatal GVHD.

Ruxolitinib Treatment Combined With RIC Reduces T and NK Cells and Allows Full Engraftment

To compare the levels of immune depletion during conditioning, we first investigated NK and T cell depletion post conditioning in WT and IL-15 KO mice. Mice were left untreated or given a MAC or RIC irradiation dose, and killed 4 days later to examine the absolute NK or CD8+ T cell numbers remaining in the BM. WT mice irradiated with RIC or MAC had a significant decrease in NK cells compared to untreated mice, but were still 10-fold higher than in untreated IL-15 KO mice (**Figure 2A**). In contrast, RIC or MAC treated WT mice had CD8+ T cell numbers similar to IL-15 KO untreated mice, and RIC treatment of IL-15 KO mice almost ablated both NK and CD8+ T cells in the BM (**Figure 2B**). In order to pharmacologically replicate the IL-15 KO phenotype, WT mice were treated with the JAK1/2 inhibitor

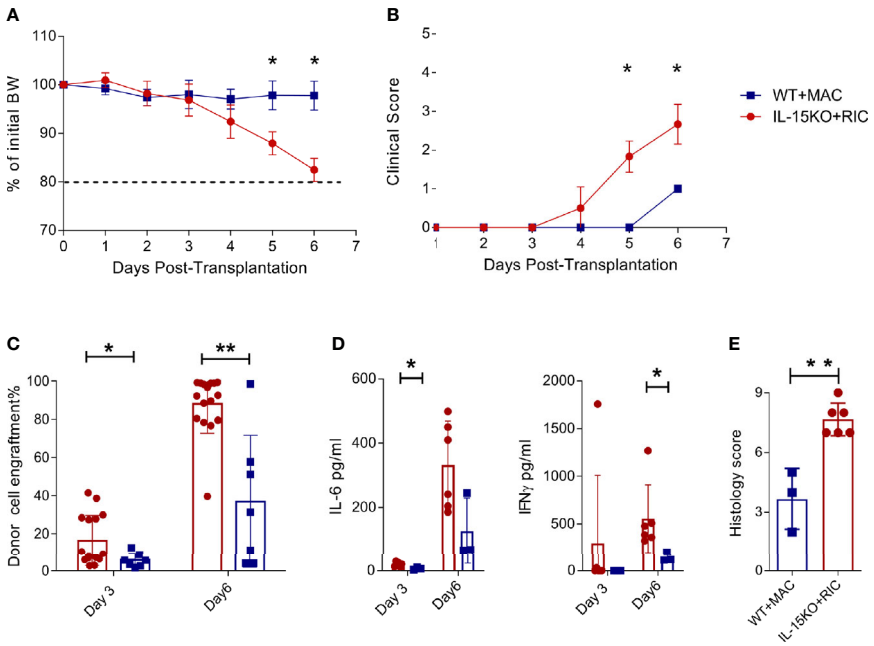

FIGURE 1 | IL-15 KO + RIC alloSCT recipients develop hyperacute GVHD. C57BL/6 WT mice were irradiated with MAC, and IL-15 KO mice were irradiated with RIC, followed by alloSCT. Mice were monitored daily for **(A)** body weight and **(B)** clinical scores, and **(C)** donor cell engraftment and **(D)** plasma cytokine concentrations of IL-6 and IFNγ on days 3 and 6. **(E)** Mice were killed on day 6 post-alloSCT, and GVHD histology was conducted on gut tissue. Statistical analysis was performed using Mann-Whitney unpaired T test. *$p < 0.05$, **$p < 0.01$.

ruxolitinib and RIC, resulting in a reduction of NK and CD8+ T cell numbers comparable to those in untreated IL-15 KO mice.

Next, we determined if transient inhibition of JAK1/2 was able to replicate the lowered engraftment barrier seen in the IL-15 KO mice, while maintaining the GVHD control of the WT mice. Depletion of recipient immunity using combination ruxolitinib and RIC was well tolerated, with minimal weight loss and low clinical scores recovering within 2 weeks post-alloSCT (**Figures 2C, D**). Mice treated with ruxolitinib and RIC engrafted by 7 days post-alloSCT, unlike vehicle and RIC treated mice which rejected the graft (**Figure 2E**). After 14 days post-alloSCT, over 80% of ruxolitinib treated mice had engrafted with donor cells, which was comparable to the MAC treated cohort, and was maintained at 70 days (**Figures 2E, F**). In comparison, the mice treated with vehicle and RIC uniformly rejected the graft and were killed on day 14 upon developing increasing clinical scores due to anaemia (**Figures 2D, E**). Donor cell engraftment in ruxolitinib treated mice was associated with moderate IFNγ and low IL-6 levels in the plasma at day 7 post-alloSCT, which abated by day 14 (**Figure 2G**). Mice killed at 70 days post-alloSCT did not develop the early gut GVHD (**Figure 2H**) that was seen in the IL-15 KO mice, however between day 30-50 post-alloSCT skin GVHD developed in approximately 25% of mice treated with ruxolitinib and RIC, which was not observed in WT mice treated with MAC (**Figure 2I**). Mice with skin GVHD had to be killed due to ulceration of the skin which developed after localised fur loss on the hind flanks.

We previously established a pre-clinical model in C57BL/6 WT mice of AML (MLL-AF9) matched to the BALB/c allogeneic donor haplotype (H2kd+), to examine the effect of venetoclax treatment with RIC on donor cell engraftment and subsequent GVT effect (8). In this study, ruxolitinib treatment improved GVT responses compared to vehicle treated controls, with some mice showing tumour control comparable to MAC treated mice (**Figure 2J**). The level of tumour response strongly correlated with the level of donor cell engraftment, with mice that had full engraftment showing complete tumour control, whereas mice that rejected the graft or had mixed chimerism (5-90% donor cells) had impaired GVT responses (**Figures 2J, K**). Overall, this suggests that transient inhibition of JAK1/2 signalling reduces the engraftment barrier presented by residual recipient NK and T cells, allowing full donor engraftment, whilst improving GVT and decreasing priming of acute GVHD onset.

Donor Cell Engraftment Is Dependent on Recipient Pre-SCT Conditioning

Our previous work has shown that pre-treatment of alloSCT recipients with short-term pharmacological inhibition of BCL2 (venetoclax) in combination with RIC permits rapid donor cell engraftment in a high percentage of mice, without graft rejection or GVHD (8). Approximately 80% of WT mice administered venetoclax for two days immediately prior to RIC and alloSCT obtained donor cell engraftment within 14 days, however approximately 40% of venetoclax-treated mice developed graft rejection after an initial period of donor

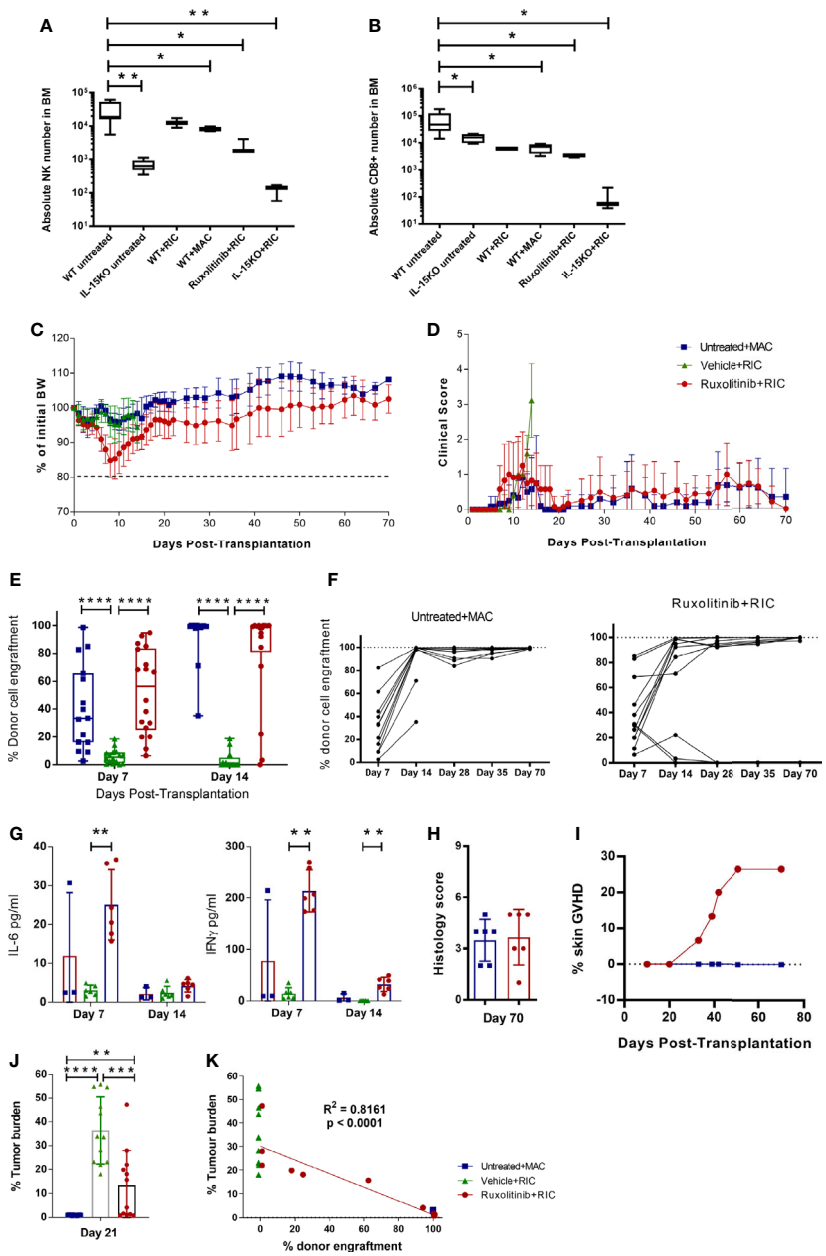

FIGURE 2 | Ruxolitinib treatment in combination with RIC mediates rapid and long-term donor cell engraftment, and permits GVT responses. Untreated WT and IL-15KO mice were compared to WT mice treated with RIC or MAC; or WT mice treated with ruxolitinib prior to RIC, or IL-15 KO mice treated with RIC. Mice were killed four days after receiving irradiation, and the absolute number of **(A)** NK cells (NKp46+CD49b+) and **(B)** CD8 (CD3+CD8+) T cells in BM were compared between different cohorts of mice (n=3-9/group). C57BL/6 WT mice were treated with ruxolitinib or vehicle for two days, and the following day treated with RIC and alloSCT. Another cohort of untreated WT mice was treated with MAC and alloSCT. Mice were monitored for **(C)** body weight and **(D)** clinical scores up to 70 days post alloSCT. **(E, F)** Donor cell engraftment (H2Kd+ cells) was monitored on days 7, 14, 28, 35 and 70 post-alloSCT in blood samples (n= 18, data combined from 3 independent experiments). **(G)** Plasma cytokine concentration of IFNγ and IL-6 was measured in blood samples collected on days 7 and 14 post-alloSCT. **(H)** Mice were killed 70 days post-alloSCT, and GVHD histology was conducted on gut tissue. **(I)** Incidence of development of skin GVHD in ruxolitinib+RIC mice compared to untreated+MAC alloSCT recipients (n=15). Mice were injected i.v. with MLL-AF9 tumour cells, and 8 days later were treated with ruxolitinib or vehicle for two days, and the following day treated with RIC and alloSCT. Another cohort of untreated WT mice was treated with MAC and alloSCT (n=12/treatment group, data combined from 2 independent experiments). **(J)** Mice were killed 21 days after alloSCT, and tumour burden was measured as a percentage of MLL-AF9+ cells in the BM. **(K)** Tumour burden was compared to donor cell engraftment between the untreated+MAC, vehicle+RIC and ruxolitinib+RIC cohorts 21 days after alloSCT. R^2 value indicates the correlation between tumour burden and donor cell engraftment in ruxolitinib+RIC alloSCT recipients. Statistical analysis was performed using Ordinary One-way Anova Holm-Sidak's multiple comparisons test **(A, B)**, Mann-Whitney unpaired T test **(E–J)**, and Pearson's Correlation coefficient **(K)**. *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.

engraftment (**Figure 3A** (8). In contrast, over 80% of ruxolitinib-treated alloSCT recipients retained long-term donor engraftment (**Figures 2F** and **3A**).

To understand the mechanism of recipient immune cell inhibition with venetoclax and ruxolitinib, WT mice were treated for two days with either venetoclax, ruxolitinib, or their respective vehicles, and then killed on day 1, 2, 3, and 7 to profile immune cell subsets in BM, spleen and liver. In contrast to ruxolitinib, venetoclax treatment rapidly depleted NK cells, including conventional (NKp46+CD49b+), immature (CD11b-CD27+), and M1 (CD11b+CD27+) and M2 (CD11b+CD27-) mature NK cells from the spleen and liver, and most strikingly from the BM (**Figures 3B–F**, **Supplementary Figure 2A**). Furthermore, venetoclax rapidly depleted CD8+ and CD4+ naïve (CD62L+CD44-) and CD8+ central memory (CD62L+ CD44+) T cells in the BM, spleen and liver (**Figures 3G–L**, **Supplementary Figure 2B**). Therefore, BCL2 inhibition affected recipient immune cell function by rapidly depleting, most notably in the BM, CD8+ naïve and central memory T cells, CD4+ naïve T cells and NK cells, whereas JAK1/2 inhibition had no significant impact on immune cell subsets.

Venetoclax and Ruxolitinib Differentially Affect MHC-II and IFN Gene Expression

The absence of change in cell subsets in ruxolitinib treated mice, despite the improved engraftment seen when these mice are used as alloSCT recipients, suggested that ruxolitinib may supress immune cell function rather than directly deplete immune cells as seen with venetoclax. Therefore, gene expression analysis was performed on BM samples from venetoclax, ruxolitinib, vehicle treated, and untreated C57BL/6 WT mice collected at days 1, 3 and 7 post-treatments, to examine which immune pathways were impacted by drug treatment. Several MHC-II genes were differentially affected by venetoclax or ruxolitinib treatment, including *H2-DMb2*, *H2-Ab1*, *H2-Eb1*, *H2-Aa*, *H2-Ob* and *CD74* (**Figure 4A**). Venetoclax downregulated relative gene

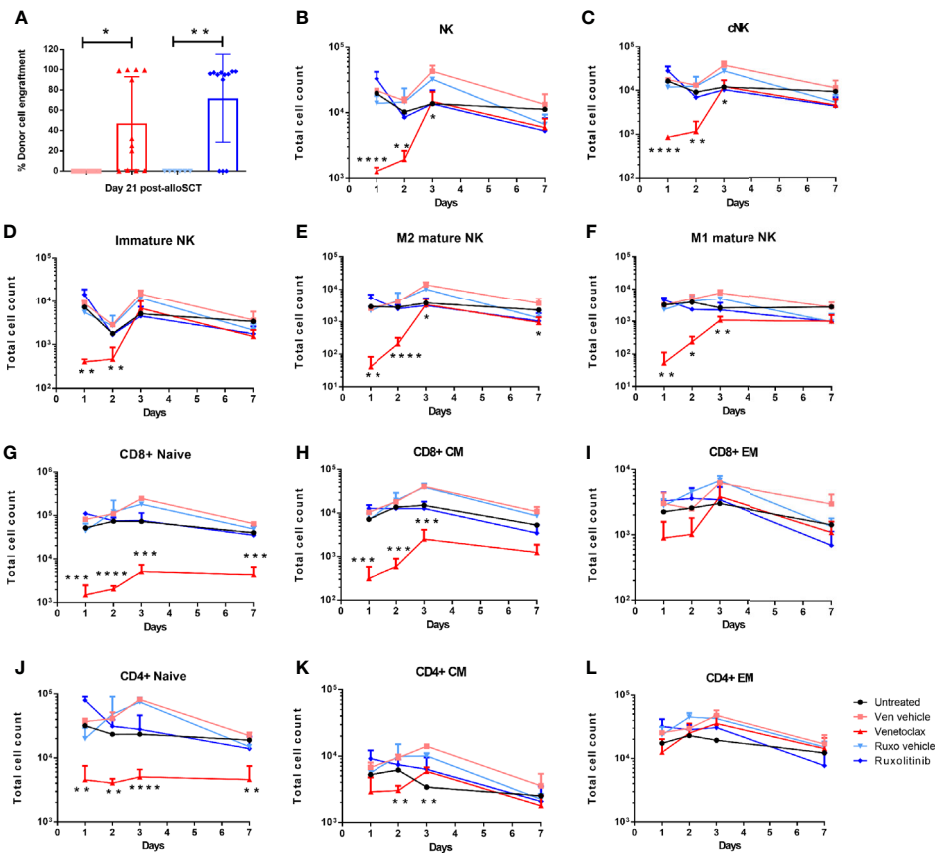

FIGURE 3 | Donor cell engraftment is dependent on recipient pre-alloSCT irradiation dose and treatment with BCL2 or JAK1/2 inhibitors. WT mice were treated with venetoclax or ruxolitinib, or their respective vehicle for two days. The following day mice were treated with RIC and alloSCT. **(A)** Donor cell engraftment (H2kd+ cells) was measured in the blood at day 21 post-alloSCT. WT mice were treated with venetoclax or ruxolitinib, or their respective vehicle for two days. Mice (n=3-4/ group) were killed on days 1, 2, 3, and 7, and BM was harvested and analysed by flow cytometry for the absolute number of **(B–F)** NK cells (NK1.1+CD3-), cNK (NKp46+CD49b+), M1 mature (CD11b+CD27+), M2 mature (CD11b+CD27-) and immature (CD11b-CD27+) NK cells; **(G–L)** naive (N; CD44-CD62L+); central memory (CM; CD44+CD62L+); effector memory (EM; CD44+CD62L-) CD4+ and CD8+ T cells. Data is representative of 3 independent experiments. Statistical analysis was performed using unpaired T test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

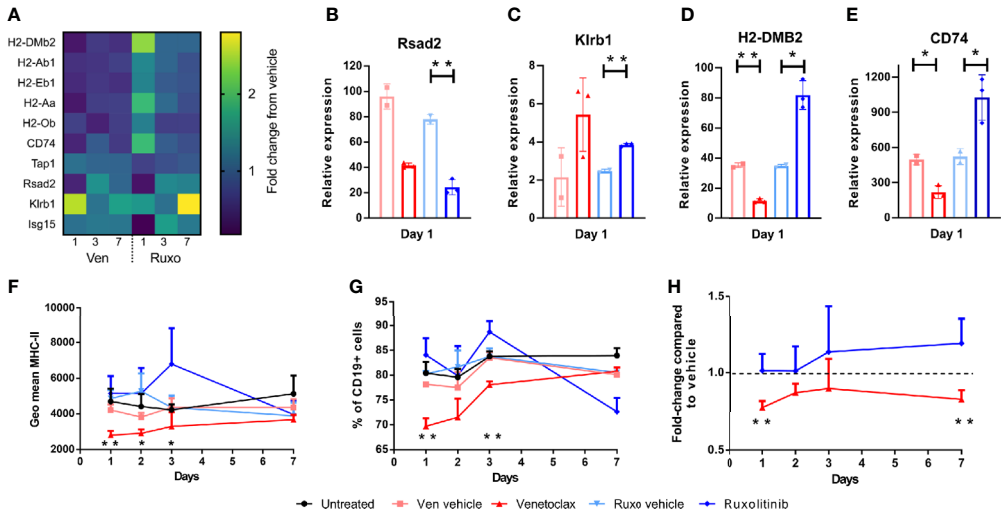

FIGURE 4 | Venetoclax and Ruxolitinib differentially affect MHC class-II and IFN-inducible gene expression. WT mice were treated with venetoclax or ruxolitinib, or their respective vehicle for two days. Mice (n=3-4/group) were killed on days 1, 3, and 7, and gene expression was determined from BM RNA using the NanoString Mouse PanCancer Immune Profiling Panel. **(A)** Heat map of relative gene expression of *H2-DMb2, H2-Ab1, H2-Eb1, H2-Aa, H2-Ob, CD74, Tap1, Rsad2, Klrb1* and *Isg15* from venetoclax and ruxolitinib-treated mice. Relative expression of *Rsad2, Klrb1, H2-DMB2* and *CD74* **(B–E)** was compared between venetoclax or ruxolitinib-treated mice and their respective vehicle on day 1 post-treatment. The geometric mean of MHC-II expression on CD19+ B cells **(F)**, percentage of CD19+ B cells expressing MHC-II **(G)**, and the fold-change of MHC-II expression on total CD45+ BM cells from venetoclax or ruxolitinib-treated mice was compared to each vehicle control group **(H)**. Statistical analysis was performed using Mann-Whitney unpaired T test **(B–E)**, and unpaired T test **(F–H)**. *p < 0.05, **p < 0.01.

expression of MHC-II genes, in contrast to ruxolitinib treatment which resulted in MCH-II upregulation. The expression of the interferon (IFN) genes *Rsad2*, *Ifit3*, *Ifnb1*, *Ifna1*, *Oas2*, *Isg15*, *Klrb1* and *Ifng* were also altered after drug treatment (**Figure 4A**). Both venetoclax and ruxolitinib treatment downregulated *Rsad2* expression which encodes Radical S-adenosyl methionine domain containing 2 (Rsad2) protein, an IFN-inducible virus inhibitory protein involved in CD4+ T cell activation (20) (**Figure 4B**). *Klrb1* encoding killer cell lectin-like receptor subfamily B member 1 (KLRB1), which inhibits IFNg production by NK cells (21), was upregulated by both venetoclax and ruxolitinib treatment (**Figure 4C**). As described above, *H2-DMB2* and *CD74* expression were downregulated by venetoclax, and upregulated by ruxolitinib (**Figures 4D, E**). The MHC-II-associated genes regulate antigen expression, and therefore likely alter alloantigen presentation in the intestinal epithelium after alloSCT (22). Subsequent flow cytometry analysis confirmed that cell surface MHC-II expression on BM CD19+ B cells, and the percentage of B cells expressing MHC-II was decreased for several days in venetoclax-treated mice, compared to untreated or vehicle treated mice, whereas expression of MHC-II increased in ruxolitinib-treated mice (**Figures 4F, G**). Furthermore, the changes to MHC-II expression were replicated in total BM CD45+ cells, as compared to each vehicle control (**Figure 4H**). Collectively, the differential effects of venetoclax and ruxolitinib on both cell type and gene expression demonstrate that these drugs work *via* different mechanisms and therefore lead to different impacts on transplant outcome when combined with RIC.

DISCUSSION

Successful donor engraftment in an alloSCT recipient requires that the conditioning regimen adequately suppresses recipient immunity to prevent donor cell rejection. This is reliably achieved in most recipients with intensive MAC regimens but at the cost of mortality and morbidity (2). Conversely, RIC regimens are safer, but associated with a higher incidence of mixed chimerism, increased rates of graft loss and poorer induction of the GVT effect with a resultant excess of relapse and poorer overall survival (5–7). Augmentation of existing RIC regimens has not been associated with improved outcomes, and strategies directed at improved donor T cell engraftment and promotion the GVT effect have been advocated (23).

We hypothesised that donor engraftment and subsequent GVT rates achieved by RIC could be improved by additional suppression of recipient immunity through either lymphocyte depletion or cytokine inhibition with either BCL2 or JAK inhibition. Similarly, we reasoned that by avoiding the tissue damage and inflammatory cytokine production associated with MAC and further suppressing JAK/STAT dependant cytokine production, in particular IL6, the rates of GVHD onset may be reduced in alloSCT recipients (24, 25).

In this study we first examined how the absence of recipient T and NK cells due to IL-15 deficiency would impact on donor cell engraftment following RIC. The resulting hyperacute, lethal gut GVHD observed in IL-15 KO recipients indicated that residual recipient immunity is necessary to prevent uncontrolled donor homeostatic T cell proliferation, activation and inflammatory

cytokine production. Given the role for residual post-conditioning recipient immunity in controlling donor engraftment, we hypothesised that a brief period of venetoclax or ruxolitinib treatment added to RIC would provide a sufficient period of immunosuppression to promote donor engraftment, whilst not full removing the regulator function of residual recipient immunity. We identified that venetoclax rapidly depleted naïve and central memory CD4+ and CD8+ T cells, NK cells, and VM T cells in the BM, spleen and liver, and we have previously demonstrated that the combination of venetoclax and RIC results in donor engraftment and GVT without the onset of GVHD (8). The incorporation of ruxolitinib into RIC of WT alloSCT recipients also resulted in NK and CD8+ T cell depletion in BM similar to that induced by MAC, and resulted in similar donor engraftment rates and associated GVT responses as seem with the MAC and ventoclax + RIC combination. However, unlike venetoclax + RIC, the ruxolitinib-containing RIC regimen did not fully avoid the onset of chronic GVHD as skin chronic GVHD was observed 1-2 months after alloSCT.

Further exploration of the venetoclax or ruxolitinib treatment of alloSCT recipients identified significant differences in gene expression within the BM of recipient mice. Reduced MHC-II expression was observed in the BM following venetoclax treatment prior to alloSCT. In contrast, MHC-II expression increased in the BM of ruxolitinib treated mice, whilst IFN gene expression decreased transiently. Ruxolitinib therapy for two days prior to transplant was insufficient to suppress IFNγ expression in the first 7 days post alloSCT (**Figure 2G**). The variation between the gene expression changes seen between venetoclax and ruxolitinib therapy is important as IFNγ-dependent MHC-II expression in recipient tissues and subsequent activation of donor CD4+ T cells is now recognised as a key priming event in the onset of GVHD (22) and may explain, in part, why ruxolitinib + RIC treated recipients developed late skin GVHD.

In our model, despite the early IFNγ cytokine rebound observed after ruxolitinib-containing RIC and the high levels of donor cell engraftment achieved by this regimen, acute GVHD was not observed. This likely reflects the absence of GVHD-promoting gut inflammation that is associated with MAC. These observations suggest that by avoiding gut toxicity through the use of ruxolitinib + RIC, acute GVHD will not be primed even following high levels of donor T cell engraftment. Although other contributors to the prevention of GVHD onset including ruxolitinib-induced decrease in dendritic cell activation (26) cannot be excluded. The potential for ruxolitinib therapy to reduce inflammatory cytokine production has resulted in pilot studies exploring its ability to improve engraftment, avoid GVHD, and replace conventional GVHD prophylaxis (27). To date, studies of this approach have been small and although associated with likely lower rates of GVHD, ongoing ruxolitinib therapy may be limited by viral activation and post-transplant cytopenias (28, 29). In contrast, our approach of transiently lowering the engraftment barrier by a short exposure of ruxolitinib prior to donor cell infusion may provide an opportunity to optimise donor engraftment, maintain GVL and avoid GVHD onset, whilst avoiding the toxicity of continuous ruxolitinib exposure.

Overall, whilst either of the targeted therapies venetoclax or ruxolitinib are able to promote increased donor engraftment in the setting of RIC and thereby avoid the toxicity and GVHD-priming effects of MAC, the mechanism of action of venetoclax including its ability to reduce MHC-II expression, added to RIC seems best placed as the combination to take forward for clinical application in order to realise the GVT benefits of alloSCT, whilst avoiding GVHD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE181060.

ETHICS STATEMENT

The animal study was reviewed and approved by Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC 12.08), and Peter MacCallum Cancer Centre Animal Ethics Committee (E607).

AUTHOR CONTRIBUTIONS

JD, KD, ML-M, AP, and RK performed experiments and analysis of results. NH provided the IL-15 KO mice. AP, EW, and NH contributed to project discussion. RK, JD, NH, and DR designed the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.749094/full#supplementary-material

Supplementary Figure 1 | IL-15 knockout mice have reduced NK and CD8+ T cells in the bone marrow. Untreated IL-15KO mice and WT C57BL/6 mice aged 8-12 weeks (n=6) were killed and cell profiles of BM were tested by flow cytometry. **(A)** The absolute number of NK cells (NKp46+CD49b+), ILC1s (NKp46+CD49a+), CD4 (CD3+CD4+) and CD8 (CD3+CD8+) T cells, B cells (CD19+), and granulocytes (CD11b+Ly6G+) were compared between WT and IL-15 KO mice. **(B)** The absolute number of mature (CD11b+CD27+/-) and immature (CD11b-CD27+) NK cells, naive (N; CD44-CD62L+); central memory (CM; CD44+CD62L+); effector memory (EM; CD44+CD62L-) CD4 and CD8 T cells; and virtual memory (VM; CD8+CD44+ CD62L+CD49d+) T cells were compared between WT and IL-15 KO mice. Statistical analysis was performed using Mann-Whitney unpaired T test.

Supplementary Figure 2 | Venetoclax treatment decreases NK and CD8+ T cells in the BM, spleen and liver. WT mice were treated with venetoclax or ruxolitinib, or their respective vehicle for two days, were killed the following day and the BM, spleen and liver was harvested and analysed by flow cytometry for the absolute number of **(A)** NK and **(B)** CD8+ T cells. Data is representative of 3 independent experiments. Statistical analysis was performed using Mann-Whitney unpaired T test.

REFERENCES

1. Gratwohl A, Pasquini MC, Aljurf M, Atsuta Y, Baldomero H, Foeken L, et al. One Million Haemopoietic Stem-Cell Transplants: A Retrospective Observational Study. *Lancet Haematol* (2015) 2(3):e91–100. doi: 10.1016/s2352-3026(15)00028-9
2. D'Souza A, Fretham C, Lee SJ, Arora M, Brunner J, Chhabra S, et al. Current Use of and Trends in Hematopoietic Cell Transplantation in the United States. *Biol Blood Marrow Transplant* (2020) 26(8):e177–82. doi: 10.1016/j.bbmt.2020.04.013
3. Phelan RA, Arora M, Chen M. *Current Use and Outcome of Hematopoietic Stem Cell Transplantation: CIBMTR US Summary Slide* (2020).
4. *Australasian Bone Marrow Transplant Recipient Registry: Annual Data Summary* (2019).
5. Scott BL, Pasquini MC, Logan BR, Wu J, Devine SM, Porter DL, et al. Myeloablative Versus Reduced-Intensity Hematopoietic Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic Syndromes. *J Clin Oncol* (2017) 35(11):1154–61. doi: 10.1200/jco.2016.70.7091
6. Wong E, Mason K, Collins J, Hockridge B, Boyd J, Gorelik A, et al. Prognostic Limitations of Donor T Cell Chimerism After Myeloablative Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic Syndromes. *Biol Blood Marrow Transplant* (2017) 23(5):840–4. doi: 10.1016/j.bbmt.2017.01.086
7. Pasic I, Paulson K, Dozois G, Schultz KR, Lipton JH, Kumar R. Inferior Outcomes With Reduced Intensity Conditioning Followed by Allogeneic Hematopoietic Cell Transplantation in Fit Individuals With Acute Lymphoblastic Leukemia: A Canadian Single-Center Study and a Comparison to Registry Data. *Leuk Lymphoma* (2021) 62:1–9. doi: 10.1080/10428194.2021.1910688
8. Jiao Y, Davis JE, Rautela J, Carrington EM, Ludford-Menting MJ, Goh W, et al. Recipient BCL2 Inhibition and NK Cell Ablation Form Part of a Reduced Intensity Conditioning Regime That Improves Allo-Bone Marrow Transplantation Outcomes. *Cell Death Differ* (2019) 26(8):1516–30. doi: 10.1038/s41418-018-0228-y
9. Davis JE, Harvey M, Gherardin NA, Koldej R, Huntington N, Neeson P, et al. A Radio-Resistant Perforin-Expressing Lymphoid Population Controls Allogeneic T Cell Engraftment, Activation, and Onset of Graft-Versus-Host Disease in Mice. *Biol Blood Marrow Transplant* (2015) 21(2):242–9. doi: 10.1016/j.bbmt.2014.11.003
10. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF, et al. A Double-Blind, Placebo-Controlled Trial of Ruxolitinib for Myelofibrosis. *N Engl J Med* (2012) 366(9):799–807. doi: 10.1056/NEJMoa1110557
11. Przepiorka D, Luo L, Subramaniam S, Qiu J, Gudi R, Cunningham LC, et al. FDA Approval Summary: Ruxolitinib for Treatment of Steroid-Refractory Acute Graft-Versus-Host Disease. *Oncologist* (2020) 25(2):e328–34. doi: 10.1634/theoncologist.2019-0627
12. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in Corticosteroid-Refractory Graft-Versus-Host Disease After Allogeneic Stem Cell Transplantation: A Multicenter Survey. *Leukemia* (2015) 29(10):2062–8. doi: 10.1038/leu.2015.212
13. Zeiser R, Socié G. The Development of Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *Blood Adv* (2020) 4(15):3789–94. doi: 10.1182/bloodadvances.2020002097
14. Jagasia M, Perales MA, Schroeder MA, Ali H, Shah NN, Chen YB, et al. Ruxolitinib for the Treatment of Steroid-Refractory Acute GVHD (REACH1): A Multicenter, Open-Label Phase 2 Trial. *Blood* (2020) 135(20):1739–49. doi: 10.1182/blood.2020004823
15. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *N Engl J Med* (2020) 382(19):1800–10. doi: 10.1056/NEJMoa1917635
16. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, et al. Reversible Defects in Natural Killer and Memory CD8 T Cell Lineages in Interleukin 15-Deficient Mice. *J Exp Med* (2000) 191(5):771–80. doi: 10.1084/jem.191.5.771
17. Nunamaker EA, Anderson RJ, Artwohl JE, Lyubimov AV, Fortman JD. Predictive Observation-Based Endpoint Criteria for Mice Receiving Total Body Irradiation. *Comp Med* (2013) 63(4):313–22.
18. Huntington ND, Puthalakath H, Gunn P, Naik E, Michalak EM, Smyth MJ, et al. Interleukin 15-Mediated Survival of Natural Killer Cells Is Determined by Interactions Among Bim, Noxa and Mcl-1. *Nat Immunol* (2007) 8(8):856–63. doi: 10.1038/ni1487
19. Delconte RB, Kolesnik TB, Dagley LF, Rautela J, Shi W, Putz EM, et al. CIS Is a Potent Checkpoint in NK Cell-Mediated Tumor Immunity. *Nat Immunol* (2016) 17(7):816–24. doi: 10.1038/ni.3470
20. Qiu LQ, Cresswell P, Chin KC. Viperin Is Required for Optimal Th2 Responses and T-Cell Receptor-Mediated Activation of NF-KappaB and AP-1. *Blood* (2009) 113(15):3520–9. doi: 10.1182/blood-2008-07-171942
21. Carlyle JR, Martin A, Mehra A, Attisano L, Tsui FW, Zúñiga-Pflücker JC. Mouse NKR-P1B, a Novel NK1.1 Antigen With Inhibitory Function. *J Immunol* (1999) 162(10):5917–23.
22. Koyama M, Mukhopadhyay P, Schuster IS, Henden AS, Hülsdünker J, Varelias A, et al. MHC Class II Antigen Presentation by the Intestinal Epithelium Initiates Graft-Versus-Host Disease and Is Influenced by the Microbiota. *Immunity* (2019) 51(5):885–98.e7. doi: 10.1016/j.immuni.2019.08.011
23. Craddock C, Jackson A, Loke J, Siddique S, Hodgkinson A, Mason J, et al. Augmented Reduced-Intensity Regimen Does Not Improve Postallogeneic Transplant Outcomes in Acute Myeloid Leukemia. *J Clin Oncol* (2021) 39(7):768–78. doi: 10.1200/jco.20.02308
24. Tawara I, Koyama M, Liu C, Toubai T, Thomas D, Evers R, et al. Interleukin-6 Modulates Graft-Versus-Host Responses After Experimental Allogeneic Bone Marrow Transplantation. *Clin Cancer Res* (2011) 17(1):77–88. doi: 10.1158/1078-0432.Ccr-10-1198
25. Kennedy GA, Tey SK, Buizen L, Varelias A, Gartlan KH, Curley C, et al. A Phase 3 Double-Blind Study of the Addition of Tocilizumab vs Placebo to Cyclosporin/Methotrexate GVHD Prophylaxis. *Blood* (2021) 137(14):1970–9. doi: 10.1182/blood.2020009050
26. Heine A, Held SA, Daecke SN, Wallner S, Yajnanarayana SP, Kurts C, et al. The JAK-Inhibitor Ruxolitinib Impairs Dendritic Cell Function *In Vitro* and *In Vivo*. *Blood* (2013) 122(7):1192–202. doi: 10.1182/blood-2013-03-484642
27. Zhao Y, Shi J, Luo Y, Gao F, Tan Y, Lai X, et al. Calcineurin Inhibitors Replacement by Ruxolitinib as Graft-Versus-Host Disease Prophylaxis for Patients After Allogeneic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2020) 26(5):e128–33. doi: 10.1016/j.bbmt.2020.01.012
28. Kröger N, Shahnaz Syed Abd Kadir S, Zabelina T, Badbaran A, Christopeit M, Ayuk F, et al. Peritransplantation Ruxolitinib Prevents Acute Graft-Versus-Host Disease in Patients With Myelofibrosis Undergoing Allogenic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2018) 24(10):2152–6. doi: 10.1016/j.bbmt.2018.05.023

29. Gupta V, Kosiorek HE, Mead A, Klisovic RB, Galvin JP, Berenzon D, et al. Ruxolitinib Therapy Followed by Reduced-Intensity Conditioning for Hematopoietic Cell Transplantation for Myelofibrosis: Myeloproliferative Disorders Research Consortium 114 Study. *Biol Blood Marrow Transplant* (2019) 25(2):256–64. doi: 10.1016/j.bbmt.2018.09.001

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REVIEW
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T Cell Subsets in Graft Versus Host Disease and Graft Versus Tumor

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Allogeneic hematopoietic cell transplantation (allo-HCT) is an essential therapeutic modality for patients with hematological malignancies and other blood disorders. Unfortunately, acute graft-versus-host disease (aGVHD) remains a major source of morbidity and mortality following allo-HCT, which limits its use in a broader spectrum of patients. Chronic graft-versus-host disease (cGVHD) also remains the most common long-term complication of allo-HCT, occurring in reportedly 30-70% of patients surviving more than 100 days. Chronic GVHD is also the leading cause of non-relapse mortality (NRM) occurring more than 2 years after HCT for malignant disease. Graft versus tumor (GVT) is a major component of the overall beneficial effects of allogeneic HCT in the treatment of hematological malignancies. Better understanding of GVHD pathogenesis is important to identify new therapeutic targets for GVHD prevention and therapy. Emerging data suggest opposing roles for different T cell subsets, e.g., IFN-γ producing CD4+ and CD8+ T cells (Th1 and Tc1), IL-4 producing T cells (Th2 and Tc2), IL-17 producing T cells (Th17 and Tc17), IL-9 producing T cells (Th9 and Tc9), IL-22 producing T cells (Th22), T follicular helper cells (Tfh), regulatory T-cells (Treg) and tissue resident memory T cells (Trm) in GVHD and GVT etiology. In this review, we first summarize the general description of the cytokine signals that promote the differentiation of T cell subsets and the roles of these T cell subsets in the pathogenesis of GVHD. Next, we extensively explore preclinical findings of T cell subsets in both GVHD/GVT animal models and humans. Finally, we address recent findings about the roles of T-cell subsets in clinical GVHD and current strategies to modulate T-cell differentiation for treating and preventing GVHD in patients. Further exploring and outlining the immune biology of T-cell differentiation in GVHD that will provide more therapeutic options for maintaining success of allo-HCT.

Keywords: graft versus host disease, graft versus tumor, tissue resident memory t cell, cell therapy, t cells subsets

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INTRODUCTION

Allogeneic hematopoietic cell transplant (allo-HCT) is a remarkably successful immunotherapy in large part due to the graft-versus-tumor (GVT) effect. Unfortunately, GVT is tethered to the pathogenesis of acute graft versus host disease (aGVHD). The detailed pathogenesis of acute GVHD (aGVHD) has recently been reviewed in depth (1, 2). Overall, T cells are indispensable mediators of

aGVHD pathogenesis since GVHD rarely develops after syngeneic or T cell-depleted transplants (3–7). Both aGVHD and GVT have been found to be initiated by antigen presenting cells (APCs) derived from the donor and from host activating donor T cells (8, 9). Such activation leads to the release of inflammatory cytokines, with subsequent proliferation of alloreactive T cells, resulting in host damage and further inflammation. Around 15-20% of hematopoietic cell transplant patients develop severe refractory GVHD leading to mortality (10, 11). Chronic GVHD (cGVHD) pathogenesis is a complex process involving both B and T cells (12). The process was reviewed recently in detail (1). Essentially, crosstalk between B and T cells leading to the proliferation of germinal centers allowing the production of allo-reactive antibodies appears to be the overlying process of the disease. The mainstays of GVHD prevention include anti-thymocyte globulin, calcineurin inhibitors and post-transplant cyclophosphamide, and first line therapies include corticosteroids in addition to calcineurin inhibitors. However, treatment with these drugs negatively affect desirable GVT (13). In addition, steroid-refractory GVHD (SR-aGVHD) patients have dismal outcomes, thus representing an urgent need for developing new treatment strategies in the field of transplant medicine (14). That said, recent breakthroughs have been made including the positive result of the randomized phase III clinical trial evaluating ruxolitinib versus best available treatment (BAT) in SR-aGVHD (15). Similarly, positive results were seen in SR-cGVHD comparing ruxolitinib versus BAT (16). The central role of T cells in the pathogenesis of GVHD has also led to extensive studies in manipulating T cell populations to reduce GVHD severity. Specific T cell subsets have been found to either exacerbate or alleviate GVHD/GVT, a finding that is currently being exploited in novel treatment options in preclinical and/or clinical studies.

T CELLS INDUCERS OF GVHD

T cells differentiation is initiated when naïve T cells are stimulated by antigens in the presence of MHC molecules under a particular milieu of cytokines their corresponding signaling pathways to develop into different T cell subsets that acquire specialized effector cell phenotypes (17). As shown in **Figure 1**, these T cell subsets are characterized by the production of signature cytokines and expression of specific transcription factors (TFs). The specific cytokines and TFs are activated by signal transducer and activator of transcription (STAT) family members to confer specialized functions to the T cell subsets. These cytokines and TFs that regulate T cells differentiation may have effects on the development of multiple T cell subsets. For example, interleukin 6 (IL-6) is essential for T follicular helper (Tfh) and T helper type 17 (Th17) differentiation through the STAT5 signaling pathway (18). Different T cell subsets have been involved in several inflammatory diseases (19, 20), and may allow the development of novel treatment strategies (21).

Figure 2 summarizes T cells subsets demonstrated or putative roles in GVHD/GVT. The gut and other tissues are damaged during irradiation and/or chemotherapy, leading to the release of various DAMPs/PAMPs, and inflammatory cytokines (22). These DAMPs, PAMPs, and cytokines activate both host and donor antigen-presenting cells (APCs), which then activate the donor T cells. The APCs are also secreting various cytokines that promotes T cell differentiation toward different T cell subsets including T helper type 1 (Th1), T helper type 2 (Th2), T helper type 17 (Th17), T helper type 9 (Th9), and regulatory T cells (Tregs). Activated T cells are able to secrete various pro-inflammatory cytokines including IFNγ, IL-17, IL-22 leading to cytolysis of cells in target tissues, mainly in the gut, liver, and skin, which can be alleviated by anti-inflammatory cytokine produced by Th2, Th9 and Treg cells, such as IL-33-producing Th9 (23).

Naïve T Cells

T cell depletion previous to HCT and the use of T cell-depleting antibodies have been used resulting in a significant reduction in GVHD (24). Anti-T-lymphocyte Globulin (ATG) has been used to prevent GVHD in the conditioning regimen but also as treatment while the patient's response is still unsatisfactory. In the steroid-refractory GVHD therapeutic setting, the response rate is reported to be 24%-41% using ATG and overall survival is poor (25). A recent phase 3 clinical trial aimed to take advantage of the benefits of T-cell depletion with respect to GVHD by using an anti-CD25 antibody (inolimomab) versus ATG, which found no difference in overall survival (26). Unfortunately, the experimental group suffered from issues with infection and relapse, common to T cell-depletion strategies leading to mortality in both arms of the study. The differences between naïve T cells and memory T cells have being investigated to determine which specific subsets of T cells were particularly inductive of pathological immune responses. Naïve T cells (T_N) are $CD45RA^+CD62L^+$ antigen inexperienced cells with a diverse TCR repertoire (27, 28). Preclinical studies have supported the role of naïve T cells (T_N) in inducing GVHD as opposed to central memory T cells (T_{CM}) (29, 30). In allogeneic mouse models of HCT, it was determined that T_N caused more severe GVHD compared to that of T_{CM} and effector memory T cells (T_{EM}) cells in isolation (29–35). It was also found in *in vitro* studies that $CD8^+$ T_N were 5-20 times more likely to be specific for a minor histocompatibility antigen than T_M (36), supporting the role of this subset in the pathogenesis of GVHD disease. Concurrently, grafts in mice performed with memory cells retained GVT activity when challenged with malignancy (29, 34, 37). A recent phase II clinical trial applied these findings to humans. Naïve T cell depletion was used to reduce GVHD in acute leukemia patients (38). Naïve T cells were depleted from peripheral blood stem cells (PBSCs) using an iron-dextran bead conjugated to a monoclonal anti-CD45RA antibody. Thirty-five patients with acute leukemia or advanced myelodysplastic syndrome received T_N-depleted HCT after myeloablative conditioning with 50 days of tacrolimus as immunosuppression. Durable engraftment was achieved in 34 out of 35 patients. Acute GVHD was not reduced in this trial. However, chronic GVHD,

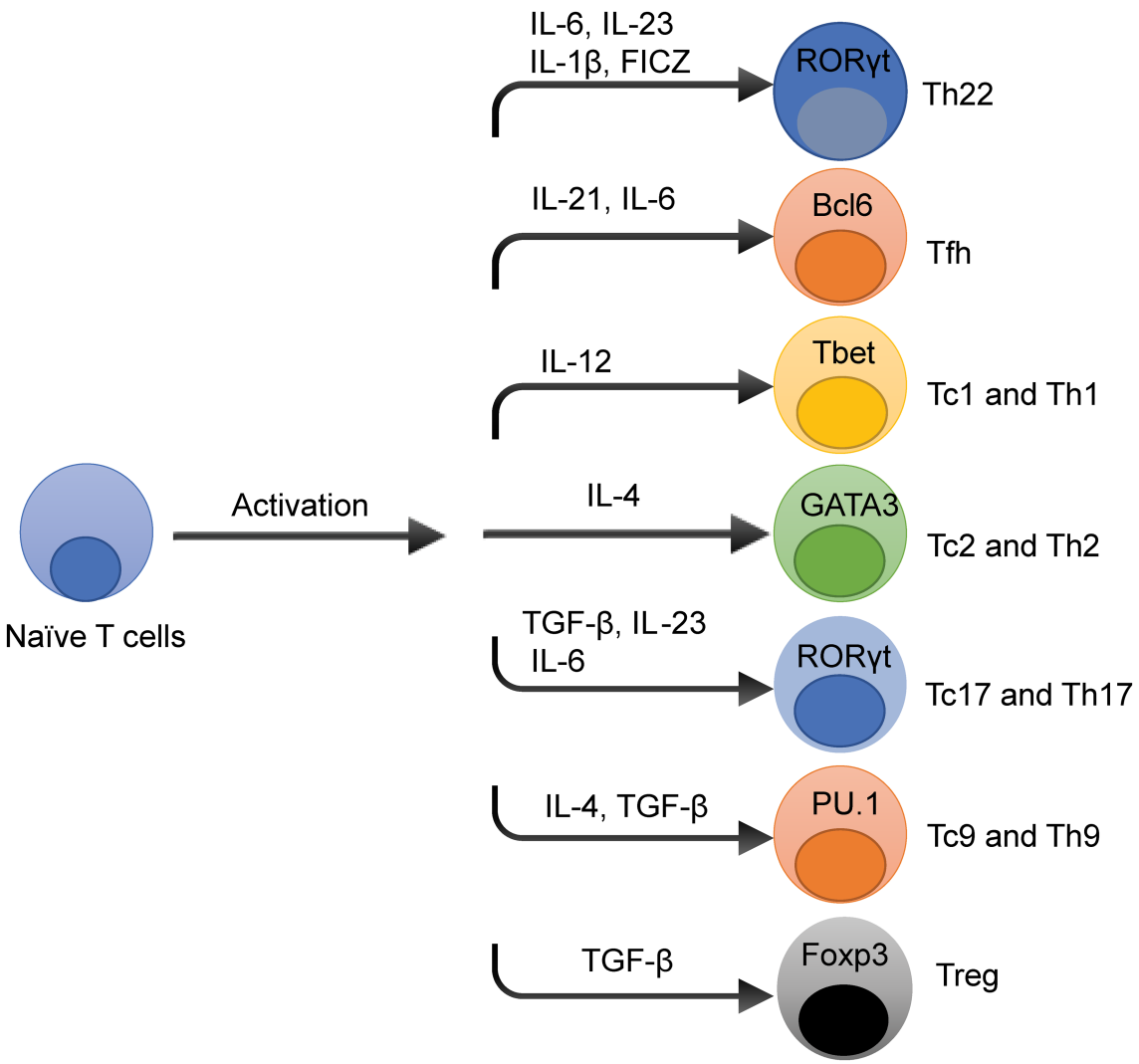

FIGURE 1 | Overview of T Cells Differentiation Pathways. The cytokine and transcription factors (TFs) niche dictates T cell differentiation in spite of the stimulation of T cell receptor signaling pathways. The prototypical cytokines and TFs that regulate each T cell subset differentiation fate are depicted. These cytokines and TFs that influence T cell differentiation have effects on the development of multiple T cell subsets, such as interleukin-6 (IL-6) is essential for T helper type 22 (Th22), T follicular helper (Tfh), and T helper 17 (Th17) cell development.

a secondary endpoint, was very low (9%) over the 2.5-year median follow up time. This method had other advantages when compared to historical controls, such as faster immune reconstitution compared to T-Cell Depleted (TCD) HCT, a 2-year disease free survival of 70% compared to 50% in TCD and 65% in T-cell-replete grafts, and a 28% relapse rate compared with 60% in TCD and 37% in T-cell replete grafts. These findings require confirmation in future randomized clinical trials.

Th1 and Tc1

The early phase of aGVHD pathogenesis is predominantly mediated by Th1/Tc1 cells, with hyperproliferation and high cytotoxicity driving disease. These cells arise in response to the transplant-conditioning-induced cytokine storm and resultant release of DAMPs and PAMPs, and they can often be targeted by standard immunosuppression regimens, which focus on the inhibition of proliferation and NFAT-driven T cell signaling (39). $CD4^+$ (Th) and $CD8^+$ (Tc) T cells are stimulated to differentiate into the Th1/Tc1 together type 1 subtype when they receive T Cell Receptor (TCR) stimulation from their specific antigen, here allo-antigen, as well as co-stimulation from a variety of different pathways. IL-12, in its activation of STAT4 has been found to be a critical component of the Th1 pathway (40, 41). In the same way, $CD8^+$ T cells are encouraged to differentiate into Tc1 cells by TCR activation, co-stimulation, and the cytokines IL-2 and IL-12 (42–44). Interferon gamma (IFNγ), a primary mediator of inflammation and tissue damage, is a primary product of activated Th1 cells (45). The defining Th1

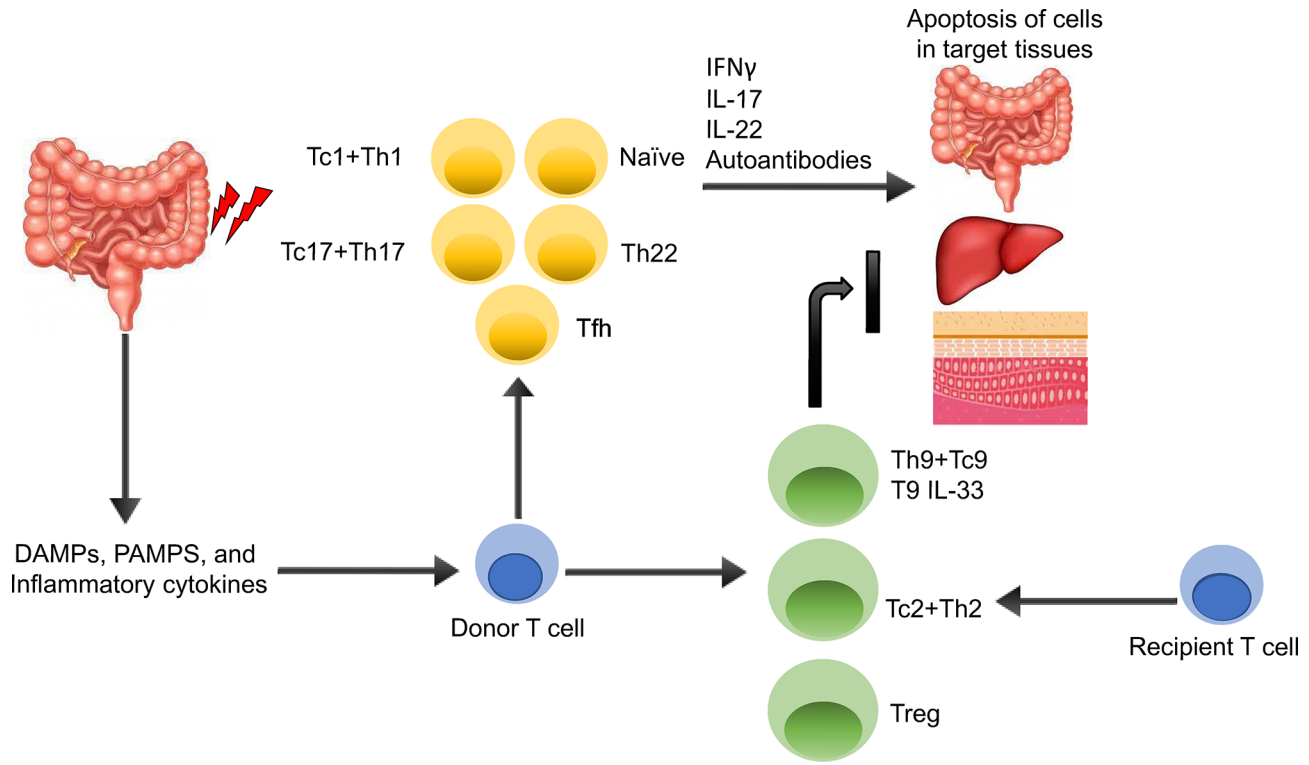

FIGURE 2 | Overview of GVHD Pathogenesis. The gut and other issues are damaged during irradiation or chemotherapy, leading to the release of various DAMPs, PAMPs, and inflammatory cytokines. These DAMPs, PAMPs, and cytokines activate both host and donor antigen-presenting cells (APCs), which then activate the donor T cells. The APCs are also secreting various cytokines that promotes T cell differentiation toward different T cell subsets including T helper type 1 (Th1), T helper type 2 (Th2), T helper type 17 (Th17), T helper type 9 (Th9), and regulatory T cells (Tregs). Activated Th1 and Th17 T cells are able to secrete various pro-inflammatory cytokines including IFNγ, IL-17, IL-22 leading to apoptosis of cells in target tissues, mainly in the gut, liver, and skin, which can be alleviated by anti-inflammatory cytokine producing Th2, Th9 and Treg cells, such as IL-33-producing Th9.

transcription factor is T-box expressed in T cells (T-bet or Tbx21) (46). Similar to Th1's, Tc1 cells also depend on T-bet as a transcription factor and are also induced by concurrent expression of eomesodermin (Eomes) (47).

Allogeneic donor Th1 and Tc1 cells have been shown in multiple experiments to induce both GVT and GVHD in mouse models and is classically thought to be the main propagator of GVHD. The IFNγ secreted by donor Th1 cells have been found to both encourage further Th1 cell differentiation and direct damage to the gut mucosa (48). Preclinical models have shown elevation of Th1-derived cytokines including tumor necrosis factor (TNF) and IFNγ in association with GVHD. The cytokines have also been implicated directly in target organ damage (48–51). T-bet is a crucial regulator of Th1 differentiation and IFNγ production, and T-bet$^{-/-}$ T cells alleviate GVHD after adoptive transfer in both major and minor MHC mismatched mouse model (52). Blockade of Th1 and Th17 differentiation by targeting T-bet and RORgammat in mice ameliorates GVHD while surprisingly not decreasing GVT activity (53). As a transcription factor, a promising strategy is to target its downstream effectors for preventing GVHD instead of directly inhibiting T-bet. In human patients suffering from acute GVHD, Th1 cytokines are found in pathologic lesions, supporting the clinical relevance of this subset in the pathogenesis of GVHD (54, 55).

The Janus Kinases (JAKs) family members (JAK-1, -2, -3, Tyrosine kinase 2) and its downstream regulators signal transducers and activators of transcription (STAT) are crucial in the pathogenesis of GVHD (56). Different JAK inhibitors, such as JAK1/2-inhibitor ruxolitinib and JAK1-inhibitor itacitinib, have been developed and applied to prevent or treat aGVHD and cGVHD with different clinical indications. JAK1/2 antagonists can suppress Th1 and Th17 cell function, activation of antigen presenting cells (APCs), MHC expression and co-stimulatory signals through inhibition of STAT1 and STAT3 signaling pathways (57). The regulatory T cell function is retained by reserving IL-2-JAK3-STAT5 signaling pathway followed JAK1/2 inhibition. In 2019, ruxolitinib (Jakafi), a JAK1/2 inhibitor, was approved by the U.S. Food and Drug Administration to treat steroid-refractory aGVHD in adults and children age 12 years and older based on the randomized phase III trial (15). It was also recently approved for steroid-refractory cGVHD in the same population based on the randomized phase III trial (16). In recently published preclinical work, the JAK1/2-inhibitor baricitinib has shown to prevent GVHD by increasing Tregs *via* the JAK3 pathway (58).

Th17 and Tc17

Th17 cells are the other major subtype of inflammatory T cell implicated in the pathogenesis of GVHD. Cytokines TFG-β1 and IL-6 ± IL-23 direct the differentiation of Th17 cells (59–63). The cells are defined by their expression of IL-17 and lack of expression of IL-4 and IFNγ (64, 65). Retinoic acid receptor-related orphan receptor gamma (RORγt) is the main transcription factor of the Th17 lineage (66). The role Th17 in the pathogenesis of aGVHD is complicated, but overall aGVHD appears to be primarily a Th1 but not Th17 process (67) while cGVHD is both a Th1 and Th17 process (68). Another Th17-like cytokine, IL-21 has also been shown in many preclinical models to induce aGVHD, either *via* knockout of IL-21 or inhibition of the IL-21 receptor (68–72). The role of Th17 cells have also been investigated in mouse models by way of their transcription factors. Multiple studies have found that the absence of both RORγt and Tbet greatly diminished the severity of aGVHD. In addition, absence of critical Th17 transcription factors led to a significant decrease in the frequency of IL-17A and TNF in subjects' serum and pathogenic lesions (73). A third method to investigate the role of the Th17 subset in the pathogenesis of GVHD has been to target the cytokines that produced Th17. While likely not specific to Th17, IL-6 inhibition in mouse models was shown to significantly decrease aGVHD severity (74, 75). In addition, blockade of IL-23 was found to diminish aGVHD severity (76–78). Finally, allogeneic donor Th17 cells have been shown to be capable of inducing lethal GVHD in isolation, but they have also been shown to be unnecessary in doing so, as the Th1/Tc1 subset is also sufficient to do so in isolation (79, 80).

In patients suffering from aGVHD, the frequency of Th17 cells in peripheral blood was increased along with the frequency of IL-17 (81). As time progresses after transplantation, Th17/Tc17 cells may become a major driving force of GVHD, secreting proinflammatory cytokines, providing a cellular reservoir for effector alloimmune cells, and supporting the Tfh-driven immune response that characterizes cGVHD (39). Indeed, Th17 cells have been even more heavily implicated in cGVHD in humans. They have been found to be present in increased frequency in the blood of cGVHD patients (81) and mixed Th1/Th17 cells were found in histological examination of cGVHD skin lesions (82). In addition, CD146 and $CCR5^+CD146^+$ CD4 T cells are present in increased frequencies in humans suffering from aGVHD and cGVHD, and these cells have been shown to be skewed toward a mixed Th1/Th17 phenotype (83, 84). In a murine model experiment, the potential application of RORγt inhibition has been studied with TMP778. Treatment resulted in a significant decrease in the observed pathology, like a group treated with an anti-IL-17 antibody (84). Furthermore, KD025, was explored in a murine model of cGVHD, which demonstrated a significant reduction in the symptoms of disease. The same study also demonstrated that KD025 inhibition decreased the production of IL-21, IL-17, and IFNγ in the PBMCs of patients suffering from GVHD (85). To follow up on these findings, a phase II clinical trial investigating ROCK2 inhibition with belumosudil (KD025) in the treatment of SR-cGVHD (NCT02841995) showed overall response rates (ORR) (95% CI) with belumosudil 200 mg once daily, 200 mg twice daily, and 400 mg once daily of 65% (38% to 86%), 69% (41% to 89%), and 62% (38% to 82%), respectively. Responses were clinically meaningful, with a median duration of response of 35 weeks, and were associated with quality-of-life improvements and corticosteroid (CS) dose reductions (86). Furthermore, the ROCKstar study showed that belumosudil showed responses for cGVHD after 2 or more prior lines of therapy (87). Based on these findings, belumosudil was recently FDA approved for patients 12 years and older who have received 2 or more prior lines of therapy (88).

Th22

Recently defined as a separate lineage from Th17 cells, Th22 cells were first described in the context of epidermis-infiltrating cells in individuals with inflammatory skin conditions that produced IL-22 and TNFα without producing IFNγ, IL-4, or IL-17 (89). Th22 cells have been shown to develop under the influence of IL-6, IL-23, IL-1β, and 6-formylindolo[3,2-B] carbazole (FICZ) *in vitro*, along with the tyrosine kinase inhibitor galunsertib. However, ideal conditions for differentiation of Th22 *in vitro* and *in vivo* have yet to be determined (90). RORγt has been established as the critical transcription factor for Th22 differentiation, while Tbet is an inhibitory transcription factor for this lineage (90). In contrast to the relatively well-established roles of Th1 and Th17 cells in the pathogenesis of GVHD, the role of Th22 cells and their trademark cytokine, IL-22, remains controversial. In murine models of aGVHD, approximately half the cytokine IL-22 was derived from Th22 cells (91). However, IL-22 has been associated with a protective effect on intestinal stem cells in an experiment that showed recipient deficiency in IL-22 led to more severe immune-mediated damage in the intestine (92). Simultaneously, it was demonstrated in a murine allo-HCT model that deficiency of IL-22 in donor T cells led to diminished aGVHD severity without inhibiting GVT (93). In line with this latter finding, exogenous injection of IL-22 into a murine model after allo-transplant was associated with increased aGVHD severity secondary to Th1 and Tc1 cell expansion, while diminishing Treg levels (94). However, the tissue protective functions of IL-22 can be decoupled from pro-inflammatory actions through structure-based design (95). Based on these findings, a study of IL-22 IgG2-Fc (F-652) along with corticosteroids for subjects with grade II-IV lower gastro-intestinal (GI) aGVHD has been conducted (NCT02406651). Preliminary results of the multicenter prospective phase 2 study showed the combination with corticosteroids was well tolerated and met primary efficacy endpoint (96). Based on these preliminary results, Genentech has sponsored an ongoing clinical trial investigating the use of IL-22Fc in addition to standard therapy for prophylaxis of aGVHD in patients undergoing allogeneic HCT (NCT04539470). Altogether, the action of IL-22 appears to depend on its source and location with donor IL-22 leading to increased aGVHD.

Tfh

Tfh cell differentiation is a multi-step process that is initiated by dendritic cell priming of a naïve $CD4^+$ cell (97). IL-6 is key to this

priming process, and its signaling will increase the key transcription factor B-cell lymphoma 6 (Bcl6) in the maturing cell (98–101). Tfh secrete IL-21 as its lead cytokine (102). IL-2 acts as an inhibitor of the Tfh pathway (103, 104). While Tfh cells have not yet been investigated for their roles in aGVHD, donor Tfh cells have been shown to induce cGVHD *via* their secretion of IL-21. This cytokine leads to the proliferation of germinal centers, differentiation of plasma cells, and the production of auto-antibodies characteristic of cGVHD (105–107). Patients with active cGVHD had a significantly lower frequency of circulating Tfh compared with patients without cGVHD which was associated with higher CXCL13 plasma levels suggesting increased homing of Tfh to secondary lymphoid organs. Further, cTfh were skewed toward a Th2/Th17 phenotype in turn promoting B-cell immunoglobulin secretion and maturation (106).

Trm

It was previously thought that T cells were exclusively found in the blood and secondary lymphoid organs at steady state. Recent observations suggest that the majority of memory T cells reside in human peripheral tissues, primarily located in the skin, gut, liver and lung. Increasing studies unraveled that tissue resident memory T (Trm) cells, representing a lineage of memory T cells, are thought to be contributors in the pathogenesis of GVHD. The Trm cells can be identified by specific markers like CD69 (108). Contribution from host T cells has been recognized recently. Pretransplant conditioning which typically consists of chemoimmunotherapeutic drugs and/or total body irradiation were thought to eliminate host T cells and therefore not play a role in GVHD, but new studies indicate that host T cells resident in peripheral tissues are highly resistant to depletion, even after high-intensity conditioning (109). In humans, host-derived Trm cells have been found in patients' skin lesions before and after allo-HCT and showed distinct transcriptomic program with RUNX3 and galectin-3 as the phenotypic signatures for these cells as compared to blood T cells (110). Similarly, host T cells were found in all skin and colon from patients with aGVHD after allo-HCT. A subset of host-derived Trm cells is highly proliferative and can be directly activated by donor-derived monocytes. These Trm cells promote the development of GVHD through production of proinflammatory cytokines such as IFNγ and IL-17 (109). Skin Trm cells are HCT conditioning resistant and can be maintained during a long period of time with replenishing T cells rapidly acquiring Trm phenotype. The role of Trm cells in other GVHD target organs is also being explored in preclinical models as well as additional functional roles. For example, murine $PSGL1^{lo}CD4^{+}$ T cells from GVHD target tissues enhance B cell differentiation into plasma cells and production of autoantibodies *via* their PD-1 interaction with PD-L2 on B cells. Similar evidence was found from humanized GVHD target tissues. In addition, human $PSGL1^{lo}CD4^{+}$ T cells were apposed with memory B cells in the liver tissues of humanized mice and cGVHD patients (111). By creating three spatiotemporal T cell compartments in non-human primates, development of pathogenic Trm into donor $CD8^{+}$ T cells after allo-HCT was observed. Results showed that by day 8 after transplant, donor T cells infiltrated into the GI tract and exhibit Trm hallmarks. The T cells displayed highly activated and cytotoxic phenotype driven by IL-15 and IL-21 signaling (112).

REGULATORY T CELLS IN GVHD

Th2

Th2 cells mainly produce IL-4, IL-5, IL-10, and IL-13 (113). GATA-binding protein 3 (GATA3) was found to be the master transcription factor for Th2 cells (114). Tc2 cells overlap with Th2 cells in many ways, including their cytokine profile and transcription factor. However, they express both less IL-4 and GATA3 than Th2 cells (115, 116). While we placed Th2 cells under the "regulatory" section due to their protective role when adoptively transferred (117, 118), their overall role is still controversial as they have also been shown to be involved in the pathogenesis of GVHD of the skin and lungs at later stages (48, 119). IL-10-producing Th2 subset has been associated with decreased GVHD in animal models. Also, the natural protective effect of Th2 cells on the gut may prove beneficial for preventing severe gut GVHD (120), the most lethal location of the disease. Additionally, Th2 and Tc2 cells have been described to mediate significantly less severe GVHD compared to Th1 and Tc1 cells after adoptive cell transfer (117, 118). However, these cells concurrently have little to no ability to kill malignant leukemia cells *in vivo* (117, 118). No conclusive results can be drawn for an association between Th2/IL-4 and cGVHD.

Rapamycin resistant T cells (Trapa) hold promise in preventing GVHD in adoptive cell transfer. Trapa cells have the advantage of being more robust *in vivo* due to their increased frequency of the T central memory phenotype (Tcm). Rapamycin resistant T cells also have the advantage of proliferating to a greater degree compared to rapamycin sensitive cells once removed from rapamycin (121). These qualities have been exploited in both preclinical and clinical studies. *Ex vivo* murine Trapa cells polarized with IL-4 toward a Th2 phenotype differentiated into the Th2-type cell and was more effective at preventing GVHD and graft rejection than control Th2 cells (122). Rapamycin-resistance in T cells has also been shown to support Treg cell populations *in vivo* in the setting of transplant, denoting another potential avenue of rapamycin and rapamycin resistance to combat GVHD (123). A phase II clinical trial investigated Th2-skewed Trapa cells used as donor leukocyte infusion (DLI) after allo-HCT. Trapa cells showed a mix of Th2 and Th1 phenotype and cumulative incidence probability of aGVHD was 20% and 40% at days 100 and 180 post-HCT, respectively. Safety was demonstrated, as none of the patients experienced transplant-related mortality (124). However, there are no phase III Trapa DLI clinical trials in process.

Th9

Th9 cells were shown to be a subset of CD4 cells unique from Th2 cells due to their significant IL-9 production and minimal IL-4 production (125). Characterization of this subset continued as transforming growth factor-beta was found to induce IL-9 expression in Th2 cells (126). A concurrent study similarly found that IL-4 along with TGF-beta led to an $IL\text{-}9^{+}$ $IL\text{-}10^{+}$ $Foxp3^{-}$ phenotype (127). Eventually, PU.1 was deciphered to be a defining transcription factor of this unique subset (128). Recently, it was described that $CD8^{+}$ cells could also differentiate into this IL-9-producing subset, representing Tc9 cells (129). Unlike the relatively straightforward role of Tregs, the function of Th9 and

Tc9 cells in the context of immunomodulation is complicated, as they have been implicated in both pro-inflammatory and anti-inflammatory actions. It was suggested that Th9 cells may prevent GVHD in an experiment that showed in a murine allogeneic model, mice treated with a co-transfer of rapamycin resistant Th9 cells showed decreased donor $CD8^+$ cell engraftment and decreased donor IFN-γ production (130). In addition, two studies suggested the importance of IL-9 specifically to immune-mediated limitation of tumor growth (131, 132).

Aside from the decreased IFN-γ with Th9 cell transfer, it was postulated that this subset may decrease GVHD through their expression of membrane-bound Stimulation-2 (ST2), the IL-33 receptor (23, 133). The IL-33/ST2 pathway has been shown to induce type 2 cytokine production, which is implicated in both supporting tissue repair and maladaptive allergic responses (134). Elevated levels of soluble ST2 (sST2), the decoy receptor, was found to be a risk factor for severe GVHD (135). Using an anti-sST2 antibody GVHD severity could be reduced. This treatment also simultaneously maintained membrane-bound ST2 expression on T cells, increasing the ratio of ST2 to sST2, as well as maintaining GVT. This combination of findings pointed to the inverse relationship between the two related receptors (136).

It has previously been described that Th2 cells express ST2 (137, 138) and that its ST2 expression is increased upon exposure to TGF-beta and IL-33. Further, it was found that IL-33 and TGF-beta treatment increased the expression of IL-9 by Th2 cells (139). With these existing data, it was hypothesized that cells polarized under Th9/Tc9 conditions with the addition of IL-33 would exhibit even greater anti-GVHD effect with maintenance of GVT (23). Indeed, this was found to be the case. Furthermore, supporting evidence was found for the mechanism of the $T9_{IL\text{-}33}$ subset's mechanism of GVHD prevention in that this subset expressed significantly more amphiregulin (AREG) on its surface than other subsets. Further research on the new T9 cell subset found that cholesterol blockade in Th9 cells with beta-cyclodextrin led to significantly increased IL-9 production as well as increased tumor killing in both a melanoma model and a metastatic lung tumor model (140). The combination of IL-33 with an anti-cholesterol agent to further enhance the desirable phenotypic characteristics of this subset is an exciting potential avenue of research that could be applied in the near future to combat GVHD while maintaining GVT. Of note, like aGVHD, sST2 is elevated in patients with cGVHD (141). However, the role of the ST2/IL-33 pathway in preclinical model of cGVHD is still under study.

Amphiregulin (AREG)-Expressing T Cell

AREG, a member of Epidermal Growth Factor (EGF) family, binds to EGF receptor and promote the proliferation of normal and malignant epithelial cells, fibroblasts and keratinocytes. Deficiency of AREG in mice showed slower clearance of helminth parasite, Trichuris muris, which was driven by Th2-biased responses (142). Recent study has revealed that IL-33 *via* its receptor ST2 enhances the production of AREG from $ST2^{hi}$ memory T helper 2 (Th2) subset, and directly involved in the reprogramming eosinophils to an inflammatory state with a boost production of osteopontin, a key profibrotic immunomodulatory protein which hence contribute to establishing of lung fibrosis (143). As described above, $T9_{IL\text{-}33}$ surface expressed amphiregulin (AREG) contributes to its GVHD prevention. Furthermore, AREG was found to be essential to the anti-GVHD effect of $T9_{IL\text{-}33}$ cells co-cultured with allogeneic colonic epithelial cells, as AREG blockade significantly increased epithelial cell damage. It was also found that AREG did not cause suppression of effector T cell subsets, explaining the ability of $T9_{IL\text{-}33}$ cells to simultaneously inhibit GVHD and maintain GVT (23). In further support of AREG's lack of suppressive activity, AREG was previously found to be inconsequential in the suppressive activity of Tregs *via* genetic ablation (144). Another recent work has revealed that *ex vivo* IL-33-stimulated Tregs (termed as $Treg_{IL\text{-}33}$) expressed higher AREG and displayed stronger immunosuppression. Adoptive transfer of $Treg_{IL\text{-}33}$ led to a marked improvement of GVHD prevention compared to either naïve control Tregs or IL-23/IL-17-stimulated $Treg_{IL\text{-}33}$. Consistently, blocking AREG with neutralizing antibody *in vivo* abolished the immunosuppression function of $Treg_{IL\text{-}33}$, which collectively suggest a critical role for AREG in IL-33/Treg-mediated GVHD control (145).

Classical Regulatory T Cells (Tregs)

In vivo, Tregs have been shown to develop under the influence of IL-2, IL-15, and TGF-β with FOXP3 as the most critical transcription factor (146–149). Tregs have an extensive experimental history with respect to GVHD, as recently reviewed (150). As predicted by their inherent biology of immunomodulation and self-tolerance, Treg populations have been shown to be decreased during GVHD, allowing for alloreactive T cells to exert their effect (151). Tregs that are FOXP3 negative are known as Type 1 regulatory (Tr1) cells (152). Tr1 cells were characterized as being generated due to alloantigen stimulation by a recipient dendritic cell as well as being stimulated by IL-27. The source of IL-27 is mainly donor macrophages in the context of allo-HCT. In conjunction with this finding, it was determined in this work that IL-6 inhibition increases the proliferation of Tr1 by increasing T cell sensitivity to IL-27 (153). Supporting the inhibitory role of Tregs in the pathogenesis of aGVHD, Tr1 deficiency has been found to exacerbate aGVHD in mouse models (153).

Naturally occurring Tregs (nTregs) have been studied for over a decade in mouse models investigating their ability to prevent aGVHD with nTreg transfer (154, 155). Recipient Treg populations have been expanded before allo-transplant in mice using tumor necrosis factor receptor-2 agonists, leading to prolonged survival and decreased aGVHD (156). Chimeric antigen receptor therapy has been applied to Tregs as well. An alloantigen (HLA-A2) specific CAR was created and applied to Tregs, thus creating an alloantigen-specific human Treg phenotype. In murine models, these CAR-T cells demonstrated superior xenogeneic GVHD prevention caused by $HLA\text{-}A2^+$ T cells compared to Treg cells expressing an irrelevant CAR (157).

In human studies, Treg cells have become the front-runner in the use of cell transfer to treat GVHD. Naturally occurring Tregs (nTregs) hold significant promise as a therapy, but nTreg use in clinic has been hindered by a limited amount of Tregs in the peripheral blood (1-2%) (158, 159) and contamination of nTregs

with $CD25^+$ T-effector or T memory cells (159–161). However, good-manufacturing practice, large-scale *ex-vivo* expansion of Tregs has been demonstrated (159). And despite these limitations, human clinical trials using Tregs have shown promising results. A phase one and dose escalation study with umbilical cord-derived nTreg cells in the prevention of GVHD was encouraging for this style of therapy in the future (162, 163). Another trial investigated the effect of early infusion of freshly sorted Tregs followed by conventional T cells (Tcons) on immune reconstitution and GVHD after haplo-identical HCT. Results of this trial showed promise for GVHD prevention, immune reconstitution, preserved GVT, and resistance to opportunistic infections (164). It is worth noticing that the role of Tregs in cGVHD pathogenesis is controversial (165). Both donor and recipient derived Tregs are known to use TGFβ as the effector of suppression in several models. In contrast to its protective role in aGVHD, Treg-produced TGFβ may exacerbate cGVHD since TGFβ can result in fibrosis of organs such as the skin and lung (120).

In the ALT-TEN trial, patients underwent haplo-identical T-cell depleted HCT combined with IL-10 pretreated T cells. The IL-10 treated cells contained Tr1 cells and T memory cells. The results demonstrated the feasibility of using Tr1 cells as a treatment for immune-mediated disorders such as aGVHD (166). The inhibitory role of IL-6 on Treg and Tr1 expansion has been explored in a phase I/II clinical trial as a potential therapeutic target for aGVHD. Anti-IL-6 tocilizumab was used in a single dose before allo-matched HCT, which showed low incidence of aGVHD with treatment, and called for further study of this method in GVHD prophylaxis (167). However, a more recent randomized phase 3 trial evaluating the addition of tocilizumab to cyclosporin and methotrexate for aGVHD prophylaxis, did not show statistically significant reduction in grade II-IV aGVHD or long-term survival (168).

T CELLS INDUCERS OF GVT

Donor grafts-derived allogeneic immune cells, particularly the T cells, recognize and eradicate leukemic cells *via* GVT reactivity, which hence could harness the power for high-risk hematological malignancies such as acute myeloid leukemia (AML) and multiple myeloma (MM). However, the normal tissues of the recipient will also be recognized and attacked by these cells also attack host normal tissues by GVHD (169). Separation of GVT reactivity from GVHD reaction is a necessary step for improving allo-HCT outcomes. Previous study indicated that Th9 cells, a unique subset of $CD4^+$ T cell that produce the pleiotropic cytokine IL-9 and boost antitumor immune responses *in vivo via* $CD8^+$ CTL-mediated antitumor immunity (131). Further study revealed that IL-9–produced $CD8^+$ T (Tc9) cells generated various cytokines and showed less cytolytic activity *in vitro* but surprisingly elicited enhanced antitumor responses against advanced tumors in OT-I/B16-OVA and Pmel-1/B16 melanoma models (170). As proof of principle of better antitumoral activity, human chimeric antigen receptor (CAR) T cells polarized and expanded under a Th9-culture condition (T9 CAR-T) showed enhanced antitumor activity against established tumors compared to IL2-polarized (T1) cells. T9 CAR-T cells secrete IL9 but little IFN-γ, express central memory phenotype and lower levels of exhaustion markers and display robust proliferative capacity (171). In allo-HCT settings, T9 cells activated with IL-33 during *in vitro* differentiation boosted their ST2 expression and IL-9 production. Adoptive cell transfer (ACT) of IL-33 activated T9 cells ($T9_{IL\text{-}33}$) decreased GVHD severity and increased GVT activity *via* two distinct mechanisms: decrease of fatal immunity by amphiregulin expression and increase of antileukemic activity *via* CD8α expression (23).

NOVEL GVHD TREATMENTS BASED ON FUNDAMENTAL T CELL BIOLOGY

Cellular Therapy

Recent reviews have summarized the scope of cellular therapies to treat GVHD (172, 173). **Table 1** list potential T-cells based cellular therapies at different stages of development. Cellular therapies are likely to expand their scope in patients with diverse diseases (174), although delivery of such "live" drugs are not easily scalable (175). Fortunately, in parallel strikes have been made in GVHD treatment with classical drugs as summarized below and in **Table 2**.

Small Molecules Inhibitors

ITK Inhibitors

Ibrutinib is an Interleukin-2-inducible T-cell kinase (ITK) and Bruton tyrosine kinase (BTK) inhibitor that hinders the survival of reactive T-cells, and B cells, respectively (176, 177). In mice, transplant of bone marrow deficient in ITK and BTK showed the importance of these molecules in the pathogenesis of cGVHD, as the transplanted mice did not experience cGVHD (25). Concurrently, mice treated with ibrutinib experienced less severe cGVHD (178). In a phase I/II study for patients with SR-cGVHD, ibrutinib was shown to significantly improve symptoms in most patients, as well as decrease the frequency of chemotactic and fibrotic factors in patients' blood (179). A significant number of adverse events (AEs) including grade ≥ 3 infectious complications were seen; however the safety profile was deemed acceptable as the AEs were similar to those observed in cGVHD patients treated with concomitant steroids (179). These studies led to the first ever drug in cGVHD to obtain the FDA breakthrough denomination.

TABLE 1 | Summary of Cellular Therapies for GVHD based on T cells subsets.

Treatment	Status
Naïve T cell depletion	Completed Phase II
Trapa DLI	Completed Phase II
Th9/TC9	Preclinical
HLA-A2 CART Treg	Preclinical
nTreg	Completed Phase I
Tr1 Expansion	Completed Phase II

TABLE 2 | Summary of recent novel small molecule treatments for GVHD.

Treatment	Mechanism	Status	Trials (examples)
Ibrutinib	BTK/ITK inhibition	Completed Phase I/II Phase III ongoing	
Ruxolitinib	JAK inhibition	Completed Phase II/III for steroid-refractory aGVHD & cGVHD	
Itacitinib	JAK inhibition	Completed Phase I, Phase III for steroid naïve patients	INCB039110
TMP778	RORγt inhibition	Preclinical	
KD025	RORγt inhibition	Phase II	NCT02841995
Tocilizumab	Anti-IL-6	Completed Phase I/II/III	
Brentuximab Vedotin	CD30 conjugated Ab	Completed Phase I	
Vedolizumab	Integrin inhibition	Halted Phase II	NCT02993783
Natalizumab	Integrin inhibition	Phase II	NCT02176031, NCT02133924

JAK Inhibitors

Janus kinases (JAKs) are tyrosine kinases that mediate cytokine-signaling in T cells, propagating survival and differentiation signals (180). The activation of a JAK leads to phosphorylation of signal transducers and activators of transcription (STATs) (181). JAK signaling has also been associated with dendritic cell function, thus amplifying this pathway's potential importance in GVHD (182, 183).

In mouse models, JAK1/2 blockade with ruxolitinib has displayed decreased IFNy Receptor (IFNyR) receptor signaling, leading to reduced severity of GVHD and preserved GVT (184, 185). In addition, JAK1/2 inhibition in mouse models led to increased frequency of Tregs and decreased frequency of inflammatory cytokines in association with the decreased severity of aGVHD (181).

Following up on findings in murine models, a preliminary trial of 6 human patients with SR-GVHD treated with ruxolitinib showed an improvement in symptoms and similar reduction of the frequency of inflammatory cytokines in peripheral blood (181). In addition, a large multicenter retrospective survey of patients who had received ruxolitinib for steroid-refractory GVHD suggested that ruxolitinib had significant efficacy (186). The results of the phase III randomized clinical trials have recently been published with an overall response of 62% in the ruxolitinib group vs. 39% in the control group ($P<0.001$) in steroid-refractory aGVHD (15), and an overall response of 50% in the ruxolitinib group vs. 26% in the control group ($P<0.001$) in steroid refractory cGVHD (16), respectively. Ruxolitinib is now the second drug to get the FDA breakthrough denomination for both cGVHD and aGVHD.

A specific blockade of JAK1 was explored in a phase I trial with itacitinib (INCB039110), which showed responses rates of 64.7% and 88.3% for steroid refractory and treatment naïve disease, respectively (187). Similar AEs were seen with this drug as with ruxolininib, including cytopenia and CMV reactivations. However, itacitinib missed the mark in phase III when given in combination with corticosteroids in patients with treatment-naïve aGVHD.

RORγt Inhibitors

TMP778

As mentioned above, one of the RORγt transcription factor small molecule inhibitors, TMP778, has showed promise in a GVHD murine model similar to an anti-IL-17 antibody (84). However, global inhibition of a transcription factor is generally too toxic to implement in clinic ad alternative have been found such as ROCK2 inhibitors upstream of transcription factors.

ROCK2 Inhibitors

Belumosudil (KD025) is a serine-threonine kinase inhibiting ROCK2 that rebalances the immune system in GVHD by downregulating pro-inflammatory Th17 cells and increasing Tregs, also acting on JAK2/JAK3 and STAT3 (85). Further, ROCK2 is an intracellular integrator of profibrotic signals. Excellent responses were seen in the phase II clinical trials (86) (87) and belumosudil was FDA approved for cGVHD patients who are received 2 prior lines of therapy as mentioned above (88).

Anti-Cytokines

Anti- IL6

The addition of a humanized anti-IL-6R mAb (Tocilizumab) to standard GVHD prophylaxis has shown in promise in reducing the incidence of aGVHD in a prospective phase I/II clinical study (167). The phase III double-blinded study of the addition of Tocilizumab vs. Placebo to cyclosporin/methotrexate GVHD Prophylaxis after HLA-Matched allo-HCT failed to meet the primary endpoint (168).

Conjugated Antibodies (Ab)

Anti-CD30 Ab

Higher frequency of $CD30^+$ CD8 T cells, plasma soluble CD30, and CD30+ lymphocytes have been demonstrated in the intestinal lesions of aGVHD patients (188). This led to the proposal of using the anti-CD30 monomethyl auristatin E (MMAE) conjugate for use in GVHD. A phase I trial for patients with SR-aGVHD showed significant toxicity associated with this drug, including neutropenic sepsis leading to death along with other grade III toxicities of headache, hypoxia, ileus, and elevated bilirubin (189).

Anti-Integrins Abs

Blockade of alpha4beta7 in the gut has been used effectively for inflammatory bowel disease as it disallows effector T cells from being trafficked to the area of inflammation. Natalizumab is one such example that has been used for autoimmune diseases such as Crohn's, however, its lack of specificity gives it the associated risk of progressive multifocal leukoencephalopathy (PML) (190).

Two phase II clinical trials are currently investigating the effectiveness of natalizumab for GVHD (NCT02176031 and NCT02133924) (14). Vedolizumab represents an example of a monoclonal antibody that is specific to the gut, as it inhibits alpha4beta7 integrin's interaction with MAdCAM-1 and therefore carries significantly lower risk for the serious adverse outcome of PML (191–193). Similarly, this integrin has been shown to be important in the pathogenesis of intestinal GVHD (184). A recent case series of 6 patients explored the use of vedolizumab for the treatment of steroid-refractory intestinal GVHD. Patients treated with vedolizumab almost universally achieved remarkable improvement in gastrointestinal GVHD symptoms, in some cases having symptoms reduced from grade IV to grade I or absent (194). However, a phase II clinical trial, NCT02993783, to evaluate the safety and efficacy of this treatment was recently terminated due to lack of efficacy. Indeed, ORR at day 28 was 50% in patients treated at 300 mg (n = 8) and 22% in patients treated at 600 mg (n = 9); 12% and 0% of patients, respectively, achieved complete response (CR). Thus, higher the dose the less efficacious vedolizumab was. Due to its mechanism of action of blocking T cell migration to the intestine, it is likely that there cannot be an effect when GVHD is already full-blown and T cells in the gut. However, it is possible that preventing effector T cells migration to the GI tract may be beneficial at early stages of GVHD or as GVHD prophylaxis.

CONCLUSION

Acute and chronic GVHD remain severe and common complications of hematopoietic stem cell transplant. Prevention and treatment of these diseases remain a critical frontier in transplant medicine. New understandings of T cell biology have led to novel treatments with a variety of targets and fundamental mechanisms. The plethora of recent human clinical trials as well as the exciting preclinical experiments have suggested the real possibility of a significant breakthrough for HSCT patients in the near future.

AUTHOR CONTRIBUTIONS

HJ, DF, and SP conceived and wrote the manuscript. All authors contributed to critical revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Magenau J, Runaas L, Reddy P. Advances in Understanding the Pathogenesis of Graft-Versus-Host Disease. *Br J Haematol* (2016) 173 (2):190–205. doi: 10.1111/bjh.13959
2. Zeiser R, Socie G, Blazar BR. Pathogenesis of Acute Graft-Versus-Host Disease: From Intestinal Microbiota Alterations to Donor T Cell Activation. *Br J Haematol* (2016) 175(2):191–207. doi: 10.1111/bjh.14295
3. Koyama M, Kuns RD, Olver SD, Raffelt NC, Wilson YA, Don AL, et al. Recipient Nonhematopoietic Antigen-Presenting Cells Are Sufficient to Induce Lethal Acute Graft-Versus-Host Disease. *Nat Med* (2011) 18 (1):135–42. doi: 10.1038/nm.2597
4. Matte CC, Liu J, Cormier J, Anderson BE, Athanasiadis I, Jain D, et al. Donor APCs Are Required for Maximal GVHD But Not for GVL. *Nat Med* (2004) 10(9):987–92. doi: 10.1038/nm1089
5. Reddy P, Maeda Y, Liu C, Krijanovski OI, Korngold R, Ferrara JL. A Crucial Role for Antigen-Presenting Cells and Alloantigen Expression in Graft-Versus-Leukemia Responses. *Nat Med* (2005) 11(11):1244–9. doi: 10.1038/nm1309
6. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of Graft Versus Host Disease by Inactivation of Host Antigen-Presenting Cells. *Science* (1999) 285(5426):412–5. doi: 10.1126/science.285.5426.412
7. Toubai T, Tawara I, Sun Y, Liu C, Nieves E, Evers R, et al. Induction of Acute GVHD by Sex-Mismatched H-Y Antigens in the Absence of Functional Radiosensitive Host Hematopoietic-Derived Antigen-Presenting Cells. *Blood* (2012) 119(16):3844–53. doi: 10.1182/blood-2011-10-384057
8. Chakraverty R, Sykes M. The Role of Antigen-Presenting Cells in Triggering Graft-Versus-Host Disease and Graft-Versus-Leukemia. *Blood* (2007) 110 (1):9–17. doi: 10.1182/blood-2006-12-022038
9. Xia G, Truitt RL, Johnson BD. Graft-Versus-Leukemia and Graft-Versus-Host Reactions After Donor Lymphocyte Infusion Are Initiated by Host-Type Antigen-Presenting Cells and Regulated by Regulatory T Cells in Early and Long-Term Chimeras. *Biol Blood Marrow Transplant* (2006) 12(4):397–407. doi: 10.1016/j.bbmt.2005.11.519
10. Blazar BR, Murphy WJ, Abedi M. Advances in Graft-Versus-Host Disease Biology and Therapy. *Nat Rev Immunol* (2012) 12(6):443–58. doi: 10.1038/nri3212
11. Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, et al. Peripheral-Blood Stem Cells Versus Bone Marrow From Unrelated Donors. *N Engl J Med* (2012) 367(16):1487–96. doi: 10.1056/NEJMoa1203517
12. MacDonald KP, Hill GR, Blazar BR. Chronic Graft-Versus-Host Disease: Biological Insights From Preclinical and Clinical Studies. *Blood* (2017) 129 (1):13–21. doi: 10.1182/blood-2016-06-686618
13. Koyama M, Hill GR. Alloantigen Presentation and Graft-Versus-Host Disease: Fuel for the Fire. *Blood* (2016) 127(24):2963–70. doi: 10.1182/blood-2016-02-697250
14. Hill L, Alousi A, Kebriaei P, Mehta R, Rezvani K, Shpall E. New and Emerging Therapies for Acute and Chronic Graft Versus Host Disease. *Ther Adv Hematol* (2018) 9(1):21–46. doi: 10.1177/2040620717741860
15. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *N Engl J Med* (2020) 382(19):1800–10. doi: 10.1056/NEJMoa1917635
16. Zeiser R, Polverelli N, Ram R, Hashmi SK, Chakraverty R, Middeke JM, et al. Ruxolitinib for Glucocorticoid-Refractory Chronic Graft-Versus-Host Disease. *N Engl J Med* (2021) 385(3):228–38. doi: 10.1056/NEJMoa2033122
17. Kaplan MH, Hufford MM, Olson MR. The Development and *In Vivo* Function of T Helper 9 Cells. *Nat Rev Immunol* (2015) 15(5):295–307. doi: 10.1038/nri3824

18. Dienz O, Rincon M. The Effects of IL-6 on CD4 T Cell Responses. *Clin Immunol* (2009) 130(1):27–33. doi: 10.1016/j.clim.2008.08.018
19. Imam T, Park S, Kaplan MH, Olson MR. Effector T Helper Cell Subsets in Inflammatory Bowel Diseases. *Front Immunol* (2018) 9:1212. doi: 10.3389/fimmu.2018.01212
20. Hirahara K, Nakayama T. CD4+ T-Cell Subsets in Inflammatory Diseases: Beyond the Th1/Th2 Paradigm. *Int Immunol* (2016) 28(4):163–71. doi: 10.1093/intimm/dxw006
21. Busch DH, Frassle SP, Sommermeyer D, Buchholz VR, Riddell SR. Role of Memory T Cell Subsets for Adoptive Immunotherapy. *Semin Immunol* (2016) 28(1):28–34. doi: 10.1016/j.smim.2016.02.001
22. Ramadan A, Paczesny S. Various Forms of Tissue Damage and Danger Signals Following Hematopoietic Stem-Cell Transplantation. *Front Immunol* (2015) 6:14. doi: 10.3389/fimmu.2015.00014
23. Ramadan A, Griesenauer B, Adom D, Kapur R, Hanenberg H, Liu C, et al. Specifically Differentiated T Cell Subset Promotes Tumor Immunity Over Fatal Immunity. *J Exp Med* (2017) 214(12):3577–96. doi: 10.1084/jem.20170041
24. Saad A, Lamb LS. *Ex Vivo* T-Cell Depletion in Allogeneic Hematopoietic Stem Cell Transplant: Past, Present and Future. *Bone Marrow Transplant* (2017) 52(9):1241–8. doi: 10.1038/bmt.2017.22
25. Arai S, Margolis J, Zahurak M, Anders V, Vogelsang GB. Poor Outcome in Steroid-Refractory Graft-Versus-Host Disease With Antithymocyte Globulin Treatment. *Biol Blood Marrow Transplant* (2002) 8(3):155–60. doi: 10.1053/bbmt.2002.v8.pm11939605
26. Socie G, Vigouroux S, Yakoub-Agha I, Bay JO, Furst S, Bilger K, et al. A Phase 3 Randomized Trial Comparing Inolimomab *vs* Usual Care in Steroid-Resistant Acute GVHD. *Blood* (2017) 129(5):643–9. doi: 10.1182/blood-2016-09-738625
27. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A Direct Estimate of the Human Alphabeta T Cell Receptor Diversity. *Science* (1999) 286(5441):958–61. doi: 10.1126/science.286.5441.958
28. Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, et al. Comprehensive Assessment of T-Cell Receptor Beta-Chain Diversity in Alphabeta T Cells. *Blood* (2009) 114(19):4099–107. doi: 10.1182/blood-2009-04-217604
29. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, Shlomchik MJ, et al. Memory CD4+ T Cells do Not Induce Graft-Versus-Host Disease. *J Clin Invest* (2003) 112(1):101–8. doi: 10.1172/JCI17601
30. Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of Allogeneic CD62L- Memory T Cells Without Graft-Versus-Host Disease. *Blood* (2004) 103(4):1534–41. doi: 10.1182/blood-2003-08-2987
31. Zhang Y, Joe G, Zhu J, Carroll R, Levine B, Hexner E, et al. Dendritic Cell-Activated CD44hiCD8+ T Cells Are Defective in Mediating Acute Graft-Versus-Host Disease But Retain Graft-Versus-Leukemia Activity. *Blood* (2004) 103(10):3970–8. doi: 10.1182/blood-2003-09-3135
32. Dutt S, Tseng D, Ermann J, George TI, Liu YP, Davis CR, et al. Naive and Memory T Cells Induce Different Types of Graft-Versus-Host Disease. *J Immunol* (2007) 179(10):6547–54. doi: 10.4049/jimmunol.179.10.6547
33. Chen BJ, Deoliveira D, Cui X, Le NT, Son J, Whitesides JF, et al. Inability of Memory T Cells to Induce Graft-Versus-Host Disease is a Result of an Abortive Alloresponse. *Blood* (2007) 109(7):3115–23. doi: 10.1182/blood-2006-04-016410
34. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, et al. Effector Memory CD4+ T Cells Mediate Graft-Versus-Leukemia Without Inducing Graft-Versus-Host Disease. *Blood* (2008) 111(4):2476–84. doi: 10.1182/blood-2007-08-109678
35. Zheng H, Matte-Martone C, Jain D, McNiff J, Shlomchik WD. Central Memory CD8+ T Cells Induce Graft-Versus-Host Disease and Mediate Graft-Versus-Leukemia. *J Immunol* (2009) 182(10):5938–48. doi: 10.4049/jimmunol.0802212
36. Bleakley M, Otterud BE, Richardt JL, Mollerup AD, Hudecek M, Nishida T, et al. Leukemia-Associated Minor Histocompatibility Antigen Discovery Using T-Cell Clones Isolated by In Vitro Stimulation of Naive CD8+ T Cells. *Blood* (2010) 115(23):4923–33. doi: 10.1182/blood-2009-12-260539
37. Li N, Matte-Martone C, Zheng H, Cui W, Venkatesan S, Tan HS, et al. Memory T Cells From Minor Histocompatibility Antigen-Vaccinated and Virus-Immune Donors Improve GVL and Immune Reconstitution. *Blood* (2011) 118(22):5965–76. doi: 10.1182/blood-2011-07-367011
38. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of Acute Leukemia Patients Transplanted With Naive T Cell-Depleted Stem Cell Grafts. *J Clin Invest* (2015) 125(7):2677–89. doi: 10.1172/JCI81229
39. Hill GR, Betts BC, Tkachev V, Kean LS, Blazar BR. Current Concepts and Advances in Graft-Versus-Host Disease Immunology. *Annu Rev Immunol* (2021) 39:19–49. doi: 10.1146/annurev-immunol-102119-073227
40. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 Responses and Enhanced Development of Th2 Cells in Stat4-Deficient Mice. *Nature* (1996) 382(6587):174–7. doi: 10.1038/382174a0
41. Magram J, Sfarra J, Connaughton S, Faherty D, Warrier R, Carvajal D, et al. IL-12-Deficient Mice are Defective But Not Devoid of Type 1 Cytokine Responses. *Ann N Y Acad Sci* (1996) 795:60–70. doi: 10.1111/j.1749-6632.1996.tb52655.x
42. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation Directs Memory Precursor and Short-Lived Effector CD8(+) T Cell Fates *via* the Graded Expression of T-Bet Transcription Factor. *Immunity* (2007) 27(2):281–95. doi: 10.1016/j.immuni.2007.07.010
43. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith KA, Ahmed R. Prolonged Interleukin-2Ralpha Expression on Virus-Specific CD8+ T Cells Favors Terminal-Effector Differentiation *In Vivo*. *Immunity* (2010) 32(1):91–103. doi: 10.1016/j.immuni.2009.11.010
44. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs That Promote the Differentiation of Effector Cytolytic T Cells. *Immunity* (2010) 32(1):79–90. doi: 10.1016/j.immuni.2009.11.012
45. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two Types of Murine Helper T Cell Clone. I. Definition According to Profiles of Lymphokine Activities and Secreted Proteins. *J Immunol* (1986) 136(7):2348–57.
46. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A Novel Transcription Factor, T-Bet, Directs Th1 Lineage Commitment. *Cell* (2000) 100(6):655–69. doi: 10.1016/S0092-8674(00)80702-3
47. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent Developments in the Transcriptional Regulation of Cytolytic Effector Cells. *Nat Rev Immunol* (2004) 4(11):900–11. doi: 10.1038/nri1490
48. Burman AC, Banovic T, Kuns RD, Clouston AD, Stanley AC, Morris ES, et al. IFNgamma Differentially Controls the Development of Idiopathic Pneumonia Syndrome and GVHD of the Gastrointestinal Tract. *Blood* (2007) 110(3):1064–72. doi: 10.1182/blood-2006-12-063982
49. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total Body Irradiation and Acute Graft-Versus-Host Disease: The Role of Gastrointestinal Damage and Inflammatory Cytokines. *Blood* (1997) 90(8):3204–13. doi: 10.1182/blood.V90.8.3204
50. Hill GR, Ferrara JL. The Primacy of the Gastrointestinal Tract as a Target Organ of Acute Graft-Versus-Host Disease: Rationale for the Use of Cytokine Shields in Allogeneic Bone Marrow Transplantation. *Blood* (2000) 95(9):2754–9. doi: 10.1182/blood.V95.9.2754.009k25_2754_2759
51. Robb RJ, Hill GR. The Interferon-Dependent Orchestration of Innate and Adaptive Immunity After Transplantation. *Blood* (2012) 119(23):5351–8. doi: 10.1182/blood-2012-02-368076
52. Fu J, Wang D, Yu Y, Heinrichs J, Wu Y, Schutt S, et al. T-Bet is Critical for the Development of Acute Graft-Versus-Host Disease Through Controlling T Cell Differentiation and Function. *J Immunol* (2015) 194(1):388–97. doi: 10.4049/jimmunol.1401618
53. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C, et al. Prevention of GVHD While Sparing GVL Effect by Targeting Th1 and Th17 Transcription Factor T-Bet and RORgammat in Mice. *Blood* (2011) 118(18):5011–20. doi: 10.1182/blood-2011-03-340315
54. Krenger W, Ferrara JL. Graft-Versus-Host Disease and the Th1/Th2 Paradigm. *Immunol Res* (1996) 15(1):50–73. doi: 10.1007/BF02918284
55. Morris ES, Hill GR. Advances in the Understanding of Acute Graft-Versus-Host Disease. *Br J Haematol* (2007) 137(1):3–19. doi: 10.1111/j.1365-2141.2007.06510.x
56. Schroeder MA, Choi J, Staser K, DiPersio JF. The Role of Janus Kinase Signaling in Graft-Versus-Host Disease and Graft Versus Leukemia. *Biol Blood Marrow Transplant* (2018) 24(6):1125–34. doi: 10.1016/j.bbmt.2017.12.797

57. Mannina D, Kroger N. Janus Kinase Inhibition for Graft-Versus-Host Disease: Current Status and Future Prospects. *Drugs* (2019) 79(14):1499–509. doi: 10.1007/s40265-019-01174-1
58. Kim S, Ashami K, Lim S, Staser K, Vij K, Santhanam S, et al. Baricitinib Prevents GvHD by Increasing Tregs *via* JAK3 and Treats Established GvHD by Promoting Intestinal Tissue Repair *via* EGFR. *Leukemia* (2021) 38:42 Z. doi: 10.1038/s41375-021-01360-9
59. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the Context of an Inflammatory Cytokine Milieu Supports *De Novo* Differentiation of IL-17-Producing T Cells. *Immunity* (2006) 24(2):179–89. doi: 10.1016/j.immuni.2006.01.001
60. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming Growth Factor-Beta Induces Development of the T (H)17 Lineage. *Nature* (2006) 441(7090):231–4. doi: 10.1038/nature04754
61. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal Developmental Pathways for the Generation of Pathogenic Effector TH17 and Regulatory T Cells. *Nature* (2006) 441(7090):235–8. doi: 10.1038/nature04753
62. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, et al. IL-21 and TGF-Beta are Required for Differentiation of Human T(H)17 Cells. *Nature* (2008) 454(7202):350–2. doi: 10.1038/nature07021
63. Huber M, Heink S, Grothe H, Guralnik A, Reinhard K, Elflein K, et al. A Th17-Like Developmental Process Leads to CD8(+) Tc17 Cells With Reduced Cytotoxic Activity. *Eur J Immunol* (2009) 39(7):1716–25. doi: 10.1002/eji.200939412
64. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A Distinct Lineage of CD4 T Cells Regulates Tissue Inflammation by Producing Interleukin 17. *Nat Immunol* (2005) 6(11):1133–41. doi: 10.1038/ni1261
65. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-Producing CD4+ Effector T Cells Develop *via* a Lineage Distinct From the T Helper Type 1 and 2 Lineages. *Nat Immunol* (2005) 6(11):1123–32. doi: 10.1038/ni1254
66. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The Orphan Nuclear Receptor RORgammat Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. *Cell* (2006) 126 (6):1121–33. doi: 10.1016/j.cell.2006.07.035
67. Murphy KM, Reiner SL. The Lineage Decisions of Helper T Cells. *Nat Rev Immunol* (2002) 2(12):933–44. doi: 10.1038/nri954
68. Hill GR, Olver SD, Kuns RD, Varelias A, Raffelt NC, Don AL, et al. Stem Cell Mobilization With G-CSF Induces Type 17 Differentiation and Promotes Scleroderma. *Blood* (2010) 116(5):819–28. doi: 10.1182/blood-2009-11-256495
69. Meguro A, Ozaki K, Hatanaka K, Oh I, Sudo K, Ohmori T, et al. Lack of IL-21 Signal Attenuates Graft-Versus-Leukemia Effect in the Absence of CD8 T-Cells. *Bone Marrow Transplant* (2011) 46(12):1557–65. doi: 10.1038/bmt.2010.342
70. Meguro A, Ozaki K, Oh I, Hatanaka K, Matsu H, Tatara R, et al. IL-21 is Critical for GVHD in a Mouse Model. *Bone Marrow Transplant* (2010) 45 (4):723–9. doi: 10.1038/bmt.2009.223
71. Bucher C, Koch L, Vogtenhuber C, Goren E, Munger M, Panoskaltsis-Mortari A, et al. IL-21 Blockade Reduces Graft-Versus-Host Disease Mortality by Supporting Inducible T Regulatory Cell Generation. *Blood* (2009) 114(26):5375–84. doi: 10.1182/blood-2009-05-221135
72. Hanash AM, Kappel LW, Yim NL, Nejat RA, Goldberg GL, Smith OM, et al. Abrogation of Donor T-Cell IL-21 Signaling Leads to Tissue-Specific Modulation of Immunity and Separation of GVHD From GVL. *Blood* (2011) 118(2):446–55. doi: 10.1182/blood-2010-07-294785
73. Fulton LM, Carlson MJ, Coghill JM, Ott LE, West ML, Panoskaltsis-Mortari A, et al. Attenuation of Acute Graft-Versus-Host Disease in the Absence of the Transcription Factor RORgammat. *J Immunol* (2012) 189(4):1765–72. doi: 10.4049/jimmunol.1200858
74. Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, et al. Blockade of Interleukin-6 Signaling Augments Regulatory T-Cell Reconstitution and Attenuates the Severity of Graft-Versus-Host Disease. *Blood* (2009) 114(4):891–900. doi: 10.1182/blood-2009-01-197178
75. Tawara I, Koyama M, Liu C, Toubai T, Thomas D, Evers R, et al. Interleukin-6 Modulates Graft-Versus-Host Responses After Experimental Allogeneic Bone Marrow Transplantation. *Clin Cancer Res* (2011) 17(1):77–88. doi: 10.1158/1078-0432.CCR-10-1198
76. Das R, Chen X, Komorowski R, Hessner MJ, Drobyski WR. Interleukin-23 Secretion by Donor Antigen-Presenting Cells is Critical for Organ-Specific Pathology in Graft-Versus-Host Disease. *Blood* (2009) 113(10):2352–62. doi: 10.1182/blood-2008-08-175448
77. Thompson JS, Chu Y, Glass JF, Brown SA. Absence of IL-23p19 in Donor Allogeneic Cells Reduces Mortality From Acute GVHD. *Bone Marrow Transplant* (2010) 45(4):712–22. doi: 10.1038/bmt.2009.215
78. Das R, Komorowski R, Hessner MJ, Subramanian H, Huettner CS, Cua D, et al. Blockade of Interleukin-23 Signaling Results in Targeted Protection of the Colon and Allows for Separation of Graft-Versus-Host and Graft-Versus-Leukemia Responses. *Blood* (2010) 115(25):5249–58. doi: 10.1182/blood-2009-11-255422
79. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, Serody JS. In Vitro-Differentiated TH17 Cells Mediate Lethal Acute Graft-Versus-Host Disease With Severe Cutaneous and Pulmonary Pathologic Manifestations. *Blood* (2009) 113(6):1365–74. doi: 10.1182/blood-2008-06-162420
80. Iclozan C, Yu Y, Liu C, Liang Y, Yi T, Anasetti C, et al. T Helper17 Cells are Sufficient But Not Necessary to Induce Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2010) 16(2):170–8. doi: 10.1016/j.bbmt.2009.09.023
81. Dander E, Balduzzi A, Zappa G, Lucchini G, Perseghin P, Andre V, et al. Interleukin-17-Producing T-Helper Cells as New Potential Player Mediating Graft-Versus-Host Disease in Patients Undergoing Allogeneic Stem-Cell Transplantation. *Transplantation* (2009) 88(11):1261–72. doi: 10.1097/TP.0b013e3181bc267e
82. Bruggen MC, Klein I, Greinix H, Bauer W, Kuzmina Z, Rabitsch W, et al. Diverse T-Cell Responses Characterize the Different Manifestations of Cutaneous Graft-Versus-Host Disease. *Blood* (2014) 123(2):290–9. doi: 10.1182/blood-2013-07-514372
83. Li W, Liu L, Gomez A, Zhang Q, Zhang J, Ramadan A, et al. A Novel Th17-Prone CD146+CCR5+ T-Cell Population As an Early Marker of Intestinal Graft-Versus-Host Disease. *Blood* (2014) 124(21):3. doi: 10.1182/blood.V124.21.3.3
84. Forcade E, Paz K, Flynn R, Griesenauer B, Amet T, Li W, et al. An Activated Th17-Prone T Cell Subset Involved in Chronic Graft-Versus-Host Disease Sensitive to Pharmacological Inhibition. *JCI Insight* (2017) 2(12):e92111. doi: 10.1172/jci.insight.92111
85. Flynn R, Paz K, Du J, Reichenbach DK, Taylor PA, Panoskaltsis-Mortari A, et al. Targeted Rho-Associated Kinase 2 Inhibition Suppresses Murine and Human Chronic GVHD Through a Stat3-Dependent Mechanism. *Blood* (2016) 127(17):2144–54. doi: 10.1182/blood-2015-10-678706
86. Jagasia M, Lazaryan A, Bachier CR, Salhotra A, Weisdorf DJ, Zoghi B, et al. ROCK2 Inhibition With Belumosudil (KD025) for the Treatment of Chronic Graft-Versus-Host Disease. *J Clin Oncol Off J Am Soc Clin Oncol* (2021) 39(17):1888–98. doi: 10.1200/JCO.20.02754
87. Cutler CS, Lee SJ, Arai S, Rotta M, Zoghi B, Lazaryan A, et al. Belumosudil for Chronic Graft-Versus-Host Disease (cGVHD) After 2 or More Prior Lines of Therapy: The ROCKstar Study. *Blood* (2021). doi: 10.1182/blood.2021012021
88. FDA. FDA Approves Belumosudil for Chronic Graft-Versus-Host Disease, in: *FDA/CEDR Resources Page*. Food and Drug Administration. Available at: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-belumosudil-chronic-graft-versus-host-disease (Accessed September 9, 2021).
89. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 Cells Represent a Distinct Human T Cell Subset Involved in Epidermal Immunity and Remodeling. *J Clin Invest* (2009) 119(12):3573–85. doi: 10.1172/JCI40202
90. Plank MW, Kaiko GE, Maltby S, Weaver J, Tay HL, Shen W, et al. Th22 Cells Form a Distinct Th Lineage From Th17 Cells In Vitro With Unique Transcriptional Properties and Tbet-Dependent Th1 Plasticity. *J Immunol* (2017) 198(5):2182–90. doi: 10.4049/jimmunol.1601480
91. Zhao K, Zhao D, Huang D, Song X, Chen C, Pan B, et al. The Identification and Characteristics of IL-22-Producing T Cells in Acute Graft-Versus-Host Disease Following Allogeneic Bone Marrow Transplantation. *Immunobiology* (2013) 218(12):1505–13. doi: 10.1016/j.imbio.2013.05.005
92. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 Protects Intestinal Stem Cells From Immune-Mediated

Tissue Damage and Regulates Sensitivity to Graft Versus Host Disease. *Immunity* (2012) 37(2):339–50. doi: 10.1016/j.immuni.2012.05.028
93. Couturier M, Lamarthée B, Arbez J, Renauld JC, Bossard C, Malard F, et al. IL-22 Deficiency in Donor T Cells Attenuates Murine Acute Graft-Versus-Host Disease Mortality While Sparing the Graft-Versus-Leukemia Effect. *Leukemia* (2013) 27:1527. doi: 10.1038/leu.2013.39
94. Zhao K, Zhao D, Huang D, Yin L, Chen C, Pan B, et al. Interleukin-22 Aggravates Murine Acute Graft-Versus-Host Disease by Expanding Effector T Cell and Reducing Regulatory T Cell. *J Interferon Cytokine Res* (2014) 34 (9):707–15. doi: 10.1089/jir.2013.0099
95. Saxton RA, Henneberg LT, Calafiore M, Su L, Jude KM, Hanash AM, et al. The Tissue Protective Functions of Interleukin-22 can be Decoupled From Pro-Inflammatory Actions Through Structure-Based Design. *Immunity* (2021) 54(4):660–72 e9. doi: 10.1016/j.immuni.2021.03.008
96. Ponce D, Alousi A, Nakamura R, Sandu K, Barker J, Shia J, et al. A Phase 2 Study of F-652, a Novel Tissue-Targeted Recombinant Human Interleukin-22 (IL-22) Dimer, for Treatment of Newly Diagnosed Acute Gvhd of the Lower GI Tract. *Biol Blood Marrow Transplant* (2021) 26(3):S51 – S2. doi: 10.1016/j.bbmt.2019.12.124
97. Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, et al. Cutting Edge: Dendritic Cell-Restricted Antigen Presentation Initiates the Follicular Helper T Cell Program But Cannot Complete Ultimate Effector Differentiation. *J Immunol* (2011) 187(3):1091–5. doi: 10.4049/jimmunol.1100853
98. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 Mediates the Development of T Follicular Helper Cells. *Science* (2009) 325(5943):1001–5. doi: 10.1126/science.1176676
99. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS Receptor Instructs T Follicular Helper Cell *Versus* Effector Cell Differentiation *via* Induction of the Transcriptional Repressor Bcl6. *Immunity* (2011) 34(6):932–46. doi: 10.1016/j.immuni.2011.03.023
100. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting Edge: STAT1 is Required for IL-6-Mediated Bcl6 Induction for Early Follicular Helper Cell Differentiation. *J Immunol* (2013) 190(7):3049–53. doi: 10.4049/jimmunol.1203032
101. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing Signals From the Bcl6 Transcription Factor and the Interleukin-2 Receptor Generate T Helper 1 Central and Effector Memory Cells. *Immunity* (2011) 35(4):583–95. doi: 10.1016/j.immuni.2011.09.009
102. Fang D, Cui K, Mao K, Hu G, Li R, Zheng M, et al. Transient T-Bet Expression Functionally Specifies a Distinct T Follicular Helper Subset. *J Exp Med* (2018) 215(11):2705–14. doi: 10.1084/jem.20180927
103. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, et al. Interleukin-2 Inhibits Germinal Center Formation by Limiting T Follicular Helper Cell Differentiation. *Immunity* (2012) 36(5):847–56. doi: 10.1016/j.immuni.2012.02.012
104. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a Potent Negative Regulator of TFH Cell Differentiation. *J Exp Med* (2012) 209 (2):243–50. doi: 10.1084/jem.20111174
105. Nguyen V, Luzina I, Rus H, Tegla C, Chen C, Rus V. IL-21 Promotes Lupus-Like Disease in Chronic Graft-Versus-Host Disease Through Both CD4 T Cell- and B Cell-Intrinsic Mechanisms. *J Immunol* (2012) 189(2):1081–93. doi: 10.4049/jimmunol.1200318
106. Forcade E, Kim HT, Cutler C, Wang K, Alho AC, Nikiforow S, et al. Circulating T Follicular Helper Cells With Increased Function During Chronic Graft-Versus-Host Disease. *Blood* (2016) 127(20):2489–97. doi: 10.1182/blood-2015-12-688895
107. Kamihara YFE, Koreth J, Liu H, Kubo T, Whangbo J, Hirakawa M, et al. *Blood*. Atlanta, GA: American Society of Hematology (2017).
108. Kumar BV, Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Rep* (2017) 20(12):2921–34. doi: 10.1016/j.celrep.2017.08.078
109. Divito SJ, Aasebo AT, Matos TR, Hsieh PC, Collin M, Elco CP, et al. Peripheral Host T Cells Survive Hematopoietic Stem Cell Transplantation and Promote Graft-Versus-Host Disease. *J Clin Invest* (2020) 130(9):4624–36. doi: 10.1172/JCI129965
110. Strobl J, Pandey RV, Krausgruber T, Bayer N, Kleissl L, Reininger B, et al. Long-Term Skin-Resident Memory T Cells Proliferate *in Situ* and are Involved in Human Graft-Versus-Host Disease. *Sci Transl Med* (2020) 12 (570):eabb7028. doi: 10.1126/scitranslmed.abb7028
111. Kong X, Zeng D, Wu X, Wang B, Yang S, Song Q, et al. Tissue-Resident PSGL1loCD4+ T Cells Promote B Cell Differentiation and Chronic Graft-Versus-Host Disease-Associated Autoimmunity. *J Clin Invest* (2021) 131(1): e135468. doi: 10.1172/JCI135468
112. Tkachev V, Kaminski J, Potter EL, Furlan SN, Yu A, Hunt DJ, et al. Spatiotemporal Single-Cell Profiling Reveals That Invasive and Tissue-Resident Memory Donor CD8(+) T Cells Drive Gastrointestinal Acute Graft-Versus-Host Disease. *Sci Transl Med* (2021) 13(576):eabc0227. doi: 10.1126/scitranslmed.abc0227
113. Nurieva RI, Chung Y. Understanding the Development and Function of T Follicular Helper Cells. *Cell Mol Immunol* (2010) 7(3):190–7. doi: 10.1038/cmi.2010.24
114. Zheng W, Flavell RA. The Transcription Factor GATA-3 is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. *Cell* (1997) 89 (4):587–96. doi: 10.1016/S0092-8674(00)80240-8
115. Croft M, Carter L, Swain SL, Dutton RW. Generation of Polarized Antigen-Specific CD8 Effector Populations: Reciprocal Action of Interleukin (IL)-4 and IL-12 in Promoting Type 2 Versus Type 1 Cytokine Profiles. *J Exp Med* (1994) 180(5):1715–28. doi: 10.1084/jem.180.5.1715
116. Omori M, Yamashita M, Inami M, Ukai-Tadenuma M, Kimura M, Nigo Y, et al. CD8 T Cell-Specific Downregulation of Histone Hyperacetylation and Gene Activation of the IL-4 Gene Locus by ROG, Repressor of GATA. *Immunity* (2003) 19(2):281–94. doi: 10.1016/S1074-7613(03)00210-3
117. Jung U, Foley JE, Erdmann AA, Eckhaus MA, Fowler DH. CD3/CD28-Costimulated T1 and T2 Subsets: Differential *In Vivo* Allosensitization Generates Distinct GVT and GVHD Effects. *Blood* (2003) 102(9):3439–46. doi: 10.1182/blood-2002-12-3936
118. Tawara I, Maeda Y, Sun Y, Lowler KP, Liu C, Toubai T, et al. Combined Th2 Cytokine Deficiency in Donor T Cells Aggravates Experimental Acute Graft-vs-Host Disease. *Exp Hematol* (2008) 36(8):988–96. doi: 10.1016/j.exphem.2008.02.010
119. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, et al. Reciprocal Differentiation and Tissue-Specific Pathogenesis of Th1, Th2, and Th17 Cells in Graft-Versus-Host Disease. *Blood* (2009) 114(14):3101–12. doi: 10.1182/blood-2009-05-219402
120. Henden AS, Hill GR. Cytokines in Graft-Versus-Host Disease. *J Immunol* (2015) 194(10):4604–12. doi: 10.4049/jimmunol.1500117
121. Jung U, Foley JE, Erdmann AA, Toda Y, Borenstein T, Mariotti J, et al. *Ex Vivo* Rapamycin Generates Th1/Tc1 or Th2/Tc2 Effector T Cells With Enhanced *In Vivo* Function and Differential Sensitivity to Post-Transplant Rapamycin Therapy. *Biol Blood Marrow Transplant* (2006) 12(9):905–18. doi: 10.1016/j.bbmt.2006.05.014
122. Mariotti J, Foley J, Ryan K, Buxhoeveden N, Kapoor V, Amarnath S, et al. Graft Rejection as a Th1-Type Process Amenable to Regulation by Donor Th2-Type Cells Through an Interleukin-4/STAT6 Pathway. *Blood* (2008) 112(12):4765–75. doi: 10.1182/blood-2008-05-154278
123. Shin HJ, Baker J, Leveson-Gower DB, Smith AT, Sega EI, Negrin RS. Rapamycin and IL-2 Reduce Lethal Acute Graft-Versus-Host Disease Associated With Increased Expansion of Donor Type CD4+CD25+Foxp3+ Regulatory T Cells. *Blood* (2011) 118(8):2342–50. doi: 10.1182/blood-2010-10-313684
124. Fowler DH, Mossoba ME, Steinberg SM, Halverson DC, Stroncek D, Khuu HM, et al. Phase 2 Clinical Trial of Rapamycin-Resistant Donor CD4+ Th2/Th1 (T-Rapa) Cells After Low-Intensity Allogeneic Hematopoietic Cell Transplantation. *Blood* (2013) 121(15):2864–74. doi: 10.1182/blood-2012-08-446872
125. Chang HC, Zhang S, Thieu VT, Slee RB, Bruns HA, Laribee RN, et al. PU.1 Expression Delineates Heterogeneity in Primary Th2 Cells. *Immunity* (2005) 22(6):693–703. doi: 10.1016/j.immuni.2005.03.016
126. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, et al. Transforming Growth Factor-Beta 'Reprograms' the Differentiation of T Helper 2 Cells and Promotes an Interleukin 9-Producing Subset. *Nat Immunol* (2008) 9(12):1341–6. doi: 10.1038/ni.1659
127. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 Inhibits TGF-Beta-Induced Foxp3+ T Cells and, Together With TGF-Beta, Generates IL-9+ IL-10+ Foxp3(-) Effector T Cells. *Nat Immunol* (2008) 9 (12):1347–55. doi: 10.1038/ni.1677

128. Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The Transcription Factor PU.1 Is Required for the Development of IL-9-Producing T Cells and Allergic Inflammation. *Nat Immunol* (2010) 11 (6):527–34. doi: 10.1038/ni.1867
129. Visekruna A, Ritter J, Scholz T, Campos L, Guralnik A, Poncette L, et al. Tc9 Cells, a New Subset of CD8(+) T Cells, Support Th2-Mediated Airway Inflammation. *Eur J Immunol* (2013) 43(3):606–18. doi: 10.1002/eji.201242825
130. Mangus CW, Massey PR, Fowler DH, Amarnath S. Rapamycin Resistant Murine Th9 Cells Have a Stable *In Vivo* Phenotype and Inhibit Graft-Versus-Host Reactivity. *PloS One* (2013) 8(8):e72305. doi: 10.1371/journal.pone.0072305
131. Lu Y, Hong S, Li H, Park J, Hong B, Wang L, et al. Th9 Cells Promote Antitumor Immune Responses *In Vivo*. *J Clin Invest* (2012) 122(11):4160–71. doi: 10.1172/JCI65459
132. Purwar R, Schlapbach C, Xiao S, Kang HS, Elyaman W, Jiang X, et al. Robust Tumor Immunity to Melanoma Mediated by Interleukin-9-Producing T Cells. *Nat Med* (2012) 18(8):1248–53. doi: 10.1038/nm.2856
133. Griesenauer B, Paczesny S. The ST2/IL-33 Axis in Immune Cells During Inflammatory Diseases. *Front Immunol* (2017) 8:475. doi: 10.3389/fimmu.2017.00475
134. Garlanda C, Dinarello CA, Mantovani A. The Interleukin-1 Family: Back to the Future. *Immunity* (2013) 39(6):1003–18. doi: 10.1016/j.immuni.2013.11.010
135. Vander Lugt MT, Braun TM, Hanash S, Ritz J, Ho VT, Antin JH, et al. ST2 as a Marker for Risk of Therapy-Resistant Graft-Versus-Host Disease and Death. *N Engl J Med* (2013) 369(6):529–39. doi: 10.1056/NEJMoa1213299
136. Zhang J, Ramadan AM, Griesenauer B, Li W, Turner MJ, Liu C, et al. ST2 Blockade Reduces Sst2-Producing T Cells While Maintaining Protective Mst2-Expressing T Cells During Graft-Versus-Host Disease. *Sci Transl Med* (2015) 7(308):308ra160. doi: 10.1126/scitranslmed.aab0166
137. Lohning M, Stroehmann A, Coyle AJ, Grogan JL, Lin S, Gutierrez-Ramos JC, et al. T1/ST2 is Preferentially Expressed on Murine Th2 Cells, Independent of Interleukin 4, Interleukin 5, and Interleukin 10, and Important for Th2 Effector Function. *Proc Natl Acad Sci USA* (1998) 95(12):6930–5. doi: 10.1073/pnas.95.12.6930
138. Xu D, Chan WL, Leung BP, Huang F, Wheeler R, Piedrafita D, et al. Selective Expression of a Stable Cell Surface Molecule on Type 2 But Not Type 1 Helper T Cells. *J Exp Med* (1998) 187(5):787–94. doi: 10.1084/jem.187.5.787
139. Blom L, Poulsen BC, Jensen BM, Hansen A, Poulsen LK. IL-33 Induces IL-9 Production in Human CD4+ T Cells and Basophils. *PloS One* (2011) 6(7): e21695. doi: 10.1371/journal.pone.0021695
140. Ma X, Bi E, Huang C, Lu Y, Xue G, Guo X, et al. Cholesterol Negatively Regulates IL-9-Producing CD8(+) T Cell Differentiation and Antitumor Activity. *J Exp Med* (2018) 215(6):1555–69. doi: 10.1084/jem.20171576
141. Yu J, Storer BE, Kushekhar K, Abu Zaid M, Zhang Q, Gafken PR, et al. Biomarker Panel for Chronic Graft-Versus-Host Disease. *J Clin Oncol Off J Am Soc Clin Oncol* (2016) 34(22):2583–90. doi: 10.1200/JCO.2015.65.9615
142. Zaiss DM, Yang L, Shah PR, Kobie JJ, Urban JF, Mosmann TR. Amphiregulin, a TH2 Cytokine Enhancing Resistance to Nematodes. *Science* (2006) 314(5806):1746. doi: 10.1126/science.1133715
143. Morimoto Y, Hirahara K, Kiuchi M, Wada T, Ichikawa T, Kanno T, et al. Amphiregulin-Producing Pathogenic Memory T Helper 2 Cells Instruct Eosinophils to Secrete Osteopontin and Facilitate Airway Fibrosis. *Immunity* (2018) 49(1):134–50 e6. doi: 10.1016/j.immuni.2018.04.023
144. Arpaia N, Green JA, Moltedo B, Arvey A, Hemmers S, Yuan S, et al. A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* (2015) 162 (5):1078–89. doi: 10.1016/j.cell.2015.08.021
145. Yang J, Ramadan A, Reichenbach DK, Loschi M, Zhang J, Griesenauer B, et al. Rorc Restrains the Potency of ST2+ Regulatory T Cells in Ameliorating Intestinal Graft-Versus-Host Disease. *JCI Insight* (2019) 4(5):e122014. doi: 10.1172/jci.insight.122014
146. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 Receptor Beta-Dependent STAT5 Activation Is Required for the Development of Foxp3+ Regulatory T Cells. *J Immunol* (2007) 178(1):280–90. doi: 10.4049/jimmunol.178.1.280
147. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A Function for Interleukin 2 in Foxp3-Expressing Regulatory T Cells. *Nat Immunol* (2005) 6 (11):1142–51. doi: 10.1038/ni1263
148. Marshall D, Sinclair C, Tung S, Seddon B. Differential Requirement for IL-2 and IL-15 During Bifurcated Development of Thymic Regulatory T Cells. *J Immunol* (2014) 193(11):5525–33. doi: 10.4049/jimmunol.1402144
149. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A Critical Function for TGF-Beta Signaling in the Development of Natural CD4+CD25+Foxp3+ Regulatory T Cells. *Nat Immunol* (2008) 9(6):632–40. doi: 10.1038/ni.1607
150. Zeiser R, Blazar BR. Acute Graft-Versus-Host Disease - Biologic Process, Prevention, and Therapy. *N Engl J Med* (2017) 377(22):2167–79. doi: 10.1056/NEJMra1609337
151. Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR. Absence of Regulatory T-Cell Control of TH1 and TH17 Cells Is Responsible for the Autoimmune-Mediated Pathology in Chronic Graft-Versus-Host Disease. *Blood* (2007) 110(10):3804–13. doi: 10.1182/blood-2007-05-091074
152. Zeng H, Zhang R, Jin B, Chen L. Type 1 Regulatory T Cells: A New Mechanism of Peripheral Immune Tolerance. *Cell Mol Immunol* (2015) 12 (5):566–71. doi: 10.1038/cmi.2015.44
153. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A, et al. Eomesodermin Promotes the Development of Type 1 Regulatory T (TR1) Cells. *Sci Immunol* (2017) 2(10):eaah7152. doi: 10.1126/sciimmunol.aah7152
154. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ Regulatory T Cells Preserve Graft-Versus-Tumor Activity While Inhibiting Graft-Versus-Host Disease After Bone Marrow Transplantation. *Nat Med* (2003) 9(9):1144–50. doi: 10.1038/nm915
155. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-Type Specific CD4+CD25+ Regulatory T Cells Favor Immune Reconstitution and Control Graft-Versus-Host Disease While Maintaining Graft-Versus-Leukemia. *J Clin Invest* (2003) 112(11):1688–96. doi: 10.1172/JCI17702
156. Chopra M, Biehl M, Steinfatt T, Brandl A, Kums J, Amich J, et al. Exogenous TNFR2 Activation Protects From Acute GvHD *via* Host T Reg Cell Expansion. *J Exp Med* (2016) 213(9):1881–900. doi: 10.1084/jem.20151563
157. MacDonald KG, Hoeppli RE, Huang Q, Gillies J, Luciani DS, Orban PC, et al. Alloantigen-Specific Regulatory T Cells Generated With a Chimeric Antigen Receptor. *J Clin Invest* (2016) 126(4):1413–24. doi: 10.1172/JCI82771
158. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high Regulatory Cells in Human Peripheral Blood. *J Immunol* (2001) 167 (3):1245–53. doi: 10.4049/jimmunol.167.3.1245
159. Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, et al. Massive *Ex Vivo* Expansion of Human Natural Regulatory T Cells (T (regs)) With Minimal Loss of *In Vivo* Functional Activity. *Sci Transl Med* (2011) 3(83):83ra41. doi: 10.1126/scitranslmed.3001809
160. June CH, Blazar BR. Clinical Application of Expanded CD4+25+ Cells. *Semin Immunol* (2006) 18(2):78–88. doi: 10.1016/j.smim.2006.01.006
161. Godfrey WR, Ge YG, Spoden DJ, Levine BL, June CH, Blazar BR, et al. *In Vitro*-Expanded Human CD4(+)CD25(+) T-Regulatory Cells can Markedly Inhibit Allogeneic Dendritic Cell-Stimulated MLR Cultures. *Blood* (2004) 104(2):453–61. doi: 10.1182/blood-2004-01-0151
162. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of *Ex Vivo* Expanded T Regulatory Cells in Adults Transplanted With Umbilical Cord Blood: Safety Profile and Detection Kinetics. *Blood* (2011) 117(3):1061–70. doi: 10.1182/blood-2010-07-293795
163. Brunstein CG, Miller JS, McKenna DH, Hippen KL, DeFor TE, Sumstad D, et al. Umbilical Cord Blood-Derived T Regulatory Cells to Prevent GVHD: Kinetics, Toxicity Profile, and Clinical Effect. *Blood* (2016) 127(8):1044–51. doi: 10.1182/blood-2015-06-653667
164. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs Prevent GVHD and Promote Immune Reconstitution in HLA-Haploidentical Transplantation. *Blood* (2011) 117(14):3921–8. doi: 10.1182/blood-2010-10-311894
165. Clark FJ, Gregg R, Piper K, Dunnion D, Freeman L, Griffiths M, et al. Chronic Graft-Versus-Host Disease is Associated With Increased Numbers of Peripheral Blood CD4+CD25high Regulatory T Cells. *Blood* (2004) 103 (6):2410–6. doi: 10.1182/blood-2003-06-2073
166. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, et al. Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated With IL-10-Anergized Donor T Cells. *Front Immunol* (2014) 5:16. doi: 10.3389/fimmu.2014.00016

167. Kennedy GA, Varelias A, Vuckovic S, Le Texier L, Gartlan KH, Zhang P, et al. Addition of Interleukin-6 Inhibition With Tocilizumab to Standard Graft-Versus-Host Disease Prophylaxis After Allogeneic Stem-Cell Transplantation: A Phase 1/2 Trial. *Lancet Oncol* (2014) 15(13):1451–9. doi: 10.1016/S1470-2045(14)71017-4
168. Kennedy GA, Tey SK, Buizen L, Varelias A, Gartlan KH, Curley C, et al. A Phase 3 Double-Blind Study of the Addition of Tocilizumab *vs* Placebo to Cyclosporin/Methotrexate GVHD Prophylaxis. *Blood* (2021) 137(14):1970–9. doi: 10.1182/blood.2020009050
169. van den Brink MR, Burakoff SJ. Cytolytic Pathways in Haematopoietic Stem-Cell Transplantation. *Nat Rev Immunol* (2002) 2(4):273–81. doi: 10.1038/nri775
170. Lu Y, Hong B, Li H, Zheng Y, Zhang M, Wang S, et al. Tumor-Specific IL-9-Producing CD8+ Tc9 Cells are Superior Effector Than Type-I Cytotoxic Tc1 Cells for Adoptive Immunotherapy of Cancers. *Proc Natl Acad Sci USA* (2014) 111(6):2265–70. doi: 10.1073/pnas.1317431111
171. Liu L, Bi E, Ma X, Xiong W, Qian J, Ye L, et al. Enhanced CAR-T Activity Against Established Tumors by Polarizing Human T Cells to Secrete Interleukin-9. *Nat Commun* (2020) 11(1):5902. doi: 10.1038/s41467-020-19672-2
172. Voermans C, Hazenberg MD. Cellular Therapies for Graft-Versus-Host Disease: A Tale of Tissue Repair and Tolerance. *Blood* (2020) 136(4):410–7. doi: 10.1182/blood.2019000951
173. Blazar BR, MacDonald KPA, Hill GR. Immune Regulatory Cell Infusion for Graft-Versus-Host Disease Prevention and Therapy. *Blood* (2018) 131 (24):2651–60. doi: 10.1182/blood-2017-11-785865
174. Weber EW, Maus MV, Mackall CL. The Emerging Landscape of Immune Cell Therapies. *Cell* (2020) 181(1):46–62. doi: 10.1016/j.cell.2020.03.001
175. Elverum K, Whitman M. Delivering Cellular and Gene Therapies to Patients: Solutions for Realizing the Potential of the Next Generation of Medicine. *Gene Ther* (2020) 27(12):537–44. doi: 10.1038/s41434-019-0074-7
176. Shinners NP, Carlesso G, Castro I, Hoek KL, Corn RA, Woodland RT, et al. Bruton's Tyrosine Kinase Mediates NF-Kappa B Activation and B Cell Survival by B Cell-Activating Factor Receptor of the TNF-R Family. *J Immunol* (2007) 179(6):3872–80. doi: 10.4049/jimmunol.179.6.3872
177. Dubovsky JA, Beckwith KA, Natarajan G, Woyach JA, Jaglowski S, Zhong Y, et al. Ibrutinib is an Irreversible Molecular Inhibitor of ITK Driving a Th1-Selective Pressure in T Lymphocytes. *Blood* (2013) 122(15):2539–49. doi: 10.1182/blood-2013-06-507947
178. Dubovsky JA, Flynn R, Du J, Harrington BK, Zhong Y, Kaffenberger B, et al. Ibrutinib Treatment Ameliorates Murine Chronic Graft-Versus-Host Disease. *J Clin Invest* (2014) 124(11):4867–76. doi: 10.1172/JCI75328
179. Miklos D, Cutler CS, Arora M, Waller EK, Jagasia M, Pusic I, et al. Ibrutinib for Chronic Graft-Versus-Host Disease After Failure of Prior Therapy. *Blood* (2017) 130(21):2243–50. doi: 10.1182/blood-2017-07-793786
180. Yamaoka K, Saharinen P, Pesu M, Holt VE 3rd, Silvennoinen O, O'Shea JJ. The Janus Kinases (Jaks). *Genome Biol* (2004) 5(12):253. doi: 10.1186/gb-2004-5-12-253
181. Spoerl S, Mathew NR, Bscheider M, Schmitt-Graeff A, Chen S, Mueller T, et al. Activity of Therapeutic JAK 1/2 Blockade in Graft-Versus-Host Disease. *Blood* (2014) 123(24):3832–42. doi: 10.1182/blood-2013-12-543736
182. Heine A, Held SA, Daecke SN, Wallner S, Yajnanarayana SP, Kurts C, et al. The JAK-Inhibitor Ruxolitinib Impairs Dendritic Cell Function *In Vitro* and *In Vivo*. *Blood* (2013) 122(7):1192–202. doi: 10.1182/blood-2013-03-484642
183. Betts BC, Abdel-Wahab O, Curran SA, St Angelo ET, Koppikar P, Heller G, et al. Janus Kinase-2 Inhibition Induces Durable Tolerance to Alloantigen by Human Dendritic Cell-Stimulated T Cells Yet Preserves Immunity to Recall Antigen. *Blood* (2011) 118(19):5330–9. doi: 10.1182/blood-2011-06-363408
184. Choi J, Ziga ED, Ritchey J, Collins L, Prior JL, Cooper ML, et al. IFNgammaR Signaling Mediates Alloreactive T-Cell Trafficking and GVHD. *Blood* (2012) 120(19):4093–103. doi: 10.1182/blood-2012-01-403196
185. Choi J, Cooper ML, Alahmari B, Ritchey J, Collins L, Holt M, et al. Pharmacologic Blockade of JAK1/JAK2 Reduces GvHD and Preserves the Graft-Versus-Leukemia Effect. *PloS One* (2014) 9(10):e109799. doi: 10.1371/journal.pone.0109799
186. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in Corticosteroid-Refractory Graft-Versus-Host Disease After Allogeneic Stem Cell Transplantation: A Multicenter Survey. *Leukemia* (2015) 29(10):2062–8. doi: 10.1038/leu.2015.212
187. Schroeder MA, Khoury HJ, Jagasia M, Ali H, Schiller GJ, Staser K, et al. A Phase 1 Trial of Itacitinib, a Selective JAK1 Inhibitor, in Patients With Acute Graft-Versus-Host Disease. *Blood Adv* (2020) 4(8):1656–69. doi: 10.1182/bloodadvances.2019001043
188. Chen YB, McDonough S, Hasserjian R, Chen H, Coughlin E, Illiano C, et al. Expression of CD30 in Patients With Acute Graft-Versus-Host Disease. *Blood* (2012) 120(3):691–6. doi: 10.1182/blood-2012-03-415422
189. Chen YB, Perales MA, Li S, Kempner M, Reynolds C, Brown J, et al. Phase 1 Multicenter Trial of Brentuximab Vedotin for Steroid-Refractory Acute Graft-Versus-Host Disease. *Blood* (2017) 129(24):3256–61. doi: 10.1182/blood-2017-03-772210
190. Schwab N, Schneider-Hohendorf T, Melzer N, Cutter G, Wiendl H. Natalizumab-Associated PML: Challenges With Incidence, Resulting Risk, and Risk Stratification. *Neurology* (2017) 88(12):1197–205. doi: 10.1212/WNL.0000000000003739
191. Lam MC, Bressler B. Vedolizumab for Ulcerative Colitis and Crohn's Disease: Results and Implications of GEMINI Studies. *Immunotherapy* (2014) 6(9):963–71. doi: 10.2217/imt.14.66
192. Milch C, Wyant T, Xu J, Parikh A, Kent W, Fox I, et al. Vedolizumab, a Monoclonal Antibody to the Gut Homing Alpha4beta7 Integrin, Does Not Affect Cerebrospinal Fluid T-Lymphocyte Immunophenotype. *J Neuroimmunol* (2013) 264(1-2):123–6. doi: 10.1016/j.jneuroim.2013.08.011
193. Soler D, Chapman T, Yang LL, Wyant T, Egan R, Fedyk ER. The Binding Specificity and Selective Antagonism of Vedolizumab, an Anti-Alpha4beta7 Integrin Therapeutic Antibody in Development for Inflammatory Bowel Diseases. *J Pharmacol Exp Ther* (2009) 330(3):864–75. doi: 10.1124/jpet.109.153973
194. Floisand Y, Lundin KEA, Lazarevic V, Kristiansen JD, Osnes LTN, Tjonnfjord GE, et al. Targeting Integrin Alpha4beta7 in Steroid-Refractory Intestinal Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2017) 23(1):172–5. doi: 10.1016/j.bbmt.2016.10.009

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Acute Graft-*Versus*-Host Disease, Infections, Vascular Events and Drug Toxicities Affecting the Central Nervous System

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Allogeneic hematopoietic cell transplantation (allo-HCT) is a curative therapy for patients with hematological malignancies. Acute Graft *versus* host diseases (GVHD) is a major immune mediated side effect of allo-HCT that can affect the central nervous system (CNS) in addition to post-allo-HCT vascular events, drug toxicity or infections. Here we summarize and discuss recent preclinical data on the CNS as a target of acute GVHD and the known mechanisms contributing to neurotoxicity with a focus on microglia and T cells. We also discuss open questions in the field and place the findings made in mouse models in a clinical context. While in mice the neurological deficits can be assessed in a controlled fashion, in patients the etiology of the CNS damage is difficult to attribute to acute GVHD *versus* infections, vascular events, and drug-induced toxicity. Ultimately, we discuss novel therapies for GVHD of the CNS. Our understanding of the biological mechanisms that lead to neurotoxicity after allo-HCT increased over the last decade. This review provides insights into CNS manifestations of GVHD *versus* other etiologies of CNS damage in mice and patients.

Keywords: GvHD, central nervous system, inflammation, drug toxicity, microglia, T cells

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INTRODUCTION

Acute graft-*versus*-host disease (GVHD) is a life-threatening complication after allogeneic hematopoietic cell transplantation (allo-HCT). About 50% of the patients with severe acute GVHD fail to respond to corticosteroids, and steroid-refractory severe GVHD has a dismal prognosis with a 1-year survival rate of less than 20% (1). GVHD was classically considered to involve the skin, intestinal tract and liver, which was termed as "tissue tropism of acute GVHD". The target organs of acute GVHD are affected by commensal bacteria that populate these locations and that may migrate through damaged epithelial barriers (2) and activate intestinal epithelium (3), neutrophils (4, 5), dendritic cells, macrophages and monocytes (6). The observation that non-sterile triggers of tissue damage such as ATP (7, 8) or uric acid (9) may contribute to GVHD support the concept that also other organs with less commensal bacteria can be affected by GVHD. There is

increasing evidence that the effects of acute GVHD are not limited to the three classical target organs, but can also occur in the central nervous system (CNS). Neurological complications were reported in 10% of the patients undergoing autologous (auto)-HCT while over 80% of allo-HCT patients experienced neurological complications at some time point (10–12) which indicates that not only the toxicity but also the allo-reactive effect of the donor immune system may contribute to neurological complications. Clinical manifestations of CNS-GVHD include seizures, reduced vision and cognitive impairment. The symptoms can resemble for example multiple sclerosis or Guillain-Barre syndrome. Risk factors for neurological complications during acute GVHD are diverse. Female gender, high doses of total body irradiation (TBI), myeloablative high dose chemotherapy-based conditioning, infections and preexisting cerebrovascular disorders are major risk factors for the development of neurological complications after allo-HCT (13–15). CNS-GVHD though considered a rare entity, significantly affects the mortality and quality of life in allo-HCT patients (13). In this review, we provide an overview on the cell types affected by CNS-GVHD and we discuss the diverse clinical manifestations of the disease as well as infections, vascular events and drug toxicities affecting the CNS.

STUDIES ON CNS-GVHD IN PRECLINICAL MODELS

Preclinical studies using mouse models of acute GVHD showed that the transfer of allogeneic T cells caused CNS infiltration by effector memory T cells (16). The allogeneic T cells infiltrated different regions of the CNS including the meninges, vasculature and parenchyma while a comparable T cell infiltration was not observed when only syngeneic T cells were transferred (16). Evidence for CNS-GVHD was not restricted to the murine model, as other investigators reported that CNS infiltration by $CD8^+$ T cells was a key feature of GVHD in non-human primates (17). Conversely, treatment of primates with immune-prophylaxis after allo-HCT reduced the abundance of T cell infiltration into the brain (17). These findings indicate that the donor T cells manage to infiltrate the CNS despite its anatomical location and immune privilege. Therefore, immune responses may evolve differently from peripheral tissues. This infiltration by T cells is likely due to disruption of the blood-brain-barrier, which under normal conditions controls the influx of immune cells into the CNS.

Though T cells play a central role for the induction of acute GVHD, other cell types also contribute to the disease. Studies reported an increase in the expression of MHC class I and II molecules in the CNS in a rat model of GVHD. Immunohistological studies revealed increased expression of host MHC in parenchymal and vascular regions along with increased infiltration of T cells (18). In line with the findings, a fivefold increase in the MHC-II expression was observed in a $CD45^{lo}CD11b^+$ microglial population which further re-iterates the involvement of microglia in CNS-GVHD pathogenesis (19). Microglial activation was not only observed in inflammatory disease of the CNS but also in several neurodegenerative diseases including Parkinsons disease and Alzheimers disease (20). Host derived IL-6 and Indoleamine 2,3 Dioxygenase-1 (IDO-1) were shown to regulate the behavior patterns and inflammation in the CNS during acute GVHD (21). Microglia and macrophages were activated and increased the production of IDO-1 which thereby resulted in behavioral deficits in a murine model of GVHD (22). Interestingly, IL-6R inhibitor treated mice had decreased infiltration of CD4 and CD8 T cells and reduced production of pro-inflammatory cytokines in CNS. Recent clinical studies showed that downstream signaling of IL-6R *via* JAK2/STAT reduced acute and chronic GVHD in patients (23–25). We have previously shown that microglia plays a central role in acute GVHD-induced neurotoxicity (26). Acute GVHD caused an amoeboid phenotype of microglia with reduced branching points and dendrites when compared to the syngeneic HCT controls in a murine model of GVHD (**Figures 1A, B**). Microglia cells that were activated during acute GVHD exhibited increased TNF expression and activated the downstream TAK/MAPK signaling. Therapeutic inhibition of TAK1 signaling by takinib reversed the microglial activation and T cell infiltration (26). Additionally the GVHD induced neurocognitive defects reduced in mice treated with takinib, suggesting a novel potential therapeutic avenue for acute GVHD of the CNS.

Consistent with the neurocognitive defects observed in mice developing GVHD, neuronal damage in the CNS was reported (16). Allogeneic T cells infiltrating the CNS induced apoptosis of neurons and neuroglia, which limited the cognitive and exploratory function in a murine model of GVHD (16). In line with the findings, an increase in the expression of c-fos was noted in several cortical regions including occipital and olfactory regions in a rat GVHD model (27). In contrast, such inflammatory effects were not observed upon transfer of syngeneic T cells (27).

Multiple effects involving endothelial damage, T cell transmigration, cytokine production and ultimately neuronal damage are involved in CNS-GVHD (**Figure 2**).

HUMAN STUDIES ON CNS-GVHD

Consistent with findings in preclinical models, human brain analysis of female sex-mismatched bone marrow transplant recipients have identified donor ($Y\text{-chromosome}^+$) derived cell infiltrates (28). In addition to this, lymphocytosis was noticed in CSF together with encephalitis with increased infiltration of T cells and gliosis with no signs of infection further confirming the occurrence of CNS-GVHD (29, 30).

Neurological deficits and MRI findings have been reported in patients developing GVHD (31).

The clinical picture of acute GVHD is often connected to neurological deficits in patients, morphological CNS white matter changes detectable by magnetic resonance imaging and intraparenchymal lymphocytic infiltration of the brain upon autopsy (31, 32). In line with the findings, studies also reported

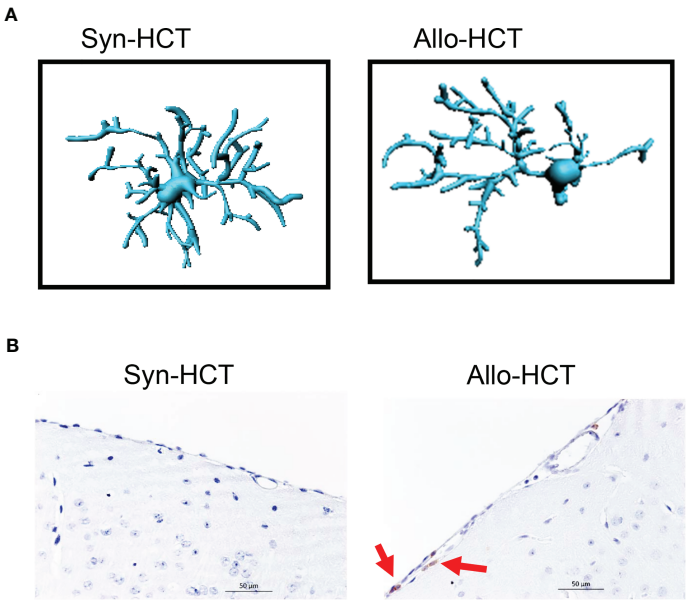

FIGURE 1 | **(A)** Microglia morphology in the CNS of mice undergoing syn-HCT or allo-HCT as previously reported (26). **(B)** Infiltration of T cells (brown) in the CNS of mice undergoing syn-HCT or allo-HCT.

neurological deficits including drowsiness, dysphoria, right dazedness and MRI findings of abnormal cerebra gyrus swelling, corpus signal, diffused white matter regions (33). Biopsy studies on GVHD brains showed axonal depletion representative of demyelination disease in a patient. CNS-GVHD is quite heterogeneous and case dependent with patients most frequently reported with delusion, hemiparesis, temporary unconsciousness and psychomotor agitation with neither T cell infiltration to the CNS nor relapse of malignancy (14, 34). On contrary, some patients also developed metabolic encephalopathy with neurological deficits ranging from vision loss, confusion to coma and death (15).

Autopsy studies revealed an increase of Iba-1$^+$ myeloid cells in the CNS of patients with GVHD when compared to the allo-HCT patients without GVHD. In addition to this the microglia from CNS-GVHD patients had increased expression of TNF (26).

Due to the rarity of CNS-GVHD and the difficulty to distinguish the disease from other mediators of CNS toxicity, biomarkers to identify CNS-GVHD would be highly desirable. IgG index in the CSF is an indicator of neurological disorders like multiple sclerosis, intrathecal inflammation (35, 36). Another study indicated that Blood Brain Barrier (BBB) impermeability, IgG –Synthesis index are early indicators of CNS demyelination (37). In addition to this, increased BBB permeability, elevated myelin basic protein in blood and CSF are some of the immune markers that could be tested for their validity as biomarkers for CNS-GVHD (36). Identifying the immune biomarkers that predict damage to neurons, glial cells and myelin membranes may help diagnose CNS-GVHD. Patients with CNS-GVHD were reported to respond to high dose corticosteroids, intravenous immunoglobulin treatments, immunosuppressive medications including methotrexate and etoposide (38). Chronic CNS-GVHD is a late complication of allo-HCT and clinical manifestations may include myasthenia gravis, myositis, demyelination, angiitis (39, 40). Patients can also present with stroke-like episodes, lacunar syndromes, multiple sclerosis-like presentations or encephalitis (30). The diagnosis of chronic CNS-GVHD is often challenging (41). The NIH Consensus Conference on criteria for clinical trials in chronic GVHD delineated three types of chronic CNS-GvHD: cerebrovascular disease, CNS demyelinating disease, and immune-mediated encephalitis (41). The NIH consensus on criteria for clinical trials in chronic GVHD recommended that the diagnosis of chronic CNS-GVHD should be made only when other organs are affected by GVHD and other neurological differential diagnoses are excluded (41). Differential diagnoses of chronic CNS-GVHD include in particular drug-induced toxicities or opportunistic infections.

NON-GVHD RELATED CAUSES FOR NEUROLOGICAL SYMPTOMS AFTER ALLO-HCT

Neurological complications after allo-HCT can have multiple etiologies such as infections, vascular events and drug-induced toxicities.

After allo-HCT, patients are immunodeficient and therefore highly susceptible to a variety of opportunistic infections caused

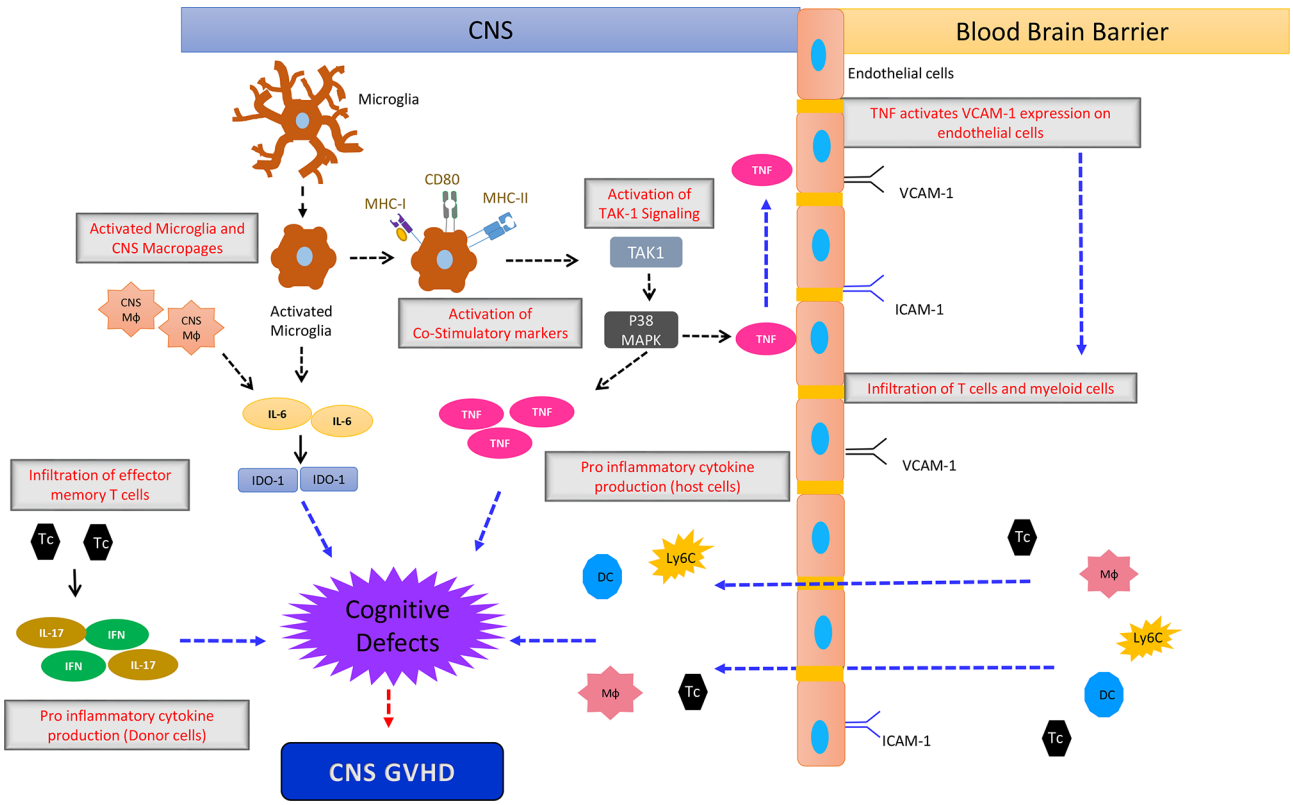

FIGURE 2 | The simplified sketch shows the proposed mechanism how CNS-GVHD evolves and contributes to neuronal damage ultimately leading to cognitive deficits. An initial event is the activation of microglia by stimuli that are not well characterized so far, being most likely damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). Activated microglia upregulates MHC I and II as well as CD80 leading to increased T cell priming. Additionally microglia- and macrophage-derived IL-6 impacting IDO-1 induces neurological defects, leading to the clinical picture of CNS GVHD. TNF derived from microglia has direct neurological toxicity. Donor T cells polarized towards Th1 and Th17 contribute to CNS GVHD as well as macrophages, monocytes and DC from the periphery. Mϕ, Macrophage; DC, Dendritic Cells; Tc, T cells; Ly6c$^+$ cells, Monocytes.

by either bacteria, fungi or viruses, which can also affect the CNS. Acute GVHD further increases the risk of opportunistic infections, which lead to neurological complications in some patients (42). CSF analysis of patients undergoing allo-HCT revealed the presence of cytomegalovirus (CMV), Epstein Bar (EBV), Human Herpes virus-6 (HHV-6), HHV-8, toxoplasma infections among others (43). Diffuse microglial hyperplasia and microglial nodular encephalopathy were reported in some patients, which indicates microglial activation in response to infectious complications during GVHD (15). Meningoencephalitis induced by Aspergillus species was observed in children and adults undergoing allo-HCT with an overall incidence rate of up to 30% (15, 44). Cerebral aspergillus infections can cause stroke like manifestations with focal deficits (45). Infections related to candida species were reported in allo-HCT patients with neurological complications ranging from vasculitis to hemorrhagic abscess (46). Bacterial infections also account for major neurological complications after allo-HCT, e.g. CNS infections with streptococcus and staphylococcus (15). Klebsiella, E coli and Listeria monocytogenes were reported to cause meningitis and brain stem encephalitis in allo-HCT patients. Toxoplasma gondii encephalitis is a rare infection after allo-HCT, mostly reported in countries with high prevalence rates of the toxoplasma (47, 48). Neurotoxoplasmosis is characterized by the presence of grey and white matter abscesses and can be diagnosed by CT or MRI scans (49). Patients undergoing allo-HCT are exposed to a variety of viruses that lead to viral encephalitis further governing the mortality and morbidity rates. HHV-6, EBV, Herpes simplex virus, CMV, John Cunningham (JC) virus, varicella zoster virus, and adenovirus are the commonly reported viral infections leading to neurological complications in GVHD patients. Progressive multifocal leukoencephalopathy is a progressive demyelinating disorder caused by JC virus primarily affecting oligodendrocytes in response to monoclonal antibodies (50). Restoration of anti-viral immune responses is the only available option for treating JC virus related infections, although tapering the immunosuppression was unsuccessful in reversing the neurological deficits in a fraction of patients (51). In addition to this, a positive correlation between CD8$^+$ T cells in the CNS and JC virus infected glial cells was reported (52). HHV-6 induced encephalitis is a serious complication observed mostly within

24 days of allo-HCT (53, 54). Patients with high levels of HHV-6 DNA in their plasma are at an increased risk of developing neurological deficits that include epilepsy, delirium, and cognitive impairment (53, 55). Pediatric patients receiving haploidentical CD45RA T cell depleted grafts presented with a high rate of HHV-6 induced encephalitis (56). Similarly, HSV related infections can affect the CNS (57). Unlike HSV, VZV infection typically affects multiple region in the CNS and the common manifestations include myelitis and vascular encephalitis. Post-transplant lymphoproliferative diseases driven by oncogenic EBV pose considerably high risks post allo-HCT (58). The infections caused by EBV are early onset and mostly donor derived and risk factors include intensity of immunosuppression and high-grade GVHD (59). The manifestations are very similar to CNS lymphomas with hypercellularity, necrosis and hemorrhages (60). CMV infections in either lungs or CNS are often associated with extremely high mortality rates in allo-HCT patients. CMV infection of the CNS is typically a late onset disease and is associated with encephalitis or polyradiculopathy (61). Umbilical cord transplantation and prolonged T cell depletion are the major risk factors associated with CMV encephalitis (62). Histological manifestations of the CMV include viral inclusion bodies in the CNS commonly referred as owls eye inclusions (63). In some patients the viral load of CMV in the CSF was higher than in the peripheral blood indicating the significance of monitoring the CMV copy levels in the CSF when CNS involved by CMV reactivation is suspected (63, 64). Allogeneic virus-specific T cells were shown to be effective against CMV and EBV (65–67) and could be used to treat neurological symptoms caused by virus infections. This strategy will be most relevant for allo-HCT patients with drug-refractory CMV infection that lack virus-specific T cells. A recent trial using stem cell-donor- or third-party-donor-derived CMV-specific T cells for the treatment of persistent CMV infections after allo-HSCT reported complete and partial virological response rates in 62.5% and 25%, respectively (68).

Vascular complications including subarachnoid, subdural, intraparenchymal and intraventricular hemorrhages were identified by autopsy studies in the CNS of allo-HCT patients (15, 69). Low platelet counts, an altered coagulation and pre-existing vascular events are risk factors contributing to hemorrhage and thrombosis post allo-HCT (70). Microvascular injury and endothelial damage leading to increased microvascular permeability were caused by calcineurin inhibitors in patients undergoing allo-HCT (71).

Medications given pre- and post-transplant also contribute to neurological deficits in patients undergoing allo-HCT. In order to suppress the immune system of the patient and to eliminate cancer cells, patients receive conditioning therapy. The type of conditioning regimen mainly depends on the underlying disease, comorbidities and the age of the patient. Conditioning regimens can include combinations of high dose TBI with cyclophosmide and cytarabine. Reduced intensity conditioning regimen (RIC) often consist of fluradabine and busulphan and minimum dose conditioning regimens use low dose TBI and busulphan (13, 72). Cyclophosphamide induces neurotoxicity by generating reactive oxygen species which further impairs the motor coordination, learning and memory in rats (73). Busulphan, an alkylating agent, is widely used for conditioning prior to allo-HCT. Busulphan penetrates the CNS as shown by active CSF drug levels and severe CNS toxicity was observed in patients treated with this agent (74). Around 2% of the allo-HCT patients treated with busulphan were reported to develop tonic clonic seizures (75, 76). A case study reported disturbances in electroenchephalography (EEG) which lasted for about 20 days upon busulphan and cyclophosphamide treatment (77). Phenytoin is effective at preventing busulphan induced seizures (78). Chemotherapy induced toxic leukoencephalopathy has an unfavorable prognosis (79). Autopsies of patients with leukoencephalopathy revealed activation of astrocytes, infiltration of activated macrophages and a decrease in microglia expressing TMEM119 along with gliosis, demyelination in white matter (80).

In addition to neurotoxicity caused by the conditioning regimen, the GVHD prophylaxis or treatment, anti-viral drugs, antibiotics and anti-fungal agents can cause toxicity to the CNS. The calcineurin inhibitors cyclosporine A (CSA) and tacrolimus are widely used for GVHD prophylaxis as they block T cell activity (81). However the expression of calcineurin is not limited to lymphocytes, but it is also expressed by CNS cells, particularly in the hippocampus (82). In the CNS calcineurin controls the function of neurons and its blockade affects the CNS function (83). Visual disturbances, increase in the occipital lobe density, cortical abnormalities, seizures, posterior reversible encephalopathy syndrome (PRES), hallucinations, motor weakness are some of the most commonly reported side effects of CSA experienced by 10-28% of the treated patients (84–87). In line with the reports, CSA treated mixed glial cultures induced cell death of neurons and oligodendrocytes indicating drug toxicity (88). While most of the side effects induced by CSA are reversible, some reports indicate that cyclosporine induced neurotoxicity might result in long-term toxicity with permanent cortical blindness (89).The mechanism of action of tacrolimus is quite similar to CSA, while some reports suggest that CSA caused milder symptoms of neurotoxicity (50). Tacrolimus induced PRES was reported in children undergoing allo-HCT for hemoglobinopathies (90–92). Recently the JAK-1 and JAK-2 inhibitor ruxolitinib has shown activity for the treatment of corticosteroid-refractory acute and chronic GVHD (23–25). A major side effects is thrombocytopenia, which may increase the risk of cerebral hemorrhage after allo-HCT.

Antimicrobials or anti infectious drugs employed in the treatment of opportunistic infections during GVHD also pose significant threat to the CNS. Neutropenia together with encephalitis induced stroke, and vertigo are the major side effects of medications including acyclovir, gancyclovir (49). In addition, thrombocytopenia induces vascular complications ranging from subdural hematoma, hemorrhages and infarct along with increased infection rate in patients post allo-HCT (49). Amphotericin B triggers confusion, Parkinsonism, visual changes and encephalopathies in some cases (49, 93). Cefepime induced seizures, encephalopathy and myoclonus were noted in some studies (49).

In aggregate, a plethora of infections, vascular events, and drug-induced toxicities can cause neurogical symptoms that need to be ruled out before diagnosing CNS-GVHD.

DIAGNOSTIC PROCEDURES THAT SHOULD BE PERFORMED IN CASE OF CNS SYMPTOMS

The NIH Consensus Conference on criteria for clinical trials in GVHD recommends the following measures in patients with suspected CNS-GVHD (41): CSF cell count, serology, culture and polymerase chain reaction for viral, bacterial or fungal DNA. Imaging should include MRI of the CNS. MRI and CSF analysis will reveal the underlying disease of the neurological symptoms in the majority of cases. CNS-GVHD is an exclusion diagnosis meaning that other causes should be excluded before immunosuppressive therapy is started. The presence of other GVHD manifestations make the diagnosis of CNS-GVHD more likely. To exclude more rare causes for neurological symptoms such as post-transplant acute limbic encephalitis in patients with anterograde amnesia, inappropriate antidiuretic hormone secretion and EEG abnormalities, it is recommended to determine HHV-6 reactivation in the CSF and perform MRI of the brain (41). In case that clinical presentation and MRI suggest an infection, but serology and PCR from CSF remain negative a biopsy of the lesion is recommended (41). In particular when chronic fungal and viral infections as well as progressive multifocal leukoencephalopathy are suspected (94). Also if relapse of the hematological malignancy in the CNS is clinically suspected a biopsy can be considered if the CSF analysis was not conclusive.

SUMMARY

Despite recent advances in the clinical management of acute GVHD, CNS-GVHD is still a life threatening complication that is often difficult to diagnose. Preclinical studies have shown that allogeneic T cells infiltrate the CNS during GVHD and activate different cell types including microglia and other myeloid cells. CNS-GVHD causes damage to neurons and endothelial cells. While CNS-GVHD accounts for some of the neurological symptoms observed after allo-HCT it is important to also consider infections, vascular events, and drug-induced toxicity. Treatment of these complications e.g. reducing CSA when CSA induced neurotoxicity is suspected could exacerbate CNS-GVHD. In case of drug toxicities the responsible drugs should be changed and avoided if CNS symptoms are severe. Therefore, to improve patient outcome it is desirable to identify biomarkers that help early identification and diagnosis of CNS-GVHD in particular when other organs are not affected by GVHD.

AUTHOR CONTRIBUTIONS

JV and RZ developed the overall concept of this article and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Zeiser R, Blazar BR. Acute Graft-Versus-Host Disease - Biologic Process, Prevention, and Therapy. *N Eng J Med* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
2. Hülsdünker J, Thomas OS, Haring E, Unger S, Gonzalo Núñez N, Tugues S, et al. Immunization Against Poly-N-Acetylglucosamine Reduces Neutrophil Activation and GVHD While Sparing Microbial Diversity. *Proc Natl Acad Sci USA* (2019) 116:20700–6. doi: 10.1073/pnas.1908549116
3. Koyama M, Mukhopadhyay P, Schuster IS, Henden AS, Hülsdünker J, Varelias A, et al. MHC Class II Antigen Presentation by the Intestinal Epithelium Initiates Graft-Versus-Host Disease and Is Influenced by the Microbiota. *Immunity* (2019) 51:885–98. doi: 10.1016/j.immuni.2019.08.011
4. Hülsdünker J, Ottmüller KJ, Neeff H, Koyama M, Gao Z, Thomas OS, et al. Neutrophils Provide Cellular Communication Between Ileum and Mesenteric Lymph Nodes at Graft-Versus-Host Disease Onset. *Blood* (2018) 131:1858–69. doi: 10.1182/blood-2017-10-812891
5. Schwab L, Goroncy L, Palaniyandi S, Gautam S, Triantafyllopoulou A, Mocsai A, et al. Neutrophil Granulocytes Recruited Upon Translocation of Intestinal Bacteria Enhance GvHD via Tissue Damage. *Nat Med* (2014) 20:648–54. doi: 10.1038/nm.3517
6. Klämbt V, Wohlfeil SA, Schwab L, Hülsdünker J, Ayata K, Apostolova P, et al. A Novel Function for P2Y2 in Myeloid Recipient-Derived Cells During GvHD. *J Immunol* (2015) 195:5795–804. doi: 10.4049/jimmunol.1501357
7. Wilhelm K, Ganesan J, Müller T, Dürr C, Grimm M, Beilhack A, et al. Graft-Versus-Host Disease Enhanced by Extracellular Adenosine Triphosphate Activating P2X7R. *Nat Med* (2010) 12:1434–8. doi: 10.1038/nm.2242
8. Zeiser R, Penack O, Holler E, Idzko M. Danger Signals Activating Innate Immunity in Graft-Versus-Host Disease. *J Mol Med* (2011) 89:833–45. doi: 10.1007/s00109-011-0767-x
9. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber F, Guarda G, et al. The Nlrp3-Inflammasome Regulates Acute Graft-Versus-Host Disease. *J Exp Med* (2013) 210:1899–910. doi: 10.1084/jem.20130084
10. Sheikh MA, Toledano M, Ahmed S, Gul Z, Hashmi SK. Noninfectious Neurologic Complications of Hematopoietic Cell Transplantation: A Systematic Review. *Hematol/Oncol Stem Cell Ther* (2021) 14(2):87–94. doi: 10.1016/j.hemonc.2020.05.006
11. Das J, Gill A, Lo C, Chan-Lam N, Price S, Wharton SB, et al. A Case of Multiple Sclerosis—Like Relapsing Remitting Encephalomyelitis Following Allogeneic Hematopoietic Stem Cell Transplantation and a Review of the Published Literature. *Front Immunol* (2020) 11:668. doi: 10.3389/fimmu.2020.00668
12. Ke P, Bao X, Zhou J, Zhu Q, Zhuang J, Hu X, et al. Central Nervous System Complications After Allogeneic Hematopoietic Stem Cell Transplantation in Children. *Acta Haematol* (2019) 142(4):217–23. doi: 10.1159/000499651
13. Siegal D, Keller A, Xu W, Bhuta S, Kim DH, Kuruvilla J, et al. Central Nervous System Complications After Allogeneic Hematopoietic Stem Cell Transplantation: Incidence, Manifestations, and Clinical Significance.

Biol Blood Marrow Transplant (2007) 13(11):1369–79. doi: 10.1016/j.bbmt.2007.07.013
14. Barba P, Piñana JL, Valcárcel D, Querol L, Martino R, Sureda A, et al. Early and Late Neurological Complications After Reduced-Intensity Conditioning Allogeneic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2009) 15(11):1439–46. doi: 10.1016/j.bbmt.2009.07.013
15. Bleggi-Torres L, De Medeiros B, Werner B, Neto J, Loddo G, Pasquini R, et al. Neuropathological Findings After Bone Marrow Transplantation: An Autopsy Study of 180 Cases. *Bone Marrow Transplant* (2000) 25(3):301–7. doi: 10.1038/sj.bmt.1702140
16. Hartrampf S, Dudakov JA, Johnson LK, Smith OM, Tsai J, Singer NV, et al. The Central Nervous System is a Target of Acute Graft Versus Host Disease in Mice. *Blood* (2013) 121:1906–10. doi: 10.1182/blood-2012-09-456590
17. Kaliyaperumal S, Watkins B, Sharma P, Furlan S, Ramakrishnan S, Giver C, et al. CD8-Predominant T-Cell CNS Infiltration Accompanies GVHD in Primates and Is Improved With Immunoprophylaxis. *Blood* (2014) 123:1967–9. doi: 10.1182/blood-2014-01-547612
18. Hickey WF, Kimura H. Graft-Vs.-Host Disease Elicits Expression of Class I and Class II Histocompatibility Antigens and the Presence of Scattered T Lymphocytes in Rat Central Nervous System. *Proc Natl Acad Sci* (1987) 84 (7):2082–6. doi: 10.1073/pnas.84.7.2082
19. Sedgwick JD, Ford AL, Foulcher E, Airriess R. Central Nervous System Microglial Cell Activation and Proliferation Follows Direct Interaction With Tissue-Infiltrating T Cell Blasts. *J Immunol* (1998) 160(11):5320–30.
20. Bachiller S, Jiménez-Ferrer I, Paulus A, Yang Y, Swanberg M, Deierborg T, et al. Microglia in Neurological Diseases: A Road Map to Brain-Disease Dependent-Inflammatory Response. *Front Cell Neurosci* (2018) 12:488. doi: 10.3389/fncel.2018.00488
21. Belle L, Koester E, Hansen E, Lawlor M, Hillard C, Drobyski WR. Host Interleukin 6 and Indoleamine 2,3 Dioxygenase Regulate Inflammation in the Brain During Graft Versus Host Disease. *Blood* (2016) 128(22):2145–5. doi: 10.1182/blood.v128.22.2145.2145
22. Belle L, Zhou V, Stuhr KL, Beatka M, Siebers EM, Knight JM, et al. Host Interleukin 6 Production Regulates Inflammation But Not Tryptophan Metabolism in the Brain During Murine GVHD. *JCI Insight* (2017) 2(14): e93726. doi: 10.1172/jci.insight.93726
23. Zeiser R, Polverelli N, Ram R, Hashmi SK, Chakraverty R, Middeke JM, et al. Ruxolitinib for Glucocorticoid-Refractory Chronic Graft-Versus-Host Disease. *N Eng J Med* (2021) 385:228–38. doi: 10.1056/NEJMoa2033122
24. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *N Eng J Med* (2020) 382:1800–10. doi: 10.1056/NEJMoa1917635
25. Zeiser R, Burchert A, Lengerke C, Verbeek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in Corticosteroid-Refractory Graft-Versus-Host Disease After Allogeneic Stem Cell Transplantation: A Multi-Center Survey. *Leukemia* (2015) 29:2062–8. doi: 10.1038/leu.2015.212
26. Mathew NR, Vinnakota JM, Apostolova P, Erny D, Hamarsheh S, Andrieux G, et al. Graft-Versus-Host Disease of the CNS Is Mediated by TNF Upregulation in Microglia. *J Clin Invest* (2020) 130:1315–29. doi: 10.1172/JCI130272
27. Furukawa H, Yamashita A, del Rey A, Besedovsky H. C-Fos Expression in the Rat Cerebral Cortex During Systemic GvH Reaction. *Neuroimmunomodulation* (2004) 11(6):425–33. doi: 10.1159/000080154
28. Unger ER, Sung JH, Manivel JC, Chenggis ML, Blazar BR, Krivit W, et al. Male Donor-Derived Cells in the Brains of Female Sexmismatched Bone Marrow Transplant Recipients: A Y-Chromosome Specific in Situ Hybridization Study. *J Neuropathol Exp Neurol* (1993) 52:460–70. doi: 10.1097/00005072-199309000-00004
29. Mariotti J, Penack O, Castagna L. Acute Graft-Versus-Host-Disease Other Than Typical Targets: Between Myths and Facts. *Transplant Cell Ther* (2021) 27(2):115–24. doi: 10.1016/j.bbmt.2020.09.033
30. Ruggiu M, Cuccuini W, Mokhtari K, Meignin V, Peffault de Latour R, Robin M, et al. Case Report: Central Nervous System Involvement of Human Graft Versus Host Disease: Report of 7 Cases and a Review of Literature. *Medicine* (2017) 96(42):e8303–3. doi: 10.1097/MD.0000000000008303
31. Shortt J, Hutton E, Faragher M, Spencer A. Central Nervous System Graft-Versus-Host Disease Post Allogeneic Stem Cell Transplant. *Br J Haematol* (2006) 132:245–7. doi: 10.1111/j.1365-2141.2005.05864.x
32. Saad AG, Alyea EP, Wen PY, DeGirolami U, Kesari S. Graft-Versus-Host Disease of the CNS After Allogeneic Bone Marrow Transplantation. *J Clin Oncol* (2009) 27:147–9. doi: 10.1200/JCO.2009.21.7919
33. Li M, Zhang Y, Guan Y, Zhang Z, Dong H, Zhao Y, et al. A Case Report of Central Nervous System Graft-Versus-Host Disease and Literature Review. *Front Neurol* (2021) 12:621392(327). doi: 10.3389/fneur.2021.621392
34. Blasiak KP, Simonetta F, Vargas M-I, Chalandon Y. Central Nervous System Graft-Versus-Host Disease (CNS-GvHD) After Allogeneic Haematopoietic Stem Cell Transplantation. *Case Rep* (2018) 2018:bcr–2017-221840. doi: 10.1136/bcr-2017-221840
35. Bonnan M, Gianoli-Guillerme M, Courtade H, Demasles S, Krim E, Marasescu R, et al. Estimation of Intrathecal IgG Synthesis: Simulation of the Risk of Underestimation. *Ann Clin Transl Neurol* (2018) 5(5):524–37. doi: 10.1002/acn3.548
36. Lyu H-R, He X-Y, Hao H-J, Lu W-Y, Jin X, Zhao Y-J, et al. Noninvasive Tools Based on Immune Biomarkers for the Diagnosis of Central Nervous System Graft-vs-Host Disease: Two Case Reports and a Review of the Literature. *World J Clin cases* (2021) 9(6):1359–66. doi: 10.12998/wjcc.v9.i6.1359
37. Zhang XH, Zhao X, Wang CC, Han W, Chen H, Chen YH, et al. IgG Synthesis Rate and Anti-Myelin Oligodendrocyte Glycoprotein Antibody in CSF may be Associated With the Onset of CNS Demyelination After Haplo-HSCT. *Ann Hematol* (2018) 97(8):1399–406. doi: 10.1007/s00277-018-3299-4
38. Ruggiu M, Cuccuini W, Mokhtari K, Meignin V, Latour R, Robin M, et al. Case Report: Central Nervous System Involvement of Human Graft Versus Host Disease. *Medicine* (2017) 96:e8303. doi: 10.1097/MD.0000000000008303
39. Hümmert MW, Stadler M, Hambach L, Gingele S, Bredt M, Wattjes MP, et al. Severe Allo-Immune Antibody-Associated Peripheral and Central Nervous System Diseases After Allogeneic Hematopoietic Stem Cell Transplantation. *Sci Rep* (2021) 11:8527. doi: 10.1038/s41598-021-87989-z
40. Das J, Gill A, Lo C, Chan-Lam N, Price S, Wharton SB, et al. A Case of Multiple Sclerosis-Like Relapsing Remitting Encephalomyelitis Following Allogeneic Hematopoietic Stem Cell Transplantation and a Review of the Published Literature. *Front Immunol* (2020) 11:668. doi: 10.3389/fimmu.2020.00668
41. Grauer O, Wolff D, Bertz H, Greinix H, Kühl JS, Lawitschka A, et al. Neurological Manifestations of Chronic Graft-Versus-Host Disease After Allogeneic Haematopoietic Stem Cell Transplantation: Report From the Consensus Conference on Clinical Practice in Chronic Graft-Versus-Host Disease. *Brain* (2010) 133:2852–65. doi: 10.1093/brain/awq245
42. Miller HK, Braun TM, Stillwell T, Harris AC, Choi S, Connelly J, et al. Infectious Risk After Allogeneic Hematopoietic Cell Transplantation Complicated by Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant J Am Soc Blood Marrow Transplant* (2017) 23(3):522–8. doi: 10.1016/j.bbmt.2016.12.630
43. Sakellari I, Gavriilaki E, Papagiannopoulos S, Gavriilaki M, Batsis I, Mallouri D, et al. Neurological Adverse Events Post Allogeneic Hematopoietic Cell Transplantation: Major Determinants of Morbidity and Mortality. *J Neurol* (2019) 266(8):1960–72. doi: 10.1007/s00415-019-09372-3
44. Dietrich U, Hettmann M, Maschke M, Doerfler A, Schwechheimer K, Forsting M. Cerebral Aspergillosis: Comparison of Radiological and Neuropathologic Findings in Patients With Bone Marrow Transplantation. *Eur Radiol* (2001) 11(7):1242–9. doi: 10.1007/s003300000756
45. Anciones C, de Felipe A, de Albóniga-Chindurza A, Acebrón F, Pián H, Masjuán J, et al. Acute Stroke as First Manifestation of Cerebral Aspergillosis. *J Stroke Cerebrovascular Dis* (2018) 27(11):3289–93. doi: 10.1016/j.jstrokecerebrovasdis.2018.07.031
46. Lai PH, Lin SM, Pan HB, Yang CF. Disseminated Miliary Cerebral Candidiasis. *AJNR Am J Neuroradiol* (1997) 18(7):1303–6.
47. Martino R, Maertens J, Bretagne S, Rovira M, Deconinck E, Ullmann A, et al. Toxoplasmosis After Hematopoietic Stem Cell Transplantation. *Clin Infect Dis* (2000) 31(5):1188–94. doi: 10.1086/317471
48. Fricker-Hidalgo H, Bulabois C-E, Brenier-Pinchart M-P, Hamidfar R, Garban F, Brion J-P, et al. Diagnosis of Toxoplasmosis After Allogeneic Stem Cell Transplantation: Results of DNA Detection and Serological Techniques. *Clin Infect Dis* (2009) 48(2):e9–15. doi: 10.1086/595709
49. Dulamea AO, Lupescu IG. Neurological Complications of Hematopoietic Cell Transplantation in Children and Adults. *Neural regener Res* (2018) 13(6):945–54. doi: 10.4103/1673-5374.233431

50. Ayas M, Al-Jefri A, Al-Seraihi A. In Cyclosporine Induced Neurotoxicity, Is Tacrolimus an Appropriate Substitute or Is It Out of the Frying Pan and Into the Fire? *Pediatr Blood Cancer* (2008) 50(2):426; author reply 427–6. doi: 10.1002/pbc.21211
51. Avivi I, Wittmann T, Henig I, Kra-Oz Z, Szwarcwort Cohen M, Zuckerman T, et al. Development of Multifocal Leukoencephalopathy in Patients Undergoing Allogeneic Stem Cell Transplantation-Can Preemptive Detection of John Cunningham Virus Be Useful? *Int J Infect Dis* (2014) 26:107–9. doi: 10.1016/j.ijid.2014.03.1381
52. Wüthrich C, Kesari S, Kim WK, Williams K, Gelman R, Elmeric D, et al. Characterization of Lymphocytic Infiltrates in Progressive Multifocal Leukoencephalopathy: Co-Localization of CD8(+) T Cells With JCV-Infected Glial Cells. *J Neurovirol* (2006) 12(2):116–28. doi: 10.1080/13550280600716604
53. Ogata M, Fukuda T, Teshima T. Human Herpesvirus-6 Encephalitis After Allogeneic Hematopoietic Cell Transplantation: What We Do and Do Not Know. *Bone Marrow Transplant* (2015) 50(8):1030–6. doi: 10.1038/bmt.2015.76
54. Zerr DM. Human Herpesvirus 6 and Central Nervous System Disease in Hematopoietic Cell Transplantation. *J Clin Virol* (2006) 37(Suppl 1):S52–6. doi: 10.1016/s1386-6532(06)70012-9
55. Zerr DM, Fann JR, Breiger D, Boeckh M, Adler AL, Xie H, et al. HHV-6 Reactivation and Its Effect on Delirium and Cognitive Functioning in Hematopoietic Cell Transplantation Recipients. *Blood J Am Soc Hematol* (2011) 117(19):5243–9. doi: 10.1182/blood-2010-10-316083
56. Inui Y, Yakushijin K, Okamura A, Tanaka Y, Shinzato I, Nomura T, et al. Human Herpesvirus 6 Encephalitis in Patients Administered Mycophenolate Mofetil as Prophylaxis for Graft-Versus-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation. *Transplant Infect Dis* (2019) 21 (1):e13024. doi: 10.1111/tid.13024
57. Zivković S. Neuroimaging and Neurologic Complications After Organ Transplantation. *J Neuroimaging* (2007) 17(2):110–23. doi: 10.1111/j.1552-6569.2007.00097.x
58. Dharnidharka VR, Webster AC, Martinez OM, Preiksaitis JK, Leblond V, Choquet S. Post-Transplant Lymphoproliferative Disorders. *Nat Rev Dis Primers* (2016) 2:15088. doi: 10.1038/nrdp.2015.88
59. Uhlin M, Wikell H, Sundin M, Blennow O, Maeurer M, Ringden O, et al. Risk Factors for Epstein-Barr Virus-Related Post-Transplant Lymphoproliferative Disease After Allogeneic Hematopoietic Stem Cell Transplantation. *Haematologica* (2014) 99(2):346–52. doi: 10.3324/haematol.2013.087338
60. Pickhardt PJ, Wippold FJ. 2nd: Neuroimaging in Posttransplantation Lymphoproliferative Disorder. *AJR Am J Roentgenol* (1999) 172(4):1117–21. doi: 10.2214/ajr.172.4.10587158
61. Zeiser R, Grüllich C, Bertz H, Pantazis G, Hufert FT, Bley TA, et al. Late Cytomegalovirus Polyradiculopathy Following Haploidentical CD34+-Selected Hematopoietic Stem Cell Transplantation. *Bone Marrow Transplant* (2004) 33:243–5. doi: 10.1038/sj.bmt.1704311
62. Reddy SM, Winston DJ, Territo MC, Schiller GJ. CMV Central Nervous System Disease in Stem-Cell Transplant Recipients: An Increasing Complication of Drug-Resistant CMV Infection and Protracted Immunodeficiency. *Bone Marrow Transplant* (2010) 45(6):979–84. doi: 10.1038/bmt.2010.35
63. Sarva H, Graber J, Remanan R, Rosenblum M, Omuro A. CMV Encephalitis in BMT Recipients. *Bone Marrow Transplant* (2012) 47(2):318–20. doi: 10.1038/bmt.2011.80
64. Ke P, Bao X, Zhou J, Li X, Zhuang J, He X, et al. Donor CMV-Specific Cytotoxic T Lymphocytes Successfully Treated Drug-Resistant Cytomegalovirus Encephalitis After Allogeneic Hematopoietic Stem Cell Transplantation. *Hematology* (2020) 25(1):43–7. doi: 10.1080/16078454.2019.1710945
65. Kaeuferle T, Krauss R, Blaeschke F, Willier S, Feuchtinger T. Strategies of Adoptive T -Cell Transfer to Treat Refractory Viral Infections Post Allogeneic Stem Cell Transplantation. *J Hematol Oncol* (2019) 12:13. doi: 10.1186/s13045-019-0701-1
66. Bollard CM, Heslop HE. T Cells for Viral Infections After Allogeneic Hematopoietic Stem Cell Transplant. *Blood* (2016) 127:3331–40. doi: 10.1182/blood-2016-01-628982
67. Einsele H, Ljungman P, Boeckh M. How I Treat CMV Reactivation After Allogeneic Hematopoietic Stem Cell Transplantation. *Blood* (2020) 135:1619–29. doi: 10.1182/blood.2019000956
68. Neuenhahn M, Albrecht J, Odendahl M, Schlott F, Dössinger G, Schiemann M, et al. Transfer of Minimally Manipulated CMV-Specific T Cells From Stem Cell or Third-Party Donors to Treat CMV Infection After Allo-HSCT. *Leukemia* (2017) 10:2161–71. doi: 10.1038/leu.2017.16
69. Taylor JW, Schiff D. *Neurologic Complications*. Germany: Springer International Publishing (2015). doi: 10.1007/978-3-319-13832-9_25
70. Balaguer-Rosello A, Bataller L, Piñana JL, Montoro J, Lorenzo I, Villalba A, et al. Noninfectious Neurologic Complications After Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2019) 25:1818–24. doi: 10.1016/j.bbmt.2019.05.024
71. Nishiguchi T, Mochizuki K, Shakudo M, Takeshita T, Hino M, Inoue Y. CNS Complications of Hematopoietic Stem Cell Transplantation. *AJR Am J Roentgenol* (2009) 192(4):1003–11. doi: 10.2214/ajr.08.1787
72. Quant E, Wen PY. Neurological Complications of Hematopoietic Stem Cell Transplantation. In: *Cancer Neurology In Clinical Practice*. Germany: Springer (2008).
73. Singh S, Kumar A. Protective Effect of Edaravone on Cyclophosphamide Induced Oxidative Stress and Neurotoxicity in Rats. *Curr Drug Saf* (2019) 14 (3):209–16. doi: 10.2174/1574886314666190506100717
74. Vassal G, Deroussent A, Hartmann O, Challine D, Benhamou E, Valteau-Couanet D, et al. Dose-Dependent Neurotoxicity of High-Dose Busulfan in Children: A Clinical and Pharmacological Study. *Cancer Res* (1990) 50 (19):6203–7.
75. McCune JS, Holmberg LA. Busulfan in Hematopoietic Stem Cell Transplant Setting. *Expert Opin Drug Metab Toxicol* (2009) 5(8):957–69. doi: 10.1517/17425250903107764
76. Caselli D, Rosati A, Faraci M, Podda M, Ripaldi M, Longoni D, et al. Risk of Seizures in Children Receiving Busulphan-Containing Regimens for Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2014) 20(2):282–5. doi: 10.1016/j.bbmt.2013.10.028
77. La Morgia C, Mondini S, Guarino M, Bonifazi F, Cirignotta F. Busulfan Neurotoxicity and EEG Abnormalities: A Case Report. *Neurol Sci* (2004) 25 (2):95–7. doi: 10.1007/s10072-004-0237-0
78. Eberly AL, Anderson GD, Bubalo JS, McCune JS. Optimal Prevention of Seizures Induced by High-Dose Busulfan. *Pharmacother: J Hum Pharmacol Drug Ther* (2008) 28(12):1502–10. doi: 10.1592/phco.28.12.1502
79. Moore-Maxwell CA, Datto MB, Hulette CM. Chemotherapy-Induced Toxic Leukoencephalopathy Causes a Wide Range of Symptoms: A Series of Four Autopsies. *Modern Pathol* (2004) 17(2):241–7. doi: 10.1038/modpathol.3800049
80. Park S-H, Lim KY, Kim S-I, Kim H, Kang J, Park JW, et al. *Toxic Leukoencephalopathy Caused by Chemotherapeutic Drugs Other Than Methotrexate*. Research Square Platform LLC (2021). doi: 10.21203/rs.3.rs-36667/v1
81. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ Regulatory T Cell Function by Calcineurin Dependent Interleukin-2 Production. *Blood* (2006) 108:390–9. doi: 10.1182/blood-2006-01-0329
82. Klee C, Draetta G, Hubbard M. Calcineurin. *Adv Enzymol* (1988) 61:149–200. doi: 10.1002/9780470123072.ch4
83. Tan TC, Robinson PJ. Mechanisms of Calcineurin Inhibitor-Induced Neurotoxicity. *Transplant Rev* (2006) 20(1):49–60. doi: 10.1016/j.trre.2006.02.005
84. Straathof K, Anoop P, Allwood Z, Silva J, Nikolajeva O, Chiesa R, et al. Long-Term Outcome Following Cyclosporine-Related Neurotoxicity in Paediatric Allogeneic Haematopoietic Stem Cell Transplantation. *Bone Marrow Transplant* (2017) 52(1):159–62. doi: 10.1038/bmt.2016.232
85. Takahata M, Hashino S, Izumiyama K, Chiba K, Suzuki S, Asaka M. Cyclosporin A-Induced Encephalopathy After Allogeneic Bone Marrow Transplantation With Prevention of Graft-Versus-Host Disease by Tacrolimus. *Bone Marrow Transplant* (2001) 28(7):713–5. doi: 10.1038/sj.bmt.1703221
86. Bechstein WO. Neurotoxicity of Calcineurin Inhibitors: Impact and Clinical Management. *Transplant Int* (2000) 13(5):313–26. doi: 10.1111/j.1432-2277.2000.tb01004.x
87. Maffini E, Festuccia M, Brunello L, Boccadoro M, Giaccone L, Bruno B. Neurologic Complications After Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2017) 23(3):388–97. doi: 10.1016/j.bbmt.2016.12.632
88. McDonald JW, Goldberg MP, Gwag BJ, Chi SI, Choi DW. Cyclosporine Induces Neuronal Apoptosis and Selective Oligodendrocyte Death in Cortical

Cultures. *Ann Neurol: Off J Am Neurol Assoc Child Neurol Soc* (1996) 40 (5):750–8. doi: 10.1002/ana.410400511
89. Casanova B, Prieto M, Deya E, Gisbert C, Mir J, Berenguer J, et al. Persistent Cortical Blindness After Cyclosporine Leukoencephalopathy. *Liver Transplant Surg* (1997) 3(6):638–40. doi: 10.1002/lt.500030614
90. Noè A, Cappelli B, Biffi A, Chiesa R, Frugnoli I, Biral E, et al. High Incidence of Severe Cyclosporine Neurotoxicity in Children Affected by Haemoglobinopaties Undergoing Myeloablative Haematopoietic Stem Cell Transplantation: Early Diagnosis and Prompt Intervention Ameliorates Neurological Outcome. *Ital J Pediatr* (2010) 36(1):1–6. doi: 10.1186/1824-7288-36-1
91. Thompson D, Harrington Y, de la Fuente J. The Incidence of Posterior Reversible Encephalopathy Syndrome Is Increased in BMT for Haemoglobinopathies. *Blood* (2013) 122(21):4579. doi: 10.1182/blood.V122.21.4579.4579
92. Gaziev J, Marziali S, Paciaroni K, Isgrò A, Di Giuliano F, Rossi G, et al. Posterior Reversible Encephalopathy Syndrome After Hematopoietic Cell Transplantation in Children With Hemoglobinopathies. *Biol Blood Marrow Transplant* (2017) 23(9):1531–40. doi: 10.1016/j.bbmt.2017.05.033
93. Duléry R. *Neurological Complications*. Germany: Springer International Publishing (2019), 403–407. doi: 10.1007/978-3-030-02278-5_53
94. Kharfan-Dabaja MA, Ayala E, Greene J, Rojiani A, Murtagh FR, Anasetti C. Two Cases of Progressive Multifocal Leukoencephalopathy After Allogeneic Hematopoietic Cell Transplantation and a Review of the Literature. *Bone Marrow Transplant* (2007) 39:101–7. doi: 10.1038/sj.bmt.1705548

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Mechanisms by Which Obesity Promotes Acute Graft-*Versus*-Host Disease in Mice

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The efficacy of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is limited by the occurrence of acute and chronic graft-*versus*-host disease (GVHD). We have recently demonstrated that obesity results in exacerbated acute gastrointestinal GVHD in both mouse models and clinical outcomes due to increased pro-inflammatory cytokine responses and microbiota alterations. We therefore wanted to delineate the role of the various parameters in obesity, adiposity, effects of high-fat (HF) diet, and the role of microbiome on GVHD pathogenesis, by taking advantage of a mouse strain resistant to diet-induced obesity (DIO). Female BALB/c mice are resistant to DIO phenotype with approximately 50% becoming DIO under HF diets. The DIO-susceptible recipients rapidly succumb to acute gut GVHD, whereas the DIO-resistant recipient littermates, which do not become obese, are partially protected from GVHD, indicating that being on HF diet alone contributes to but is not the primary driver of GVHD. Microbiome assessment revealed restricted diversity in both cohorts of mice, but coprophagy normalizes the microbiota in mice housed together. We then individually housed DIO-resistant, DIO-susceptible, and lean control mice. Notably, each of the individually housed groups demonstrates marked restricted diversity that has been shown to occur from the stress of single housing. Despite the restricted microbiome diversity, the GVHD pathogenesis profile remains consistent in the group-housed mice, with the lean control single-housed mice exhibiting no acute GVHD and DIO-resistant recipients showing again partial protection. These results demonstrate that the deleterious effects of obesity on acute

gut GVHD are critically dependent on adiposity with the HF diet also playing a lesser role, and the microbiome alterations with obesity instead appear to fuel ongoing acute GVHD processes.

Keywords: obesity, microbiome, GVHD, high-fat (HF) diet, cytokine storm

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is used for the treatment of a variety of hematopoietic disorders ranging from cancer to aplastic anemia. However, the key limitation to allo-HSCT is the development of graft-*versus*-host-disease (GVHD) that can manifest through either acute or chronic pathologies resulting in significant morbidity. A hallmark of acute GVHD is that of an inflammatory cascade resulting in a "cytokine storm" or pro-inflammatory cytokines attack multiple organs including the gastrointestinal (GI) tract, liver, lung, and skin, with chronic GVHD being more delayed and fibrotic in nature. The acute GVHD process driven by alloreactive donor T cells is fueled in part by the cytoreductive conditioning applied that causes tissue damage to susceptible organs (i.e., the GI system) and pro-inflammatory cytokine induction. Different mouse models can mirror these pathologic processes and can be highly selective depending on the strain combination for modeling acute or chronic GVHD.

Obesity, which is characterized as body mass index (BMI) greater than 30, is known to modulate immune responses and induce a meta-inflammatory state that has been linked to worse outcomes in various disease states (1, 2). Due to its rising prevalence in the US population, understanding the effect of obesity on health outcomes is critical. We have observed that obesity exacerbates immune dysregulation in mice and promotes a GVHD-like lethal pro-inflammatory cytokine storm following strong systemic immunotherapy or lipopolysaccharide (LPS) injection (3). In the context of allo-HSCT, we have recently demonstrated that mice with diet-induced obesity (DIO) placed on high-fat (HF) diets developed more severe acute GVHD due in part to the induction of a heightened pro-inflammatory cytokine storm (4). These effects were also observed clinically in high-BMI HSCT recipients (4–7). Interestingly, it was also observed that obesity resulted in restricted microbiome diversity and increased gut permeability, which may account for the specific targeting of the gut for acute GVHD attack (4, 8–10). Prophylactic treatment with antibiotics could partially protect the DIO mice from acute GVHD, indicating that the microbiome likely plays a role (4). The microbiome has been under intensive study in HSCT, particularly clinically, with outcomes being linked to specific deleterious and beneficial bacteria species (4, 11–14). Also, the reduction of microbiome diversity has been previously reported to be a negative prognostic indicator following HSCT (11, 14). However, with the DIO mouse model, it was not clear whether the microbiome alterations were primarily responsible for the increased GVHD observed. With HF diets, there is markedly increased adiposity that may be equally as important as immunomodulators fueling pro-inflammatory cytokine responses (3), but there is evidence that the diet itself may also affect immune and in particular T-cell responses due to free fatty acid metabolism (15, 16). We therefore wanted to delineate in obesity which parameters were the major drivers in the augmented acute gut GVHD observed.

Here, we attempt to discern the individual contributions of adiposity, diet, and microbiome on obesity-associated poor GVHD outcome. For these studies, the BALB/c DIO mouse model was chosen because when placed on an HF diet, only half of the female mice become obese even after several months of exposure (17, 18). The other half of the mice, which do not become obese, have weights similar to control mice fed a low-fat diet. This provides a unique opportunity to delineate the individual roles of obesity based on weight and diet on obesity-associated poor GVHD outcome. Using a major histocompatibility complex (MHC)-matched but minor histocompatibility antigen (mHA) mismatch strain combination where normally only later chronic GVHD occurs (19, 20), we observed that DIO-susceptible (DIO-S) female mice succumb with nearly 100% lethality to rapid acute gut GVHD, whereas the normal-weight DIO-resistant (DIO-R) female littermates fed the same diet also displayed increased acute GVHD but had improved outcomes, with more than 50% of mice recovering but later developing similar chronic GVHD as the lean recipients. As both co-housed DIO-S and DIO-R mice had similar microbiome profiles and decreased microbiota diversity when compared to lean controls, we then wanted to assess the role of the microbiome by single housing the mice on the different diets. Surprisingly, single cage housing resulted in marked microbiome reductions in diversity in all cohorts. After allo-HSCT in singly housed mice, the patterns of acute GVHD susceptibility remained the same as the group-housed recipients (with no GVHD in the lean recipients and comparable GVHD outcomes in the DIO-S and DIO-R cohorts), indicating that reduction of microbiome diversity alone was not sufficient to drive acute GVHD susceptibility. These data highlight the dynamics between obesity, diet, and microbiome and demonstrate that adiposity is the major driver for increased gut GVHD, although high-fat (HF) diet exposure can play a role to a lesser extent.

METHODS

Mice and Allo-HSCT

In this study, 6–8-week-old female BALB/c and C57BL/6 mice were obtained from Taconic Farms. Mice were placed on an HF diet or low-fat diet (D12492 or D12450J, Research Diets, Inc.). In

our singly housed mouse model, 6- to 8-week-old female BALB/c mice were placed in individual cages with low-fat or HF diet for at least 4 months to establish their body weight and gain phenotype before being used for HSCT.

The 8–10-week-old female B10.D2 mice were obtained from Jackson Laboratory and were used as donor mice. In order to create the MHC matched, mHA GVHD model, BALB/c mice ($H2^d$) received lethal total body irradiation (TBI) (800 cGy; ^{137}Cs source) and underwent transplantation with bone marrow cells with or without splenocytes (25×10^6) from the donor B10.D2 mice ($H2^d$) as described previously (4, 19). All mice were maintained at the University of California (UC), Davis Medical Center's vivarium in accordance with Institutional Animal Care and Use Committee (IACUC) standards.

Acute GVHD and Chronic GVHD Clinical Score Criteria

Acute GVHD clinical scores were determined based on weight loss (0–2), hunching (0–2), diarrhea (0–2), fur texture (0–2), and skin integrity (0–2). Mice were euthanized if they had a total score over 7 out of 10 or showed severe hunching (4, 21).

For sclerodermatous chronic GVHD, BALB/c mice were monitored for skin clinical scores and body weight loss post-allo-HSCT as described previously (4, 20). Briefly, skin clinical scores were assigned as follows: 0, healthy appearance; 1, skin lesions with alopecia area <1 cm^2; 2, skin lesions with alopecia area of 1–2 cm^2; 3, skin lesions with alopecia area >2 cm^2. Tail, ear, or paw scaling represented an additional 0.3 point for each lesion. Mice with a clinical skin score >3.3 (on a scale of 0–3.9) or with severe ischemic skin and tail lesions and hunching were euthanized per guidelines.

Magnetic Resonance Imaging

Mice were anesthetized with isoflurane and oxygen and then scanned on a Biospec 70/30 7.0-Tesla small-animal magnetic resonance imaging (MRI) system (Bruker Biospin Inc.) using a 60-mm quadrature transmitter/receiver coil for whole-body imaging. The scanning protocol consisted of the multislice with multi-echo spin-echo sequence with a single echo and with respiratory gating to minimize breathing artifacts. Scan parameters were echo time (TE) of 7.062 and repetition time (TR) of 775, conducted with and without chemical-selective fat suppression. Slice images were obtained in the coronal direction to improve spatial resolution while keeping scan time and TR at minimum. The in-plane matrix was 200 × 267 with a resolution of 0.3 × 0.3 mm. Forty-four slices were acquired with a slice thickness of 0.6 mm. Field of view was 6 cm × 8 cm × 2.64 cm. Difference images were generated by subtracting the fat-suppressed images from the non–fat-suppressed images to identify the three-dimensional distribution of fat deposits. Physiological monitoring (temperature and respiration) was used during the entire scan to ensure consistency and animal physiological stability.

Histology and Histopathology Scores

Tissues harvested from mice were placed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were evaluated and graded by a board-certified veterinary pathologist in a single-blinded fashion. GI pathology was scored on a scale of 0–3 based on goblet cell loss, gland epithelial piling, and karyomegaly. Images were visualized with a Vanox AHBS3 microscope with an SPlan Apo 20×/0.70 NA objective (Olympus, NY, USA). Images were acquired with a SPOT RT color digital camera using SPOT version 4.0.2 software (Diagnostic Instruments, MI, USA).

Fibrotic skin samples were assessed by Masson's trichrome staining with a Masson's 2000 Trichrome kit (SKU# KTMTR2; American MasterTech, Lodi, CA, USA). Images were visualized with a Vanox AHBS3 microscope with an SPlan Apo 20×/0.70 numerical aperture (NA) objective (Olympus, NY, USA) and acquired with a SPOT RT color digital camera using SPOT version 4.0.2 software (Diagnostic Instruments, MI, USA).

Cytometric Bead Array

Serum cytokines were measured by cytometric bead array (CBA) flex set kits (BD Biosciences, San Jose, CA): mouse tumor necrosis factor (TNF) (Cat# 558299), mouse interleukin (IL)-6 (Cat# 558301). Serum samples were diluted 1:4 using assay diluent solution provided in the kit. Capture beads and detection beads were added as described in the user guide. Cytokine concentration was measured by flow cytometry.

Antibodies and Flow Cytometry Analysis

Single-cell suspensions (1 million cells) were first incubated with Fc Block (BD Pharmingen, San Diego, CA, USA) for 10 min and then coincubated with antibodies for 20 min at 4°C, followed by washing with staining buffer (phosphate-buffered saline + 1% fetal bovine serum). Flow cytometry analysis was performed with the LSR Fortessa cell analyzer (BD Biosciences, San Jose, CA, USA), and data were analyzed using FlowJo v10 software (FlowJo, Ashland, OR, USA). We used the following fluorochrome-conjugated monoclonal antibodies purchased from BioLegend (San Diego, CA, USA): CD45–Pacific Blue (30-F11), CD19–Brilliant Violet 785 (6D5), CD11c–phycoerythrin (PE)/Cy7 (N418), I-A/I-E–APC (allophycocyamin)/Cy7 (M5/114.15.2). We used the following fluorochrome-conjugated monoclonal antibodies purchased from BD Biosciences (San Jose, CA, USA): CD229.1-PE (30C7).

Mouse Microbiome Analysis

Microbiome data are uploaded into a biorepository with Bioproject ID PRJNA758120. DNA was isolated using the Qiagen DNeasy PowerSoil kit (Qiagen) with the following modifications. After addition of buffer C1, samples were incubated at 65°C for 10 min and subjected to homogenization using a Biospec Mini-Beadbeater (Biospec Products) for 2 min. An additional wash step with 100% ethanol was included preceding the wash with kit buffer C5. Samples were eluted in 100 µl of buffer C6. Primers 319F (**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**(spacer)*GT*ACTCCTACGGGAGGCAGCAGT) and 806R (**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**(spacer)*CC*GGACTACNVGGGTWTCTAAT) were used to amplify the V3–V4 domain of the 16S rRNA using a two-step PCR procedure. In step 1 of the

amplification procedure, both forward and reverse primers contained an Illumina tag sequence (bold), a variable-length spacer (no spacer, C, TC, or ATC for 319F; no spacer, G, TG, or ATG for 806R) to increase diversity and improve the quality of the sequencing run, a linker sequence (italicized), and the 16S target sequence (underlined). Each 25-µl PCR reaction contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 µM final concentration of each primer, and 1 µl of DNA for each sample. PCR conditions were as follows: an initial incubation at 95°C for 3 min, followed by 25 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 3 min. In step 2, each sample was barcoded with a unique forward and reverse barcode combination using forward primers (**AATGATACG GCGACCACCGAGATCTACAC**NNNNNNNNTCGTCGGC AGCGTC) with an Illumina P5 adapter sequence (bold), a unique 8-nt barcode (N), a partial matching sequence of the forward adapter used in step 1 (underlined), and reverse primers (**CAAGCAGAAGACGGCATACGAGAT**NNNNNNNNGT CTCGTGGGCTCGG) with an Illumina P7 adapter sequence (bold), unique 8-nt barcode (N), and a partial matching sequence of the reverse adapter used in step 1 (underlined). The PCR reaction in step two contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 µM final concentration of each uniquely barcoded primer, and 1 µl of the product from the PCR reaction in step 1 diluted at a 10:1 ratio in water. PCR conditions were as follows: an initial incubation at 95°C for 3 min, followed by eight cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 3 min. The final product was quantified on the Qubit instrument using the Qubit Broad Range DNA kit (Invitrogen), and individual amplicons were pooled in equal concentrations. The pooled library was cleaned utilizing Ampure XP beads (Beckman Coulter), then the band of interest was further subjected to isolation *via* gel electrophoresis on a 1.5% Blue Pippin HT gel (Sage Science). The library was quantified *via* qPCR followed by 300-bp paired-end sequencing using an Illumina MiSeq instrument (Illumina) in the Genome Center DNA Technologies Core, University of California, Davis. DNA extractions and library preparation were performed by the UC Davis Host Microbe Systems Biology Core Facility.

Heatmaps were generated with R package "pheatmap". Boxplots and volcano plots were created with R. The linear discriminate effect size (LefSe) program was used to ascertain any significant differences in taxonomic abundance (22). The LefSe program uses the Kruskal–Wallis sum-rank test to detect taxa with significant differential abundance in relation to class, and then biological significance is determined by pairwise tests between subclasses using the Wilcoxon rank-sum test. Finally, linear discriminate analysis is used to estimate the effect size of each differentially abundant taxa, and taxanomic cladograms were generated to highlight significant differences in taxa.

Statistical Analysis

Acute GVHD clinical scores and skin clinical scores were analyzed by two-way analysis of variance (ANOVA) with Tukey's *post-hoc* test for comparison among groups. Flow cytometry data were analyzed using the Student's *t*-test. A p-value <0.05 was considered significant. Survival curves were plotted on a Kaplan–Meier curve and analyzed by a log-rank test. The data were graphed and statistically analyzed using GraphPad Prism (GraphPad Software, Inc., CA, USA).

RESULTS

High Body Weight, Not High-Fat Diet Exposure, Is the Major Driver of Lethal GVHD Post-HSCT in Mice

BALB/c mice have been shown to be relatively resistant to HF diet weight gain, with some gaining weight (DIO-S) and some maintaining weight values comparable to mice on control diets (DIO-R) in the same cage (17). After 4 months on HF diet, we also observed that there was a weight range only in BALB/c mice that could be stratified into two cohorts: DIO-S [mean body weights larger than the standard deviation (SD) of body weights of control mice by 5 times, body weights are at least 35 g] and DIO-R (less than 3× SD of control mice mean body weights, lower than 30 g; **Figures 1A, B**; **Supplemental Figure S1A**). Body weight gain kinetics also showed a significant difference between DIO-S and DIO-R mice (**Supplemental Figure S1B**). MRI scan and visceral fat quantification also revealed high body fat content in DIO-S mice compared to DIO-R or control mice (**Figures 1C–E**). These mice were then divided into cohorts to assess effects on GVHD outcome compared to mice on the control diet. We used the well-established MHC-matched but minor mismatch strain combination model of B10.D2 ($H\text{-}2^d$) bone marrow cells and splenocytes into lethally irradiated BALB/c mice ($H\text{-}2^d$) that normally results in a later sclerodermatous chronic GVHD (23, 24). However, using DIO recipients, we observed that obesity triggered a rapidly lethal acute GVHD targeting GI tract in this strain combination along with other mouse GVHD models (4). Now, comparing DIO-S and DIO-R mice of the same strain, we observed that the DIO-S mice again all significantly succumbed to lethal GVHD, while all the lean control mice survived (**Figure 1F**). The DIO-R mice with comparable body weights as the control lean mice all showed acute GVHD symptoms with decreased survival but to a much lesser extent as the DIO-S mice (**Figure 1F**). Clinical symptoms of the DIO-S and DIO-R mice also showed that they developed acute GVHD with diarrhea, body weight loss, ruffled fur, and severe hunching (**Supplemental Figures S1C, D**). Cytokine assessment confirmed evidence of heightened TNF and significantly increased IL-6 in the serum of DIO-R and DIO-S mice early after HSCT (**Figures 1G, H**). Notably, high pro-inflammatory cytokine levels of IL-6 correlate with decreased acute GVHD survival in the mice (**Figure 1I**). Based on the clinical symptoms, we examined the GI tract by histopathology and observed marked pathology affecting the small intestine and the colon of the DIO-S mice and DIO-R mice posttransplant with goblet cell loss, multifocal lamina propria inflammation (**Figures 2A, B**) in agreement with previous results (4). Gene expression assessment revealed a significant increase of pro-

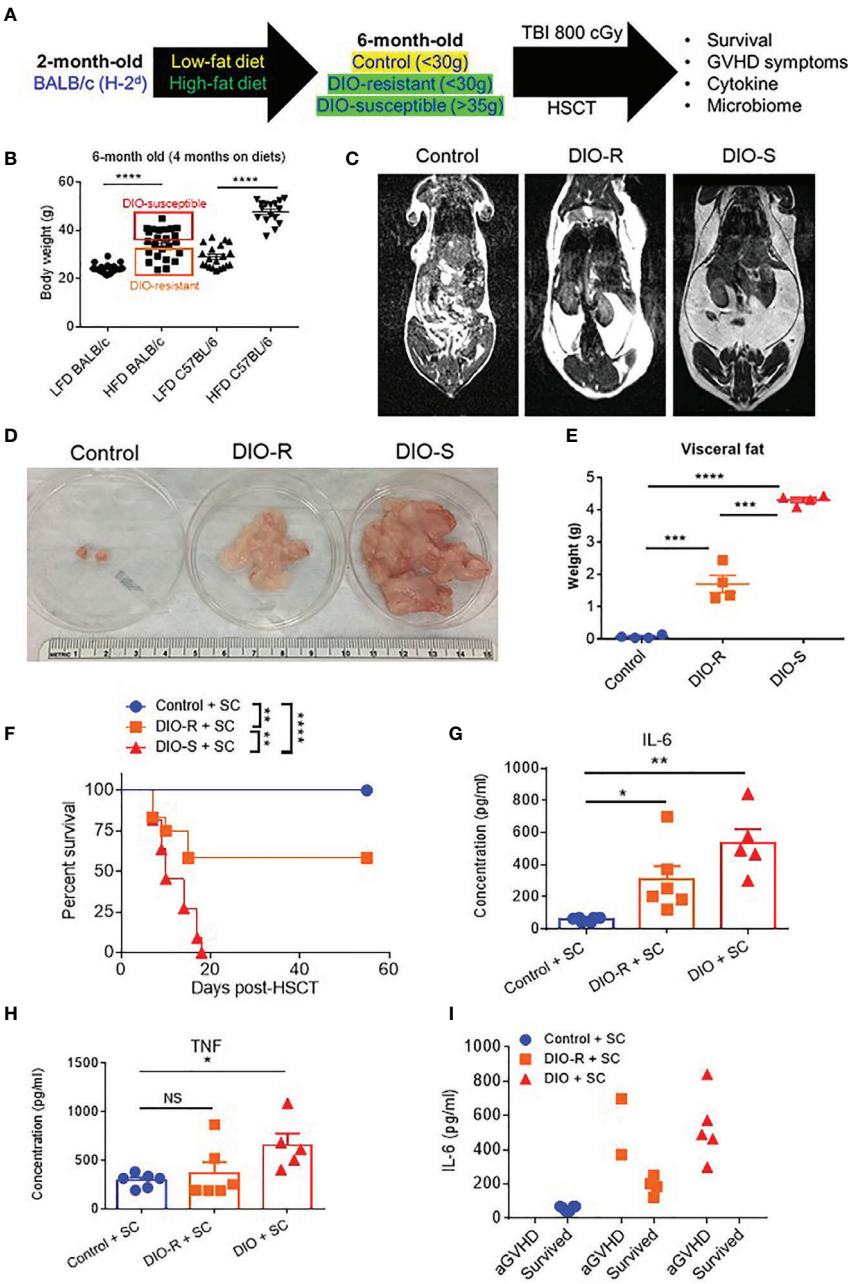

FIGURE 1 | **(A)** Lethally irradiated control, DIO-R, and DIO-S BALB/c mice received 8 million bone marrow cells and 25 million splenocytes from donor B10.D2 mice. **(B)** Body weights of BALB/c and C57BL/6 mice after 4 months on LF or HF diet (n=16-24/group). **(C)** Magnetic resonance imaging scans of control, DIO-R, and DIO-S mice. Fat tissue is white. **(D)** Representative images of visceral fat content of control, DIO-R, and DIO-S mice. **(E)** Quantification of visceral fat content of control, DIO-R, and DIO-S mice (n=4/group). **(F)** Survival rate post-HSCT (n=12/group). **(G)** Serum IL-6 and **(H)** TNF concentrations at day 7 post-HSCT (n=5-6/group). **(I)** Correlation between acute GVHD outcomes and IL-6 levels (n=5-6/group). Graphs depict mean ±s.e.m. Survival curve **(F)** was plotted on a Kaplan-Meier curve and analyzed by a log-rank test. One-way ANOVA test was used in **(B, F, G)**. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, NS, not significant.

apoptotic gene Caspase 3 in the gut of DIO-S mice (**Supplemental Figure S1E**). Activated dendritic cells (MHC II^+ $CD11c^+$) were also significantly higher in the mesenteric lymph nodes of DIO-S mice (**Supplemental Figures S2A, B**). Interestingly, the surviving DIO-R and control recipients later developed typical sclerodermatous GVHD associated with this model (**Figures 2C–E**). Taken together, these results demonstrate that obesity correlates with a paradigm shift in GVHD pathogenesis, shifting a typical chronic GVHD into a lethal acute GI tract GVHD in an MHC-matched, mHA-mismatched model and that body weight and not diet appears to be the major driver correlating with GVHD outcome.

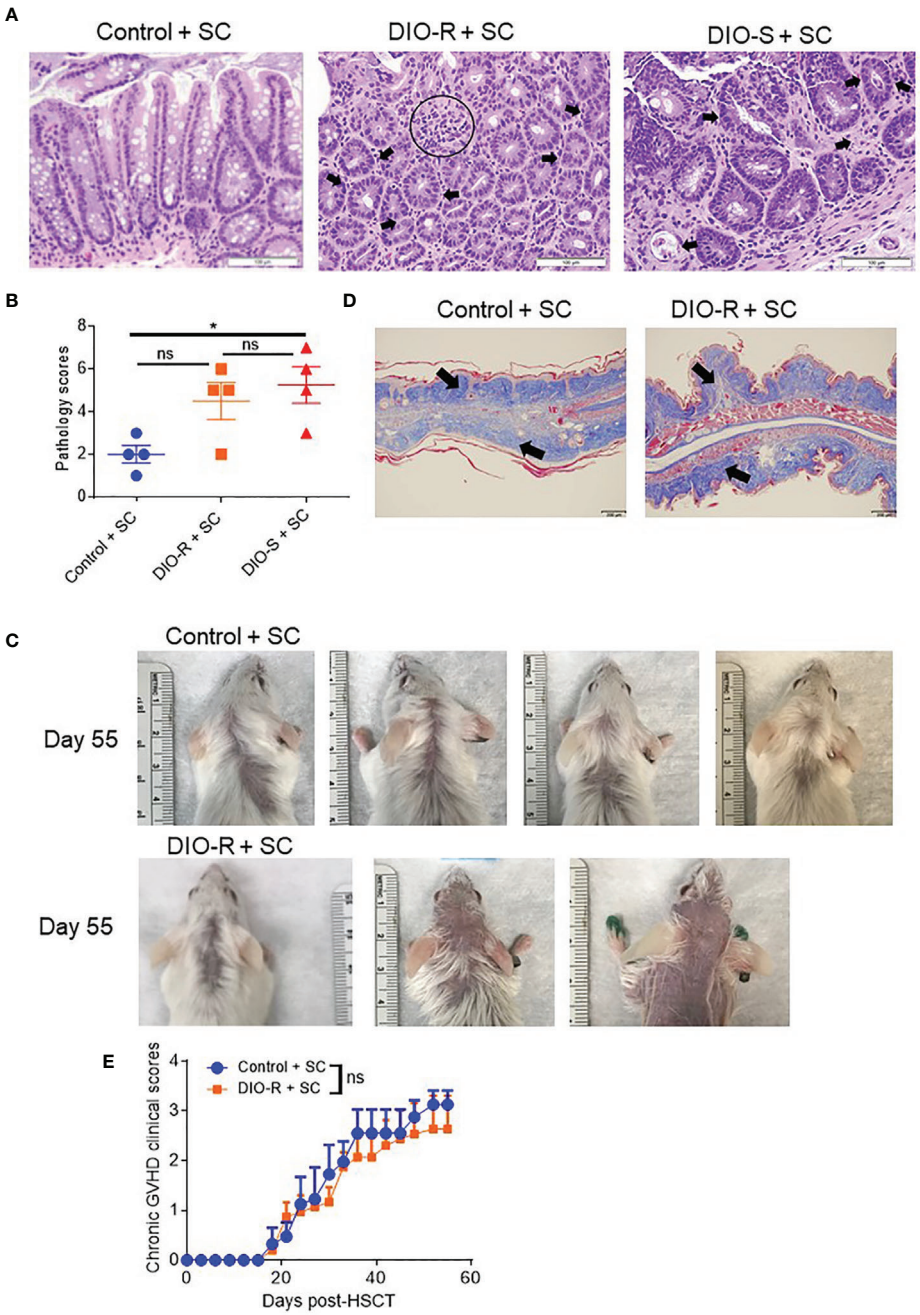

FIGURE 2 | **(A)** Representative images of H&E staining from colon samples at day 7 post-HSCT. The scale bar is 100μm. Circle indicates severe goblet cell loss and mild, multifocal lamina proprial inflammation. Arrows indicate piling of glandular epithelial cells, degenerate crypts, apoptotic crypt abscess and rare karyomegalic cells. **(B)** Pathology scores of samples from **(A)** (n=4/group). **(C)** Representative images of sclerodermatous GVHD with alopecia at day 55 post-HSCT. **(D)** Representative photos of sclerodermatous GVHD with tissue fibrosis by Trichrome staining. The scale bar is 200μm. Arrows indicate collagen deposition (blue). **(E)** Chronic GVHD clinical scores post-HSCT (n=3-4/group). Bar graphs depict mean ±s.e.m. One-way ANOVA test was used in **(B)**. Unpaired Student's t test was used in **(E)**. *$p < 0.05$, NS, not significant.

High-Fat Diet Exposure Results in Less Diverse Microbiota

We and others have observed that obesity induces microbiome changes in both mice and humans, resulting in marked decreases in microbial diversity that can correlate with poorer prognoses in allo-HSCT (4, 8, 9, 25). We next assessed the role of HF diet consumption on microbiome alterations in DIO-R and DIO-S cohorts. Indeed, microbiome profiles of both DIO-R and DIO-S mice shared a high level of similarity at multiple taxonomic units (**Figure 3A**). Principle component analysis showed closely

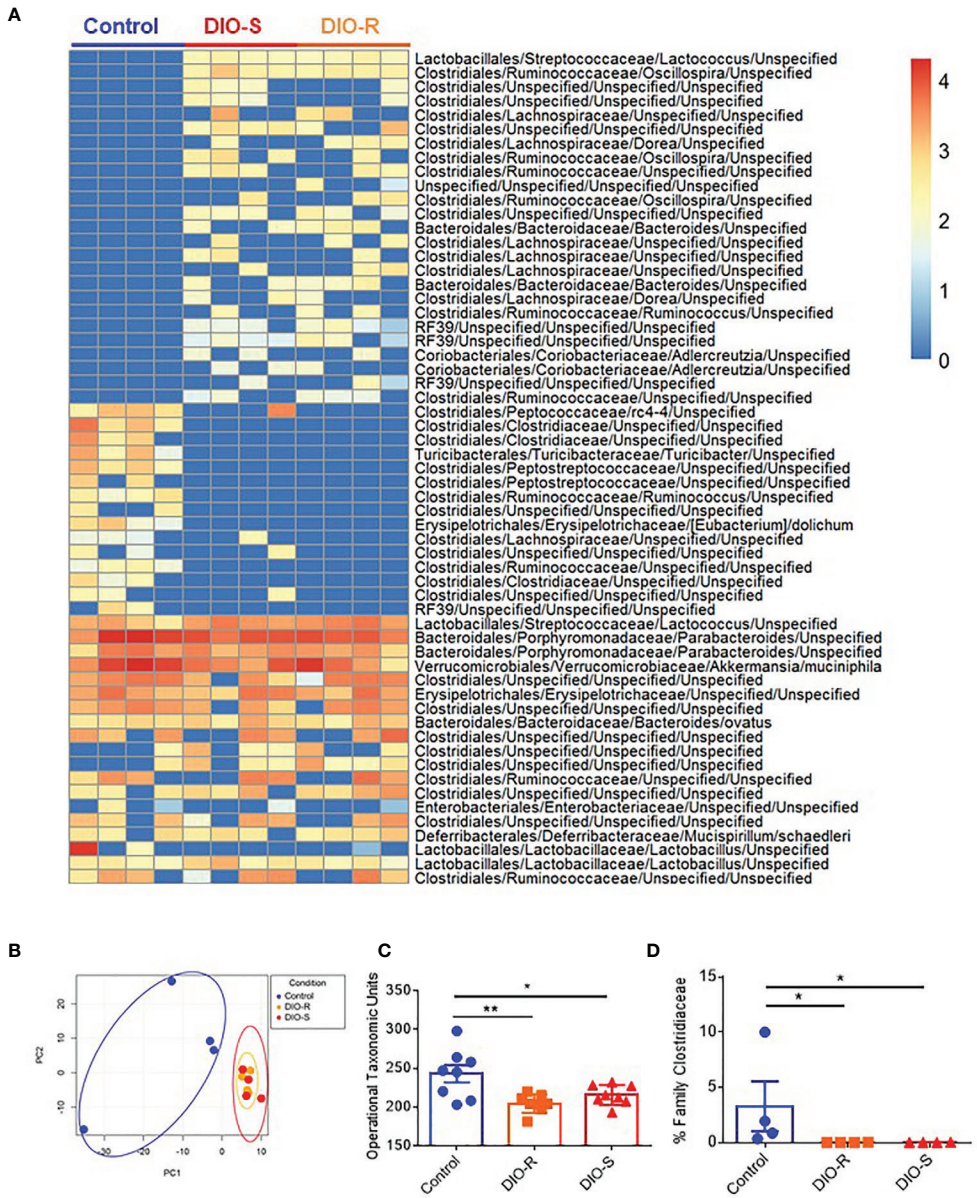

FIGURE 3 | **(A)** Taxonomic profiles of resting control, DIO-R, and DIO-S BALB/c mice (n=4/group). **(B)** Principle component analysis (PCA) of microbiome profiles of control, DIO-R, and DIO-S BALB/c mice (n=4/group). **(C)** Operational taxonomic units (OTUs) in control, DIO-R, and DIO-S BALB/c mice (n=8/group). **(D)** Abundance of family Clostridiaceae of control, DIO-R, and DIO-S BALB/c mice. Bar graphs depict mean ±s.e.m. One-way ANOVA test was used in **(C, D)**. *p < 0.05, **p < 0.01.

distributed microbiome profiles of DIO-R and DIO-S mice (**Figure 3B**). Resting DIO-R and DIO-S mice displayed a less-diverse microbiome compared to the control (**Figure 3C**) that could be correlated with poor HSCT outcome compared to control diet recipients (4, 11, 14). Interestingly, the family *Clostridiaceae* abundance, which has been shown to correlate with better GVHD outcomes (13), was reduced in both DIO-R and DIO-S mice compared to the control mice (**Figure 3D**). These results indicated that exposure to HF diet alone regardless of body weight or adiposity can modify the gut microbiota and potentially contribute to acute GVHD pathogenesis post-HSCT but, given the comparable microbiome profiles yet different GVHD severities, was not the dominant driver in outcome.

Singly Housed Mice Reproduce the Obesity-Resistant Phenotype and GVHD Outcomes Observed in Co-Housed Mice

Given that our data were obtained from co-housed mice (in each cage, there are both DIO-S and DIO-R mice) and that due to the coprophagy of mice that has been reported to normalize a

microbiome within a cage (26), we wanted to address if singly housing the BALB/c mice prior to placing on the diets would allow for better representation of the potential microbiome alterations that can occur with HF diet and weight gain and if social hierarchy status influenced body weight gain on HF diets (**Figure 4A**). Mice were singly housed at 6–8 weeks old and given control or HF diets, with their body weights monitored. Surprisingly, singly housed BALB/c mice fed with HF diet still displayed the same obesity-resistant and obesity-susceptible phenotypes, indicating that social hierarchy and social status do not impact weight gain (**Figure 4B**). Following allo-HSCT, the patterns of acute GVHD onset also remained the same, as the singly housed DIO-S and DIO-R mice had similar outcomes compared to group-housed littermates, with the DIO-S having higher acute GVHD clinical scores and lower survival compared to DIO-R (**Figures 4C, D**). These GVHD outcomes were again correlated with IL-6 and TNF levels (**Figures 4E–G**). Similarly, the lean control mice and surviving singly housed DIO-R mice developed sclerodermatous GVHD symptoms with alopecia later at 8 weeks after HSCT (**Figures 5A–C**). These results indicate that body weight gain is the primary driver in acute gut GVHD pathology following allo-HSCT.

Singly Housing Normalizes Microbiome Profiles Between High-Fat and Low-Fat Diet-Fed Mice

We then characterized the microbiome status of the singly housed control, DIO-R, and DIO-S mice, as there would be no normalization as seen in the group-housed cohorts. It has been previously reported that cold temperature can markedly reduce microbiome diversity in mice, and the stresses of social isolation are likely responsible for the effects seen in single-housed cohorts (27). Our data showed that the taxon abundances in all three groups (single-housed control, DIO-R, and DIO-S) were very different when compared to their co-housed counterparts, yet DIO-R and DIO-S were similar to each other (**Figure 6A**). Principal component analysis revealed that the microbiome profiles of singly housed DIO-S and DIO-R mice were distributed in a scattered pattern (**Figure 6B**). Surprisingly, in contrast to the marked effect that mice on HF diets had on the microbiome compared to control diet mice, we observed similar microbiome alterations occurring between the microbiome of singly housed DIO-R, DIO-S, and control mice displaying similar reductions in diversity. Diversity assessment showed that all the singly housed mice had more restricted microbiome diversity compared to the co-housed mice, even more so than of that group-housed HF diet-fed mice (**Figure 6C**). However, because the singly housed control diet mice did not show evidence of the acute GVHD seen in the HF mice but also had significantly lower operational taxonomic units (OTUs) compared to group-housed control, these results suggest that microbiome diversity alone might not be a crucial indicator for acute GVHD outcomes in the absence of obesity. Interestingly, abundance of family Clostridiaceae in singly housed mice still stayed undetectable compared to high mean levels observed in the control mice (**Figure 6D**). These data suggest that HF diet consumption and fat content might play a more important role than simply microbiome alterations in order to induce the acute gut GVHD pathogenesis in mice.

DISCUSSION

To our knowledge, our results are the first to delineate the roles of body weight *vs.* HF diet exposure in conjunction with the microbiome on acute GVHD processes. We used a strain combination for allo-HSCT in which only HF diet and obesity induce acute gut GVHD, where, normally, chronic skin GVHD would result. We also took advantage of DIO resistance of BALB/c mice for distinguishing the role of adiposity *vs.* the diet itself on GVHD induction. Furthermore, the use of microbiome assessment involving group-housed *vs.* single-housed cohorts allowed for delineation of reductions in microbiome diversity in the development of acute GVHD in these mice due to the effects of single-housing stress in inducing microbiome alterations in mice. These results indicate that adiposity itself is the primary driver for the acute gut GVHD pathogenesis, while the diet itself does appear to exert some effects, as reflected in the survival and pathology observed. In contrast, using single-housed control mice on low-fat diet, where microbiome diversity reductions still occurred, had no effect on acute GVHD induction, indicating that while the microbiome alterations can indeed play a role in perhaps fueling ongoing acute GVHD processes, these alone are not sufficient for its induction in this strain combination. This is in agreement with our recent study in which prophylactic antibiotics could only partially ameliorate acute GVHD in DIO recipients (4) but perhaps surprising that obesity itself was the primary driver for induction of gut GVHD processes perhaps due to increased gut damage resulting from the cytoreductive conditioning in obese mice.

With the increasing incidence of obesity in the US and other countries in the world, as well as increased consumption of foods with HF content, our study raises salient points of how the metabolically unhealthy body condition critically contributes to poor overall survival and GVHD outcomes in the settings of allo-HSCT particularly impacting the gastrointestinal system. There are multiple factors that potentially play major roles in inducing acute gut GVHD in recipients with prolonged HF diet consumption and body fat deposition including, but not limited to, increased damage by cytoreductive conditioning and production of pro-inflammatory cytokines after radiation and HSCT exacerbating cytokine storm (4); the impaired intestinal barrier and integrity accommodate bacterial translocation to the bloodstream, causing systemic sepsis (4); the activation of T cells due to increased fatty acid metabolism (15, 28); the activation of antigen-presenting cells, i.e., dendritic cells, in the mesenteric lymph nodes that in turn recruit and further activate donor T cells to trigger greater tissue damage and inflammation (29, 30); and the reduced diversity of microbiota and taxa alterations that likely all contribute for acute gut GVHD pathogenesis.

HF diets have been shown to induce dysbiosis in the gut flora that contributes to low-grade inflammation, impaired antimicrobial peptide production, decreased tight junction

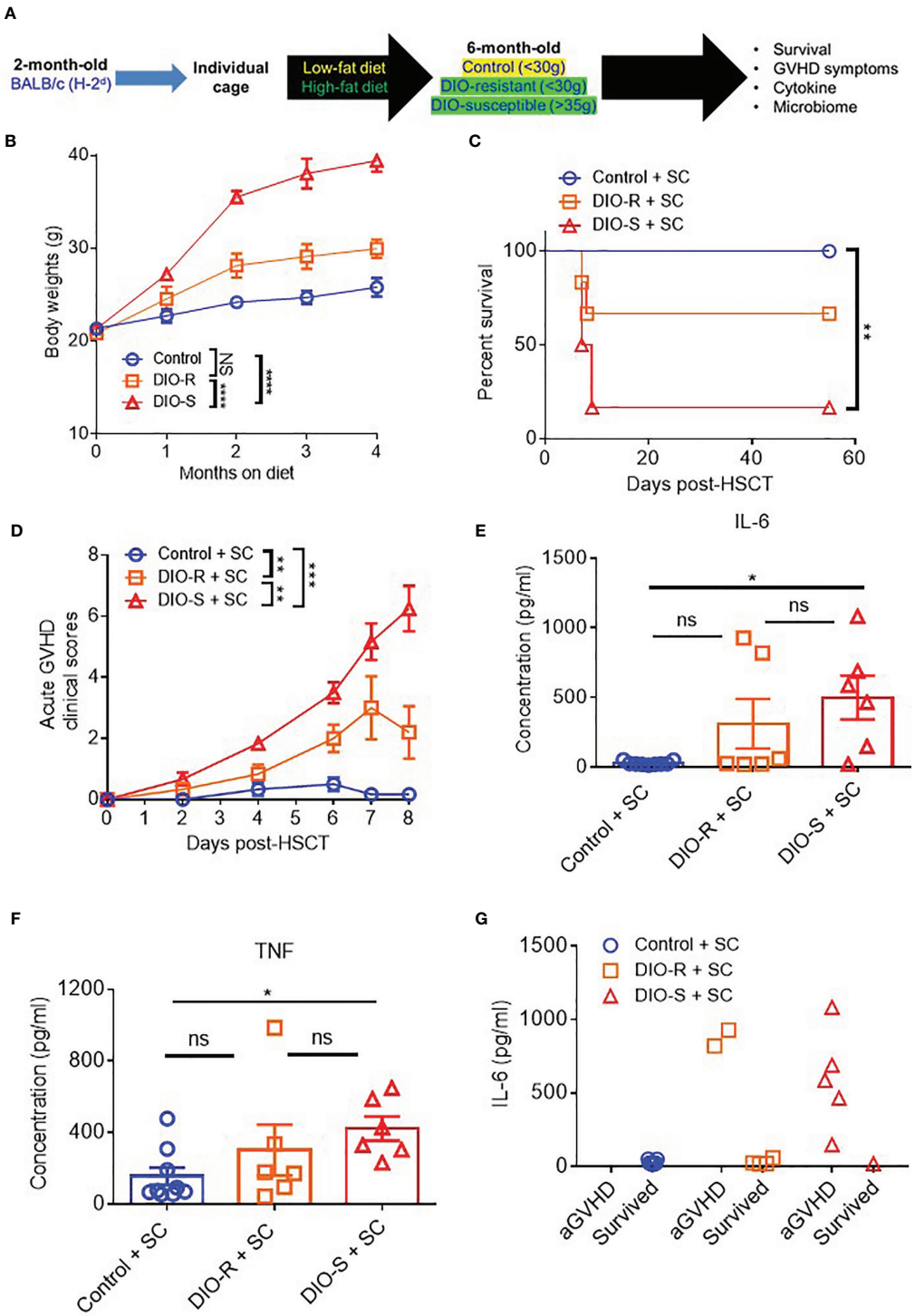

FIGURE 4 | **(A)** 8-week-old BALB/c mice were individually housed and put on LF or HF diet for 4 months. Mice were then lethally irradiated and received 8 million bone marrow cells and 25 million splenocytes from donor B10.D2 mice. **(B)** Kinetics of body weight gain of BALB/c mice on LF or HF diet. **(C)** Survival rate post-HSCT (n=6-9/group). **(D)** aGVHD clinical scores post-HSCT (n=6-9/group). **(E)** Serum IL-6 and **(F)** TNF concentrations at day 7 post-HSCT (n=6-9/group). **(G)** Correlation between acute GVHD outcomes and IL-6 levels (n=6-9/group). Bar graphs depict mean ±s.e.m. Survival curve **(C)** was plotted on a Kaplan-Meier curve and analyzed by a log-rank test. Body weght curve **(B)** and clinical scores **(D)** were analyzed by 2-way analysis of variance (ANOVA) with Tukey's post hoc test for comparison among groups. One-way ANOVA test was used in **(E, F)**. *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant.

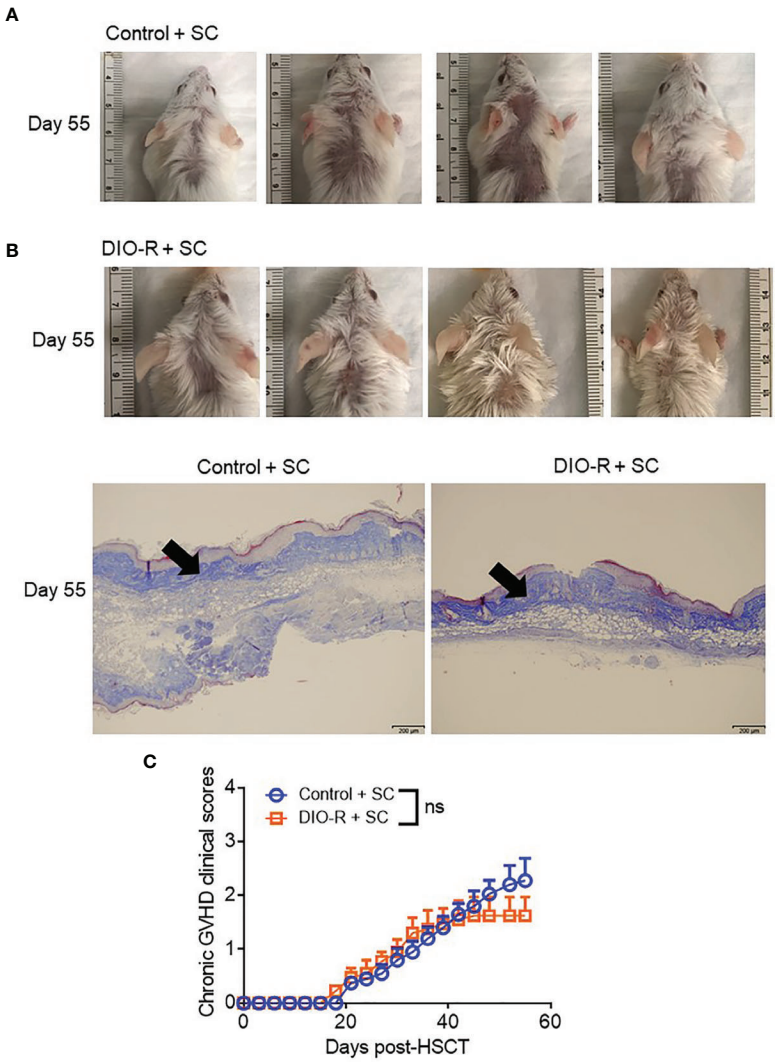

FIGURE 5 | **(A)** Representative images of sclerodermatous GVHD with alopecia at day 55 post-HSCT. **(B)** Representative photos of sclerodermatous GVHD with tissue fibrosis by Trichrome staining. The scale bar is 200μm. Arrows indicate collagen deposition (blue). **(C)** Chronic GVHD clinical scores post-HSCT (n=4/group). Bar graphs depict mean ±s.e.m. Unpaired Student's t test was used in **(C)**. NS, not significant.

protein expression, and mucus layer destruction (8, 9, 25, 31). These changes can lead to bacterial translocation from the intestine into the bloodstream, Toll-like receptor-mediated inflammation due to the release of pathogen-associated molecular patterns (PAMPs) (i.e., endotoxin, LPS, flagellin) or metabolites (i.e., bile acid) (10) that can facilitate more pro-inflammatory cytokine production, intestinal barrier deregulation, and continued GVHD-mediated gut pathology.

In assessing the complex role of the microbiome in GVHD, attention has centered on roles of certain bacterial flora or strains and the impact of reduced microbial diversity that has been well-documented to occur in obesity (4, 8, 9, 25). The results indicate that reductions alone in microbiome diversity cannot induce acute GVHD in this strain combination, although it is likely that it augments the impact of obesity and diet since antibiotics have been demonstrated to partially protect these DIO recipients (4). Similarly, the reduced GVHD occurring in the DIO-R mice suggests that diet can also play a role, although more detailed assessment of adiposity is needed to rule out that the DIO-R mice, while comparable in body weight to low-fat diet-fed mice, still have modest increases in adiposity that could be sufficient for the effects observed. All of the results presented would indicate that body fat alone and neither diet nor microbiome diversity is sufficient for the increased lethal acute gut GVHD observed. These data are also in agreement with studies demonstrating mice placed on HF diets for shorter periods of time in which body weight has not been significantly altered yet microbiome alterations have occurred, but no GVHD effects resulted (4). As the restricted microbiome diversity observed in single-housed mice regardless of diet is likely due to stress, it will be important

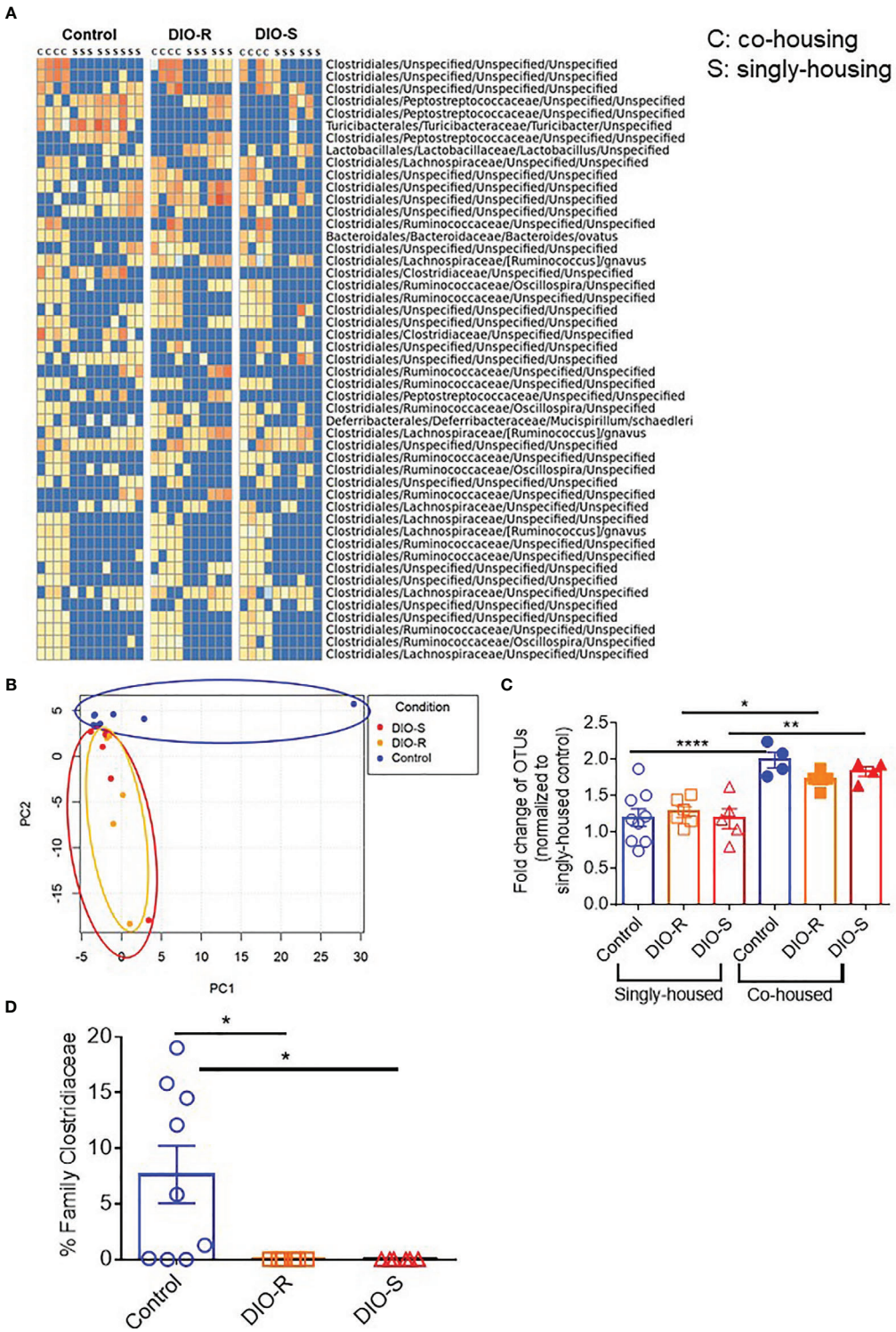

FIGURE 6 | **(A)** Taxonomic profiles of resting control, DIO-R, and DIO-S BALB/c mice being co-housed or singly housed for 4 months (n=4-9/group). **(B)** Principle component analysis (PCA) of microbiome profiles of singly-housed control, DIO-R, and DIO-S BALB/c mice (n=6-9/group). **(C)** Fold change of OTUs in singly-housed versus co-housed control, DIO-R, and DIO-S BALB/c mice (n=4-9/group). **(D)** Abundance of family Clostridiaceae of singly-housed control, DIO-R, and DIO-S BALB/c mice (n=6-9/group). Bar graphs depict mean ±s.e.m. One-way ANOVA test was used in **(C, D)**. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

to carefully delineate the impact of stress on microbiome composition, as it may also have a bearing on GVHD outcome. The results presented therefore demonstrate the critical role of body fat deposition on acute gut GVHD induction over that of diet and microbiome alterations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://www.ncbi.nlm.nih.gov/bioproject/758120, PRJNA758120.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC) standards.

AUTHOR CONTRIBUTIONS

LK and LV designed and performed experiments, analyzed results, and co-wrote the article. CD performed experiments and edited the article. EC performed histology scores and edited the article. LK, AM, and EM performed microbiome data analysis. EM, AM, and BB edited the article. WM directed the project, designed experiments, interpreted results, and co-wrote the article. 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.2021.752484/full#supplementary-material

Supplementary Figure 1 | (A) Representative images of control, DIO-R, and DIO-S mice. **(B)** Kinetics of body weight gain of BALB/c mice on LF or HF diet. **(C)** Acute GVHD clinical scores post-HSCT (n=12/group). **(D)** Body weights (g) of co-housed control, DIO-R, and DIO-S post-HSCT. **(E)** Quantification of gene expression of caspase-3 in the ileum of control, DIO-R, and DIO-S mice at 48 hours after radiation (n=3/group). Graphs depict mean ±s.e.m. Body weght curve **(B)** and clinical scores **(C)** were analyzed by 2-way analysis of variance (ANOVA) with Tukey's post hoc test for comparison among groups. Unpaired Student's t test was used in **(E)**. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, NS, not significant.

Supplementary Figure 2 | (A) Flow cytometry gating strategy on activated dendritic cells in the mesenteric lymph nodes 4 days after HSCT. **(B)** Quantification of numbers of activated dendritic cells in the mesenteric lymph nodes 4 days after HSCT (n=4/group). Graphs depict mean ±s.e.m. One-way ANOVA test was used in **(B)**. *$p < 0.05$, **$p < 0.01$.

REFERENCES

1. de Heredia FP, Gomez-Martinez S, Marcos A. Obesity, Inflammation and the Immune System. *Proc Nutr Soc* (2012) 71(2):332–8. doi: 10.1017/S0029665112000092
2. Milner JJ, Beck MA. The Impact of Obesity on the Immune Response to Infection. *Proc Nutr Soc* (2012) 71(2):298–306. doi: 10.1017/S0029665112000158
3. Mirsoian A, Bouchlaka MN, Sckisel GD, Chen M, Pai CC, Maverakis E, et al. Adiposity Induces Lethal Cytokine Storm After Systemic Administration of Stimulatory Immunotherapy Regimens in Aged Mice. *J Exp Med* (2014) 211 (12):2373–83. doi: 10.1084/jem.20140116
4. Khuat LT, Le CT, Pai CS, Shields-Cutler RR, Holtan SG, Rashidi A, et al. Obesity Induces Gut Microbiota Alterations and Augments Acute Graft-*Versus*-Host Disease After Allogeneic Stem Cell Transplantation. *Sci Transl Med* (2020) 12(571):eaay7713. doi: 10.1126/scitranslmed.aay7713
5. Navarro WH, Loberiza FRJr., Bajorunaite R, van Besien K, Vose JM, Lazarus HM, et al. Effect of Body Mass Index on Mortality of Patients With Lymphoma Undergoing Autologous Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant* (2006) 12(5):541–51. doi: 10.1016/j.bbmt.2005.12.033
6. Fuji S, Kim SW, Yoshimura K, Akiyama H, Okamoto S, Sao H, et al. Possible Association Between Obesity and Posttransplantation Complications Including Infectious Diseases and Acute Graft-*Versus*-Host Disease. *Biol Blood Marrow Transplant* (2009) 15(1):73–82. doi: 10.1016/j.bbmt.2008.10.029
7. Pereira AZ, de Almeida-Pitito B, Eugenio GC, Ruscitto do Prado R, Silva CC, Hamerschlak N. Impact of Obesity and Visceral Fat on Mortality in Hematopoietic Stem Cell Transplantation. *JPEN J Parenter Enteral Nutr* (2020). doi: 10.1002/jpen.2048
8. Boudry G, Hamilton MK, Chichlowski M, Wickramasinghe S, Barile D, Kalanetra KM, et al. Bovine Milk Oligosaccharides Decrease Gut Permeability and Improve Inflammation and Microbial Dysbiosis in Diet-Induced Obese Mice. *J Dairy Sci* (2017) 100(4):2471–81. doi: 10.3168/jds.2016-11890
9. Hamilton MK, Ronveaux CC, Rust BM, Newman JW, Hawley M, Barile D, et al. Prebiotic Milk Oligosaccharides Prevent Development of Obese Phenotype, Impairment of Gut Permeability, and Microbial Dysbiosis in High Fat-Fed Mice. *Am J Physiol Gastrointest Liver Physiol* (2017) 312(5): G474–87. doi: 10.1152/ajpgi.00427.2016
10. Murakami Y, Tanabe S, Suzuki T. High-Fat Diet-Induced Intestinal Hyperpermeability is Associated With Increased Bile Acids in the Large Intestine of Mice. *J Food Sci* (2016) 81(1):H216–222. doi: 10.1111/1750-3841.13166
11. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of Intestinal Inflammation by Microbiota Following Allogeneic Bone Marrow Transplantation. *J Exp Med* (2012) 209(5):903–11. doi: 10.1084/jem.20112408
12. Jenq RR, Taur Y, Devlin SM, Ponce DM, Goldberg JD, Ahr KF, et al. Intestinal Blautia Is Associated With Reduced Death From Graft-*Versus*-Host Disease. *Biol Blood Marrow Transplant* (2015) 21(8):1373–83. doi: 10.1016/j.bbmt.2015.04.016

13. Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut Microbiome-Derived Metabolites Modulate Intestinal Epithelial Cell Damage and Mitigate Graft-*Versus*-Host Disease. *Nat Immunol* (2016) 17 (5):505–13. doi: 10.1038/ni.3400
14. Taur Y, Jenq RR, Perales MA, Littmann ER, Morjaria S, Ling L, et al. The Effects of Intestinal Tract Bacterial Diversity on Mortality Following Allogeneic Hematopoietic Stem Cell Transplantation. *Blood* (2014) 124 (7):1174–82. doi: 10.1182/blood-2014-02-554725
15. Byersdorfer CA, Tkachev V, Opipari AW, Goodell S, Swanson J, Sandquist S, et al. Effector T Cells Require Fatty Acid Metabolism During Murine Graft-*Versus*-Host Disease. *Blood* (2013) 122(18):3230–7. doi: 10.1182/blood-2013-04-495515
16. Rebeles J, Green WD, Alwarawrah Y, Nichols AG, Eisner W, Danzaki K, et al. Obesity-Induced Changes in T-Cell Metabolism Are Associated With Impaired Memory T-Cell Response to Influenza and Are Not Reversed With Weight Loss. *J Infect Dis* (2019) 219(10):1652–61. doi: 10.1093/infdis/jiy700
17. James BR, Tomanek-Chalkley A, Askeland EJ, Kucaba T, Griffith TS, Norian LA. Diet-Induced Obesity Alters Dendritic Cell Function in the Presence and Absence of Tumor Growth. *J Immunol* (2012) 189(3):1311–21. doi: 10.4049/jimmunol.1100587
18. Boi SK, Buchta CM, Pearson NA, Francis MB, Meyerholz DK, Grobe JL, et al. Obesity Alters Immune and Metabolic Profiles: New Insight From Obese-Resistant Mice on High-Fat Diet. *Obes (Silver Spring)* (2016) 24(10):2140–9. doi: 10.1002/oby.21620
19. Pai CC, Chen M, Mirsoian A, Grossenbacher SK, Tellez J, Ames E, et al. Treatment of Chronic Graft-*Versus*-Host Disease With Bortezomib. *Blood* (2014) 124(10):1677–88. doi: 10.1182/blood-2014-02-554279
20. Pai CS, Khuat LT, Chen M, Murphy WJ, Abedi M. Therapeutic Effects of a NEDD8-Activating Enzyme Inhibitor, Pevonedistat, on Sclerodermatous Graft-*Versus*-Host Disease in Mice. *Biol Blood Marrow Transplant* (2017) 23(1):30–7. doi: 10.1016/j.bbmt.2016.10.022
21. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J Jr, Crawford JM, et al. An Experimental Model of Idiopathic Pneumonia Syndrome After Bone Marrow Transplantation: I. *Roles Minor H Antigens endotoxin. Blood* (1996) 88(8):3230–9. doi: 10.1182/blood.V88.8.3230.bloodjournal8883230
22. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic Biomarker Discovery and Explanation. *Genome Biol* (2011) 12 (6):R60. doi: 10.1186/gb-2011-12-6-r60
23. Hamilton BL. L3T4-Positive T Cells Participate in the Induction of Graft-vs-Host Disease in Response to Minor Histocompatibility Antigens. *J Immunol* (1987) 139(8):2511–5.
24. Schroeder MA, DiPersio JF. Mouse Models of Graft-*Versus*-Host Disease: Advances and Limitations. *Dis Model Mech* (2011) 4(3):318–33. doi: 10.1242/dmm.006668
25. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity Alters Gut Microbial Ecology. *Proc Natl Acad Sci USA* (2005) 102 (31):11070–5. doi: 10.1073/pnas.0504978102
26. Ericsson AC, Franklin CL. Manipulating the Gut Microbiota: Methods and Challenges. *ILAR J* (2015) 56(2):205–17. doi: 10.1093/ilar/ilv021
27. Worthmann A, John C, Ruhlemann MC, Baguhl M, Heinsen FA, Schaltenberg N, et al. Cold-Induced Conversion of Cholesterol to Bile Acids in Mice Shapes the Gut Microbiome and Promotes Adaptive Thermogenesis. *Nat Med* (2017) 23(7):839–49. doi: 10.1038/nm.4357
28. Sikder K, Shukla SK, Patel N, Singh H, Rafiq K. High Fat Diet Upregulates Fatty Acid Oxidation and Ketogenesis via Intervention of PPAR-Gamma. *Cell Physiol Biochem* (2018) 48(3):1317–31. doi: 10.1159/000492091
29. Chen Y, Tian J, Tian X, Tang X, Rui K, Tong J, et al. Adipose Tissue Dendritic Cells Enhances Inflammation by Prompting the Generation of Th17 Cells. *PloS One* (2014) 9(3):e92450. doi: 10.1371/journal.pone.0092450
30. Cheng L, Jin H, Qiang Y, Wu S, Yan C, Han M, et al. High Fat Diet Exacerbates Dextran Sulfate Sodium Induced Colitis Through Disturbing Mucosal Dendritic Cell Homeostasis. *Int Immunopharmacol* (2016) 40:1–10. doi: 10.1016/j.intimp.2016.08.018
31. Ahmad R, Rah B, Bastola D, Dhawan P, Singh AB. Obesity-Induces Organ and Tissue Specific Tight Junction Restructuring and Barrier Deregulation by Claudin Switching. *Sci Rep* (2017) 7(1):5125. doi: 10.1038/s41598-017-04989-8

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Mesenchymal Stromal Cells for the Treatment of Graft Versus Host Disease

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Graft versus host disease (GvHD) is a life-threating complication of allogeneic hematopoietic stem cell transplantation, which is initially treated with high dose corticosteroids. Approximately 50% of acute GvHD cases are resistant to steroid treatment, and two-year mortality rates in those steroid-resistant patients exceed 80%. Chronic GvHD necessitates prolonged corticosteroid use, which is typically associated with limited efficacy and troublesome adverse effects. No agent has yet been established as an optimal second line therapy for either acute or chronic GvHD, but mesenchymal stromal cells (MSCs) have shown substantial promise. MSCs promote an immunosuppressive and immunoregulatory environment *via* multifactorial mechanisms, including: secretion of proteins/peptides/hormones; transfer of mitochondria; and transfer of exosomes or microvesicles containing RNA and other molecules. A large number of clinical studies have investigated MSCs from various sources as a treatment for acute and/or chronic GvHD. MSCs are generally safe and well tolerated, and most clinical studies have generated encouraging efficacy results, but response rates have varied. Confounding factors include variability in MSC donor types, production methodology and dose regimens, as well as variations in study design. It is well-established that extensive culture expansion of primary donor-derived MSCs leads to marked changes in functionality, and that there is a high level of inter-donor variability in MSC properties. However, recent manufacturing innovations may be capable of overcoming these problems. Further adequately powered prospective studies are required to confirm efficacy and establish the place of MSC therapy in the treatment of this condition.

Keywords: graft versus host disease (GvHD), mesenchymal stromal (stem) cell (MSC), stem cell, bone marrow transplant (BMT), allogeneic

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) offers a potentially curative option for conditions including hematological malignancies. However, its benefits are often limited by serious complications, including graft versus host disease (GvHD).

GvHD arises from donor T-lymphocytes attacking host tissues. Features of GvHD may be categorized as either acute or chronic, which were historically distinguished by the time of occurrence (<100 or >100 days post-transplant) (1). However, this is likely an over-

simplification, because acute GvHD may persist beyond 100 days, and there may be overlap between acute and chronic syndromes (2). Both acute and chronic GvHD commonly affect the skin. Chronic GvHD is characterized by an initial lichenoid stage, similar to acute skin GvHD (3), often followed by a distinct sclerotic stage (4). Other organs typically affected by acute GvHD are the liver and gastrointestinal tract. Chronic GvHD may affect almost any organ in the body (5).

GvHD is the cause of death in 8-16% of adult allogeneic HSCT recipients (6). It should be noted that these figures likely underestimate the extent to which GvHD contributes to post-transplant mortality, given that GvHD may also predispose HSCT recipients to other common causes of death, such as organ failure, infection and hemorrhage.

Acute GvHD is typically staged and graded according to criteria established at the 1994 Consensus Conference on Acute GvHD Grading (7). In most clinical trials, response to treatment is measured based on improvement in the severity of GvHD by at least one grade (Partial Response; PR) and/or resolution of all acute GvHD signs or symptoms, i.e. a return to Grade 0 (Complete Response; CR). The term Overall Response (OR) rate refers to the sum of PR and CR rates. Similarly, consensus criteria have also been developed for chronic GvHD, under which a global severity score is based on two different scores of the severity of cutaneous disease (8).

The prophylaxis and management of GvHD is complex, and approaches vary substantially between centers worldwide (9). Corticosteroids remain the mainstay of first-line treatment for both acute and chronic GvHD. Approximately 50% of acute GvHD cases prove to be resistant to high doses of steroids, and the prognosis in those patients is extremely poor, with two-year overall survival (OS) rates below 20% (10). In moderate-severe chronic GvHD, systemic steroid treatment for at least one year is typically required, with approximately 50-60% of patients requiring secondary "steroid-sparing" treatment (such as antithymocyte globulin (ATG), extracorporeal photopheresis (ECP) or mycophenolate mofetil), and more than 10% requiring systemic treatment for over seven years (11). Even when steroid treatment is effective in chronic GvHD patients, it may be associated with severe adverse effects, especially when administered systemically for lengthy periods.

Diverse second-line agents have been investigated for the treatment of GvHD after the failure of steroids. In 2019, the Janus kinase inhibitor ruxolitinib (Jakafi®, Incyte Corporation) was approved in the USA for the treatment of steroid resistant acute GvHD (SR-aGvHD) (12). In July 2021, the rho kinase (ROCK) inhibitor belumosudil (Rezurock™, Kadmon Pharmaceuticals) was approved in the USA, for the treatment of chronic GvHD after failure of at least two prior lines of systemic therapy (13). Both of these recent approvals apply to adults and children over 12 years of age only. Other agents investigated for acute GvHD include ATG, anti-CD26 antibodies, and ECP (14, 15). An even wider range of agents has been investigated for chronic GvHD, including Janus kinase inhibitors, tyrosine kinase inhibitors, proteasome inhibitors, monoclonal antibodies, and fusion proteins (16). However, to date no agent has been established as an optimal second line therapy for either acute or chronic GvHD, and there remains a need for new therapies with superior safety and efficacy profiles.

The subject of this review is the use of mesenchymal stromal cells (MSCs) in the context of GvHD. Over the past two decades, there has been extensive interest in the potential therapeutic use of MSCs in a wide range of clinical settings (17), including in support of HSCT (18) and in the treatment of GvHD (19).

MECHANISM OF ACTION

MSCs lack human leucocyte antigen (HLA) Class II expression, which allows allogeneic administration without donor-recipient matching. MSCs exert multifactorial effects, including: paracrine activity involving secretion of proteins/peptides and hormones; transfer of mitochondria by way of tunneling nanotubes or microvesicles; and transfer of exosomes or microvesicles containing RNA and other molecules (**Figure 1**) (20, 21).

MSCs promote an immunosuppressive and immunoregulatory environment, by secretion of cytokines, chemokines, growth factors and extracellular vesicles (22, 23). Notably, MSCs constitutively secrete indoleamine 2,3-dioxygenase (IDO), and activation of MSCs by inflammatory cytokines including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) leads to upregulation of this IDO secretion (24, 25). IDO, in turn, leads to suppression of allogeneic T cell proliferation (26). Further immunomodulatory effects of MSCs are mediated *via* effects on B cells, natural killer cells, monocytes and dendritic cells (24). An interesting idea is that apoptosis of MSCs *in vivo* contributes to their immunomodulatory effects, a phenomenon that may be mediated through the production and release of apoptotic extracellular vesicles (27). Additionally, when undergoing apoptosis, MSCs induce IDO production in recipient phagocytes (28). It has also been shown that amelioration of GvHD in a humanized mouse model was associated with altered phosphorylation and cellular localization of the T cell-specific kinase, Protein Kinase C theta (PKCθ) (29).

Aside from immunomodulation, MSCs have also been shown to limit tissue damage and stimulate tissue repair, primarily as a result of paracrine effects on other endogenous recipient cells (20).

CLINICAL EXPERIENCE

The use of MSCs to treat GvHD in a human subject was first reported in 2004 by Le Blanc *et al*, of Karolinska Institutet, Sweden (30). After receiving an allogeneic HSCT from a HLA-matched, unrelated donor, a nine-year-old boy developed Grade IV acute GvHD, which was refractory to treatment with corticosteroids and several second-line agents. The authors reported that the other 24 patients at their center who had experienced such severe acute GvHD had all died within 6 months, with a median survival of just 2 months. In this case, the patient was treated with two intravenous (IV) infusions

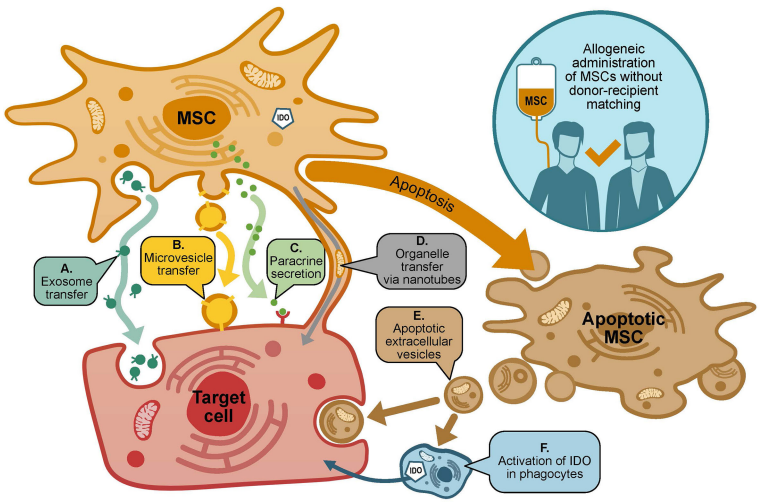

FIGURE 1 | Mechanisms of action of MSCs in GvHD. MSCs may exert many effects on target cells *via* diverse potentially-overlapping mechanisms. Target cells include (i) donor and host immune cells, including T cells, B cells, NK cells, monocytes and dendritic cells; and (ii) host cells susceptible to damage by GvHD, e.g. cells of the skin, gastrointestinal tract and liver. Potential mechanisms through which MSCs may act include **(A, B)**: transfer of exosomes or microvesicles containing RNA and other molecules; **(C)** paracrine activity including secretion of proteins (including IDO), peptides and hormones; **(D)** transfer of organelles *via* tunneling nanotubes; **(E, F)** MSC apoptosis results in the release of apoptotic extracellular vesicles that act on target cells, as well as induction of IDO production in recipient phagocytes.

of bone-marrow-derived allogeneic MSCs (on Days 73 and 150 post HSCT) from a related donor (his mother). The first infusion was followed by a marked but incomplete improvement, while after the second infusion the patient appeared to have recovered completely, and he remained alive and well after one year.

Since that initial case report, numerous clinical trials have been conducted to investigate MSCs as a treatment for GvHD. As of 30 June 2021, a total of 43 interventional clinical trials/expanded access programs involving MSCs as a treatment for GvHD have been registered on clinicaltrials.gov. Of those studies, 19 are complete, and papers summarizing the results of 10 have been published. Papers summarizing a further 17 studies involving patients with GvHD that were not registered on clinicaltrials.gov have also been published (studies conducted outside of the USA are not required to be registered on clinicaltrials.gov). **Table 1** summarizes the overall characteristics of the published and unpublished studies registered on clinicaltrials.gov.

The vast majority of studies have involved allogeneic MSCs, with bone marrow being the most common tissue source. A small number of trials have used MSCs derived from cord blood or adipose tissue, and a single study to date has been undertaken using iPSC-derived MSCs. The trials have been conducted by a wide range of sponsors, including both academic and commercial institutions.

As summarized in **Table 2**, most (n=18) published studies included only patients with SR-aGvHD. The patient population varied across other studies: five included patients with either SR-aGvHD or chronic GvHD; two included only patients with chronic GvHD; one included patients with either SR-aGvHD or newly diagnosed acute GvHD; and one included only patients with newly diagnosed acute GvHD.

TABLE 1 | Overall Summary of Characteristics of Clinical Studies of MSCs in GvHD (n = 60).

Study Type	n	Age group	n	MSC Source	n
Phase 1	12	Adults only	23	Bone marrow	42
Phase 1/2	15	Adults and children	32	Cord blood	7
Phase 2	13	Children only	5	Adipose tissue	2
Phase 2/3	4			iPSCs	1
Phase 3	6	**Registered on clinicaltrials.gov**	43	Not stated	8
Compassionate use	10	Not yet recruiting	3		
		Recruiting	5	**MSC Donor Type**	
GvHD Type		Active, not recruiting	1	Allogeneic	1
Acute GvHD	40	Withdrawn/terminated	2	Autologous	57
Acute or chronic GvHD	10	Complete	19	Not stated	2
Chronic GvHD	10	Unknown	13		

TABLE 2 | Summary of Published Clinical Studies of MSCs in GvHD.

First-line Treatment of Acute GvHD, in Combination With Corticosteroids

Reference	Study Type[a]	MSC source [dose[b]] (# of infusions]	Group (if applicable)	D28 Response	OS	
				CR	OR	
Kebriaei et al. (31)	Phase 2 CT (n=32; age: 34-67)	BM [2 or 8] (2)	Low dose High dose	88% 67%	88% 100%	69% (D90) (pooled cohorts)
Soder et al. (32)	Phase 1 CT (n=5; age: 35-63)	UCB [2 or 10] (2)	Low dose: High dose:	50% 33%	100% 33%	67% (D180) 33% (D180)

Treatment of SR-aGvHD

Reference	Study Type[a]	MSC source [dose[b]] (# of infusions]	Group (if applicable)	D28 Response		Response (other timepoints)		OS
				CR	OR	CR	OR	
von Bonin et al. (33)	Compassionate use (n=13; age: 21-69)	BM [0.6-1.1] (1-5)		8%	54%			31%*
Lucchini et al. (34)	Compassionate use (n=8; age: 4-15)	BM [0.7-1.6] (1-2)		24%	71%			63% (1y)
Muroi et al. (35)	Phase 1/2 CT (n=14; age: 4-62)	BM [2] (8-12)		75%	93%			57% (2y)
Introna et al. (36)	Phase 1 CT (n=40; age: 1-65)	BM [1 ± 0.5] (≥2)		28%	68%			50% (1y); 38% (2y)
Zhao et al. (37)	Phase 2 CT (n=47; age: 14-54) BAT control	BM [1] (2-8)	MSCs: Controls:	36% 26%	75% 42%			45% (3y) 23% (3y)
Muroi et al. (38)	Phase 2/3 CT (n=25; age: 5-66)	BM [2] (8-12)		24%	60%			48% (1y)
Salmenniemi et al. (39)	Phase 1 CT (n=26; age: 2-66)	BM [2] (1-6)		27%	62%			42%*
Bader et al. (40)	Compassionate use (n=69; age: 1-78)	BM [1-2] (1-4)		32%	83%			71% (6 m)
Kebriaei et al. (41)	Phase 3 CT (n=260; age: 0-70) BAT control	BM [2] (8-12)	MSCs: Controls:	37% 32%	58% 54%			34% (D180) 42% (D180)
Kurtzberg et al. (42)	Compassionate use (n=241; age: 0-18)	BM [2] (8-12)		14%	65%			67% (D100)
Kurtzberg et al. (43)	Phase 3 CT (n=54; age: 0-17)	BM [2] (8-12)		30%	70%			69% (D180)
Bloor et al. (44)	Phase 1 CT (n=15; age: 21-66)	iPSC [1 or 2] (2)	Low dose: High dose:	13% 57%	63% 86%	50% (D100) 57% (D100)	88% (D100) 86% (D100)	88% (D100) 86% (D100)
Soder et al. (32)	Phase 1 CT (n=5; age: 48-73)	UCB [2 or 10] (2)	Low dose: High dose:	33% 50%	100% 50%			100% (D180) 50% (D180)
Prasad et al. (45)	Compassionate use (n=12; age: 0-15)	BM [2 or 8] (8-12)				17% (D32); 58% (D60)	67% (D32); 75% (D60)	58% (D100); 40% (2y)
Sánchez-Guijo et al. (46)	Phase 2 CT (n=25; age: 20-65)	BM [0.7-1.3] (2-4)				46% (D60)	71% (D60)	44% (1y)
Ringden et al. (47)	Phase 1 CT (n=8; age: 3-61)	BM [0.7-9] (1-2)				75%*	75%*	38% (2y)
Le Blanc et al. (48)	Phase 2 CT (n=55; age: 0-64)	BM [0.4-9] (1-5)				55%*	71%*	35% (2y)
Arima et al. (49)	Phase 1 CT (n=3; age: 39-64)	BM [0.5] (1)				0%*	33%*	0% (2y)
Perez-Simon et al. (50)	Phase 1/2 CT (n=10; age: 18-65)	BM [0.6-2.9] (1-4)				10%*	70%*	20%*
Herrmann et al. (51)	Phase 1 CT (n=12; age: 21-58)	BM [1.7-2.3] (2-19)				58%*	92%*	50% (3y)
Ball et al. (52)	Compassionate use (n=37, age: 0-18)	BM [0.9-3] (1-19)				65%*	86%*	51%*
Resnick et al. (53)	Compassionate use (n=50, age: 1-69)	BM [0.3-2.3] (1-4)				34%*	66%*	
von Dalowski et al. (54)	Compassionate use (n=58; age: 19-71)	BM [0.5-2.1] (1-6)				9%*	47%*	19% (1y); 17% (2y)
Dotoli et al. (55)	Compassionate use (n=46; age: 1-78)	BM [1-29.8] (1-7)				7%*	50%*	20% (1y); 17% (2y)

Treatment of Chronic GvHD

Reference	Study Type[a]	MSC source [dose[b]] (# of infusions]	Group (if applicable)	CR	OR	OS
Ringden et al. (47)	Compassionate use (n=1; age: 27)	BM [0·6] (1)		No response	No response	0% (1y)
Lucchini et al. (34)	Compassionate use (n=5; age: 5-12)	BM [0.7-1.4] (1-4)		40% (D28)	80% (D28)	100%*

(Continued)

TABLE 2 | Continued

First-line Treatment of Acute GvHD, in Combination With Corticosteroids						
Reference	**Study Type[a]**	**MSC source [dose[b]] (# of infusions)**	**Group (if applicable)**	**D28 Response**	**OS**	
				CR	**OR**	
Perez-Simon et al. (50)	Phase 1/2 CT (n=10; age: 21-66)	BM [0.2-1.2] (1-4)		13%*	50%*	63%*
Herrmann et al. (51)	Phase 1 CT (n=12; age: 31-53)	BM [1.7-2.3] (2-19)		29%*	57%*	29% (1y)
Jurado et al. (56)	Phase 1/2 CT (n=14; age: 24-60)	UCB [1 or 3] (1)	Low dose:	57% (pooled cohorts) (1y)	67% (1y)	67% (1y)
			High dose:	80% (1y)	80% (1y)	
Salmenniemi et al. (39)	Compassionate use (n=4; age: 37-63)	BM [2] (1-6)		No response	No response	25%* (3m)
Boberg et al. (57)	Phase 1 CT (n=11; age: 20-61)	BM [2] (6-9)		Not reported	55%*	82%*

a. No internal control group unless stated.

b. Dose expressed as 10^6 cells/kg.

CR, complete response; OR, overall response; OS, overall survival; CT, clinical trial; BAT, best available therapy; BM, bone marrow; AT, adipose tissue; UCB, umbilical cord blood; iPSC, induced pluripotent stem cell; y, year; D, day.

** Timeframe for assessment not specified, and duration of follow-up varied between patients in some cases.*

DISCUSSION

There is broad consensus that MSCs are generally safe and well tolerated (17, 21). None of the published studies of MSCs in GvHD reviewed here identified any significant safety issues. This is consistent with the wider experience of MSCs in the treatment of other conditions. A systematic review of MSCs in 55 clinical trials, in which 2,696 patients received MSC treatment, found an association between MSCs and transient fever, but not with acute infusional toxicity, infection, thrombotic/embolic events, death or malignancy (58).

Clinical studies of MSCs for SR-aGvHD in particular have generated encouraging efficacy results, but response rates have varied. For example, four studies of MSCs in SR-aGvHD have reported D28 OR rates exceeding 80% (32, 35, 40, 44) and a further three have reported D28 OR rates of at least 70% (34, 37, 43). However, a number of other published studies have reported lower D28 OR rates, ranging from 50-68% (33, 36, 38, 39, 41, 42).

There has been even greater variability in D28 CR rates, with an overall range of 8-75% reported in SR-aGvHD patients (32–44). Notably, CR and OR rates do not necessarily correlate: one study that reported a very high D28 OR rate (93%) also reported a very high D28 CR rate (75%), while another study reported a D28 OR rate of 100%, but a CR rate of just 33% (32, 35). While the latter study had a very small sample size (n=5), this inconsistency was also evident in larger studies: in a compassionate use study with the commercial MSC product remestemcel-L (n=241) the D28 OR and CR rates were 65% and 14% (i.e. 21% of responders were complete responders) (42); while in a Phase 3 clinical trial with the same product (n=260), the D28 OR and CR rates were 58% and 37% (i.e. 64% of responders were complete responders).

Overall, outcomes with MSCs compare favorably to those reported with other second-line agents. In clinical trials in patients with SR-aGvHD, ruxolitinib treatment led to D28 OR rates of 55-62% and D28 CR rates of 27-34% (59, 60); etanercept treatment led to D28 OR rates of 50-53% and D28 CR rates of 0-20% (61, 62); while one-month OR and CR rates in patients treated with ECP were <50% and 33%, respectively (63). It should also be noted that some safety concerns have been associated with ruxolitinib and etanercept.

Caution must be exercised in comparing results between studies. Confounding factors include variability in MSC donor types, MSC dose per infusion, and number of infusions per patient – even within the same trial in some instances. A further issue is that there is no universally accepted definition of steroid-resistance. Clinical trials in SR-aGvHD typically require patients to have failed to respond despite treatment with corticosteroids, but the minimum period of treatment required varies between trials [e.g. 3 days (48) or 7 days (41)]. Additionally, many of the published studies have been compassionate use programs rather than formal, prospective clinical trials, while most of the clinical trials have been open-label studies with no control group.

Importantly, there has also been a lack of standardization on the timeframe for assessment of outcome measures. The most common timepoint to assess acute GvHD response has been 28 days, and for that reason D28 CR and OR rates are shown in separate columns in **Table 2**. However, some studies assessed CR and OR at different specified timepoints, while in many studies the timeframe for response assessment was not specified, meaning that a response at any time during follow-up was counted. In studies where response rates were assessed at more than one timepoint, there was a marked increase in response rates at later timepoints (44, 45). Consequently, D28 response rates cannot be compared with response rates at later timepoints, or response rates at unspecified timepoints.

Similarly, many studies have reported OS rates at the time of last follow-up, and the duration of follow-up per patient has

typically varied both within and between studies. Furthermore, some studies have reported OS only at early timepoints such as D100 or D180, which are likely to be too soon to draw any conclusions.

Greater standardization in the design of future clinical trials would facilitate more robust evaluation of the efficacy of potential GvHD treatments. In recent years, D28 OR rate has been the primary endpoint in several Phase 2 and 3 clinical trials in SR-aGvHD, including those that supported FDA approval of ruxolitinib (43, 59, 60). Although not necessarily the primary endpoint, this outcome measure has also been reported in numerous other MSC trials, along with D28 CR rate (32–42, 44). Another important consideration is what, if any, control group to include. Until recently, as there were no treatments specifically approved for SR-aGvHD, the only ethical control options in SR-aGvHD trials were: a best available therapy (BAT) control group; an external control group; or no control group. Each of those options had limitations, but in light of the recent FDA approval, the possibility now exists to conduct trials in SR-aGvHD with ruxolitinib as a control. A proposed Phase 3 trial of a monoclonal antibody-based treatment in SR-aGvHD was recently registered on clinicaltrials.gov, which aims to demonstrate that the investigational agent is superior to ruxolitinib based on D28 CR rate (64). A similar design may be suitable for late-stage trials of MSC products, while a non-inferiority design might also be sufficient to support approval, especially as MSCs appear to have a very good safety profile. However, the inclusion of a ruxolitinib control might remain challenging in multinational studies, as ruxolitinib is not yet approved for the treatment of SR-aGvHD in the European Union or many other jurisdictions.

Another notable variable lies in the MSC manufacturing processes used. Academic studies have typically used minimally expanded bone marrow-derived MSCs, but most commercially-sponsored studies have utilized bone marrow-derived MSCs that were extensively expanded using industrial-scale processes.

The initial trial with a commercially produced bone marrow-derived product (remestemcel-L) in acute GvHD showed positive results (31). However, the results of that study are difficult to interpret in the context of other published studies, as it involved first-line treatment of acute GvHD in combination with corticosteroids, rather than treatment of SR-aGvHD. Additionally, the study did not include a control group, and first-line acute GvHD treatment with corticosteroids in the absence of MSCs has been shown to result in CR and OR rates as high as 69% and 78%, respectively (65). A subsequent randomized-controlled Phase 3 trial of remestemcel-L in patients with SR-aGvHD was completed in 2009, but with disappointing outcomes, which were belatedly published in 2020 (41). The trial found that remestemcel-L treatment led to significantly improved OR and durable CR rates in patients with liver GvHD. There was also a higher OR rate in children treated with remestemcel-L compared to controls. Nonetheless, the trial failed to meet its primary endpoint – there was no statistical difference between the durable complete response rate in patients treated with remestemcel-L in comparison to those treated with placebo. In more recent years, further trials with remestemcel-L in SR-aGvHD have been completed, including: two single arm, open-label clinical trials in adults and children in Japan (n=14 and n=25, respectively) (35, 38); a single arm, open-label clinical trial in children in the USA (n=51) (43) and a large compassionate use study in children in the USA (n=241) (42). Those studies have generated more positive results, but CR rates in particular have been mixed (24-75% in Japan, and 14-30% in the USA).

A number of suggestions have been offered to explain the apparent inconsistency in outcomes between trials. A review published in 2013 observed that the most striking difference between academic and commercial MSC treatments was the extent of MSC expansion – ranging from the production of 5-10 doses per bone marrow donation at academic centers, to 10,000 doses with remestemcel-L (66). There is a substantial body of evidence in the literature demonstrating that extensive culture expansion of bone marrow-derived MSCs leads to marked changes in functionality (67, 68). There is also evidence that clinical efficacy of MSCs is impaired even by modest levels of expansion (69).

It is well-established that there is a high level of inter-donor variability in MSC properties. For example, MSC gene expression, differentiation, proliferation and colony-forming capacity vary markedly between donors (67, 70). The susceptibility of MSCs to activation by IFN-γ and TNF-α, and the consequent upregulation of IDO expression and suppression of T cell proliferation, is also donor-dependent (24, 71, 72). With respect to processes that rely on isolation of MSCs from random donors, this variability may lead to an unpredictable variability in efficacy – between, and potentially within, studies.

It has also been suggested that cryopreserving MSCs and then administering the cells immediately post-thaw may impair their functionality (66). However, the same approach has been used in the majority of clinical trials involving allogeneic MSCs, many of which have generated positive results.

A number of groups have attempted to circumvent the challenges associated with inter-donor variability and extensive MSC expansion using novel manufacturing approaches. One such approach is to generate an MSC bank from pooled bone marrow donations from multiple donors. There is evidence that this approach, known as the "MSC-FFM" method, can facilitate consistency within an MSC bank, with encouraging clinical trial results (40). By pooling donations, a larger quantity of MSCs can be produced compared to a single-donor bank with a similar level of expansion. However, based on the upper end of the dose regimen range used in the initial clinical trial, we calculate that each bank would only suffice for the treatment of approximately 175 patients. There is also a need to investigate consistency between banks produced using this method.

An alternative approach is to rely on pluripotent stem cells (PSCs) as a starting material for the production of MSCs. PSCs have the capacity to replicate indefinitely without loss of pluripotency, in addition to the ability to differentiate into any adult cell type. This means that a single bank of PSCs has the

potential to give rise to an effectively limitless number of therapeutic cells. There are two types of PSCs: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). While both types of PSCs have broadly similar properties, research and commercialisation of ESC-based therapies has been hampered by ethical controversy and political/funding constraints. These issues do not apply to iPSCs, which are derived from adult cells. The generation of human iPSCs was first reported by two independent groups in 2007 (73, 74). To illustrate their enormous self-replication capacity, it has been reported that even after $10^{(71)}$-fold expansion in culture, iPSCs retain their ability to differentiate into all three germ-layers (75). We have conducted a Phase 1 clinical trial in SR-aGvHD with iPSC-derived MSCs produced using a proprietary process (Cymerus™, Cynata Therapeutics Limited) (44). In contrast to processes reliant on the isolation of primary MSCs from donated tissue, a single iPSC bank has the capacity to produce 29 million clinical doses (each containing $1x10^8$ MSCs) using this process, at current scale. Thus, problems associated with inter-donor variability would be virtually eliminated. Furthermore, as this process achieves its scale by expansion at the iPSC stage, and prior to differentiation of the cells into MSCs, it involves relatively little expansion at the MSC stage. This is expected to minimise the type of functional changes that have been observed after extensive expansion of primary MSCs.

The small number of published studies in chronic GvHD comprise compassionate use studies or Phase 1/2 clinical trials, and all have had small sample sizes (n=1-14). Results have been mixed, with responses of 0-57% (CR) and 0-80% (OR), at various, and in some cases unspecified, timepoints (34, 39, 47, 50, 51, 56, 57). It is difficult to draw conclusions from this limited dataset.

As represented in **Figure 1**, multifactorial effects of MSCs have been identified, which include transfer of exosomes, microvesicles and organelles, and paracrine activity mediated by secretion of immunomodulatory molecules (20–26). In recent years, it has been suggested that the immunomodulatory effects of MSCs result in part from apoptosis, and the subsequent release of apoptotic extracellular vesicles and activation of IDO production in macrophages (27). In addition to the fact that MSCs act in numerous different ways, a further complication is that MSCs target a wide range of cells *in vivo*. In the context of GvHD, the target cells fall into two main categories: (i) immune cells from the host and HSCT donor; and (ii) cells that are damaged by GvHD, such as cells of the skin, liver and gastrointestinal tract. It may be that this diverse arsenal of mechanisms gives MSCs an advantage over more conventional single-target therapeutic agents, especially against a disease such as GvHD, which itself is underpinned by complex pathology involving a multitude of cell types and pathways. However, this also makes it extremely challenging to comprehensively elucidate the mechanisms of action of MSCs, either in general or with respect to the treatment of GvHD in particular. An improved understanding of MSC mechanisms of action would be beneficial for the clinical community, as well as providing a basis for the development of *in vitro* potency assays, to help identify and address problems with MSC variability.

CONCLUSION

A substantial body of evidence suggests that MSCs have a beneficial effect in treating SR-aGvHD. Recent innovations may be capable of overcoming problems associated with inter-donor variability and functional changes associated with extensive culture expansion. Further adequately powered prospective studies are required to confirm efficacy and establish the place of MSC therapy in the treatment of this condition.

Experience to date with MSCs as a treatment for chronic GvHD is much more limited. The prevalence of clinical investigation of MSCs for acute GvHD versus chronic GvHD might suggest that the clinical community has identified more promise in the former, but further investigation in chronic GvHD appears to be warranted.

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KK and JR drafted and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Garnett C, Apperley JF, Pavlů J. Treatment and Management of Graft-Versus-Host Disease: Improving Response and Survival. *Ther Adv Hematol* (2013) 4(6):366–78. doi: 10.1177/2040620713489842

2. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-Versus-Host Disease: I. Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant* (2005) 11:945–56. doi: 10.1016/j.bbmt.2005.09.004

3. Aractingi S, Chosidow O. Cutaneous Graft-Versus-Host Disease. *Arch Dermatol* (1998) 134(5):602–12. doi: 10.1001/archderm.134.5.602
4. Inamoto Y, Storer BE, Petersdorf EW, Nelson JL, Lee SJ, Carpenter PA, et al. Incidence, Risk Factors, and Outcomes of Sclerosis in Patients With Chronic Graft-Versus-Host Disease. *Blood* (2013) 121(25):5098–103. doi: 10.1182/blood-2012-10-464198
5. Socié G, Ritz J. Current Issues in Chronic Graft-Versus-Host Disease. *Blood* (2014) 124(3):374–84. doi: 10.1182/blood-2014-01-514752
6. Phelan R, Arora M, Chen M. Current Use and Outcome of Hematopoietic Stem Cell Transplantation: CIBMTR US Summary Slides. (2020). Available at: https://www.cibmtr.org/ReferenceCenter/SlidesReports/SummarySlides/Pages/index.aspx.
7. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant* (1995) 15(6):825–8.
8. Jagasia MH, Greinix HT, Arora M, Williams KM, Wolff D, Cowen EW, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-Versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant* (2015) 21(3):389–401. doi: 10.1016/j.bbmt.2014.12.001
9. Penack O, Marchetti M, Ruutu T, Aljurf M, Bacigalupo A, Bonifazi F, et al. Prophylaxis and Management of Graft Versus Host Disease After Stem-Cell Transplantation for Haematological Malignancies: Updated Consensus Recommendations of the European Society for Blood and Marrow Transplantation. *Lancet Haematol* (2020) 7(2):e157–67. doi: 10.1016/S2352-3026(19)30256-X
10. Westin JR, Saliba RM, De Lima M, Alousi A, Hosing C, Qazilbash MH, et al. Steroid-Refractory Acute GVHD: Predictors and Outcomes. *Adv Hematol* (2011) 2011:601953. doi: 10.1155/2011/601953
11. Flowers MED, Martin PJ. How We Treat Chronic Graft-Versus-Host Disease. *Blood* (2015) 125(4):606–15. doi: 10.1182/blood-2014-08-551994
12. Food and Drug Administration. *FDA Approves Ruxolitinib for Acute Graft-Versus-Host Disease* (2019). Available at: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-ruxolitinib-acute-graft-versus-host-disease (Accessed September 29, 2021).
13. Food and Drug Administration. *FDA Approves Belumosudil for Chronic Graft-Versus-Host Disease* (2021). Available at: https://www.fda.gov/drugs/drug-approvals-and-databases/fda-approves-belumosudil-chronic-graft-versus-host-disease (Accessed September 29, 2021).
14. Mannina D, Kröger N. Janus Kinase Inhibition for Graft-Versus-Host Disease: Current Status and Future Prospects. *Drugs* (2019) 79(14):1499–509. doi: 10.1007/s40265-019-01174-1
15. Cottliar JA. *Atlas of Graft Versus Host Disease*. Cham, Switzerland: Springer International Publishing AG (2017).
16. Saidu NEB, Bonini C, Dickinson A, Grce M, Inngjerdingen M, Koehl U, et al. New Approaches for the Treatment of Chronic Graft-Versus-Host Disease: Current Status and Future Directions. *Front Immunol* (2020) 11:578314. doi: 10.3389/fimmu.2020.578314
17. Galderisi U, Peluso G, Di Bernardo G. Clinical Trials Based on Mesenchymal Stromal Cells are Exponentially Increasing: Where are We in Recent Years? *Stem Cell Rev Rep* (2021) 16:1–14. doi: 10.1007/s12015-021-10231-w
18. Crippa S, Santi L, Berti M, De Ponti G, Bernardo ME. Role of Ex Vivo Expanded Mesenchymal Stromal Cells in Determining Hematopoietic Stem Cell Transplantation Outcome. *Front Cell Dev Biol* (2021) 9:663316. doi: 10.3389/fcell.2021.663316
19. Introna M, Golay J. Tolerance to Bone Marrow Transplantation: Do Mesenchymal Stromal Cells Still Have a Future for Acute or Chronic GvHD? *Front Immunol* (2020) 11:609063. doi: 10.3389/fimmu.2020.609063
20. Spees JL, Lee RH, Gregory CA. Mechanisms of Mesenchymal Stem/Stromal Cell Function. *Stem Cell Res Ther* (2016) 7:125. doi: 10.1186/s13287-016-0363-7
21. Sharma RR, Pollock K, Hubel A, McKenna D. Mesenchymal Stem or Stromal Cells: A Review of Clinical Applications and Manufacturing Practices. *Transfusion* (2014) 54(5):1418–37. doi: 10.1111/trf.12421
22. Galipeau J, Sensébé L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell* (2018) 22(6):824–33. doi: 10.1016/j.stem.2018.05.004
23. Harrell CR, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Mesenchymal Stem Cell-Derived Exosomes and Other Extracellular Vesicles as New Remedies in the Therapy of Inflammatory Diseases. *Cells* (2019) 8(12):E1605. doi: 10.3390/cells8121605
24. Weiss ARR, Dahlke MH. Front. Immunomodulation by Mesenchymal Stem Cells (MSCs): Mechanisms of Action of Living, Apoptotic, and Dead MSCs. *Front Immunol* (2019) 10:1191. doi: 10.3389/fimmu.2019.01191
25. Martin I, Galipeau J, Kessler C, Le Blanc K, Dazzi F. Challenges for Mesenchymal Stromal Cell Therapies. *Sci Transl Med* (2019) 20 11(480):pii: eaat2189. doi: 10.1126/scitranslmed.aat2189
26. Terness P, Bauer TM, Röse L, Dufter C, Watzlik A, Simon H, et al. Inhibition of Allogeneic T Cell Proliferation by Indoleamine 2,3-Dioxygenase–expressing Dendritic Cells. *J Exp Med* (2002) 196(4):447–57. doi: 10.1084/jem.20020052
27. Fu Y, Sui B, Xiang L, Yan X, Wu D, Shi S, et al. Emerging Understanding of Apoptosis in Mediating Mesenchymal Stem Cell Therapy. *Cell Death Dis* (2021) 12:596. doi: 10.1038/s41419-021-03883-6
28. Galleu A, Riffo-Vasquez Y, Trento C, Lomas C, Dolcetti L, Cheung TS, et al. Apoptosis in Mesenchymal Stromal Cells Induces *In Vivo* Recipient-Mediated Immunomodulation. *Sci Transl Med* (2017) 9(416):7828. doi: 10.1126/scitranslmed.aam7828
29. Ozay EI, Vijayaraghavan J, Gonzalez-Perez G, Shanthalingam S, Sherman HL, Garrigan DT, et al. Cymerus™ iPSC-MSCs Significantly Prolong Survival in a Pre-Clinical, Humanized Mouse Model of Graft-Vs-Host Disease. *Stem Cell Res* (2019) 35:101401. doi: 10.1016/j.scr.2019.101401
30. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of Severe Acute Graft-Versus-Host Disease With Third Party Haploidentical Mesenchymal Stem Cells. *Lancet* (2004) 363:1439–41. doi: 10.1016/S0140-6736(04)16104-7
31. Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, et al. Adult Human Mesenchymal Stem Cells Added to Corticosteroid Therapy for the Treatment of Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2009) 15:804 11. doi: 10.1016/j.bbmt.2008.03.012
32. Soder RP, Dawn B, Weiss ML, Dunavin N, Weir S, Mitchell J, et al. A Phase I Study to Evaluate Two Doses of Wharton's Jelly-Derived Mesenchymal Stromal Cells for the Treatment of *De Novo* High-Risk or Steroid-Refractory Acute Graft Versus Host Disease. *Stem Cell Rev Rep* (2020) 16:979–91. doi: 10.1007/s12015-020-10015-8
33. von Bonin M, Stölzel F, Goedecke A, Richter K, Wuschek N, Hölig K, et al. Treatment of Refractory Acute GVHD With Third-Party MSC Expanded in Platelet Lysate-Containing Medium. *Bone Marrow Transplant* (2009) 43:245–51. doi: 10.1038/bmt.2008.316
34. Lucchini G, Introna M, Dander E, Rovelli A, Balduzzi A, Bonanomi S, et al. Platelet-Lysate-Expanded Mesenchymal Stromal Cells as a Salvage Therapy for Severe Resistant Graft-Versus-Host Disease in a Pediatric Population. *Biol Blood Marrow Transplant* (2010) 16:1293–301. doi: 10.1016/j.bbmt.2010.03.017
35. Muroi K, Miyamura K, Ohashi K, Murata M, Eto T, Kobayashi N, et al. Unrelated Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells for Steroid-Refractory Acute Graft-Versus-Host Disease: A Phase I/II Study. *Int J Hematol* (2013) 98:206–13. doi: 10.1007/s12185-013-1399-4
36. Introna M, Lucchini G, Dander E, Galimberti S, Rovelli A, Balduzzi A. Treatment of Graft Versus Host Disease With Mesenchymal Stromal Cells: A Phase I Study on 40 Adult and Pediatric Patients. *Biol Blood Marrow Transplant* (2014) 20:375–81. doi: 10.1016/j.bbmt.2013.11.033
37. Zhao K, Lou R, Huang F, Peng Y, Jiang Z, Huang K, et al. Immunomodulation Effects of Mesenchymal Stromal Cells on Acute Graft-Versus-Host Disease After Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2015) 21:97–104. doi: 10.1016/j.bbmt.2014.09.030
38. Muroi K, Miyamura K, Okada M, Yamashita T, Murata M, Ishikawa T, et al. Bone Marrow–Derived Mesenchymal Stem Cells (JR–031) for Steroid –Refractory Grade III or IV Acute Graft–Versus–Host Disease: A Phase II/III Study. *Int J Hematol* (2016) 103:243–50. doi: 10.1007/s12185-015-1915-9
39. Salmenniemi U, Itälä-Remes M, Nystedt J, Putkonen M, Niittyvuopio R, Vettenranta K, et al. Good Responses But High TRM in Adult Patients After

MSC Therapy for GvHD. *Bone Marrow Transplant* (2017) 52:606–8. doi: 10.1038/bmt.2016.317
40. Bader P, Kuçi Z, Bakhtiar S, Basu O, Bug G, Dennis M, et al. Effective Treatment of Steroid and Therapy-Refractory Acute Graft Versus-Host Disease With a Novel Mesenchymal Stromal Cell Product (MSC-FFM). *Bone Marrow Transplant* (2018) 53:852–62. doi: 10.1038/s41409-018-0102-z
41. Kebriaei P, Hayes J, Daly A, Uberti J, Marks DI, Soiffer R, et al. A Phase 3 Randomized Study of Remestemcel-L Versus Placebo Added to Second-Line Therapy in Patients With Steroid-Refractory Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2020) 26(5):835–44. doi: 10.1016/j.bbmt.2019.08.029
42. Kurtzberg J, Prockop D, Chaudhury S, Horn B, Nemecek E, Prasad V, et al. Study 275: Updated Expanded Access Program for Remestemcel-L in Steroid-Refractory Acute Graft-Versus-Host Disease in Children. *Biol Blood Marrow Transplant* (2020) 26:855–64. doi: 10.1016/j.bbmt.2020.01.026
43. Kurtzberg J, Abdel-Azim H, Carpenter P, Chaudhury S, Horn B, Mahadeo K, et al. A Phase 3, Single-Arm, Prospective Study of Remestemcel-L, *Ex Vivo* Culture-Expanded Adult Human Mesenchymal Stromal Cells for the Treatment of Pediatric Patients Who Failed to Respond to Steroid Treatment for Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2020) 26:845–54. doi: 10.1016/j.bbmt.2020.01.018
44. Bloor AJC, Patel A, Griffin JE, Gilleece MH, Radia R, Yeung DT, et al. Production, Safety and Efficacy of iPSC-Derived Mesenchymal Stromal Cells in Acute Steroid-Resistant Graft Versus Host Disease: A Phase I, Multicenter, Open-Label, Dose-Escalation Study. *Nat Med* (2020) 26:1720–5. doi: 10.1038/s41591-020-1050-x
45. Prasad VK, Lucas KG, Kleiner GI, Talano JAM, Jacobsohn D, Broadwater G, et al. Efficacy and Safety of *Ex Vivo* Cultured Adult Human Mesenchymal Stem Cells (Prochymal™) in Pediatric Patients With Severe Refractory Acute Graft-Versus-Host Disease in a Compassionate Use Study. *Biol Blood Marrow Transplant* (2011) 17(4):534–41. doi: 10.1016/j.bbmt.2010.04.014
46. Sánchez-Guijo F, Caballero-Velázquez T, López-Villar O, Redondo A, Parody R, Martínez C, et al. Sequential Third-Party Mesenchymal Stromal Cell Therapy for Refractory Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2014) 20:1580–5. doi: 10.1016/j.bbmt.2014.06.015
47. Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lönnies H, et al. Mesenchymal Stem Cells for Treatment of Therapy-Resistant Graft-Versus-Host Disease. *Transplantation* (2006) 81:1390–7. doi: 10.1097/01.tp.0000214462.63943.14
48. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal Stem Cells for Treatment of Steroid-Resistant, Severe, Acute Graft-Versus-Host Disease: A Phase II Study. *Lancet* (2008) 371:1579–86. doi: 10.1016/S0140-6736(08)60690-X
49. Arima N, Nakamura F, Fukunaga A, Hirata H, Machida H, Kouno S, et al. Single Intra-Arterial Injection of Mesenchymal Stromal Cells for Treatment of Steroid-Refractory Acute Graft-Versus-Host Disease: A Pilot Study. *Cytotherapy* (2010) 12:265–8. doi: 10.3109/14653240903390795
50. Pérez-Simon JA, López-Villar O, Andreu EJ, Rifón J, Muntion S, Diez Campelo M, et al. Mesenchymal Stem Cells Expanded *In Vitro* With Human Serum for the Treatment of Acute and Chronic Graft-Versus-Host Disease: Results of a Phase I/II Clinical Trial. *Haematologica* (2011) 96:1072–6. doi: 10.3324/haematol.2010.038356
51. Herrmann R, Sturm M, Shaw K, Purtill D, Cooney J, Wright M, et al. Mesenchymal Stromal Cell Therapy for Steroid-Refractory Acute and Chronic Graft Versus Host Disease: A Phase 1 Study. *Int J Hematol* (2012) 95:182–8. doi: 10.1007/s12185-011-0989-2
52. Ball LM, Bernardo ME, Roelofs H, van Tol MJD, Contoli B, Zwaginga JJ, et al. Multiple Infusions of Mesenchymal Stromal Cells Induce Sustained Remission in Children With Steroid-Refractory, Grade III–IV Acute Graft-Versus-Host Disease. *Br J Haematol* (2013) 163:501–9. doi: 10.1111/bjh.12545
53. Resnick IB, Barkats C, Shapira MY, Stepensky P, Bloom AI, Shimoni A, et al. Treatment of Severe Steroid Resistant Acute GVHD With Mesenchymal Stromal Cells (MSC). *Am J Blood Res* (2013) 3:225–38.
54. Von Dalowski F, Kramer M, Wermke M, Wehner R, Röllig C, Alakel N, et al. Mesenchymal Stromal Cells for Treatment of Acute Steroid-Refractory Graft Versus Host Disease: Clinical Responses and Long-Term Outcome. *Stem Cells* (2016) 34:357–66. doi: 10.1002/stem.2224
55. Dotoli GM, De Santis GC, Orellana MD, de Lima Prata K, Caruso SR, Fernandes TR, et al. Mesenchymal Stromal Cell Infusion to Treat Steroid-Refractory Acute GvHD III/IV After Hematopoietic Stem Cell Transplantation. *Bone Marrow Transplant* (2017) 52:859–62. doi: 10.1038/bmt.2017.35
56. Jurado M, de la Mata C, Ruiz García A, López-Fernández E, Espinosa O, Remigia MJ, et al. Adipose Tissue-Derived Mesenchymal Stromal Cells as Part of Therapy for Chronic Graft-Versus-Host Disease: A Phase I/II Study. *Cytotherapy* (2017) 19:927–36. doi: 10.1016/j.jcyt.2017.05.002
57. Boberg E, von Bahr L, Afram G, Lindström C, Ljungman P, Heldring N, et al. Treatment of Chronic GvHD With Mesenchymal Stromal Cells Induces Durable Responses: A Phase II Study. *Stem Cells Transl Med* (2020) 9:1190–202. doi: 10.1002/sctm.20-0099
58. Thompson M, Mei SHJ, Wolfe D, Champagne J, Fergusson D, Stewart DJ, et al. Cell Therapy With Intravascular Administration of Mesenchymal Stromal Cells Continues to Appear Safe: An Updated Systematic Review and Meta-Analysis. *EClinicalMedicine* (2020) 19:100249. doi: 10.1016/j.eclinm.2019.100249
59. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *N Engl J Med* (2020) 382(19):1800–10. doi: 10.1056/NEJMoa1917635
60. Jagasia M, Perales MA, Schroeder MA, Ali H, Shah NN, Chen YB, et al. Ruxolitinib for the Treatment of Steroid-Refractory Acute GVHD (REACH1): A Multicenter, Open-Label, Phase 2 Trial. *Blood* (2020) 135(20):1739–49. doi: 10.1182/blood.2020004823
61. De Jong CN, Saes L, Klerk CPW, van der Klift M, Cornelissen JJ, Broers AEC. Etanercept for Steroid-Refractory Acute Graft-Versus-Host Disease: A Single Center Experience. *PloS One* (2017) 12(10):e0187184. doi: 10.1371/journal.pone.0187184
62. Park JH, Lee HJ, Kim SR, Song GW, Lee SK, Park SY, et al. Etanercept for Steroid-Refractory Acute Graft Versus Host Disease Following Allogeneic Hematopoietic Stem Cell Transplantation. *Korean J Intern Med* (2014) 29(5):6306. doi: 10.3904/kjim.2014.29.5.630
63. Greinix HT, Volc-Platzer B, Kahls P, Fischer G, Rosenmayr A, Keil F, et al. Extracorporeal Photochemotherapy in the Treatment of Severe Steroid-Refractory Acute Graft-Versus-Host Disease: A Pilot Study. *Blood* (2000) 96:2426–31. doi: 10.1182/blood.V96.7.2426
64. US National Library of Medicine. A Study to Compare T-Guard vs Ruxolitinib for Treatment of Steroid-Refractory Acute Graft-Versus-Host Disease (SR-aGVHD) (BMT CTN 2002) (2002)(2021). Available at: https://clinicaltrials.gov/ct2/show/NCT04934670 (Accessed September 29, 2021).
65. Martin PJ, Rizzo JD, Wingard JR, Ballen K, Curtin PT, Cutler C, et al. First- and Second-Line Systemic Treatment of Acute Graft-Versus-Host Disease: Recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant* (2012) 18:1150–63. doi: 10.1016/j.bbmt.2012.04.005
66. Galipeau J. The Mesenchymal Stromal Cells Dilemma–Does a Negative Phase III Trial of Random Donor Mesenchymal Stromal Cells in Steroid-Resistant Graft-Versus-Host Disease Represent a Death Knell or a Bump in the Road? *Cytotherapy* (2013) 15(1):2–8. doi: 10.1016/j.jcyt.2012.10.002
67. Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, et al. Replicative Senescence of Mesenchymal Stem Cells: A Continuous and Organized Process. *PloS One* (2008) 3(5):e2213. doi: 10.1371/journal.pone.0002213
68. De Witte SFH, Lambert EE, Merino A, Strini T, Douben HJCW, O'Flynn L, et al. Aging of Bone Marrow– and Umbilical Cord–Derived Mesenchymal Stromal Cells During Expansion. *Cytotherapy* (2017) 19:798–807. doi: 10.1016/j.jcyt.2017.03.071
69. von Bahr L, Sundberg B, Lönnies L, Sander B, Karbach H, Hägglund H, et al. Long-Term Complications, Immunologic Effects, and Role of Passage for Outcome in Mesenchymal Stromal Cell Therapy. *Biol Blood Marrow Transplant* (2012) 18(4):557–64. doi: 10.1016/j.bbmt.2011.07.023
70. Siegel G, Kluba T, Hermanutz-Klein U, Bieback K, Northoff H, Schäfer R. Phenotype, Donor Age and Gender Affect Function of Human Bone Marrow-Derived Mesenchymal Stromal Cells. *BMC Med* (2013) 11:146. doi: 10.1186/1741-7015-11-146

71. Wegmeyer H, Bröske AM, Leddin M, Kuentzer K, Nisslbeck AK, Hupfeld J, et al. Mesenchymal Stromal Cell Characteristics Vary Depending on Their Origin. *Stem Cells Dev* (2013) 22(19):2606–18. doi: 10.1089/scd.2013.0016
72. Ketterl N, Brachtl G, Schuh C, Bieback K, Schallmoser K, Reinisch A, et al. A Robust Potency Assay Highlights Significant Donor Variation of Human Mesenchymal Stem/Progenitor Cell Immune Modulatory Capacity and Extended Radio-Resistance. *Stem Cell Res Ther* (2015) 6:236. doi: 10.1186/s13287-015-0233-8
73. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of Pluripotent Stem Cells From Adult Human Fibroblasts by Defined Factors. *Cell* (2007) 131:861–72. doi: 10.1016/j.cell.2007.11.019
74. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced Pluripotent Stem Cell Lines Derived From Human Somatic Cells. *Science* (2007) 318:1917–20. doi: 10.1126/science.1151526
75. Lei Y, Schaffer DV. A Fully Defined and Scalable 3D Culture System for Human Pluripotent Stem Cell Expansion and Differentiation. *Proc Natl Acad Sci USA* (2013) 24:110:E5039–5048. doi: 10.1073/pnas.1309408110

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REVIEW
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Translational Clinical Strategies for the Prevention of Gastrointestinal Tract Graft *Versus* Host Disease

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Graft versus host disease (GVHD) is the major non-relapse complication associated with allogeneic hematopoietic stem cell transplantation (HSCT). Unfortunately, GVHD occurs in roughly half of patients following this therapy and can induce severe life-threatening side effects and premature mortality. The pathophysiology of GVHD is driven by alloreactive donor T cells that induce a proinflammatory environment to cause pathological damage in the skin, gastrointestinal (GI) tract, lung, and liver during the acute phase of this disease. Recent work has demonstrated that the GI tract is a pivotal target organ and a primary driver of morbidity and mortality in patients. Prevention of this complication has therefore emerged as an important goal of prophylaxis strategies given the primacy of this tissue site in GVHD pathophysiology. In this review, we summarize foundational pre-clinical studies that have been conducted in animal models to prevent GI tract GVHD and examine the efficacy of these approaches upon subsequent translation into the clinic. Specifically, we focus on therapies designed to block inflammatory cytokine pathways, inhibit cellular trafficking of alloreactive donor T cells to the GI tract, and reconstitute impaired regulatory networks for the prevention of GVHD in the GI tract.

Keywords: graft versus host disease, inflammatory cytokines, gastrointestinal tract, translational clinical trials, allogeneic hematopoietic stem cell transplantation, mouse models

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INTRODUCTION

Graft versus host disease (GVHD) is the major non-relapse cause of morbidity and mortality occurring after allogenic hematopoietic stem cell transplantation (HSCT) (1, 2). GVHD consists of both acute and chronic phases, which have distinguishing temporal and pathophysiological characteristics (3–5). Acute GVHD primarily targets the skin, gastrointestinal (GI) tract, lung, and liver, with the GI tract being the primary target organ that determines subsequent morbidity in patients (6). Involvement of this tissue site can be attributed to the conditioning regimen that licenses the gut to release damage (DAMPS) and pattern-associated molecular patterns (PAMPs) that activate and recruit innate immune cells (7). These cells then lead to the activation and clonal expansion of alloreactive T cells, which perpetuate a proinflammatory cascade that ultimately results in pathological damage (8). Ultimately, GVHD in the GI tract can result in protracted immune

suppression, infectious complications due to compromised mucosal integrity, and prolonged hospitalization.

Corticosteroids have long been first line therapy for patients with acute GVHD in the GI tract as they function to inhibit inflammatory pathways and cytokine production (9, 10). However, clinical responses do not occur in all patients as up to 50% can become refractory to systemic therapy (11). In addition, corticosteroids have side effects, which can be disabling and life threatening, including diabetes, infectious complications, and myopathy (12). For these patients, secondary agents for steroid resistant disease are much less effective and mortality is unacceptably high. Thus, prevention of this complication has emerged as a primary goal in the field in order to circumvent the need for prolonged immune suppressive therapy in patients who develop GVHD in this tissue site.

Amelioration of this complication in humans is therefore dependent upon increasing our understanding of the pathophysiology of GI GVHD. To unravel pathophysiologic mechanisms by which this disease is propagated and devise potentially translatable clinical strategies, animal models, primarily using mice, have been employed to examine how dysregulation of the immune system occurs in this setting (7, 13–16). From this work, a number of strategies have been examined that include the blockade of inflammatory cytokine pathways, the alteration of T cell trafficking into the GI tract, and the re-establishment of competent regulatory mechanisms (**Figure 1**). In this review, we highlight recent pre-clinical studies in each of these areas and examine the results from the subsequent clinical trials that have emerged as a direct translation of this work in human allogeneic HSCT recipients.

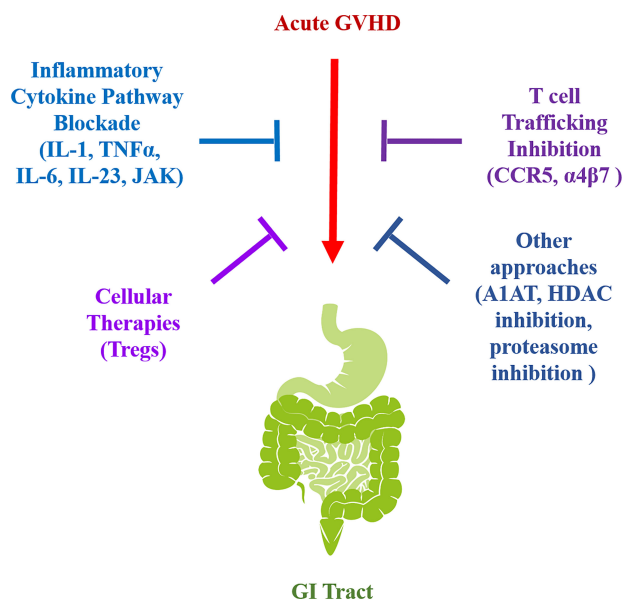

FIGURE 1 | Graphical schematic summarizing the translational strategies for GI GVHD prevention.

BLOCKADE OF INFLAMMATORY CYTOKINE PATHWAYS

Interleukin 1

Interleukin-1 (IL-1) was the first interleukin to be described and exhibits a myriad of functions that are critical for inflammation. IL-1, along with 10 other members, comprise the IL-1 super family since they possess a highly conserved gene structure and are primarily clustered in a 400kb region of human chromosome 2 (17, 18). While primarily relevant for promoting the activity of innate immune myeloid cells, IL-1 also plays a key role in the differentiation of T_H17 cells (19). The role of IL-1 has been explored preclinically in several immune-mediated diseases such as inflammatory bowel disease, asthma, and psoriasis but has mainly been tested clinically in rheumatoid arthritis with modest results (20).

McCarthy et al. first demonstrated that IL-1 could be a critical modulator of acute GVHD in murine studies. IL-1α was observed to be elevated in the skin of mice with GVHD and inhibition of IL-1 signaling with an IL-1R antagonist reduced GVHD mortality without impairing engraftment (21). Subsequently, Abhyankar et al. revealed that mRNA levels of IL-1 transcripts were increased several hundred-fold in GVHD target organs and also reported that IL-1R antagonist treatment could reduce mortality (22). Unfortunately, a later study showed only transient benefits of IL-1R antagonists in a minor antigen-disparate murine model and no effects in a fully MHC disparate model (23), suggesting that other pro-inflammatory cytokines may be able to compensate for deficiencies in IL-1 signaling during acute GVHD.

More recently, Park and colleagues evaluated the mechanism for how IL-1 blockade alleviates GVHD severity (24). They demonstrated that pretreatment of donor cells with an IL-1R antibody increased the proportion of Tregs to Th17 cells in host organs. Moreover, they observed decreased numbers of T cells and improved pathology in the GI tract, implicating a role for IL-1 in mediating intestinal inflammation during GVHD. In addition, Jankovic et al. demonstrated that early blockade of IL-1β as well as genetic deficiency of IL-1R in donor dendritic cells and T cells both improved GVHD-induced mortality (25). Correspondingly, immunohistochemical staining with IL-1β of intestinal biopsies revealed that the density of IL-1β staining correlated with augmented GVHD grades in patients. Altogether, these preclinical data suggested that IL-1 antagonism could improve GI GVHD by influencing donor T cell phenotypes and infiltration into the gut.

Based on preclinical data indicating that IL-1 inhibition could be beneficial for GVHD, Antin et al. tested whether treatment with a recombinant human IL-1 receptor antagonist could be beneficial for the prevention of acute GVHD in a phase I/II trial. They observed stage-specific improvements of GVHD in the skin, liver and particularly the GI tract (82% of patients) as well as demonstrated that the treatment was safe (26). Another phase I/II clinical study also showed improvements in 8/14 GVHD patients treated with a recombinant human IL-1 receptor antagonist, but only 33% of patients with GI-tract involvement

displayed improvement (27). Due to these preliminary results, Antin et al. performed a larger scale double-blind, randomized, placebo-controlled study including 186 patients who underwent allogenic stem cell transplantation. Either IL-1R antagonist or placebo was given to patients from day -4 to 10 days after conditioning with cyclophosphamide and total body irradiation. Disappointingly, they found that there was no statistically significant difference in the percentage of the patients in the IL-1R antagonist versus placebo group that developed moderate to severe GVHD. Furthermore, there was no difference in hematologic recovery, toxicity, or overall survival (28). Based largely on these negative results, specific inhibition of IL-1 signaling has largely been abandoned as a therapeutic approach to prevent GVHD globally and more specifically in the GI tract.

Tumor Necrosis Factor-Alpha

TNF-α, a pleiotropic inflammatory cytokine involved in the pathogenesis of rheumatoid arthritis, psoriasis, inflammatory bowel disease, and cancer also plays a role in acute GVHD. While TNFα is primarily produced by macrophages and monocytes during acute inflammation, context-dependent insults can induce TNFα to be released by lymphocytes, endothelial cells and other cell types as well. TNFα signaling occurs through 2 receptors; TNFR1, which is ubiquitously expressed and promotes inflammation and tissue damage, and TNFR2, which is restricted to a few cell types and responsible for homeostatic functions (29).

One of the first pre-clinical studies to evaluate the role of TNFα in GVHD was performed in 1987. Piguet et al. utilized a semi-allogenic murine model to assess whether administration of anti-TNFα antibodies eight days after GVHD induction could be beneficial. This therapeutic approach limited skin epidermal necrosis, reduced gut epithelial cell damage, and decreased mortality (30). The authors attributed the inflammatory effects of TNFα in the GI tract to be mediated by donor lymphocytes and potentially due to the increase of Ia expression on the gut mucosal epithelium. Moreover, pathological analysis revealed that TNFα induced gut dilatation with marked flattening of the villi and elevation of the crypts (30). More recent studies have expanded upon those initial results to help define the mechanisms of TNF-α during different stages of GVHD. Schmaltz et al. demonstrated that allogeneic T cells deficient in TNF induced significantly less morbidity and mortality compared to control T cells. Moreover, TNF deficiency in donor T cells induced reduced histological damage in the lower GI tract (31). Additional mechanistic studies by Stickel et al. demonstrated that miR-146a regulates the transcription of TNF levels and that T cells deficient in miR-146a induced augmented levels of TNF-α and worsened GVHD severity. Correspondingly, overexpression of miR-146a in donor T cells reduced TNFα levels and pathological damage in the small intestine and colon (32). Collectively, these preclinical studies provided rationale for utilizing anti-TNF therapies to treat GVHD patients with GI tract involvement.

Early clinical studies helped to define the kinetics of TNFα production following GVHD. This work demonstrated that systemic TNFα levels were increased during the conditioning phase (33) as well as early post transplantation (34, 35). Moreover, Holler et al. demonstrated that augmented levels of TNFα preceded complications of bone marrow transplantation and correlated with the development of acute GVHD symptoms, indicating that anti-TNFα therapy could be a promising option for GVHD prophylaxis.

Administration of infliximab, a chimeric IgG1 monoclonal antibody which binds to soluble and transmembrane human TNF-α, has been given to patients for GVHD treatment with some success (36), but there is limited clinical data on the efficacy of infliximab for GVHD prophylaxis, particularly in the GI tract. Hamadani et al. conducted a prospective trial of infliximab for the prophylaxis of GVHD (37). Infliximab or placebo was administered one day prior to conditioning, but unfortunately treated patients exhibited similar incidences of grade II-IV acute GVHD compared to the control group (both ~36%). Choi et al. performed a phase II clinical trial at two centers to test whether etanercept, which is a soluble receptor that binds to both TNFα and TNFβ, could reduce TNFR1 levels, ameliorate GVHD occurrence and improve survival (38). Surprisingly, etanercept did not influence TNFR1 levels in patients who received TBI-based conditioning but was rather effective in patients who received non-TBI based regiments. Etanercept treated patients who were not conditioned with TBI exhibited relatively low rates of grade III or IV GVHD (16%). Moreover, they reported that lower TNFR1 levels correlated with GVHD mortality. Unfortunately, this study did not test whether etanercept ameliorated the severity of GI GVHD. Overall, there have been only limited and inconclusive data that TNFα targeting strategies are efficacious for acute GVHD and none evaluating the prevention of GI GVHD.

Interleukin 6

IL-6 is a pleiotropic cytokine and plays a critical role in regulating acute and chronic inflammation, hematopoiesis, metabolic control, and metabolism. IL-6 can be produced by a variety of cells including fibroblasts, muscle cells, keratinocytes, monocytes, macrophages, and endothelial cells (39). During acute inflammation, monocytes and macrophages rapidly produce IL-6 in response to PAMPs and DAMPs. Moreover, IL-6 contributes to the differentiation of T_H17 cells and plays an integral role in skewing naïve T cells towards proinflammatory phenotypes limiting regulatory T cell (Treg) differentiation. Several studies have identified a role for IL-6 and members of the IL-6 superfamily (IL-11, IL-23, IL-27, and IL-31) in contributing to autoimmune disorders, cancer, and GVHD (40–42).

Pre-clinical murine studies by Chen et al. demonstrated that antibody-mediated blockade of IL-6R reduced pathologic damage associated with GVHD. Specifically, histological analyses revealed that IL-6R inhibition had a profound effect on minimizing inflammation within the colon. Mechanistically, inhibition of IL-6 signaling augmented the generation of Tregs and correspondingly reduced T_H1 and T_H17 cell expansion (42). Interestingly, the colon displayed the highest levels of IL-6 and IL-6R expression after GVHD. Both donor and host production

of IL-6 appeared to be important as transplantation with IL-$6^{-/-}$ recipient or donor mice had no protective effect on GVHD mortality. While Chen et al. demonstrated the importance of IL-6 in both the donor and recipient directions, another report observed that IL-6 deficiency in donor T cells was sufficient to protect mice from the effects of GVHD (43). The experimental designs of these studies however differed with respect to radiation dose, length of IL-6 inhibition, and purity of T cells in the transplant inoculum. This study also confirmed that administration of an anti-IL-6R antibody protected animals from lethal GVHD and reduced pathological damage in the GI tract, although there was no effect on Treg reconstitution.

Recently, the role of IL-6 during the pathophysiology of acute GVHD was further defined (44). This study sought to identify the cell types responsible for IL-6 signaling that perpetuate gut-associated GVHD. The authors conducted studies in which the IL-6R was specifically deleted from intestinal cells using Villin-Cre mice. They observed that this had no effect on acute GVHD pathology in the GI tract indicating that IL-6R expression in the GI tract was dispensable. Rather, subsequent experiments revealed that IL-6 secretion by recipient DCs was critical for initiating GVHD by way of classical signaling upon interactions with donor T cells. In fact, deletion of DC produced IL-6 specifically prevented the differentiation of pro-inflammatory donor T_H17 and T_H22 cells and subsequent damage to the GI tract. Overall, this study further confirmed a role for IL-6 in acute GVHD pathophysiology in the GI tract.

Tocilizumab is a humanized monoclonal antibody that binds to both membrane-bound and soluble forms of the IL-6R and was initially approved for the therapy of patients with rheumatoid arthritis (45). Based on preclinical data, studies sought to determine whether the prophylactic administration of tocilizumab could prevent the development of lower GI tract GVHD. To that end, a study from Australia showed that administration of tocilizumab in addition to standard immune suppression resulted in a very low incidence of both grades II-IV (12%) and III-IV (4%) acute GVHD. There was also a low incidence of GI tract involvement (8%) reported in a heterogeneous group of patients that included those that received reduced intensity and myeloablative conditioning regimens (46). A subsequent phase II trial designed in similar fashion to that of Kennedy and colleagues also administered a single dose of tocilizumab as prophylaxis to patients that also received standard immune suppression. Following treatment with tocilizumab, only 3% and 6% of patients displayed grade III-IV acute GVHD by days 100 and 180, respectively. Importantly, no patient developed lower GI tract disease within the first 100 days, providing evidence that tocilizumab was effective for the prevention of GI tract GVHD in humans (47).

A more recent phase III trial administered standard immune suppression plus either tocilizumab versus placebo to a heterogeneous group of patients in Australia. Patients received either reduced intensity or myeloablative conditioning regimens followed by transplantation of peripheral stem cell grafts from matched sibling or unrelated donors. The results of this study showed a non-significant trend towards improvements in grade II-IV acute GVHD and acute GVHD-free survival. There were no statistically significant reductions in moderate to severe GVHD in any specific tissue sites, including the GI tract, although there was a trend towards more favorable outcomes in tocilizumab-treated patients. Limitations of the study were the lack of a centralized GVHD grading committee across all centers, the fact that the control group fared much better than in earlier publications with respect to acute GVHD-free survival, and concerns that the study was under powered to detect more modest differences in experimental end points (48). Collectively, these studies support further research designed to determine whether blockade of IL-6 signaling is efficacious for the prevention of GVHD within the GI tract in humans.

Interleukin 23

IL-23 is a pro-inflammatory cytokine that is a member of the IL-12 family that includes IL-27, IL-35, and IL-39 and is primarily produced by dendritic cells and macrophage/monocyte populations. IL-23 regulates T cell and natural killer cell responses as well as induces the differentiation of T_H1 cells and prolongs their survival. IL-23 shares a p40 subunit with IL-12 but also has a unique p19 subunit as well. Members of the IL-12 family have been demonstrated to play a pro-inflammatory role in autoimmunity as well as bacterial and parasite-induced infections (49).

With respect to GVHD pathophysiology, several reports have identified that inhibition of IL-23 signaling with either antibody-based or genetic strategies reduces the severity of GVHD without compromising GVL effects in murine transplantation models. Importantly, these studies demonstrated that there was preferential protection from pathological damage within the GI tract (50, 51). These findings indicated that IL-23 has an important organ-specific role within the context of a systemic inflammatory disorder. More recently, additional studies demonstrated that blockade of the IL-23 receptor (IL-23R) by either antibody or genetic approaches also reduced overall GVHD mortality and protected animals from pathological damage in the GI tract (52). This was attributable to a population of $CD4^+$ $IL\text{-}23R^+$ T cells that directly mediated tissue damage. Further examination uncovered a subset of $CD4^+$ T cells that not only co-expressed the IL-23R but also express the beta 2 integrin CD11c and gut homing molecules $\alpha4\beta7$ and CCR9. These cells constituted a colitogenic $CD4^+$ T cell population that possessed an innate-like gene signature, suggesting that these cells serve as an important bridge between the innate and adaptive arms of the immune system and are positioned to mediate early inflammatory events. More recently, Bastian et al. confirmed that IL-23R alpha was required for the induction of GVHD development and that absence of IL-23R signaling in both $CD4^+$ and $CD8^+$ T cells resulted in a decrease in the production of GM-CSF and IFN-γ in the GI tract, further corroborating the importance of IL-23 signaling during acute GVHD (53).

From a translational perspective, ustekinumab which blocks the common p40 subunit shared by IL-12 and IL-23 has been administered to allogeneic HSCT recipients to prevent GVHD. In a randomized, blinded, placebo-controlled trial, Pidala and

colleagues demonstrated that ustekinumab was effective in suppressing IL-12/IL23p40 levels and reducing the levels of IL-17 and IFN-alpha. However, ustekunimab-treated patients had no difference in the incidence of grades II-IV acute or chronic GVHD, and there was no specific protective effect noted in the GI tract (54). Another follow-up randomized trial to address this question and determine whether this antibody can prevent GVHD has recently opened (NCT04572815). While there have not been any published studies that examined whether selective blockade of IL-23 can mitigate the severity of GVHD, a phase I-II clinical trial utilizing the p19-specific antibody tildrakizumab is currently under way (NCT04112810).

Janus Kinase Inhibition

The JAK-STAT pathway involves a family of intracellular tyrosine kinases that regulate the function of key inflammatory cytokine signaling pathways (55). This family includes four JAK and seven STAT proteins which together respond to cues outside of the nucleus to ultimately facilitate transcription of immune-related genes responsible for regulating inflammation (Aaronson et al., 2002 Science).

Several preclinical studies have demonstrated a role for JAK-STAT signaling in mediating cytokine release and inducing GVHD target organ damage (56–58). For example, Ma et al. showed that abrogating JAK/STAT1 signaling in donor T cells could ameliorate GVHD and that transplantation of Stat1-deficient donor cells resulted in enhanced protection in the small intestine and colon (57). Subsequent studies substantiated that work by demonstrating that pharmacological inhibition of JAK1/2 with ruxolitinib could reduce GVHD while preserving graft versus tumor responses (59, 60). Carniti and colleagues observed that ruxolitnib improved overall survival and reduced pathological damage in target organs that included the small and large intestine. Protection in the GI tract was attributable to a reduction in T cell and macrophage infiltration that was due, in part, to reduced CXCR3 expression on allogeneic T cells (60).

Other JAK inhibitors have also been utilized in pre-clinical studies to prevent acute GVHD. Choi et al. administered baricitinib, a selective inhibitor of JAK1 and JAK2, and demonstrated that this agent could prevent GVHD by expanding the Treg pool and downregulating CXCR3 expression on T_H1 and T_H2 cells (61). Interestingly, baricitinib was superior to ruxolitnib in preventing GVHD-induced mortality. More recently, Sun and colleagues utilized a highly selective JAK1 inhibitor (SHR0302) (62) and demonstrated improved overall survival when compared to vehicle treated controls (63). SHR0302 also reduced the infiltration of immune cells into the GI tract through reduction of CXCR3 expression on donor T cells as well as mitigated the release of the proinflammatory cytokines, IL-6, IFN-γ, and TNF-α.

Cumulative preclinical work and the success of JAK inhibitors as salvage therapy for GVHD treatment in patients (64, 65) ultimately led to the FDA approval of ruxolitnib for the treatment of steroid refractory acute GVHD (66). This success has also been the impetus for clinical trials designed to assess whether JAK inhibition could be successful for GVHD prophylaxis. To that end, a recent trial revealed that the JAK1 inhibitor itacitinib was well tolerated and displayed efficacy in steroid refractory acute GVHD (67). Consequently, there are now several ongoing trials designed to examine the efficacy of itacitinib for acute GVHD prophylaxis (NCT04339101), (NCT03755414) and (NCT04859946). Results from these trials should help delineate whether JAK targeting strategies are efficacious for acute GVHD prophylaxis and if administration of this class of agents prevents pathological damage in the GI tract.

INHIBITION OF T CELL TRAFFICKING

CCR5

Trafficking of donor T cells into the GI tract and the establishment of tissue residency have been shown to be critical events in the pathophysiology of GVHD in this tissue site (68). Consequently, strategies to prevent donor T cell trafficking into the GI tract have been examined as an approach to mitigate pathological damage. CCR5 is a chemokine primarily expressed on the surfaces of T cells, NK cells, and macrophages. It facilitates immune cell trafficking through the cognate ligands CCL3, CCL4 and CCL5, which can be expressed in inflammatory sites. Several studies have identified that CCR5 facilitates migration of memory CD8 T cells during viral infections (69, 70), Tregs in tumor progression (71), and NK cells in murine models of hepatitis (72).

In transplantation studies, Murai et al. demonstrated in a parent to F1 model that disrupting a gene encoding CCR5 could prevent the recruitment of donor T cells into Peyer's patches (PPs) and reduce acute GVHD. They concluded that donor cytotoxic T cells utilize CCR5 to enter the gut and that the PP is an essential site for initiating GVHD (73). Conversely, Welniak and colleagues showed that transplantation of CCR5 knockout donor cells into lethally irradiated MHC-mismatched recipients increased T cell produced IFNγ and TNFα in the GI tract and induced pathological damage in the gut (74). In a subsequent study, Wysocki et al. identified a critical role for CCR5 expression on donor $CD4^+$ $CD25^+$ Tregs. Specifically, CCR5 expression on donor Tregs seemed to be essential for entry into the lung, liver, spleen, and mesenteric lymph nodes (75). Collectively, these results suggest that the role of CCR5 during GVHD appear to be model and perhaps cell dependent.

Reshef et al. examined the effect of the CCR5 antagonist, maraviroc, on lymphocyte function and chemotaxis *in vitro* as well as performed a phase 1/2 study on 38 high-risk patients who received standard immune suppression along with maraviroc as GVHD prophylaxis (76). They observed that maraviroc inhibited lymphocyte chemotaxis and noted a low incidence of grades II to IV acute GVHD (15 and 24% on days 100 and 180, respectively). Only 9% of patients developed GVHD in the GI tract within the first year. Moy et al. also demonstrated that maraviroc treatment resulted in a lower incidence of acute GVHD and reduced levels of the gut-specific marker Reg3a, which is associated with epithelial integrity (77). More recently, Reshef et al. performed

a subsequent phase II trial to examine the efficacy of an extended course of maraviroc in 37 patients. They found that the rate of grade II-IV acute GVHD was 22 ± 7% and the grade III-IV acute GVHD was 5 ± 4% at 180 days, while noting that GVHD of the GI tract was uncommon (78). They concluded that compared to the prior short-course treatment study, an extended course of maraviroc could result in significantly higher GVHD-free, relapse-free survival. The requirement for an extended course of maraviroc suggested that more prolonged inhibition of CCR5 signaling might be required for durable prevention of GVHD of the GI tract. Despite those promising clinical results, however, a recent trial evaluating maraviroc for GVHD prophylaxis did not demonstrate superior protection from acute GVHD when combined with standard immune suppression. Specifically, Bolaños-Meade et al. conducted a randomized phase II trial in which one of the arms examined the efficacy of maraviroc, tacrolimus and methotrexate as GVHD prophylaxis (79). This studied revealed that there was no difference in the incidence of grade III or IV acute GVHD or overall survival in these patients when compared to those treated with tacrolimus and methotrexate alone, which represented the control group. Whether a more extended course of maraviroc could be required to achieve GVHD prophylaxis in the GI tract in some patients has not been formally examined in a randomized setting.

α4β7 (Lymphocyte Peyer Patch Adhesion Molecule)

Lymphocyte Peyer patch adhesion molecule (LPAM), also known as α4β7 integrin, is responsible for homing into gut-associated lymphoid tissue. When expressed on T lymphocytes, α4β7 integrin licenses cells to bind to mucosal addressin cell adhesion molecule (MAdCAM), which is chiefly expressed on high endothelial venules of mucosal lymphoid organs as well as intestinal lamina propria (80). Given the importance of this ligand/receptor interaction, numerous investigators have explored the role of α4β7 integrin in propagating GVHD, particularly with regards to inflammation in the GI tract.

Several pre-clinical studies have been conducted to examine the role of α4β7/MAdCAM in the pathophysiology of GVHD. Petrovic et al. showed that transplantation of allogeneic $\alpha4\beta7^{-/-}$ T cells resulted in significantly reduced GVHD-induced mortality compared to wild type T cells which was attributed to delayed homing to the intestines and liver (81). In addition, Waldman et al. also explored the role of α4β7 in GVHD by transplanting β7-deficient allogeneic T cells into conditioned mice. Despite β7-deficient T cells having intact activation, proliferation, cytokine production, and cytotoxicity, they induced less GVHD morbidity and mortality compared to wild type T cells due to their inability to traffic to the liver and the gut (82). Utilizing an MHC-mismatched murine transplantation model, Dutt and colleagues demonstrated that genetic deletion of α4β7 integrin alone was insufficient to protect mice from lethal GVHD; but rather the deletion of both α4β7 and CD62L together were required to protect mice from GVHD (83). This study suggests that α4β7 and L-selectin may have additive effects in influencing T cell homing to the gut. Another report demonstrated that inhibition of MAdCAM-1 reduced the recruitment of donor $CD8^+$ T cells into the intestine and alleviated GVHD by limiting intestinal injury (84). They also demonstrated that delayed administration of an anti-MAdCAM-1 antibody reduced intestine-infiltrating $\alpha4\beta7^+$ $CD8^+$ T cells without compromising anti-leukemic effects. Collectively, these studies indicated that both CD4 and CD8 cells utilize α4β7 integrin to enter the GI tract during GVHD. Recent work by Fu and colleagues utilized 3D imaging to visualize intricate allogeneic T cell spatial localization within the GI tract following GVHD (85). These data demonstrated that intestinal stem cells were the primary target of alloreactive donor T cells. Moreover, they demonstrated that this process is dependent on β7 integrin and MAdCAM-1 interactions as inhibition with anti-MAdCAM-1 antibody reduced donor T cell invasion into the lower crypt regions of the mucosa and attenuated GI tract damage.

In clinical studies, Chen et al. examined the peripheral blood of patients with symptoms of acute GVHD before treatment (86). The collected samples were subcategorized into three groups: intestinal GVHD, skin GVHD, and no GVHD. Interestingly, they reported that patients with intestinal GVHD had a significantly higher percentage of α4β7 integrin-expressing memory $CD8^+$ T cells (7.7%) compared to patients with skin GVHD (1.3%) and no GVHD (1.0%). α4β7 was not differentially expressed on any $CD4^+$ or $CD8^+$ T cell subsets that were analyzed. Therefore, this study highlights the importance of α4β7 expression on $CD8^+$ T cells particularly for propagating human GVHD symptoms within the gut.

Vedolizumab, a monoclonal antibody that binds to α4β7, has been approved for treatment of ulcerative colitis and Crohn's disease and, more recently, has been examined as a treatment for steroid refractory GI GVHD with variable results (87, 88). Danylesko et al. retrospectively analyzed the efficacy of vedolizumab in 29 patients from three transplant centers, 24 of which displayed histopathology associated with gut GVHD (89). An overall response rate of 79% was observed with 28% of patients having a complete response, despite treatment being administered mainly as second- or third-line therapy. Notably, a large percentage (69%) of patients who received early administration of vedolizumab were able to have immunosuppression discontinued altogether, supporting the premise that vedolizumab was most effective for patients with steroid refractory severe GI GVHD when administered soon after onset. Recent findings from Mehta et al. substantiated this conclusion that early treatment with vedolizumab for GVHD may be necessary for optimal results as vedolizumab treatment as a secondary or tertiary treatment for grade III of IV patients who were refractory to ruxolitinib, displayed minimal response rates (90). More recently, Fløisand et al. conducted another clinical trial to evaluate the efficacy of vedolizumab for steroid refractory intestinal GVHD and observed a response rate in over two-thirds of participants (91). Unfortunately, the study did not evaluate GVHD prophylaxis and had to be discontinued prematurely as vedolizumab did not meet the primary efficacy endpoint at 28 days.

These data demonstrate that delayed α4β7 inhibition has limited efficacy for the treatment of acute GVHD patients who are steroid refractory. This could be due to the fact that there are multiple trafficking mechanisms employed by allogeneic T cells that contribute to lower GI tract damage, or that pathological damage facilitated by T cell entry into the gut occurs rapidly after transplant and may be dispensable at later time points. Cumulatively, these data suggest that earlier intervention may be necessary and that vedolizumab may be better suited as a preventive therapy rather than as steroid-refractory secondary treatment for gut-associated acute GVHD. To that end, there is currently a trial evaluating the efficacy of vedolizumab for acute GVHD prophylaxis (NCT03657160). This trial will assess the effect of vedolizumab on decreasing the incidence of GI-acute GVHD and acute GVHD-induced mortality 6 months after transplant. Results from this study will help to determine whether administration of vedolizumab could be appropriate for GI GVHD prevention.

CELLULAR THERAPY

Regulatory T Cells

Regulatory $CD4^+$ T cells are immunosuppressive lymphocytes that express high levels of the IL-2 receptor alpha-chain CD25, as well as the fork-head box transcription factor, Foxp3 (92). Natural Tregs (nTregs) arise in the thymus and comprise a small percentage of the total $CD4^+$ T cell population that is present in the periphery. These cells are responsible for maintaining immune homeostasis and promoting tolerance to self-antigens to prevent autoimmunity (93). Due to their low frequency, it can be difficult to obtain high numbers of nTregs. However, Tregs can also be induced (iTregs) in the presence of TGF-β and IL-2 from conventional $CD4^+$ T cells and have been employed to mitigate inflammation caused by effector T cells (94, 95). While it remains a challenge to maintain the immunosuppressive functions of iTregs *in vivo*, the relative ease of expansion and potent anti-inflammatory properties have generated interest in elucidating their potential therapeutic role for GVHD (96).

Taylor et al. performed experiments to deplete $CD4^+$ $CD25^+$ before allogeneic T cell transfer and to deplete Tregs *in vivo* by administering a CD25-depleting antibody (97). Both depletion strategies increased allogeneic T cell mediated GVHD. Moreover, transplant of cultured $CD4^+$ $CD25^+$ cells with allogeneic T cells before transplant significantly inhibited lethal GVHD *in vivo*. Subsequently, they demonstrated that high levels of L-selectin on Tregs were required for them to inhibit allogeneic T cell responses and limit GVHD (98). Importantly, Tregs that prevented GVHD did not interfere with GVL effects (99). Tawara et al. identified that IL-10, the major anti-inflammatory cytokine produced by Tregs, did not prevent disease or pathology in the gut when administered exogenously, but that Treg-derived IL-10 was able to induce GI protection and improve mortality (100). Moreover, they demonstrated that host APCs are required to facilitate the expansion of donor regulatory IL-10 producing T cells during GVHD and yield benefits in the GI tract (101).

Brunstein et al. conducted a study in which they enriched $CD4^+$ $CD25^+$ $Foxp3^+$ cells from umbilical cord blood before transplantation into 23 acute GVHD patients (102). They observed that patients treated with these cells had reduced levels of grade II-IV GVHD patients compared to those that did not receive Treg therapy (43% versus 61%). The same group performed a similar study that resulted in only 9% of treated patients developing grade II-IV acute GVHD at 100 days compared to 45% in control patients. Contemporaneously, Di Ianni and colleagues evaluated whether infusion of donor $CD4^+$ $CD25^+$ Tregs could prevent acute GVHD in patients who received haploidentical transplants (103). Strikingly, of the 28 patients who received transplants, 26 achieved engraftment and only two developed grade III or IV GVHD. Unfortunately, neither of these studies analyzed organ-specific effects.

More recently, Meyer et al. performed a phase I/II study to test whether administration of human leukocyte-matched Tregs with CD34-selected hematopoietic cells and conventional T cells could prevent acute GVHD in patients undergoing myeloablative HCTs for hematological malignancies (104). They reported that of the 12 patients who received highly pure cryopreserved (n=5) or fresh (n=7) Tregs (<90%), only two acquired grade III or IV GVHD, with only one developing GI GVHD. Interestingly, none of the seven patients who received fresh Tregs developed acute or chronic GVHD, suggesting that fresh cells may be more efficacious for transplant. While findings from this trial are promising, the small number of patients in this study make it difficult to draw meaningful conclusions. Notably, the same group has a follow up trial underway that should involve more patients (NCT04013685).

Macmillan performed a phase I study to determine the safety and efficacy of induced Tregs (iTregs) on GVHD prophylaxis in adults with high-risk malignancy (105). They reported that iTregs could be safely infused into the adults and circulated for up to multiple weeks. Only three out of 14 patients developed acute GVHD with one experiencing grade IV lower GI involvement following transplant. Moreover, they found that 11% of the iTregs were $CD103^+$, which is noteworthy given that CD103 is an integrin that is associated with gut homing in T cells. While these trials show some promise, optimizing the ability of Tregs to maintain their functions *in vivo* under inflammatory conditions and improving their gut-homing capabilities will be critical for preventing GI tract acute GVHD. Currently there is an active phase I trial to administer *ex-vivo* expanded donor regulatory cells for the prevention of acute GVHD (NCT01795573). This trial is designed to co-culture recipient dendritic cells and donor Tregs prior to allogeneic stem cell transplantation to determine whether the incidence of acute GVHD is reduced.

OTHER APPROACHES

α1-antitrypsin

α1-antitrypsin (A1AT), is a protease inhibitor produced by the liver and can inactivate serine proteases produced by myeloid

cells and suppress their ability to produce pro-inflammatory cytokines. Pre-clinical studies have been performed which have revealed unique mechanisms for how A1AT influences acute GVHD. Marcondes et al., 2011 showed that A1AT could mediate protection by first demonstrating that it suppresses IL-32 and T cell proliferation *in vitro* (106). Utilization of an MHC-minor antigen model revealed that A1AT reduced several inflammatory cytokines including IL-1β and TNFα. This decrease in inflammatory cytokines resulted in a reduction in interstitial gastritis, crypt loss, and apoptosis in the duodenum, which ameliorated GVHD-induced mortality.

With respect to GVHD prevention, Gergoudis et al. performed a biomarker-guided preemptive study examining whether administration of A1AT could reduce the incidence of GVHD in patients deemed to be at high risk for steroid-resistant complications (107). Thirty patients that were identified as high risk for steroid refractory acute GVHD determined by a composite risk score that included measurement of Reg3α and ST2. Prior data have shown that these biomarkers in particular are predictive for the development of GI GVHD, making them surrogate candidates for prophylactic intervention. Results from this study were comparatively analyzed against a contemporaneous historical control population that did not receive A1AT therapy. Unfortunately, this study revealed that there was no reduction in GVHD incidence compared to the control group, indicating that A1AT administration had no impact on preventing the emergence of steroid refractory GVHD. Overall, while there has been some evidence that A1AT therapy could be beneficial for steroid resistant acute GVHD treatment (108), there have been no strict prophylaxis studies that have proven that A1AT can prevent GVHD arising in the GI tract.

Histone Deacetylase Inhibition

Histone deacetylases (HDACs) play a key role in regulating gene transcription by acting as transcriptional repressors to remove acetyl groups and promote chromatin condensation (109). HDAC inhibitors are chemical compounds that irreversibly block the action of HDACs to uncoil condensed chromatin and allow for post-translational modifications of genes. In particular, HDAC inhibitors have been demonstrated to play a role as antitumor agents by inducing cell cycle arrest and apoptosis (110). Moreover, HDAC inhibitors have been utilized to treat various neurodegenerative diseases (111), to improve depressive behaviors and stabilize epileptic events (112). Recently, HDAC inhibitors have been highlighted for their ability to alleviate inflammation within the gastrointestinal tract (113) due to their ability to quell NF-κB-mediated cytokine release (114) and promote epithelial regeneration (115). Due to these properties, HDAC inhibition has been explored as a therapeutic strategy for ameliorating GI GVHD both preclinically and clinically (116–118).

Reddy and colleagues were the first to evaluate whether the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) could improve GVHD-induced morbidity and mortality in an MHC-mismatched murine model of the disease (119). They demonstrated that SAHA could reduce serum levels of proinflammatory cytokines TNFα, IL-1β, and IFN-γ. Moreover, SAHA limited severe villous blunting, crypt destruction and inflammation in the small intestine that was observed in vehicle treated mice, which resulted in improved survival. Importantly, these benefits did not seem to compromise GVL effects as they identified HDAC inhibitors as novel therapeutic agents for GVHD. A subsequent study performed by Reddy et al. expanded upon the mechanism of HDAC inhibition for GVHD by reporting that pretreatment of DCs with HDAC inhibitors could reduce TLR-mediated secretion of proinflammatory cytokines, increase indoleamine 2,3-dioxygenase (IDO) and suppress activation markers CD40 and CD80 (120). Furthermore, injection of DCs cells *ex vivo* with HDAC inhibitors before transplant was sufficient to protect mice from GVHD. These findings illustrate the prominent role HDACs play in regulating DC function to aggravate intestinal damage associated with GVHD. Another group corroborated the benefits of HDAC inhibition for GVHD by elaborating on its mechanism for protection of GVHD mice (56). Leng et al. identified that SAHA could limit GVHD-induced mortality by limiting TNFα and IL-1β levels through the phosphorylation of STAT1 in the liver and spleen. Whether or not HDAC inhibition-mediated prevention of STAT1 phosphorylation is an important mechanism for protecting the GI tract during GVHD was not evaluated.

The pre-clinical success observed with HDAC inhibition laid the foundation for testing whether this therapy could be beneficial for GVHD prophylaxis. Choi et al. performed a phase I/II trial to evaluate whether the HDAC inhibitor, vorinostat, could reduce the incidence of GVHD if administered 10 days before transplantation until day 100 in patients with high-risk hematological malignant disease who received stem cell grafts from matched related donors after reduced intensity conditioning (121). They found that vorinostat, in addition to standard GVHD prophylaxis was both safe and reduced the incidence of grades II-IV GVHD (22%) by day 100 compared to historical controls. However, of the patients that developed GVHD, most of them were reported to have GI GVHD. A second trial by the same group tested whether vorinostat, along with standard prophylactic agents, could prevent acute GVHD in recipients of unrelated stem cell grafts that received myeloablative conditioning (122). This study also identified that vorinostat was safe and resulted in grade II-IV GVHD occurring in 22% of patients, with only 8% exhibiting grade III-IV GVHD. Moreover, they performed correlative analyses in PBMCs from these patients to find that IL-6, Reg3α, and ST2 (all markers associated with GI GVHD) correlated with reduced GVHD in patients at day 30 after transplant. This study also revealed that only 11% of patients displayed GI GVHD at day 100. Together, these trials provide evidence that vorinostat has promise for the prevention of GVHD in the GI tract.

A second HDAC inhibitor, panobinostat, was recently evaluated both in a phase I trial for GVHD treatment (123) and in a phase II trial for GVHD prophylaxis (124). In the prophylactic trial, intervention with panobinostat began at -5 days before transplant and was continually administered for 28 weeks in patients with acute myeloid leukemia (n= 18), myelodysplastic syndrome (n = 13) and other malignancies (n = 8). The cumulative incidence rate of grade II-IV acute GVHD at 100 days was only 18.4% and the one-year overall

survival was 89.5%. Importantly, of the patients who developed acute GVHD and received the full treatment of panobinostat, none developed greater than grade I GI GVHD. In addition, they observed a decrease in plasma IL-6 levels in treated patients at day 90 compared to controls but did not witness any differences in Reg3α and ST2 at day 28, unlike the vorinostat study. Overall, reports from clinical trials utilizing vorinostat and panobinostat indicate that HDAC inhibition could be an appropriate preventative strategy for GI tract GVHD.

Proteasome Inhibition

Proteasomes are large catalytic protein complexes that cleave and degrade misfolded, damaged or erroneous proteins into peptides (125). They can also play a role in inducing activation of NFκB-dependent signaling pathways that are responsible for preventing apoptosis and promoting the release of proinflammatory cytokines. Due to these capabilities, unregulated proteasome activity has been demonstrated to correlate with the severity of autoimmune diseases and cancer (126). Proteome inhibitors have been demonstrated to be effective as anti-tumor agents (127) and to reduce NFκB –mediated inflammation in models of psoriasis (128) and asthma (129) as well. For these reasons, there has been interest in investigating whether proteasome inhibition could be efficacious for GVHD prophylaxis.

Bortezomib, a boronic acid dipeptide derivative, was the first proteasome inhibitor to be approved by the US Food and Drug Administration (FDA) in 2003 (130) and was initially clinically approved as a therapy for multiple myeloma patients due to its growth-inhibitory and anti-apoptotic effects (131). Sun and colleagues performed seminal experiments to test whether bortezomib could have prophylactic effects for acute GVHD (132). They found that bortezomib could promote the apoptosis of alloreactive T cells *in vitro* and protect mice from acute GVHD *in vivo* without adversely affecting donor reconstitution when administered at the time of transplant. Subsequently, Vodanovic-Janovic et al. evaluated whether administration of bortezomib could protect mice from GVHD (133). They reported that early post-transplant therapy with bortezomib improved GVHD-free survival without compromising donor engraftment; however, extended administration of bortezomib exacerbated pathological damage in the colon and resulted in early mortality due to gut toxicity. This study indicated that while early post-transplant proteasome inhibition may be beneficial, more protracted administration exacerbated GVHD-induced immune-mediated damage in the GI tract.

Sun et al. corroborated these findings and demonstrated that prolonged bortezomib administration increased serum levels of TNFα and IFNγ in multiple murine models of GVHD and led to early mortality (134). The detrimental effect of bortezomib on allogenic T cells appeared to be CD4 mediated and TNFα dependent as mice transplanted with TNFα deficient donor CD4 T cells were resistant to the toxic effects of bortezomib. More recently, Li and colleagues (135) reported that early doses of bortezomib on days 0 and 1 after transplant prevented pathological damage in the GI tract and improved survival. This benefit corresponded with decreased serum levels of IL-2, TNFα and IFNγ. Overall, these preclinical studies indicated that bortezomib administration could protect the GI tract from GVHD but that this was schedule dependent.

Based on preclinical results indicating a protective role of bortezomib administration for acute GVHD, Koreth and colleagues conducted a phase I/II trial to test whether a short course of bortezomib could be an applicable intervention for GVHD prophylaxis following transplantation from HLA-mismatched unrelated donors for patients with hematologic malignancies (136). Of the patients who were given bortezomib, 22% displayed grade II to IV acute GVHD at day 180 and importantly, these patients did not experience augmented GI toxicity. Two subsequent phase II trials were performed by Koreth and colleagues to evaluate the prophylactic effects of bortezomib on GVHD. The first involved administration of short-course bortezomib for patients who underwent myeloablative conditioning and included both HLA-matched and HLA-mismatched donors (137). Bortezomib was effective in generating a low incidence of grade II to IV acute GVHD involving the skin, liver and/or lower GI tract with only 12% of patients displaying grade III to IV acute GVHD. The second was an open-label three-arm phase II randomized control trial in patients who received reduced intensity conditioning and allogenic transplants lacking HLA-matched donors (138). Unfortunately, this trial reported that bortezomib-based regimens did not lower GVHD incidence compared to control regimens. A more recent phase II randomized trial compared multiple interventions, one including bortezomib in addition to standard immunosuppression, to evaluate its role in GVHD prophylaxis, which involved both HLA-matched and HLA-mismatched donors (79). Similarly, this study demonstrated that bortezomib had no beneficial effect on reducing the incidence of grades II-IV acute GVHD when compared to standard immune suppression alone. While none of these studies specifically examined the GI tract for organ-specific protective effects, the lack of any overall reduction in acute GVHD argues against any protective effect in this tissue site.

CONCLUSIONS

The GI tract is the major site of morbidity and mortality associated with the development of acute GVHD. Unfortunately, a significant percentage of patients fail to respond to first line therapy with corticosteroids and require second line therapy for steroid refractory disease. Outcomes for these patients is significantly worse and a substantial proportion of these patients ultimately do not respond to salvage therapy. In addition, patients who develop GI GVHD often require hospitalization and are at risk for secondary infections due to compromised epithelial barrier integrity, which adversely impacts quality of life and can also result in premature fatality. Consequently, prevention of this complication, particularly within the GI tract, is critically vital to improve overall treatment outcomes and should be a primary goal of GVHD prophylaxis strategies.

To that end, preclinical studies have identified that inhibition of inflammatory cytokine pathways, blockade of gut homing molecules that are expressed on the surface of alloreactive donor

T cells, and reconstitution of regulatory pathways as potential therapeutic strategies that have shown promise and led to translation in human clinical trials. Unfortunately, many of these strategies, while promising in animal studies, have not translated well into the clinic. Reasons for this are not entirely clear but are likely multifactorial and related to limitations of mouse models that do not fully replicate the complexity of human allogeneic stem cell transplantation with respect to recipient age, stem cell source, conditioning regimen intensity, and MHC disparity which all impact GVHD severity. In other cases, some of these approaches have only recently entered clinical trials for GVHD prophylaxis (e.g., blockade of α4β7 integrin and IL-23 signaling) so the verdict is still out on whether they will be efficacious for prevention of GI tract GVHD. To date, blockade of IL-6 signaling, administration of Treg infusions, and histone deacetylase inhibition have reported clinical outcomes in which there appears to be a reduction in GI tract GVHD; however, definitive data are still lacking with these approaches. Thus, additional investigations are required to clearly identify effective prophylactic strategies that will ameliorate toxicity to this important tissue site, and secondarily lead to an improvement in overall transplant outcomes.

AUTHOR CONTRIBUTIONS

AR and WD wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Shlomchik WD. Graft-Versus-Host Disease. *Nat Rev Immunol* (2007) 7:340–52. doi: 10.1038/nri2000
2. Blazar BR, Murphy WJ, Abedi M. Advances in Graft-Versus-Host Disease Biology and Therapy. *Nat Rev Immunol* (2012) 12:443–58. doi: 10.1038/nri3212
3. Zeiser R, Socié G, Blazar BR. Pathogenesis of Acute Graft-Versus-Host Disease: From Intestinal Microbiota Alterations to Donor T Cell Activation. *Br J Haematol* (2016) 175:191–207. doi: 10.1111/bjh.14295
4. Vriesendorp HM, Heidt PJ. History of Graft-Versus-Host Disease. *Exp Hematol* (2016) 44:674–88. doi: 10.1016/j.exphem.2016.05.011
5. Socié G, Ritz J. Current Issues in Chronic Graft-Versus-Host Disease. *Blood* (2014) 124:374–84. doi: 10.1182/blood-2014-01-514752
6. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-Versus-Host Disease. *Lancet* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
7. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total Body Irradiation and Acute Graft-Versus-Host Disease: The Role of Gastrointestinal Damage and Inflammatory Cytokines. *Blood* (1997) 90:3204–13. doi: 10.1182/blood.V90.8.3204
8. Baker MB, Altman NH, Podack ER, Levy RB. The Role of Cell-Mediated Cytotoxicity in Acute GVHD After MHC-Matched Allogeneic Bone Marrow Transplantation in Mice. *J Exp Med* (1996) 183:2645–56. doi: 10.1084/jem.183.6.2645
9. Dignan FL, Clark A, Amrolia P, Cornish J, Jackson G, Mahendra P, et al. Diagnosis and Management of Acute Graft-Versus-Host Disease. *Br J Haematol* (2012) 158:30–45. doi: 10.1111/j.1365-2141.2012.09129.x
10. Martin PJ, Rizzo JD, Wingard JR, Ballen K, Curtin PT, Cutler C, et al. First- and Second-Line Systemic Treatment of Acute Graft-Versus-Host Disease: Recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant* (2012) 18:1150–63. doi: 10.1016/j.bbmt.2012.04.005
11. Malard F, Huang XJ, Sim JPY. Treatment and Unmet Needs in Steroid-Refractory Acute Graft-Versus-Host Disease. *Leukemia* (2020) 34:1229–40. doi: 10.1038/s41375-020-0804-2
12. García-Cadenas I, Rivera I, Martino R, Esquirol A, Barba P, Novelli S, et al. Patterns of Infection and Infection-Related Mortality in Patients With Steroid-Refractory Acute Graft Versus Host Disease. *Bone Marrow Transplant* (2017) 52:107–13. doi: 10.1038/bmt.2016.225
13. Schroeder MA, DiPersio JF. Mouse Models of Graft-Versus-Host Disease: Advances and Limitations. *Dis Model Mech* (2011) 4:318–33. doi: 10.1242/dmm.006668
14. Schwarte S, Hoffmann MW. Influence of Radiation Protocols on Graft-vs-Host Disease Incidence After Bone-Marrow Transplantation in Experimental Models. *Methods Mol Med* (2005) 109:445–58. doi: 10.1385/1-59259-862-5:445
15. Schwarte S, Bremer M, Fruehauf J, Sorge Y, Skubich S, Hoffmann MW. Radiation Protocols Determine Acute Graft-Versus-Host Disease Incidence After Allogeneic Bone Marrow Transplantation in Murine Models. *Int J Radiat Biol* (2007) 83:625–36. doi: 10.1080/09553000701534572
16. Korngold R. Lethal Graft-Versus-Host Disease in Mice Directed to Multiple Minor Histocompatibility Antigens: Features of CD8+ and CD4+ T Cell Responses. *Bone Marrow Transplant* (1992) 9:355–64.
17. Taylor SL, Renshaw BR, Garka KE, Smith DE, Sims JE. Genomic Organization of the Interleukin-1 Locus. *Genomics* (2002) 79:726–33. doi: 10.1006/geno.2002.6752
18. Nicklin MJ, Barton JL, Nguyen M, FitzGerald MG, Duff GW, Kornman KA. A Sequence-Based Map of the Nine Genes of the Human Interleukin-1 Cluster. *Genomics* (2002) 79:718–25. doi: 10.1006/geno.2002.6751
19. Sims JE, Smith DE. The IL-1 Family: Regulators of Immunity. *Nat Rev Immunol* (2010) 10:89–102. doi: 10.1038/nri2691
20. Mertens M, Singh JA. Anakinra for Rheumatoid Arthritis: A Systematic Review. *J Rheumatol* (2009) 36:1118–25. doi: 10.3899/jrheum.090074
21. McCarthy PL, Abhyankar S, Neben S, Newman G, Sieff C, Thompson RC, et al. Inhibition of Interleukin-1 by an Interleukin-1 Receptor Antagonist Prevents Graft-Versus-Host Disease. *Blood* (1991) 78:1915–8. doi: 10.1182/blood.V78.8.1915.1915
22. Abhyankar S, Gilliland DG, Ferrara JL. Interleukin-1 is a Critical Effector Molecule During Cytokine Dysregulation in Graft Versus Host Disease to Minor Histocompatibility Antigens. *Transplantation* (1993) 56:1518–23. doi: 10.1097/00007890-199312000-00045
23. Vallera DA, Taylor PA, Vannice JL, Panoskaltsis-Mortari A, Blazar BR. Interleukin-1 or Tumor Necrosis Factor-Alpha Antagonists do Not Inhibit Graft-Versus-Host Disease Induced Across the Major Histocompatibility Barrier in Mice. *Transplantation* (1995) 60:1371–4.
24. Park MJ, Lee SH, Lee EJ, Kim EK, Choi JY, Cho ML. IL-1 Receptor Blockade Alleviates Graft-Versus-Host Disease Through Downregulation of an Interleukin-1β-Dependent Glycolytic Pathway in Th17 Cells. *Mediators Inflammation* (2015) 2015:631384. doi: 10.1155/2015/631384
25. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber FC, Guarda G, et al. The Nlrp3 Inflammasome Regulates Acute Graft-Versus-Host Disease. *J Exp Med* (2013) 210:1899–910. doi: 10.1084/jem.20130084
26. Antin JH, Weinstein HJ, Guinan EC, McCarthy P, Bierer BE, Gilliland DG, et al. Recombinant Human Interleukin-1 Receptor Antagonist in the Treatment of Steroid-Resistant Graft-Versus-Host Disease. *Blood* (1994) 84:1342–8. doi: 10.1182/blood.V84.4.1342.1342
27. McCarthy PL, Williams L, Harris-Bacile M, Yen J, Przepiorka D, Ippoliti C, et al. A Clinical Phase I/II Study of Recombinant Human Interleukin-1 Receptor in Glucocorticoid-Resistant Graft-Versus-Host Disease. *Transplantation* (1996) 62:626–31. doi: 10.1097/00007890-199609150-00015

28. Antin JH, Weisdorf D, Neuberg D, Nicklow R, Clouthier S, Lee SJ, et al. Interleukin-1 Blockade Does Not Prevent Acute Graft-Versus-Host Disease: Results of a Randomized, Double-Blind, Placebo-Controlled Trial of Interleukin-1 Receptor Antagonist in Allogeneic Bone Marrow Transplantation. *Blood* (2002) 100:3479–82. doi: 10.1182/blood-2002-03-0985
29. Bradley JR. TNF-Mediated Inflammatory Disease. *J Pathol* (2008) 214:149–60. doi: 10.1002/path.2287
30. Piguet PF, Grau GE, Allet B, Vassalli P. Tumor Necrosis Factor/Cachectin is an Effector of Skin and Gut Lesions of the Acute Phase of Graft-vs.-Host Disease. *J Exp Med* (1987) 166:1280–9. doi: 10.1084/jem.166.5.1280
31. Schmaltz C, Alpdogan O, Muriglan SJ, Kappel BJ, Rotolo JA, Ricchetti ET, et al. Donor T Cell-Derived TNF is Required for Graft-Versus-Host Disease and Graft-Versus-Tumor Activity After Bone Marrow Transplantation. *Blood* (2003) 101:2440–5. doi: 10.1182/blood-2002-07-2109
32. Stickel N, Prinz G, Pfeifer D, Hasselblatt P, Schmitt-Graeff A, Follo M, et al. MiR-146a Regulates the TRAF6/TNF-Axis in Donor T Cells During GVHD. *Blood* (2014) 124:2586–95. doi: 10.1182/blood-2014-04-569046
33. Deeg HJ, Spitzer TR, Cottler-Fox M, Cahill R, Pickle LW. Conditioning-Related Toxicity and Acute Graft-Versus-Host Disease in Patients Given Methotrexate/Cyclosporine Prophylaxis. *Bone Marrow Transplant* (1991) 7:193–8.
34. Min CK, Lee WY, Min DJ, Lee DG, Kim YJ, Park YH, et al. The Kinetics of Circulating Cytokines Including IL-6, TNF-Alpha, IL-8 and IL-10 Following Allogeneic Hematopoietic Stem Cell Transplantation. *Bone Marrow Transplant* (2001) 28:935–40. doi: 10.1038/sj.bmt.1703258
35. Holler E, Kolb HJ, Möller A, Kempeni J, Liesenfeld S, Pechumer H, et al. Increased Serum Levels of Tumor Necrosis Factor Alpha Precede Major Complications of Bone Marrow Transplantation. *Blood* (1990) 75:1011–6. doi: 10.1182/blood.V75.4.1011.1011
36. Marty FM, Lee SJ, Fahey MM, Alyea EP, Soiffer RJ, Antin JH, et al. Infliximab Use in Patients With Severe Graft-Versus-Host Disease and Other Emerging Risk Factors of Non-Candida Invasive Fungal Infections in Allogeneic Hematopoietic Stem Cell Transplant Recipients: A Cohort Study. *Blood* (2003) 102:2768–76. doi: 10.1182/blood-2003-01-0267
37. Hamadani M, Hofmeister CC, Jansak B, Phillips G, Elder P, Blum W, et al. Addition of Infliximab to Standard Acute Graft-Versus-Host Disease Prophylaxis Following Allogeneic Peripheral Blood Cell Transplantation. *Biol Blood Marrow Transplant* (2008) 14:783–9. doi: 10.1016/j.bbmt.2008.04.006
38. Choi SW, Stiff P, Cooke K, Ferrara JL, Braun T, Kitko C, et al. TNF-Inhibition With Etanercept for Graft-Versus-Host Disease Prevention in High-Risk HCT: Lower TNFR1 Levels Correlate With Better Outcomes. *Biol Blood Marrow Transplant* (2012) 18:1525–32. doi: 10.1016/j.bbmt.2012.03.013
39. Akira S, Taga T, Kishimoto T. Interleukin-6 in Biology and Medicine. *Adv Immunol* (1993) 54:1–78. doi: 10.1016/S0065-2776(08)60532-5
40. Jordan SC, Choi J, Kim I, Wu G, Toyoda M, Shin B, et al. Interleukin-6, A Cytokine Critical to Mediation of Inflammation, Autoimmunity and Allograft Rejection: Therapeutic Implications of IL-6 Receptor Blockade. *Transplantation* (2017) 101:32–44. doi: 10.1097/TP.0000000000001452
41. Ho LJ, Luo SF, Lai JH. Biological Effects of Interleukin-6: Clinical Applications in Autoimmune Diseases and Cancers. *Biochem Pharmacol* (2015) 97:16–26. doi: 10.1016/j.bcp.2015.06.009
42. Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, et al. Blockade of Interleukin-6 Signaling Augments Regulatory T-Cell Reconstitution and Attenuates the Severity of Graft-Versus-Host Disease. *Blood* (2009) 114:891–900. doi: 10.1182/blood-2009-01-197178
43. Tawara I, Koyama M, Liu C, Toubai T, Thomas D, Evers R, et al. Interleukin-6 Modulates Graft-Versus-Host Responses After Experimental Allogeneic Bone Marrow Transplantation. *Clin Cancer Res* (2011) 17:77–88. doi: 10.1158/1078-0432.CCR-10-1198
44. Wilkinson AN, Chang K, Kuns RD, Henden AS, Minnie SA, Ensbey KS, et al. IL-6 Dysregulation Originates in Dendritic Cells and Mediates Graft-Versus-Host Disease *via* Classical Signaling. *Blood* (2019) 134:2092–106. doi: 10.1182/blood.2019000396
45. Yao X, Huang J, Zhong H, Shen N, Faggioni R, Fung M, et al. Targeting Interleukin-6 in Inflammatory Autoimmune Diseases and Cancers. *Pharmacol Ther* (2014) 141:125–39. doi: 10.1016/j.pharmthera.2013.09.004
46. Kennedy GA, Tey SK, Buizen L, Varelias A, Gartlan KH, Curley C, et al. Addition of Interleukin-6 Inhibition With Tocilizumab to Standard Graft-Versus-Host Disease Prophylaxis After Allogeneic Stem-Cell Transplantation: A Phase 1/2 Trial. *Lancet Oncol* (2014) 15:1451–9. doi: 10.1016/S1470-2045(14)71017-4
47. Drobyski WR, Szabo A, Zhu F, Keever-Taylor C, Hebert KM, Dunn R, et al. Tocilizumab, Tacrolimus and Methotrexate for the Prevention of Acute Graft-. *Haematologica* (2018) 103:717–27. doi: 10.3324/haematol.2017.183434
48. Kennedy GA, Tey SK, Buizen L, Varelias A, Gartlan KH, Curley C, et al. A Phase 3 Double-Blind Study of the Addition of Tocilizumab vs Placebo to Cyclosporin/Methotrexate GVHD Prophylaxis. *Blood* (2021) 137:1970–9. doi: 10.1182/blood.2020009050
49. Trinchieri G. Interleukin-12: A Cytokine at the Interface of Inflammation and Immunity. *Adv Immunol* (1998) 70:83–243. doi: 10.1016/S0065-2776(08)60387-9
50. Das R, Komorowski R, Hessner MJ, Subramanian H, Huettner CS, Cua D, et al. Blockade of Interleukin-23 Signaling Results in Targeted Protection of the Colon and Allows for Separation of Graft-Versus-Host and Graft-Versus-Leukemia Responses. *Blood* (2010) 115:5249–58. doi: 10.1182/blood-2009-11-255422
51. Das R, Chen X, Komorowski R, Hessner MJ, Drobyski WR. Interleukin-23 Secretion by Donor Antigen-Presenting Cells is Critical for Organ-Specific Pathology in Graft-Versus-Host Disease. *Blood* (2009) 113:2352–62. doi: 10.1182/blood-2008-08-175448
52. Zhou V, Agle K, Chen X, Beres A, Komorowski R, Belle L, et al. A Colitogenic Memory CD4+ T Cell Population Mediates Gastrointestinal Graft-Versus-Host Disease. *J Clin Invest* (2016) 126:3541–55. doi: 10.1172/JCI80874
53. Bastian D, Sui X, Nguyen HD, Wu Y, Schutt S, Tian L, et al. Interleukin-23 Receptor Signaling by Interleukin-39 Potentiates T Cell Pathogenicity in Acute Graft-Versus-Host Disease. *Am J Transplant* (2021) 21(11):3538–49. doi: 10.1111/ajt.16624
54. Pidala J, Beato F, Kim J, Betts B, Jim H, Sagatys E, et al. IL-12/IL-23p40 Neutralization Blocks Th1/Th17 Response After Allogeneic Hematopoietic Cell Transplantation. *Haematologica* (2018) 103:531–9. doi: 10.3324/haematol.2017.171199
55. O'Shea JJ, Murray PJ. Cytokine Signaling Modules in Inflammatory Responses. *Immunity* (2008) 28:477–87. doi: 10.1016/j.immuni.2008.03.002
56. Leng C, Gries M, Ziegler J, Lokshin A, Mascagni P, Lentzsch S, et al. Reduction of Graft-Versus-Host Disease by Histone Deacetylase Inhibitor Suberonylanilide Hydroxamic Acid is Associated With Modulation of Inflammatory Cytokine Milieu and Involves Inhibition of STAT1. *Exp Hematol* (2006) 34:776–87. doi: 10.1016/j.exphem.2006.02.014
57. Ma H, Lu C, Ziegler J, Liu A, Sepulveda A, Okada H, et al. Absence of Stat1 in Donor CD4$^+$ T Cells Promotes the Expansion of Tregs and Reduces Graft-Versus-Host Disease in Mice. *J Clin Invest* (2011) 121:2554–69. doi: 10.1172/JCI43706
58. Sugerman PB, Faber SB, Willis LM, Petrovic A, Murphy GF, Pappo J, et al. Kinetics of Gene Expression in Murine Cutaneous Graft-Versus-Host Disease. *Am J Pathol* (2004) 164:2189–202. doi: 10.1016/S0002-9440(10)63776-5
59. Choi J, Cooper ML, Alahmari B, Ritchey J, Collins L, Holt M, et al. Pharmacologic Blockade of JAK1/JAK2 Reduces GvHD and Preserves the Graft-Versus-Leukemia Effect. *PloS One* (2014) 9:e109799. doi: 10.1371/journal.pone.0109799
60. Carniti C, Gimondi S, Vendramin A, Recordati C, Confalonieri D, Bermema A, et al. Pharmacologic Inhibition of JAK1/JAK2 Signaling Reduces Experimental Murine Acute GVHD While Preserving GVT Effects. *Clin Cancer Res* (2015) 21:3740–9. doi: 10.1158/1078-0432.CCR-14-2758
61. Choi J, Cooper ML, Staser K, Ashami K, Vij KR, Wang B, et al. Baricitinib-Induced Blockade of Interferon Gamma Receptor and Interleukin-6 Receptor for the Prevention and Treatment of Graft-Versus-Host Disease. *Leukemia* (2018) 32:2483–94. doi: 10.1038/s41375-018-0123-z
62. Wu H, Yan S, Chen J, Luo X, Li P, Jia X, et al. JAK1-STAT3 Blockade by JAK Inhibitor SHR0302 Attenuates Inflammatory Responses of Adjuvant-Induced Arthritis Rats and Decreases Th17 and Total B Cells. *Joint Bone Spine* (2016) 83:525–32. doi: 10.1016/j.jbspin.2015.09.002
63. Sun X, He Q, Yang J, Wang A, Zhang F, Qui H, et al. Preventive and Therapeutic Effects of a Novel JAK Inhibitor SHR0302 in Acute Graft-Versus-Host Disease. *Cell Transplant* (2021) 30:9636897211033778. doi: 10.1177/09636897211033778

64. Zeiser R, Burchert A, Lengerke C, Verbek M, Maas-Bauer K, Metzelder SK, et al. Ruxolitinib in Corticosteroid-Refractory Graft-Versus-Host Disease After Allogeneic Stem Cell Transplantation: A Multicenter Survey. *Leukemia* (2015) 29:2062–8. doi: 10.1038/leu.2015.212
65. Spoerl S, Mathew NR, Bscheider M, Schmitt-Graeff A, Chen S, Mueller T, et al. Activity of Therapeutic JAK 1/2 Blockade in Graft-Versus-Host Disease. *Blood* (2014) 123:3832–42. doi: 10.1182/blood-2013-12-543736
66. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-Versus-Host Disease. *N Engl J Med* (2020) 382:1800–10. doi: 10.1056/NEJMoa1917635
67. Schroeder MA, Khoury HJ, Jagasia M, Ali H, Schiller GJ, Staser K, et al. A Phase 1 Trial of Itacitinib, a Selective JAK1 Inhibitor, in Patients With Acute Graft-Versus-Host Disease. *Blood Adv* (2020) 4:1656–69. doi: 10.1182/bloodadvances.2019001043
68. Tkachev V, Kaminski J, Potter EL, Furlan SN, Yu A, Hunt DJ, et al. Spatiotemporal Single-Cell Profiling Reveals That Invasive and Tissue-Resident Memory Donor CD8. *Sci Transl Med* (2021) 13(576):eabc0227. doi: 10.1126/scitranslmed.abc0227
69. Kohlmeier JE, Miller SC, Smith J, Lu B, Gerard C, Cookenham T, et al. The Chemokine Receptor CCR5 Plays a Key Role in the Early Memory CD8+ T Cell Response to Respiratory Virus Infections. *Immunity* (2008) 29:101–13. doi: 10.1016/j.immuni.2008.05.011
70. Fukada K, Sobao Y, Tomiyama H, Oka S, Takiguchi M. Functional Expression of the Chemokine Receptor CCR5 on Virus Epitope-Specific Memory and Effector CD8+ T Cells. *J Immunol* (2002) 168:2225–32. doi: 10.4049/jimmunol.168.5.2225
71. Tan MC, Goedegebuure PS, Belt BA, Flaherty B, Sankpal N, Gillanders WE, et al. Disruption of CCR5-Dependent Homing of Regulatory T Cells Inhibits Tumor Growth in a Murine Model of Pancreatic Cancer. *J Immunol* (2009) 182:1746–55. doi: 10.4049/jimmunol.182.3.1746
72. Ajuebor MN, Wondimu Z, Hogaboam CM, Le T, Proudfoot AE, Swain MG. CCR5 Deficiency Drives Enhanced Natural Killer Cell Trafficking to and Activation Within the Liver in Murine T Cell-Mediated Hepatitis. *Am J Pathol* (2007) 170:1975–88. doi: 10.2353/ajpath.2007.060690
73. Murai M, Yoneyama H, Ezaki T, Suematsu M, Terashima Y, Harada A, et al. Peyer's Patch is the Essential Site in Initiating Murine Acute and Lethal Graft-Versus-Host Reaction. *Nat Immunol* (2003) 4:154–60. doi: 10.1038/ni879
74. Welniak LA, Wang Z, Sun K, Kuziel W, Anver MR, Blazar BR, et al. An Absence of CCR5 on Donor Cells Results in Acceleration of Acute Graft-vs-Host Disease. *Exp Hematol* (2004) 32:318–24. doi: 10.1016/j.exphem.2003.12.003
75. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, Su L, et al. Critical Role for CCR5 in the Function of Donor CD4+CD25+ Regulatory T Cells During Acute Graft-Versus-Host Disease. *Blood* (2005) 106:3300–7. doi: 10.1182/blood-2005-04-1632
76. Reshef R, Luger SM, Hexner EO, Loren AW, Frey NV, Nasta SD, et al. Blockade of Lymphocyte Chemotaxis in Visceral Graft-Versus-Host Disease. *N Engl J Med* (2012) 367:135–45. doi: 10.1056/NEJMoa1201248
77. Moy RH, Huffman AP, Richman LP, Crisalli L, Wang XK, Hoxie JA, et al. Clinical and Immunologic Impact of CCR5 Blockade in Graft-Versus-Host Disease Prophylaxis. *Blood* (2017) 129:906–16. doi: 10.1182/blood-2016-08-735076
78. Reshef R, Ganetsky A, Acosta EP, Blauser R, Crisalli L, McGraw J, et al. Extended CCR5 Blockade for Graft-Versus-Host Disease Prophylaxis Improves Outcomes of Reduced-Intensity Unrelated Donor Hematopoietic Cell Transplantation: A Phase II Clinical Trial. *Biol Blood Marrow Transplant* (2019) 25:515–21. doi: 10.1016/j.bbmt.2018.09.034
79. Bolaños-Meade J, Reshef R, Fraser R, Fei M, Abhyankar S, Al-Kadhimi Z, et al. Three Prophylaxis Regimens (Tacrolimus, Mycophenolate Mofetil, and Cyclophosphamide; Tacrolimus, Methotrexate, and Bortezomib; or Tacrolimus, Methotrexate, and Maraviroc) Versus Tacrolimus and Methotrexate for Prevention of Graft-Versus-Host Disease With Haemopoietic Cell Transplantation With Reduced-Intensity Conditioning: A Randomised Phase 2 Trial With a Non-Randomised Contemporaneous Control Group (BMT CTN 1203). *Lancet Haematol* (2019) 6:e132–43. doi: 10.1016/S2352-3026(18)30221-7
80. Williams MB, Butcher EC. Homing of Naive and Memory T Lymphocyte Subsets to Peyer's Patches, Lymph Nodes, and Spleen. *J Immunol* (1997) 159:1746–52.
81. Petrovic A, Alpdogan O, Willis LM, Eng JM, Greenberg AS, Kappel BJ, et al. LPAM (Alpha 4 Beta 7 Integrin) is an Important Homing Integrin on Alloreactive T Cells in the Development of Intestinal Graft-Versus-Host Disease. *Blood* (2004) 103:1542–7. doi: 10.1182/blood-2003-03-0957
82. Waldman E, Lu SX, Hubbard VM, Kochman AA, Eng JM, Terwey T, et al. Absence of Beta7 Integrin Results in Less Graft-Versus-Host Disease Because of Decreased Homing of Alloreactive T Cells to Intestine. *Blood* (2006) 107:1703–11. doi: 10.1182/blood-2005-08-3445
83. Dutt S, Ermann J, Tseng D, Liu YP, George TI, Fathman CG, et al. L-Selectin and Beta7 Integrin on Donor CD4 T Cells are Required for the Early Migration to Host Mesenteric Lymph Nodes and Acute Colitis of Graft-Versus-Host Disease. *Blood* (2005) 106:4009–15. doi: 10.1182/blood-2005-06-2339
84. Ueha S, Murai M, Yoneyama H, Kitabatake M, Imai T, Shimaoka T, et al. Intervention of MAdCAM-1 or Fractalkine Alleviates Graft-Versus-Host Reaction Associated Intestinal Injury While Preserving Graft-Versus-Tumor Effects. *J Leukoc Biol* (2007) 81:176–85. doi: 10.1189/jlb.0306231
85. Fu YY, Egorova A, Sobieski C, Kuttiyara J, Calafiore M, Takashima S, et al. T Cell Recruitment to the Intestinal Stem Cell Compartment Drives Immune-Mediated Intestinal Damage After Allogeneic Transplantation. *Immunity* (2019) 51:90–103.e103. doi: 10.1016/j.immuni.2019.06.003
86. Chen YB, McDonough S, Chen H, Kennedy J, Illiano C, Attar EC, et al. Expression of α4β7 Integrin on Memory CD8(+) T Cells at the Presentation of Acute Intestinal GVHD. *Bone Marrow Transplant* (2013) 48:598–603. doi: 10.1038/bmt.2012.191
87. Fløisand Y, Lazarevic VL, Maertens J, Mattsson J, Shah NN, Zachée P, et al. Safety and Effectiveness of Vedolizumab in Patients With Steroid-Refractory Gastrointestinal Acute Graft-Versus-Host Disease: A Retrospective Record Review. *Biol Blood Marrow Transplant* (2019) 25:720–7. doi: 10.1016/j.bbmt.2018.11.013
88. Coltoff A, Lancman G, Kim S, Steinberg A. Vedolizumab for Treatment of Steroid-Refractory Lower Gastrointestinal Acute Graft-Versus-Host Disease. *Bone Marrow Transplant* (2018) 53:900–4. doi: 10.1038/s41409-018-0094-8
89. Danylesko I, Bukauskas A, Paulson M, Peceliunas V, Gedde-Dahl DYT, Shimoni A, et al. Anti-α4β7 Integrin Monoclonal Antibody (Vedolizumab) for the Treatment of Steroid-Resistant Severe Intestinal Acute Graft-Versus-Host Disease. *Bone Marrow Transplant* (2019) 54:987–93. doi: 10.1038/s41409-018-0364-5
90. Mehta RS, Saliba RM, Jan A, Shigle TL, Wang E, Nieto Y, et al. Vedolizumab for Steroid Refractory Lower Gastrointestinal Tract Graft-Versus-Host Disease. *Transplant Cell Ther* (2021) 27:272.e271–272.e275. doi: 10.1016/j.jtct.2020.12.011
91. Fløisand Y, Schroeder MA, Chevallier P, Selleslag D, Devine S, Renteria A, et al. A Phase 2a Randomized Clinical Trial of Intravenous Vedolizumab for the Treatment of Steroid-Refractory Intestinal Acute Graft-Versus-Host Disease. *Bone Marrow Transplant* (2021) 56(10):2477–88. doi: 10.1038/s41409-021-01356-0
92. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY, et al. Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3. *Immunity* (2005) 22:329–41. doi: 10.1016/j.immuni.2005.01.016
93. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, et al. Foxp3+ CD25+ CD4+ Natural Regulatory T Cells in Dominant Self-Tolerance and Autoimmune Disease. *Immunol Rev* (2006) 212:8–27. doi: 10.1111/j.0105-2896.2006.00427.x
94. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-Beta Induction of Transcription Factor Foxp3. *J Exp Med* (2003) 198:1875–86. doi: 10.1084/jem.20030152
95. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting Edge: TGF-Beta Induces a Regulatory Phenotype in CD4+CD25- T Cells Through Foxp3 Induction and Down-Regulation of Smad7. *J Immunol* (2004) 172:5149–53. doi: 10.4049/jimmunol.172.9.5149
96. Beres AJ, Drobyski WR. The Role of Regulatory T Cells in the Biology of Graft Versus Host Disease. *Front Immunol* (2013) 4:163. doi: 10.3389/fimmu.2013.00163
97. Taylor PA, Lees CJ, Blazar BR. The Infusion of Ex Vivo Activated and Expanded CD4(+)CD25(+) Immune Regulatory Cells Inhibits Graft-

Versus-Host Disease Lethality. *Blood* (2002) 99:3493–9. doi: 10.1182/blood.V99.10.3493
98. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, Lucas PJ, Gress RE, Levine BL, et al. L-Selectin(hi) But Not the L-Selectin(Lo) CD4+25+ T-Regulatory Cells are Potent Inhibitors of GVHD and BM Graft Rejection. *Blood* (2004) 104:3804–12. doi: 10.1182/blood-2004-05-1850
99. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ Regulatory T Cells Preserve Graft-Versus-Tumor Activity While Inhibiting Graft-Versus-Host Disease After Bone Marrow Transplantation. *Nat Med* (2003) 9:1144–50. doi: 10.1038/nm915
100. Tawara I, Sun Y, Liu C, Toubai T, Nieves E, Evers R, et al. Donor- But Not Host-Derived Interleukin-10 Contributes to the Regulation of Experimental Graft-Versus-Host Disease. *J Leukoc Biol* (2012) 91:667–75. doi: 10.1189/jlb.1011510
101. Tawara I, Shlomchik WD, Jones A, Zou W, Nieves E, Liu C, et al. A Crucial Role for Host APCs in the Induction of Donor CD4+CD25+ Regulatory T Cell-Mediated Suppression of Experimental Graft-Versus-Host Disease. *J Immunol* (2010) 185:3866–72. doi: 10.4049/jimmunol.1001625
102. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of Ex Vivo Expanded T Regulatory Cells in Adults Transplanted With Umbilical Cord Blood: Safety Profile and Detection Kinetics. *Blood* (2011) 117:1061–70. doi: 10.1182/blood-2010-07-293795
103. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Del Papa B, Perruccio K, et al. Immunoselection and Clinical Use of T Regulatory Cells in HLA-Haploidentical Stem Cell Transplantation. *Best Pract Res Clin Haematol* (2011) 24:459–66. doi: 10.1016/j.beha.2011.05.005
104. Meyer EH, Laport G, Xie BJ, MacDonald K, Heydari K, Sahaf B, et al. Transplantation of Donor Grafts With Defined Ratio of Conventional and Regulatory T Cells in HLA-Matched Recipients. *JCI Insight* (2019) 4(10): e127244. doi: 10.1172/jci.insight.127244
105. MacMillan ML, Hippen KL, McKenna DH, Kadidlo D, Sumstad D, DeFor TE, et al. First-In-Human Phase 1 Trial of Induced Regulatory T Cells for Graft-Versus-Host Disease Prophylaxis in HLA-Matched Siblings. *Blood Adv* (2021) 5:1425–36. doi: 10.1182/bloodadvances.2020003219
106. Marcondes AM, Li X, Tabellini L, Bartenstein M, Kabacka J, Sale GE, et al. Inhibition of IL-32 Activation by α-1 Antitrypsin Suppresses Alloreactivity and Increases Survival in an Allogeneic Murine Marrow Transplantation Model. *Blood* (2011) 118:5031–9. doi: 10.1182/blood-2011-07-365247
107. Gergoudis SC, DeFilipp Z, Özbek U, Sandhu KS, Etra AM, Choe HT, et al. Biomarker-Guided Preemption of Steroid-Refractory Graft-Versus-Host Disease With α-1-Antitrypsin. *Blood Adv* (2020) 4:6098–105. doi: 10.1182/bloodadvances.2020003336
108. Marcondes AM, Hockenbery D, Lesnikova M, Dinarello CA, Woolfrey A, Gernsheimer T, et al. Response of Steroid-Refractory Acute GVHD to α1-Antitrypsin. *Biol Blood Marrow Transplant* (2016) 22:1596–601. doi: 10.1016/j.bbmt.2016.05.011
109. Xu WS, Parmigiani RB, Marks PA. Histone Deacetylase Inhibitors: Molecular Mechanisms of Action. *Oncogene* (2007) 26:5541–52. doi: 10.1038/sj.onc.1210620
110. Johnstone RW. Histone-Deacetylase Inhibitors: Novel Drugs for the Treatment of Cancer. *Nat Rev Drug Discov* (2002) 1:287–99. doi: 10.1038/nrd772
111. Hahnen E, Hauke J, Tränkle C, Eyüpoglu IY, Wirth B, Blümcke I, et al. Histone Deacetylase Inhibitors: Possible Implications for Neurodegenerative Disorders. *Expert Opin Investig Drugs* (2008) 17:169–84. doi: 10.1517/13543784.17.2.169
112. Machado-Vieira R, Ibrahim L, Zarate CA. Histone Deacetylases and Mood Disorders: Epigenetic Programming in Gene-Environment Interactions. *CNS Neurosci Ther* (2011) 17:699–704. doi: 10.1111/j.1755-5949.2010.00203.x
113. Salim SY, Söderholm JD. Importance of Disrupted Intestinal Barrier in Inflammatory Bowel Diseases. *Inflamm Bowel Dis* (2011) 17:362–81. doi: 10.1002/ibd.21403
114. Joshi M, Reddy ND, Kumar N, Sumalatha S, Chamallamudi MR. Cinnamyl Sulfonamide Hydroxamate Derivatives Inhibited LPS-Stimulated NF-kB Expression in RAW 264.7 Cells. *Curr Pharm Des* (2020) 26:4934–43. doi: 10.2174/1381612826666200625101442
115. Friedrich M, Gerbeth L, Gerling M, Rosenthal R, Steiger K, Weidinger C, et al. HDAC Inhibitors Promote Intestinal Epithelial Regeneration *via* Autocrine Tgfβ1 Signalling in Inflammation. *Mucosal Immunol* (2019) 12:656–67. doi: 10.1038/s41385-019-0135-7
116. Choi S, Reddy P. HDAC Inhibition and Graft Versus Host Disease. *Mol Med* (2011) 17:404–16. doi: 10.2119/molmed.2011.00007
117. Kim S, Santhanam S, Lim S, Choi J. Targeting Histone Deacetylases to Modulate Graft-Versus-Host Disease and Graft-Versus-Leukemia. *Int J Mol Sci* (2020) 21(12):4281. doi: 10.3390/ijms21124281
118. Xu X, Li X, Zhao Y, Huang H. Immunomodulatory Effects of Histone Deacetylation Inhibitors in Graft-Vs.-Host Disease After Allogeneic Stem Cell Transplantation. *Front Immunol* (2021) 12:641910. doi: 10.3389/fimmu.2021.641910
119. Reddy P, Maeda Y, Hotary K, Liu C, Reznikov LL, Dinarello CA, et al. Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid Reduces Acute Graft-Versus-Host Disease and Preserves Graft-Versus-Leukemia Effect. *Proc Natl Acad Sci USA* (2004) 101:3921–6. doi: 10.1073/pnas.0400380101
120. Reddy P, Sun Y, Toubai T, Duran-Struuck R, Clouthier SG, Weisiger E, et al. Histone Deacetylase Inhibition Modulates Indoleamine 2,3-Dioxygenase-Dependent DC Functions and Regulates Experimental Graft-Versus-Host Disease in Mice. *J Clin Invest* (2008) 118:2562–73. doi: 10.1172/JCI34712
121. Choi SW, Braun T, Chang L, Ferrara JL, Pawarode A, Magenau JM, et al. Vorinostat Plus Tacrolimus and Mycophenolate to Prevent Graft-Versus-Host Disease After Related-Donor Reduced-Intensity Conditioning Allogeneic Haemopoietic Stem-Cell Transplantation: A Phase 1/2 Trial. *Lancet Oncol* (2014) 15:87–95. doi: 10.1016/S1470-2045(13)70512-6
122. Choi SW, Braun T, Henig I, Gatza E, Magenau J, Parkin B, et al. Vorinostat Plus Tacrolimus/Methotrexate to Prevent GVHD After Myeloablative Conditioning, Unrelated Donor HCT. *Blood* (2017) 130:1760–7. doi: 10.1182/blood-2017-06-790469
123. Perez L, Fernandez H, Horna P, Riches M, Locke F, Field T, et al. Phase I Trial of Histone Deacetylase Inhibitor Panobinostat in Addition to Glucocorticoids for Primary Therapy of Acute Graft-Versus-Host Disease. *Bone Marrow Transplant* (2018) 53:1434–44. doi: 10.1038/s41409-018-0163-z
124. Perez L, Fernandez H, Kharfan-Dabaja M, Khimani F, Betts B, Mishra A, et al. A Phase 2 Trial of the Histone Deacetylase Inhibitor Panobinostat for Graft-Versus-Host Disease Prevention. *Blood Adv* (2021) 5:2740–50. doi: 10.1182/bloodadvances.2021004225
125. Fricker LD. Proteasome Inhibitor Drugs. *Annu Rev Pharmacol Toxicol* (2020) 60:457–76. doi: 10.1146/annurev-pharmtox-010919-023603
126. Wang J, Maldonado MA. The Ubiquitin-Proteasome System and its Role in Inflammatory and Autoimmune Diseases. *Cell Mol Immunol* (2006) 3:255–61.
127. Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, et al. Proteasome Inhibitors: A Novel Class of Potent and Effective Antitumor Agents. *Cancer Res* (1999) 59:2615–22.
128. Zollner TM, Podda M, Pien C, Elliott PJ, Kaufmann R, Boehncke WH. Proteasome Inhibition Reduces Superantigen-Mediated T Cell Activation and the Severity of Psoriasis in a SCID-Hu Model. *J Clin Invest* (2002) 109:671–9. doi: 10.1172/JCI0212736
129. Elliott PJ, Pien CS, McCormack TA, Chapman ID, Adams J. Proteasome Inhibition: A Novel Mechanism to Combat Asthma. *J Allergy Clin Immunol* (1999) 104:294–300. doi: 10.1016/S0091-6749(99)70369-6
130. Adams J, Kauffman M. Development of the Proteasome Inhibitor Velcade (Bortezomib). *Cancer Invest* (2004) 22:304–11. doi: 10.1081/CNV-120030218
131. Richardson PG, Hideshima T, Anderson KC. Bortezomib (PS-341): A Novel, First-in-Class Proteasome Inhibitor for the Treatment of Multiple Myeloma and Other Cancers. *Cancer Control* (2003) 10:361–9. doi: 10.1177/107327480301000502
132. Sun K, Li M, Sayers TJ, Welniak LA, Murphy WJ. Inhibition of Acute Graft-Versus-Host Disease With Retention of Graft-Versus-Tumor Effects by the Proteasome Inhibitor Bortezomib. *Proc Natl Acad Sci USA* (2004) 101:8120–5. doi: 10.1073/pnas.0401563101
133. Vodanovic-Jankovic S, Hari P, Jacobs P, Komorowski R, Drobyski WR. NF-kappaB as a Target for the Prevention of Graft-Versus-Host Disease: Comparative Efficacy of Bortezomib and PS-1145. *Blood* (2006) 107:827–34. doi: 10.1182/blood-2005-05-1820
134. Sun K, Li M, Sayers TJ, Welniak LA, Murphy WJ. Differential Effects of Donor T-Cell Cytokines on Outcome With Continuous Bortezomib Administration After Allogeneic Bone Marrow Transplantation. *Blood* (2008) 112:1522–9. doi: 10.1182/blood-2008-03-143461

135. Li Z, Wu Q, Yan Z, Li D, Lu G, Mou W, et al. The Protection and Therapy Effects of Bortezomib in Murine Acute Graft-Versus-Host Disease. *Transplant Proc* (2013) 45:2527–35. doi: 10.1016/j.transproceed.2013.03.042
136. Koreth J, Stevenson KE, Kim HT, McDonough SM, Bindra B, Armand P, et al. Bortezomib-Based Graft-Versus-Host Disease Prophylaxis in HLA-Mismatched Unrelated Donor Transplantation. *J Clin Oncol* (2012) 30:3202–8. doi: 10.1200/JCO.2012.42.0984
137. Koreth J, Kim HT, Lange PB, Bindra B, Reynolds CG, Chammas MJ, et al. A Bortezomib-Based Regimen Offers Promising Survival and Graft-Versus-Host Disease Prophylaxis in Myeloablative HLA-Mismatched and Unrelated Donor Transplantation: A Phase II Trial. *Biol Blood Marrow Transplant* (2015) 21:1907–13. doi: 10.1016/j.bbmt.2015.05.027
138. Koreth J, Kim HT, Lange PB, Poryanda SJ, Reynolds CG, Rai SC, et al. Bortezomib-Based Immunosuppression After Reduced-Intensity Conditioning Hematopoietic Stem Cell Transplantation: Randomized Phase II Results. *Haematologica* (2018) 103:522–30. doi: 10.3324/haematol.2017.176859

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Off-the-Shelf Partial HLA Matching SARS-CoV-2 Antigen Specific T Cell Therapy: A New Possibility for COVID-19 Treatment

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Background: Immunological characteristics of COVID-19 show pathological hyperinflammation associated with lymphopenia and dysfunctional T cell responses. These features provide a rationale for restoring functional T cell immunity in COVID-19 patients by adoptive transfer of SARS-CoV-2 specific T cells.

Methods: To generate SARS-CoV-2 specific T cells, we isolated peripheral blood mononuclear cells from 7 COVID-19 recovered and 13 unexposed donors. Consequently, we stimulated cells with SARS-CoV-2 peptide mixtures covering spike, membrane and nucleocapsid proteins. Then, we culture expanded cells with IL-2 for 21 days. We assessed immunophenotypes, cytokine profiles, antigen specificity of the final cell products.

Results: Our results show that SARS-CoV-2 specific T cells could be expanded in both COVID-19 recovered and unexposed groups. Immunophenotypes were similar in both groups showing CD4+ T cell dominance, but CD8+ and CD3+CD56+ T cells were also present. Antigen specificity was determined by ELISPOT, intracellular cytokine assay, and cytotoxicity assays. One out of 14 individuals who were previously unexposed to SARS-CoV-2 failed to show antigen specificity. Moreover, ex-vivo expanded SARS-CoV-2 specific T cells mainly consisted of central and effector memory subsets with reduced alloreactivity against HLA-unmatched cells suggesting the possibility for the development of third-party partial HLA-matching products.

Discussion: In conclusion, our findings show that SARS-CoV-2 specific T cell can be readily expanded from both COVID-19 and unexposed individuals and can therefore be manufactured as a biopharmaceutical product to treat severe COVID-19 patients.

One Sentence Summary: Ex-vivo expanded SARS-CoV-2 antigen specific T cells developed as third-party partial HLA-matching products may be a promising approach for treating severe COVID-19 patients that do not respond to previous treatment options.

Keywords: COVID19, T cell therapy, HLA sharing, virus specific T cells, viral immunity

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has caused significant medical, social, and economic disruptions worldwide. The recent development of vaccines by global pharmaceutical companies has brought us a step closer to eradicating the virus. However, while vaccination can prevent future infections, there have already been more than 3,025,835 deaths due to the absence of appropriate therapeutic measures, and this number continues to increase (1). Approximately 20% of COVID-19 patients develop life-threatening pneumonia, which requires extensive medical care. An increasing number of cases, along with a limited number of available intensive care units and medical teams, has led to a collapse of healthcare systems in many countries (2).

In this study, we investigated the role of T cell responses in viral immunity and hypothesized that adoptive T cell therapy may be a promising and safe approach to combat SARS-CoV-2. Virus-specific T cells (VST) have been used in the treatment of various infectious diseases (3). However, the majority of VST therapies have been focused on latent DNA-virus infections, especially in the hematopoietic stem cell transplantation setting (4, 5), instead of acute infections. COVID-19 is a challenging clinical situation for VST application. COVID-19 is an acute viral infection associated with pro-inflammatory cytokine storms that may negatively impact the infused T cells. Moreover, T cells are more readily expanded in convalescent donors in the presence of memory T cells and, therefore, the donor-sources for SARS-CoV-2-specific T cells may be limited. It has been reported, however, that unexposed healthy individuals contain cross-reactive T cells against common coronaviruses that may respond to SARS-CoV-2 (6, 7). Researchers have already attempted to use VSTs to treat high-risk COVID19 patients in the absence of other effective treatments. These studies have adopted various T-cell production methods, including culture-expansion (8, 9) and automatic selection methods (10, 11). Regardless of the method used, CD4+ T cells, especially type 1 helper T (Th1) cells, dominated over CD8+ T cells in SARS-CoV-2 specific T-cell immunity (6, 7, 12).

In this study, we aimed to demonstrate the effects of culture-expanded SARS-CoV-2-specific T cells from both COVID-19 recovered and unexposed individuals. We demonstrated that these ex-vivo expanded cells were cytotoxic to SARS-CoV-2 protein-expressing target cells and may also exhibit immunosuppressive properties to inhibit pro-inflammatory cytokines. We suggest two different clinical applications of these cells, either as personalized autologous vaccines or as third-party off-the-shelf bioproducts.

MATERIALS AND METHODS

Donors

Seven recovered COVID-19 patients, who were treated at Seoul St. Mary's Hospital, were recruited for blood donations. Thirteen randomly selected blood donors, unexposed to SARS-CoV-2, were also recruited. All donors fulfilled the blood bank eligibility criteria and were examined for clinical signs of COVID-19, including fever and respiratory symptoms, in accordance with the hospital's quarantine regulations. All donors signed consent forms approved by the Institutional Review Board of Seoul St. Mary's Hospital, and all procedures were performed in accordance with the Declaration of Helsinki (KC20TSSI0274, KC20TSSI0872).

For recovered donors, individuals who have been quarantined after confirmation of COIVD-19 or who have received treatment and met all the release criteria from public quarantine was defined as recovered. Clinically, individuals did not show fever and showed improvement in clinical symptoms even without taking antipyretic drugs. Furthermore, recruited individuals must have had confirmed negative PCR test results at least twice at 24-hour intervals. For unexposed donors, individuals must have no fever or respiratory symptoms and must be SARS-CoV-2 Ab IgG antibody negative. For both recovered and unexposed donors the exclusion criteria include those who are HIV antibody positive, syphilis positive, hepatitis B or C carriers or actively infected with other serious infections requiring medical treatment.

We obtained either 50 cc of whole blood or one blood volume of leukapheresis from each donor. This study was approved by the Clinical Research Information Service, Republic of Korea, and registered in the World Health Organization Registry Network (KCT0005370, KCT0005864). Donor characteristics are shown in **Table 1**.

Human Leukocyte Antigen (HLA) Testing

For HLA typing of donors, an aliquot of the peripheral blood was sent to the Catholic Hematopoietic Stem Cell Bank for analysis. The sequence-based typing (SBT) method was used as previously described (13). DNA was extracted from whole blood in ethylenediaminetetraacetic acid (EDTA)-coated blood containers. The AlleleSEQR kit (Abbott, Chicago, IL, USA) was used for HLA Class I and Class II genotyping, according to the manufacturer's instructions. Sequencing was performed using the ABI 3130XL genetic analyzer (Applied Biosystems, Waltham, MA, USA) with POP 6 polymer for exons 2, 3, and 4 of HLA-A, B, and C, and for exon 2 and codon 86 for HLA-DRB1. HLA types were analyzed using SBTengine (Genome Diagnostics B.V., Utrecht, Netherlands), and reanalyzed using Assign SBT v3.5

TABLE 1 | Donor characteristics.

Donor No.	COVID19	Sex/ Age	SARS-CoV2IgG/ IgM*	COVID19 severity	COVID19 treatment used	Days to recovery from diagnosis	Days from recovery to cell production
1	Recovered	F/61	3+/1+	Mild	Steroids, Haloxin, Kaletra	23	103
2	Recovered	M/61	4+/1+	Severe	Steroids, Haloxin, Kaletra	31	90
3	Recovered	M/43	1+/-	Mild	Steroids, Haloxin, Kaletra	14	113
4	Unexposed	M/29	-/-	N.A	N.A	N.A	N.A
5	Unexposed	F/33	-/-	N.A	N.A	N.A	N.A
6	Unexposed	F/27	-/-	N.A	N.A	N.A	N.A
7	Unexposed	F/23	-/-	N.A	N.A	N.A	N.A
8	Unexposed	M/33	-/-	N.A	N.A	N.A	N.A
9	Unexposed	F/30	-/-	N.A	N.A	N.A	N.A
10	Unexposed	M/32	-/-	N.A	N.A	N.A	N.A
11	Unexposed	F/27	-/-	N.A	N.A	N.A	N.A
12	Unexposed	F/27	-/-	N.A	N.A	N.A	N.A
13	Unexposed	M/25	-/-	N.A	N.A	N.A	N.A
14	Recovered	F/58	N.T.	Mild	Steroids, Haloxin, Kaletra	26	87
15	Recovered	F/60	4+/-	Mild	Steroids	15	78
16	Recovered	M/23	-/+	Mild	None	Unknown	Unknown
17	Recovered	M/23	2+/-	Mild	None	Unknown	Unknown
18	Unexposed	F/29	-/-	N.A	N.A	N.A	N.A
19	Unexposed	F/64	-/-	N.A	N.A	N.A	N.A
20	Unexposed	F/39	-/-	N.A	N.A	N.A	N.A

N.A, not applicable; N.T, not tested.

**) Test results from SGTi-flex COVID-19 IgM/IgG assay (Sugentech Inc., Korea) were used.*

(Conexio Genomics, Applecross, Australia). The HLA types of donors are listed in **Table 2**.

Anti-SARS-CoV-2 IgG and IgM Detection

We used different commercial assays according to the manufacturer's instructions to detect Anti-SARS-CoV-2 IgG and IgM. We used SGTi-flex COVID-19 IgM/IgG assay (Sugentech Inc., Korea), SARS-CoV-2 IgG assay (Abbott, Chicago, IL, USA), Elecsys Anti-SARS-CoV-2 assay (Roche Diagnostics, Basel, Switzerland), and ADVIA Centaur SARS-CoV-2 Total assay (Siemens, Munich, Germany).

SARS-CoV-2 Specific T Cell (SARS-CoV-2 Specific CTL) Generation

To generate SARS-CoV-2 specific CTLs, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient centrifugation. Then, at least $1x10^6$ PBMCs were seeded in an appropriate well plate or flask, depending on the starting cell numbers, at a cell density of $1x10^7$ cells/mL. PBMCs were stimulated with Peptivators for SARS-COV-2 Spike (S), Membrane (M), and Nucleocapsid (N) (1 ug/mL; Miltenyi Biotec, Bergisch Gladbach, Germany) proteins. Peptivator is a pool of lypophilized peptides, consisting of 15-mer sequences

TABLE 2 | HLA types of Donors.

Donor No.	HLA-A		HLA-B		HLA-C		HLA-DRB	
1	02:01	33:03	55:02	58:01	03:02	03:03	12:01	13:02
2	24:02	26:02	15:07	51:02	03:03	15:02	04:03	09:01
3	11:01	24:02	54:01	54:01	01:02	01:02	04:05	08:03
4	02:01	33:03	15:07	58:01	03:02	03:03	04:03	13:02
5	24:02	30:01	07:02	40:06	07:02	08:01	01:01	08:03
6	24:02	30:01	13:02	40:02	03:04	06:02	07:01	11:01
7	24:02	26:02	51:01	54:01	07:02	14:02	11:01	14:05
8	02:01	02:01	07:02	15:11	03:03	07:02	09:01	15:01
9	24:02	33:03	40:06	44:03	01:02	14:03	09:01	13:02
10	02:06	33:03	44:03	54:01	01:02	14:03	13:02	15:01
11	02:07	24:02	15:11	46:01	01:02	03:03	09:01	09:01
12	02:01	24:02	27:05	40:06	01:02	02:02	14:05	15:02
13	24:02	24:02	15:07	56:01	01:02	03:03	04:03	14:54
14	02:01	11:01	51:01	15:11	03:03	15:02	04:05	14:05
15	02:01	30:01	40:06	44:03	03:03	07:06	0701	14:03
16	24:02	33:03	35:01	44:03	03:04	07:06	04:03	07:01
17	02:01	03:01	13:01	27:05	02:02	03:04	04:05	15:02
18	02:06	30:01	13:02	51:01	06:01	14:02	04:05	11:01
19	24:02	24:01	51:01	54:01	01:02	14:02	04:05	12:01
20	24:02	33:03	44:03	51:01	07:06	14:02	07:01	12:01

with 11 amino acids overlap, covering the entire sequence of each protein. Peptivator for Spike protein only covers the immunodominant sequence domains of the spike glycoprotein. On the same day, the cells were further stimulated with 50ng/mL of recombinant human interferon-gamma (rhIFN-γ; R&D systems, Minneapolis, MN, USA). A few days later, the cells were expanded using 60ng/mL of recombinant human interleukin-2 (rhIL-2; R&D systems), applied every 3–4 days for 3–4 weeks. Cells were suspended and maintained in 5% human serum (Sigma-Aldrich, St. Louis, MO, USA) containing AIM-V medium (Gibco; Thermo Fisher Scientific, Wilmington, DE, USA) at 37°C under 5% (v/v) CO_2. Cells were later harvested and cryopreserved for further characterization.

Six out of twenty leukapheresis products were produced at the cell processing facility of the Catholic Institute of Cell Therapy, at the Catholic University of Korea, in accordance with good manufacturing practices (GMPs). The same manufacturing method was performed using animal-free reagents. On the day of harvest, phenotypes and functional potencies of the cells were characterized. The final products were also tested for sterility, mycoplasma, endotoxins and adventitious viruses.

Preparation of PHA-Blasts

Phytohaemagglutinin (PHA)-induced blasts were prepared from autologous PBMCs stimulated with PHA (3 μg/mL; Sigma-Aldrich) and rhIL-2 (25 IU/mL) in 5% human serum containing AIM-V medium for 3–4 days. For peptide pulsing, PHA-blasts ($1x10^7$/mL) were harvested and incubated with CMV pp65 peptivator (Miltenyi Biotec) at a concentration of 2 μg/mL and incubated at 37°C, under 5% (v/v) CO_2, for 2 hours.

Immunophenotyping

Immunophenotyping of PBMCs was performed by staining for various surface-marker combinations using the following fluorescence-conjugated antibodies: CCR7 (3D12), CD3 (UCHT1), CD4 (SK3), CD8 (SK1), CD14(61D3), CD16 (CB16), CD19 (HIB19), CD45RA (H100), CD45RO (UCHL1), CD56 (TULY56, CMSSB), CD57 (QA17A04; Biolegend, San Diego, CA, USA), CD62L (DREG-56), Lag3 (3DS223H), PD-1 (EH-12.2H7; Biolegend), and Tim3 (F38-2E2). All antibodies were purchased from eBioscience, Inc. (San Diego, CA, USA), unless mentioned otherwise. Cells were washed once with flow cytometry staining buffer, pelleted, and antibodies were added prior to incubation in the dark, at room temperature for 15 minutes. The cells were then washed and analyzed. Flow cytometry gating strategy for various lymphocytes were first based on a low forward scatter (FSC) and low side scatter (SSC) gating of lymphocytes. Then, T cell markers CD3, CD4, CD8; NK cell marker CD56 were used to identify the major lymphocyte subsets. Various markers were used to assess activation, cytokine production, and exhaustion.

Intracellular Cytokine Staining Following Peptide Stimulation

Expanded cells were stimulated overnight with SARS-COV-2 S, M, and N peptivators (1 μg/mL each; Miltenyi Biotec). Protein transport inhibitor containing monensin (BD GolgiStop; Pharmingen, San Diego, CA, USA) was added in the last 4h of incubation at 37°C, under 5% (v/v) CO_2. Positive controls were stimulated with cell stimulation cocktails containing phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin (Invitrogen Corp.; Waltham, MA, USA) while negative controls were not peptide-stimulated. Cells were first stained for surface markers: CD3, CD56, CD8, and CD4. Intracellular IFN-γ and TNF-α, IL-2 staining was performed according to the manufacturer's instructions for the kit (eBioscience). Flow cytometry was performed using the BD Biosciences Fortessa cytometer.

Activation-Induced Marker (AIM) Assay

Expanded cells were stimulated with SARS-COV-2 S, M, and N peptivator (1 μg/mL each; Miltenyi Biotec). Unstimulated cells were used as negative controls. Following overnight stimulation, cells were surface stained for CD3, CD4, CD8, CD56, CD137 (4B4), CD154 (14–21), CD25 (BC96), CD38 (HIT2), CD69 (FN50), and HLA-DR (L243). Flow cytometry was performed using the BD Biosciences Fortessa cytometer.

Foxp3 Staining for Treg Cell Detection

To detect Treg cells, Foxp3 staining was performed using the eBioscience Foxp3 staining kit. Expanded cells were stained for surface markers: CD3, CD25, CD127 (EBioRDR5; eBioscience), CD4, and CD8. The cells were resuspended in 500 μL fixation/permeabilization buffer and incubated for 30 minutes at 4°C, and then washed in permeabilization buffer. The cells were then pelleted and incubated with FoxP3 (PCH10; eBioscience) antibodies for 30 minutes at 4°C. Flow cytometry was performed using the BD Biosciences Fortessa cytometer. In some experiments, the cells were stimulated with peptides overnight, prior to Foxp3 staining.

Cell Proliferation Assay

To measure alloreactivity and cell proliferation, expanded CTLs were labeled with CellTrace Cell Proliferation kit (Invitrogen) according to the manufacturer's instructions. The SARS-CoV-2-specific CTLs were incubated with CellTrace CFSE dye for 20 minutes at room temperature in the dark. Cells were then washed with cell culture medium to remove remaining free dye from the solution. Labeled cells were pelleted by centrifugation and resuspended in cell culture medium, and then incubated for at least 10 minutes before cell stimulation. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelled SARS-CoV-2-specific CTLs were co-cultured for 5 days with allogeneic PBMCs or non-CD4, HLA-DR+ sorted antigen presenting cells. Acquisition and analysis were completed using the FACS Canto cytometer (BD Biosciences).

Cytotoxicity *Assay*

Flow cytometry-based cytotoxicity assay was performed as previously described (22). The target cells ($1x10^6$/mL) were labeled with CFSE in complete culture medium at room temperature, under 5% CO_2 for 20 minutes, as per the manufacturer's protocols. Target cells ($1x10^5$) were incubated overnight with the effector cells at different effector:target ratios.

Prior to flow cytometric acquisition, cells were stained with 7-Aminoactinomycin D (7-AAD; eBioscience) and cytotoxicity was evaluated using a FACS Canto cytometer (BD Biosciences). Target cells were gated on CFSE+ cells and then examined for cell death by uptake of 7-AAD. The percentage of effector cell mediated cytotoxicity was then calculated using the following equation:

$$Cytotoxicity\ (\%) = \frac{(\text{Dead target cells}(\%) - \text{spontaneous deaths}\ (\%)) \times 100}{(100 - \text{spontaneous deaths}\ (\%))}$$

ELISPOT Assay

For detection of IFN-γ secreting cells, ELISPOT assays were performed using the BD ELISPOT assay kit, according to the manufacturer's instructions. The final T cell products were serially diluted from $1x10^5$ to $1.25x10^4$ cells/well. SARS-CoV-2 antigen specific activity was measured using SARS-CoV-2 S, M, N peptivators (1 μg/mL each; Miltenyi Biotec). Each culture condition was run in triplicate. The number of spots corresponding to IFN-γ secreting cells was counted using an AID-ELISPOT-Reader.

Luminex Multiplex Cytokine Assay

Concentrations of the following immune molecules were determined using a magnetic bead-based immunoassay: CD40L, CD137 (4-1BB), CD152 (CTLA4), GM-CSF, Granzyme B, ICAM-1, IFN-α, IFN-β, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-6, 7, IL-8 (CXCL8), IL-9, IL-10, IL-12p70, IL-15, IL-17A, IL-21, IL-23, perforin, and TNF-α (Procartaplex; Thermo Fisher Scientific, Wilmington, DE, USA). Cell culture supernatant samples were obtained from SARS-CoV-2-specific CTLs before and after SARS-CoV-2 peptide stimulation. The median fluorescence intensities of analytes were detected using the flow-based MAGPIX System (MilliporeSigma, Burlington, MA, USA). Cytokine concentrations were calculated using Luminex xPONENT v. 4.2 software, using a standard curve derived from known reference concentrations supplied by the manufacturer. A five-parameter model was used to interpolate the final concentrations. Values were expressed as pg/mL.

Data Analysis

Flow cytometry data were analyzed using FlowJo 9.9.6 (FlowJo, LLC., Ashland, OR, USA). Prism (GraphPad Software Inc., San Diego, CA, USA) was used for plotting data and statistical analyses. Data were presented as means ± ranges, unless indicated otherwise. The number of donors was denoted by *n*.

Statistical Analysis

Data were presented as means ± standard deviations (SDs) unless indicated. Mann–Whitney U test or Student's t-test were used for comparison between two groups, while the Kruskal–Wallis test was used for comparison between multiple groups. Statistical analyses were performed using the SPSS Statistics software (version 16.0; IBM Inc., Armonk, NY, USA). P-values < .05 were considered significant.

RESULTS

Dominant CD4+ Phenotypes in *Ex Vivo* Culture-Expanded SARS-CoV-2 Specific T Cells Mainly Consisting of Effector Memory Cells

We collected either 50 cc of peripheral blood, or one blood volume leukapheresis from both COVID-19 recovered and unexposed donors. Following isolation, PBMCs were stimulated with SARS-CoV-2 S, M, and N peptides along with IFN-γ. We then induced cell proliferation by addition of IL-2 and expanded the cultures for 21 days (**Figure 1A**). While the expansion rate varied among individuals, overall expansion seemed to be more effective in COVID-19 recovered individuals (range: 1.20–166.50, median: 23.7) compared to unexposed individuals (range: 0.0129–130, median: 7.8; **Figures 1B, C**), but the differences were not statistically significant. We investigated the immune cell subsets in the final cell products of COVID-19 recovered and unexposed individuals (**Figure 1D**). The cells showed prominent CD3+ T cell immunophenotype (median: 93.8 and 93.9 in recovered and unexposed, respectively) with the presence of CD3+CD56+ T cells (median: 18.5 and 6.07 in recovered and unexposed, respectively). CD14+ monocytes, CD3-CD56+ natural killer cells, and CD19+ B cells were either absent or less than 5% (**Figure 1C**). There were no significant differences in immunophenotypes of the final cell products between recovered and unexposed individuals. Within CD3+ T cells, we observed slightly higher levels of CD4+ T cells (median 58.6 and 46.3 in recovered and unexposed, respectively) compared to CD8+ T cells (median: 25.8 and 24.7 in recovered and unexposed, respectively). We examined the changes in immunophenotypes before culture, and post-culture on days 4, 7, 14, and 21. We observed a decrease in all immune cell subsets until day 7 of culture, suggesting that cell deaths were not affected by the added SARS-CoV-2 peptides and cytokines (**Supplementary Figure 1**).

Moreover, the memory T cell subtypes of CD4+ and CD8+ in recovered and unexposed individuals showed reduced levels of CD45RA+CD62L+ naive T cells and increased levels of CD45RA+CD62L− central memory and CD45RA−CD62L− effector memory T cells (**Figure 1E**). Final products from COVID-19 recovered individuals showed higher central memory CD4+ and CD8+ T cell levels, compared to unexposed individuals; however, the differences were not significant. Final products from unexposed individuals showed significantly higher levels of CD45RA+CD62L− terminally-differentiated memory CD4+ T cells, but comparable CD8+ T cell levels. There was an overall decrease in naive CD4+ and CD8+ T cells, until there were almost none present on the day of harvest (**Supplementary Figure 1B**). On the other hand, effector memory CD4+ T cells showed a sudden increase during the last week of culture, accompanied by a drop in central memory CD4+ T cell levels. For CD8+ T cells, effector memory and terminally-differentiated effector memory cells showed a steady increase, but central memory

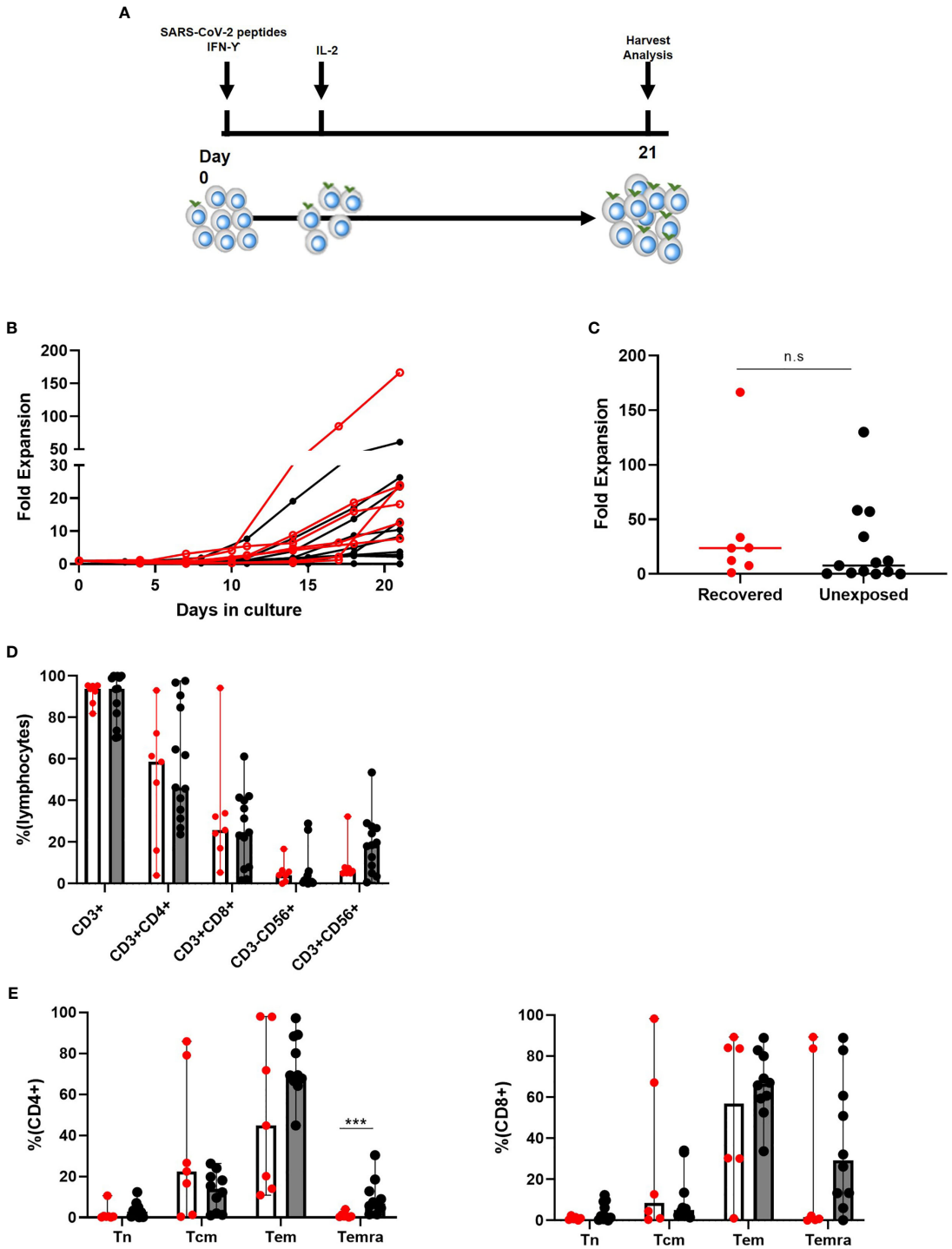

FIGURE 1 | Characteristics of SARS-CoV-2-specific T cells from recovered (n = 7) and unexposed (n = 13) individuals. **(A)** Generation method. **(B, C)** Fold expansion of cells after 21 days of culture (Red indicates COVID-19 recovered individuals; black indicates unexposed individuals). **(D)** Immunophenotypic analysis: COVID19-recovered (red circles with white bar) and unexposed (black circles with gray bars) individuals. **(E)** Memory type of CD4+ and CD8+ T cells. Tn: CD45RA +CD62L+ naive T cells; Tcm: CD45RA–CD62L+ central memory T cells; Tem: CD45RA–CD62L– effector memory T cells; Temra: CD45RA+CD62l– terminally differentiated effector memory T cells; *n.s.*, not significant. ***p <.001. The bar indicates median and the error bars indicate range.

CD8+ T cells showed a rapid drop during the last week of culture.

Therefore, expansion of SARS-CoV-2 specific T cells were feasible in both recovered and unexposed individuals and these final T cell products consisted of mainly CD4+ and also CD8+ effector memory T cells with the presence of CD3+CD56+ T cells.

Expanded SARS-CoV-2 Specific T Cells From Recovered Individuals Showed Higher Antigen-Specificity Against SARS-CoV-2 S, M, and N Proteins

Next, we determined whether the expanded cells were specific to SARS-CoV-2 S, M, and N proteins by detecting anti-viral cytokine production following antigenic stimulation. We

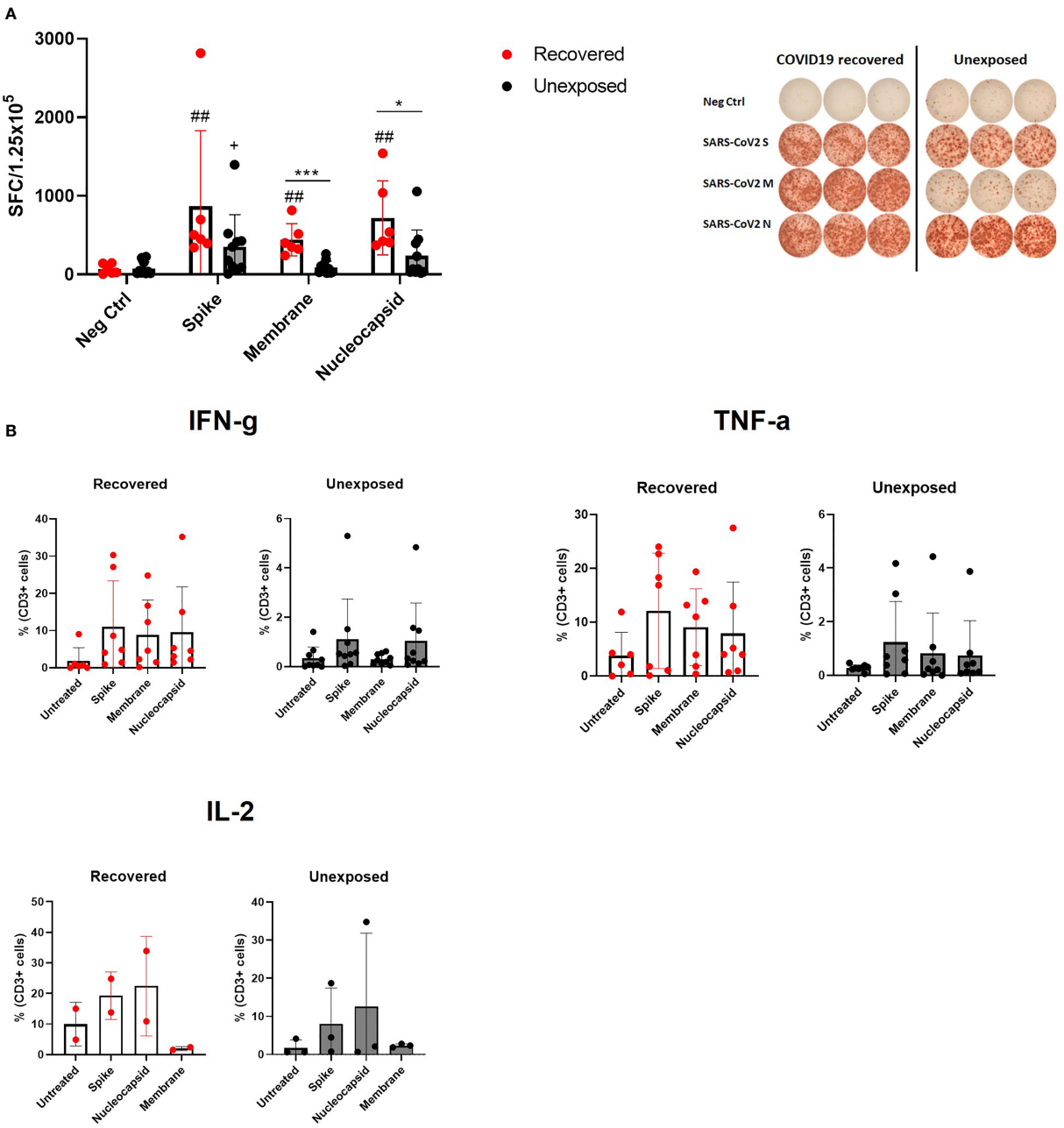

FIGURE 2 | Continued

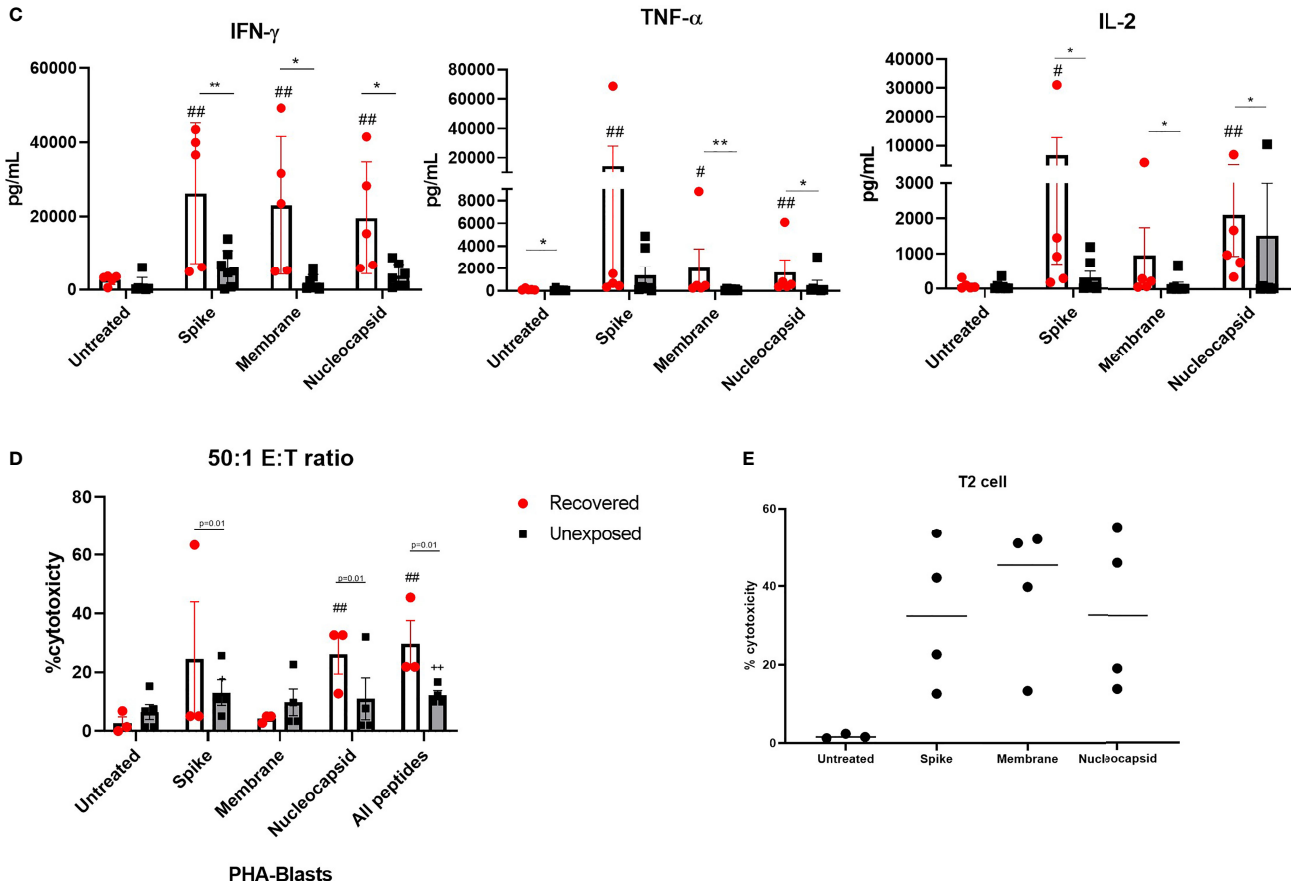

FIGURE 2 | Antigen specificity of SARS-CoV-2-specific T cells for SARS-CoV-2 proteins. **(A)** IFN-γ ELISPOT assay against S, M, and N peptide mixtures covered viral proteins in COVID19 recovered (n=6, red circles with white bar) and unexposed (n=9, black circles with gray bar) individuals. The number of spot-forming cells per 1.25 x 10^5 cells are shown. Neg Ctrl indicates negative controls, in which the cells were treated with fresh complete medium only. SARS-CoV-2-specific T cells were treated with S, M, or N peptide mixtures for 24 hours. Right panel shows a representative ELISPOT result between COVID-recovered and unexposed individual **(B)** Flow cytometric analysis of CD3+ T cell intracellular cytokine production from recovered (n=6) and unexposed (n=9) individuals. **(C)** Supernatant multiplex assays to measure cytokine production from recovered (n=6, red circles with white bar) and unexposed (n=9, black circles with gray bar) individuals. Untreated group was the negative control group. **(D)** Specific cytotoxic activity of SARS-CoV-2 specific T cells on autologous non-pulsed (untreated) and SARS-CoV-2 peptide pulsed PHA-induced blasts. Cells were co-cultured at a 50:1 effector to target ratio for 4 hours, and cell deaths were determined using flow cytometry. Cytotoxicity of SARS-CoV-2 T cells produced from recovered (n=3, red circles with white bar) and unexposed (n=5, black circles with gray bar) individuals were compared. **(E)** Cytotoxic effect against SARS-CoV_2 peptide pulse T2 cell line was evaluated. SARS-CoV-2 specific T cells (n=4) were co-cultured at 50:1 effector to target ratio overnight and cell death was determined using flow cytometry. Each circle represents a donor. Statistical comparisons between unexposed and recovered individual groups are indicated as follows *p <.05; **p <.01; ***p <.001. Statistical comparisons between recovered negative control and peptide mixture treated groups are indicated as follows #p<.05; ##p<.01. Statistical comparisons between unexposed negative control and peptide mixture treated groups are indicated as follows +p<.05; ++p<.01.

performed ELISPOT assays to determine IFN-γ secreting cells against each SARS-CoV-2 protein. All but two unexposed donors showed IFN-γ secreting cells against at least one protein among three of SARS-CoV-2 S, M, and N proteins. In other words, 18 out of 20 SARS-CoV-2 specific T cell products showed IFN- γ production against one or more SARS-CoV-2 proteins. The numbers of IFN-γ secreting cells against S-glycoprotein were similar between COVID-19 recovered and unexposed individuals. However, the numbers of IFN-γ secreting cells against M and N proteins were significantly higher in COVID-19 recovered individuals (**Figure 2A**). Prior to culture, the numbers of IFN-γ secreting cells were higher in recovered individuals, whereas almost none were detected in unexposed individuals (**Supplementary Figure 2A**). However, intracellular cytokine secreting assay by flow cytometry showed that the antigen-specific cytokine production between recovered and unexposed individuals were almost similar with no significant differences prior to culture (**Supplementary Figure 2B**). After 21 days of culture, antigen-specific cytokine production including IFN-γ, TNF-α, and IL-2 were expanded with significantly elevated levels in the recovered group (**Figure 2B**). CD4+, CD8+, and CD3+CD56+ T cells were mainly responsible for IFN-γ and TNF-α secretion, whereas CD8+ T cells and a proportion of CD3-CD56+ were the source of IL-2 production (**Supplementary Figure 2C**). Similar to the ELISPOT results, IFN-γ secreting

cells were increased against S and N peptide mixtures. Moreover, TNF-α cells and IL-2 secreting cells were responsive to S and N restimulation. The cytokine production in the supernatants was similar to the previous results (**Figure 2C**), but IFN-γ production against M peptide stimulation was higher in the culture supernatant. The levels of cytokine production in response to antigen re-stimulation was significantly higher in COVID-19 recovered individuals compared to unexposed individuals' SARS-CoV-2 T cell product.

To test for direct cytotoxic effects, we used SARS-CoV-2 peptide pulsed autologous PHA-blasts as an alternative to SARS-CoV-2 infected cells. We observed higher cytotoxicity with SARS-CoV-2 peptide pulsed PHA-blasts compared to unpulsed PHA-blasts (**Figure 2D**). There was a slight increase in cytotoxicity when all three peptides were pulsed in comparison to single peptide pulsing. While the trend indicated that the cytotoxic effects of SARS-CoV-2 specific T cells from recovered individuals tend to be higher than those from unexposed individuals, a broad variation of cytotoxicity between donors still exist for direct comparison. In addition, we co-cultured HLA-A2 expressing SARS-CoV-2 specific T cells with SARS-CoV-2 peptide-pulsed T2 cell line (**Figure 2E**) demonstrating high anti-viral cytotoxic effects. While the direct cytotoxic effects of SARS-CoV-2 specific T cells differed between each individual, granzyme B and perforin secreting cells were consistently upregulated in the cells expanded from both recovered and unexposed individuals. CD8+ T cells were the major source of granzyme B and perforin prior to culture; however, All major immune subsets of the final products were able to produce these cytotoxic molecules (**Supplementary Figures 3A, B**). Moreover, granzyme B and perforin levels

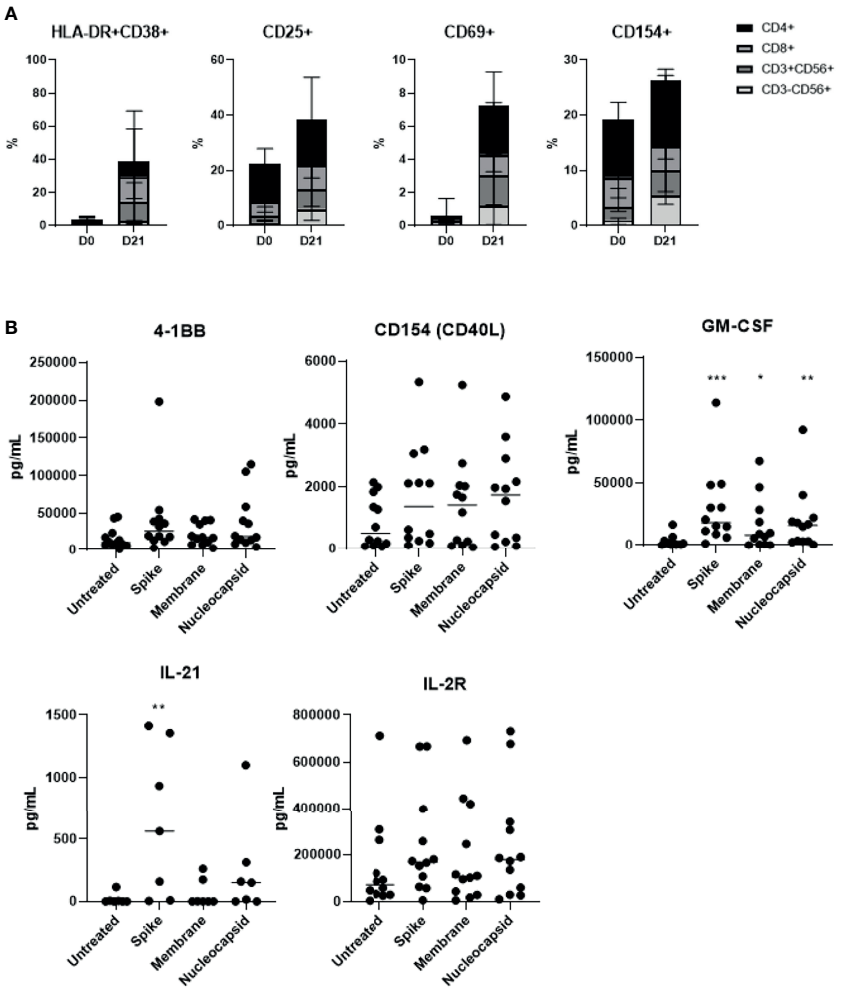

FIGURE 3 | SARS-CoV-2-specific T cells showed high activation marker levels, which were further up-regulated in response to antigenic stimulation (n=15, 6 recovered and 9 unexposed individuals). **(A)** HLA–DR+CD38+, CD25+, CD69+ and CD154+ for all lymphocyte subsets were measured prior to and after 21 days of culture. **(B)** SARS-CoV-2-specific T cells were treated with S, M, or N peptide mixtures for at least 18 hours, and the supernatant was collected to measure T-cell activation-related cytokine production from 5 unexposed and 7 recovered individuals. *p <.05; **p <.01; ***p <.001.

were produced at similar levels in the final cell products from both unexposed and COVID-19 recovered individuals (**Supplementary Figure 3C**).

Our observations indicate that SARS-CoV-2 specific T cells show antigen specificity through cytokine production in response to antigenic restimulation, direct cytotoxic effects against antigen expressing target cells and also the secretion of cytotoxic molecules. However, SARS-CoV-2 specific T cells cultured from recovered individuals had a stronger antigen-specific cytokine response.

SARS-CoV-2 Specific T Cells Showed Activated Markers Associated With Immunogenicity

We determined activation markers of the final cell products and were able to observe an activated phenotype with up-regulated levels of HLA-DR+CD38+, CD25, CD69, and CD154 in all three major T cell subsets, following 21 days of culture (**Figure 3A**). These changes following SARS-CoV-2 peptide antigenic stimulation were minimal and not significant suggesting the activated nature of the product. Furthermore, we examined cytokines involved in T cell activation, such as 4-1BB, CD40L (CD154), and IL-2R, and those that promote immunogenicity, such as GM-CSF and IL-21 (**Figure 3B**). Most of these cytokines increased following S-glycoprotein stimulation. The levels of 4-1BB remained upregulated, indicating overall T cell activation within the products. Slightly higher activated and immunogenic phenotype was observed from the final SARS-CoV-2 specific T cell product produced from COVID-19 recovered individuals than unexposed individuals; however, there was no statistical significance (**Supplementary Figure 4**). The results suggest that the SARS-CoV-2 specific T cells regardless of donor origin possess a memory T cell phenotype that is associated with the activated markers and moreover, produce immunogenic cytokines that may potentially induce anti-SARS-CoV-2 immunity.

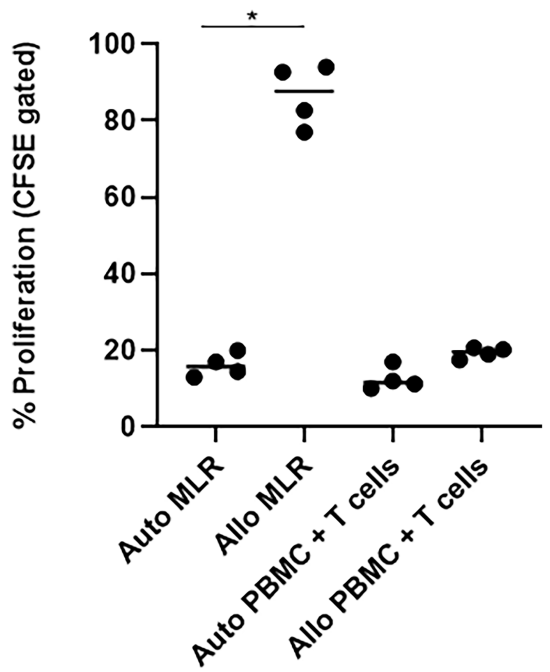

FIGURE 4 | SARS-CoV-2 specific T cells (n=4) did not show alloreactivity against HLA-unmatched PBMCs. T cells, SARS-CoV-2-specific T cells; auto, autologous; allo, allogeneic; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells. Autologous mixed lymphocyte group was used as negative control, while allogeneic mixed lymphocyte group was used as positive control. *p <.05.

Third-Party SARS-CoV-2-Specific T Cells Did Not Show Alloreactivity in a Mixed Lymphocyte Reaction

We assessed the allogeneicity of SARS-CoV-2 specific T cells against autologous and HLA-mismatched allogenic PBMCs by CFSE proliferation assay. As a control, we co-cultured HLA-mismatched PBMCs from two different donors, or CFSE-labelled and un-labelled PBMCs from the same donor for five days (**Figure 4**). The CFSE-labelled responder cells showed active proliferation in the allogeneic condition, suggesting alloreactive T cells against the respective stimulating antigen-presenting cells. In contrast, when we co-cultured SARS-CoV-2 specific T cells against HLA-mismatched allogeneic PBMCs, the proliferation was minimal compared to the allogeneic control, and similar to the autologous control, suggesting that the ex-vivo expanded SARS-CoV-2 specific T cells did not show alloreactivity.

SARS-CoV-2 Specific T Cells Contained Regulatory T Cells and Expressed Inhibitory Markers

With the observation that SARS-CoV-2 specific T cells showed high expression of CD4 and CD25, we examined the presence of CD4+CD25+CD127− cells within the final products (**Figure 5A**). CD4+CD25+CD127 levels (median: 6.365; range: 0.59–66.72) varied between donors, but there were no significant differences between the donor groups (**Supplementary Figure 5A**). The majority of CD4+ T cells were CD4+CD25 −CD127+ cells (Tcons). Majority of the CD4+CD25+CD127- cells were regulatory T cells (Tregs) confirmed by Foxp3 expression (**Figure 5B**). Next, we examined three representative inhibitory markers: PD-1, Tim-3, and LAG-3. All three markers were upregulated following 21 days of culture (**Supplementary Figure 5B**). Although PD-1 expression was similar in both donor groups, Tim-3 and LAG-3 levels were up-regulated in all four subsets especially in the products of recovered individuals (**Figure 5C**). We investigated further potentially relevant anti-inflammatory cytokines. Similarly, IL-4 and IL-10 were found to be increased in response to antigenic stimulation especially in the products of COVID-19 recovered individuals (**Figure 5D**). Since we did not confirm all relevant exhaustion markers and transcription factors, it is unclear whether these cells were strongly activated,

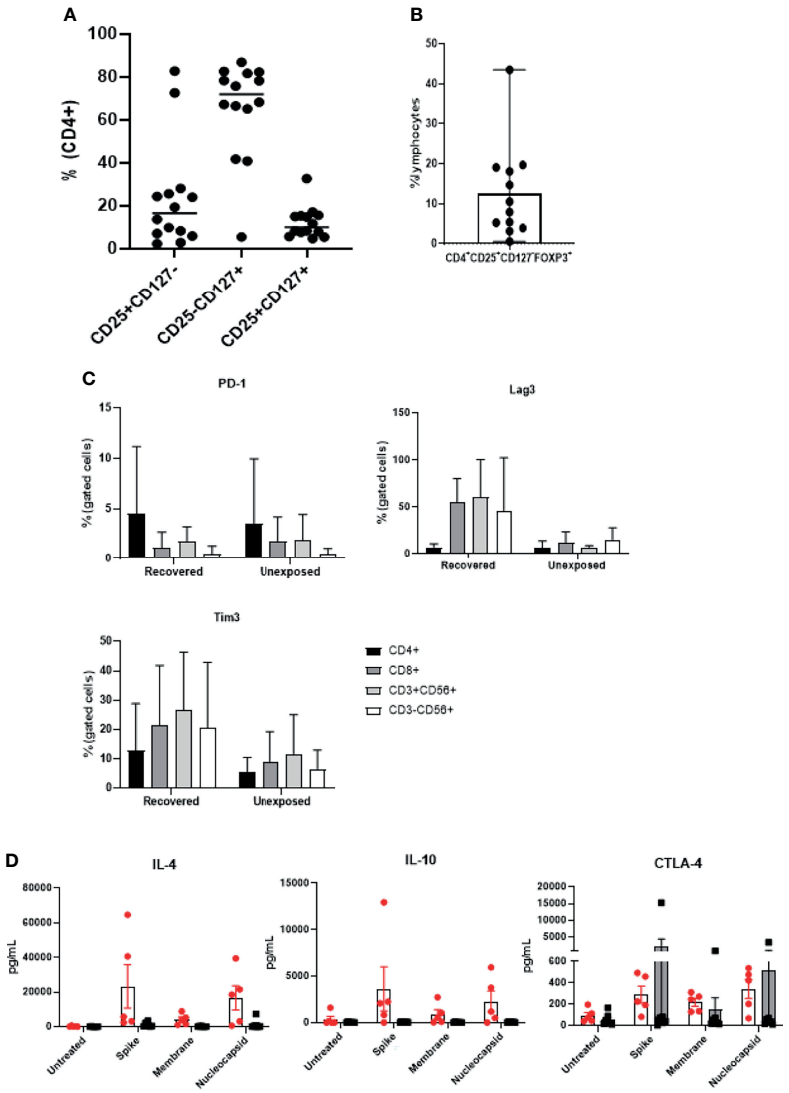

FIGURE 5 | Expanded cell products show presence of Tregs and inhibitory markers. (n=14) **(A)** The percentages of CD4+CD25^{high}CD127^{low}, CD4+CD25^{low}CD127^{high}; Tcons, and CD4+CD25^{high}CD127^{high} cells are indicated from 9 unexposed and 5 recovered individuals) **(B)** Percentage of CD4+CD25^{high}CD127^{low} Foxp3+ Treg ratio was determined (n=12). Each circle represents a cell product produced from a single donor. **(C)** Flow cytometric analysis of immune checkpoint marker (PD-1+, Tim-3+, and LAG-3+) expression of each lymphocyte subset was measured from recovered (n=6) and unexposed (n=9) individuals. **(D)** SARS-CoV-2-specific T cells were treated with S, M, or N peptide mixtures for at least 18 hours, and the supernatant from recovered (n=6, red circles with white bar) and unexposed (n=9, black circles with gray bar) individuals was collected to measure anti-inflammatory cytokine production.

exhausted, or possessed inhibitory functions, and further studies will be required for better characterization of these cells.

DISCUSSION

The emergence of the COVID-19 pandemic in 2019 has led to global efforts to develop effective vaccines and treatments. Unlike previous viral outbreaks, SARS-CoV-2 is known to be highly contagious and can spread at unprecedented rates. While the vaccination process has been accelerated worldwide to achieve herd immunity, there has been little progress in COVID-19 treatment options (23).

Acute respiratory distress syndrome (ARDS), respiratory failure, and cytokine storm syndrome due to a hyper-inflammatory cytokine profile, are the leading causes of mortality in COVID-19 (24, 25). Adjuvant treatments, such as corticosteroids and immunomodulators are being used to alleviate hyper-inflammation. Evidence suggests that both dexamethasone (26, 27) and anti-IL-6 antibodies (28, 29) can improve COVID-19 survival and mortality rates. These approaches treat COVID-19 complications instead of

eliminating viral replication or virus-infected cells. In fact, these anti-inflammatory drugs may adversely affect the anti-viral immune responses. To combat SARS-CoV-2 infections directly, genetically modified neutralizing antibodies have been developed that bind to the receptor-binding domains of the S protein of SARS-CoV-2, block S-protein attachment to human ACE2 receptors, and inhibit virus entry into cells (30, 31). However, antibodies are only effective when viruses are rapidly replicating within the body, which is usually one week after infection. Patients with severe ARDS or pneumonia do not respond well to antibody treatment, as the viruses are not present in the blood stream (14). Experience with human coronaviruses, including MERS-CoV and SARS-CoV-2, suggests that antibody titers decline over time (15). Therefore, once viral infection is established, cellular immunity is the final and the most important line of defense.

In our study, we suggest a treatment approach that relies on the most fundamental concept in viral immunology: adaptive immunity, specifically T cells. The main purpose of SARS-CoV-2-specific T cell therapy is to reverse actively and restore inadequate T cell responses, to improve viral control while minimizing excessive inflammatory responses. Our results demonstrated that manufacturing antigen-specific T cells from either COVID-19 recovered or unexposed individuals is feasible, and reproducible within 21 days. Our ex vivo expanded SARS-CoV-2 antigen-specific memory T cell products were polyclonal, containing both CD4+, CD8+, and CD56+ T cells (**Figure 1**), with antigen-specific IFN-γ, TNF-α, and IL-2 production, leading to direct cytotoxic effects against target cells (**Figure 2**). These T cells showed a functionally-activated phenotype, producing cytokines that may participate in recruitment of immune cells and induce anti-viral immunity (**Figure 3**). However, they did not induce an allogeneic response against HLA-mismatched target cells, suggesting a possibility for off-the-shelf third-party products. A proportion of the cell products consisted of CD4+CD25^{high}CD127^{low} Foxp3-expressing Tregs (**Figure 5**). However, because we did not study the functionality of these Tregs, further studies need to confirm whether these cells do exhibit anti-inflammatory effects. In addition, Tim-3 and Lag-3 in particular were upregulated in the products from COVID-19 recovered individuals. The co-expression of the two markers associated with the presence of Tregs and secretion of IL-4 and IL-10 seem to suggest immunosuppressive capabilities. Whether these marker indicate functionality, hyperactivation or

TABLE 3 | Published pre-clinical studies on SARS-CoV-2 specific T cells.

Country	Donor	Generation method	Main Immunophenotypical characteristics of final cell product	Reference
United States	COVID-19 convalescent or vaccinated individual	*Ex-Vivo* expansion	–	(21)
Spain	COVID-19 convalescent or unexposed individual	*Ex-Vivo* expansion	CD3+CD4+	(8)
Spain	COVID-19 convalescent individual	Separation of memory T cells	CD3+CD4+CD8+ CD45RA-	(11)
Germany	COVID-19 convalescent individual	Separation of IFN-γ+ producing cells responding to SARS-COV-2 peptides followed by *ex-Vivo* expansion	CD3+CD4+CD8+ CD45RA- IFN-γ+	(10)
United States	COVID-19 convalescent or unexposed individual	*Ex-Vivo* expansion	CD4 dominant T cells	(9)
United States	COVID-19 convalescent or unexposed individual	*Ex-Vivo* expansion	NR3C1 gene transduced T cells	(32)
Singapore	COVID-19 patients	Separation of IFN-γ+ producing cells responding to SARS-COV-2 peptides	CD3+CD4+,CD8+,CD56+ CD45RA- IFN-γ+	(33)

TABLE 4 | Registered clinical trials using SARS-CoV-2 T cells to treat COVID-19.

Country	Phase	# of patients	Target Patients	Cell Dose	Generation Method	Status	Clinical trial identifier number
Singapore	I/II	18	Severe COVID-19	Unknown	Cell Separation	Recruiting	NCT04457726
Germany	I/II	51	Severe COVID-19	1000-5000 cells/kg	Cell Separation	Not yet recruiting	NCT0476186
United States	I	16	SARS-COV-2 infected cancer patients	Unknown	*Ex-Vivo* expansion	Recruiting	NCT04742595
United States	I/II	58	Severe COVID-19	1, 2, 4x10^7/m^2	*Ex-Vivo* expansion	Recruiting	NCT04401410
United States	I	24	Elderly patients with severe COVID-19	Unknown	Unknown	Not yet recruiting	NCT04765449
Spain	I/II	58	Severe COVID-19	Unknown (dose-escalation is included)	Cell Separation	Recruiting	NCT04578210

TABLE 5 | GMP production of SARS-CoV-2 specific T cells.

Donor #	Starting Material	Fold Expansion	Theoretical number of doses that can be produced*
16	Leukapheresis	3.5	294 doses
17	Leukapheresis	1.2	713 doses
18	Whole Blood	8.4	47 doses
19	Whole Blood	10.4	40 doses
20	Leukapheresis	7.8	265 doses

**One dose was considered as 2x10^7/m^2 body surface area. Body surface area of an average adult male 1.9m^2 was used for calculation.*

exhaustion remains to be elucidated and we hope to address this in future studies. Nonetheless, the heterogenous characteristics of our SARS-CoV-2-specific T cell products were unique and important for COVID-19, which requires varying immunological strategies.

SARS-CoV-2 specific T cells as a therapeutic option have been previously reported (**Table 3**), and a few clinical trials are registered and open for recruitment (**Table 4**). The majority of these clinical trials concentrate on treating patients with severe disease, or at a high risk for developing severe disease, such as cancer patients and older adults. There are two main methods for SARS-CoV-2 T cell generation: the ex-vivo expansion method, and direct selection of T cells. Each method has its advantages and disadvantages. Direct selection of T cells responding to SARS-CoV-2, or selection of memory T cells is convenient in emergency situations. However, it is not scalable because the final cell product numbers are relatively low. Ex-vivo expansion methods are more time-consuming and require more experience. However, mass production is possible and, depending on the method used, has the potential to treat hundreds to thousands of patients, using only a few donors. Furthermore, establishment of a mini bank for T cells with common HLA types may allow third-party off-the-shelf use of SARS-CoV-2-specific T cells (16). We have produced clinical-grade SARS-COV-2-specific T cells at a GMP facility. While the expansion rates showed donor variabilities, between 40 and 713 doses could be produced, considering a dose of $2x10^7$ cells/m^2 for an average adult (**Table 5**).

Because antigen-specific cytokine production tends to be higher in recovered individuals, the use of T cells generated from recovered individuals may be more useful as a therapeutic drug. However, cells generated from unexposed individuals may still be applied as a personalized T cell vaccines especially for immunocompromised individuals and those who are at a high risk for vaccine adverse effects. While, our results also showed comparable expansion from unexposed individuals, the antigen-specific cytokine production and cytotoxicity remained low compared to cells produced from COVID-19 recovered individuals, possibly due to cross-reactivity with other common corona viruses (7). We hypothesized that once the autologous SARS-CoV-2 T cells are infused into a healthy individual, the cells would circulate in the bloodstream and lymphatics to induce a vaccine-like anti-viral immune response. Because our products were predominantly CD4+ T cells, which produced IL-21, they may potentially interact with B cells to promote production of neutralizing antibodies. However, this remains to be elucidated and requires investigation in a clinical trial.

More recently, with on-going variant outbreaks due to mutations in the SARS-CoV-2 gene, therapeutic options that specifically target the spike glycoprotein have become ineffective. Based on our results, ex vivo expanded SARS-CoV-2 specific T cells can target three major SARS-CoV-2 peptides including membrane and nucleocapsid proteins. This implies that cell therapy can be a resistant and effective approach against future SARS-CoV-2 mutations.

Our study had some limitations, which may need to be addressed in future studies. Further functional analyses of the produced cells need to be performed. The cells exhibited a heterogenous population that included antigen-specific, as well as antigen-independent activated cells. While our results showed antigen-specific cytokine production, the cytotoxicity effects remained moderate at high effector to target ratios, possibly due to CD4 dominance of the cell products. Further studies are required to clarify the mechanism of action of each cell population. For instance, CD4 and CD8 cells may be isolated to determine their individual cytotoxicities. In addition, due to limited access to MHC class I and II pentamers at the time of the study, we could not directly enumerate antigen-specific T cells. Sorting antigen-specific and non-specific cells may help determine their roles in viral control. Detailed phenotypical analyses are also needed, especially for CD4+ T cells, as our observations have suggested the presence of follicular helper T cells within the product. Moreover, Tregs and the inhibitory markers induced in the final product need to be analyzed to determine whether they demonstrated functional exhaustion or immunosuppressive capacity. Our study also indicated the possibility of inducing SARS-CoV-2-specific T cells from SARS-CoV-2 unexposed donors. Larger-scale studies with more unexposed donors are required to investigate this possibility further. With an increasing number of vaccinated individuals, it will be intriguing to see whether vaccinations affect the manufacture of SARS-CoV-2-specific T cells.

The pivotal role of T-cell immunity in viral infections is well known, and has been used in clinical practice for over 20 years (17). Adoptive transfer of VSTs has proven to be safe, with minimal adverse events (18). Advances in manufacturing methods have significantly reduced production time (19), and commercialization is possible by third-party VST banks (16, 20). Currently, there are no effective treatments for hospitalized COVID-19 patients. Therefore, efforts must be directed toward developing newer therapeutic approaches.

In conclusion, we believe that development of treatment strategies for hospitalized COVID-19 patients is a matter of some urgency. SARS-CoV-2-specific T cells are a promising approach for treating COVID-19, one that can directly eliminate infected cells, regulate hyper-inflammatory responses, and provide long-term anti-viral immunity.

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 author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Seoul St. Mary's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SGC designed the study. NK, J-ML, E-JO, and K-II collected data. S-GC, NK, J-ML, E-JO, and K-II analyzed and interpreted data. NK, J-ML, E-JO, K-II, and S-GC wrote and revised the manuscript. NK, J-ML, E-JO, K-II, and S-GC approved the final version of 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.2021.751869/full#supplementary-material

Supplementary Figure 1 | Kinetics of immune cell subsets during 21 days of culture (n=5). Changes in **(A)** immunophenotype, **(B)** CD4+, and **(C)** CD8+ memory T cells during culture of SARS-CoV-2-specific T cells from 3 unexposed and 2 recovered individuals.

Supplementary Figure 2 | Antigen specificity of SARS-CoV-2 specific T cells prior to culture from unexposed (n=3) and recovered (n=2) individuals through **(A)** ELISPOT assay and **(B)** intracellular cytokine staining using flow cytometry. **(C)** Following 21 days of culture, different cell subsets including CD8+, CD4+, CD3+CD56+ and CD3-CD56+ produced antigen specific IFN-ϒ TNF-α and IL-2 in response to peptide re-stimulation. The difference in cytokine production levels are shown between recovered (n= 7) and unexposed (n=8) individuals.

Supplementary Figure 3 | SARS-CoV-2 specific T cells show robust production of cytotoxic molecules, granzyme B and perforin (n=15). **(A)** Ex-vivo culture induces granzyme B and perforin in SARS-CoV-2-specific T cells. **(B)** SARS-CoV-2 specific T cells (n=15) were treated with S, M, or N peptide mixtures for at least 18 hours. Stimulated cells were collected for flow cytometric analysis of granzyme-b-producing, and granzyme-b- and perforin-producing cells of each lymphocyte subset. The levels remain unchanged after antigenic re-stimulation. **(C)** Cell culture supernatants from recovered (n=6, red circles with white bar) and unexposed (n=9, black circles with gray bar) individuals after re-stimulation were collected for granzyme B and perforin measurement. Results represent data from 3 unexposed and 2 recovered individuals. *p <.05; **p <.01; *p <.001.

Supplementary Figure 4 | Activation markers and related cytokine production of SARS-CoV-2 specific T cells between unexposed and recovered individuals (n=15). **(A)** HLA–DR+CD38+, CD25+, CD69+ and CD154+ for all lymphocyte subsets were measured prior to, and after 21 days of culture. **(B)** SARS-CoV-2-specific T cells from recovered (n=6, red circles with white bar) and unexposed (n=9, black circles with gray bar) individuals were treated with S, M, or N peptide mixtures for at least 18 hours, and the supernatant was collected to measure T-cell activation-related cytokine production. Results represent data from 9 unexposed and 6 recovered individuals.

Supplementary Figure 5 | (A) The percentages of $CD4+CD25^{high}CD127^{low}$, $CD4+CD25^{low}CD127^{high}$; Tcons, and $CD4+CD25^{high}CD127^{high}$ cells were compared between recovered (n=5) and unexposed (n=9) individuals. **(B)** Inhibitory markers including PD-1, Tim-3, and LAG-3 were upregulated following cell culture in each lymphocyte subset.

REFERENCES

1. *WHO Coronavirus Disease (COVID-19) Dashboard 2021*. Available at: https://covid19.who.int/?adgroupsurvey={adgroupsurvey}&gclid=CjwKCAiAgc-ABhA7EiwAjev-jxA5tl8AwQfk_FOQN0JiFjFPZg0ve5QpZ7Mb7zV_vrI3Er4L29gHVhoCwUUQAvD_BwE.
2. Wu J, Li J, Zhu G, Zhang Y, Bi Z, Yu Y, et al. Clinical Features of Maintenance Hemodialysis Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *Clin J Am Soc Nephrol* (2020) 15(8):1139–45. doi: 10.2215/CJN.04160320
3. Barrett AJ, Prockop S, Bollard CM. Virus-Specific T Cells: Broadening Applicability. *Biol Blood Marrow Transplant* (2018) 24(1):13–8. doi: 10.1016/j.bbmt.2017.10.004
4. Bollard CM, Heslop HE. T Cells for Viral Infections After Allogeneic Hematopoietic Stem Cell Transplant. *Blood* (2016) 127(26):3331–40. doi: 10.1182/blood-2016-01-628982
5. Ottaviano G, Chiesa R, Feuchtinger T, Vickers MA, Dickinson A, Gennery AR, et al. Adoptive T Cell Therapy Strategies for Viral Infections in Patients Receiving Haematopoietic Stem Cell Transplantation. *Cells* (2019) 8(1):1–12. doi: 10.3390/cells8010047
6. Braun J, Loyal L, Frentsch M, Wendisch D, Georg P, Kurth F, et al. SARS-CoV-2-Reactive T Cells in Healthy Donors and Patients With COVID-19. *Nature* (2020) 587(7833):270–4. doi: 10.1038/s41586-020-2598-9
7. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans With COVID-19 Disease and Unexposed Individuals. *Cell* (2020) 181(7):1489–501 e15. doi: 10.1016/j.cell.2020.05.015
8. Guerreiro M, Aguilar-Gallardo C, Montoro J, Frances-Gomez C, Latorre V, Luna I, et al. Adoptive Transfer of Ex Vivo Expanded SARS-CoV-2-Specific Cytotoxic Lymphocytes: A Viable Strategy for COVID-19 Immunosuppressed Patients? *Transpl Infect Dis* (2021) 23(4):e13602. doi: 10.1111/tid.13602
9. Keller MD, Harris KM, Jensen-Wachspress MA, Kankate VV, Lang H, Lazarski CA, et al. SARS-CoV-2-Specific T Cells are Rapidly Expanded for Therapeutic Use and Target Conserved Regions of the Membrane Protein. *Blood* (2020) 136(25):2905–17. doi: 10.1182/blood.2020008488
10. Cooper RS, Fraser AR, Smith L, Burgoyne P, Imlach SN, Jarvis LM, et al. Rapid GMP-Compliant Expansion of SARS-CoV-2-Specific T Cells From Convalescent Donors for Use as an Allogeneic Cell Therapy for COVID-19. *Front Immunol* (2020) 11:598402. doi: 10.3389/fimmu.2020.598402
11. Ferreras C, Pascual-Miguel B, Mestre-Duran C, Navarro-Zapata A, Clares-Villa L, Martin-Cortazar C, et al. SARS-CoV-2-Specific Memory T Lymphocytes From COVID-19 Convalescent Donors: Identification, Biobanking, and Large-Scale Production for Adoptive Cell Therapy. *Front Cell Dev Biol* (2021) 9:620730. doi: 10.3389/fcell.2021.620730
12. Weiskopf D, Schmitz KS, Raadsen MP, Grifoni A, Okba NMA, Endeman H, et al. Phenotype and Kinetics of SARS-CoV-2-Specific T Cells in COVID-19 Patients With Acute Respiratory Distress Syndrome. *Sci Immunol* (2020) 5(48):1–10. doi: 10.1126/sciimmunol.abd2071
13. Min GJ, Kim HJ, Kim TG, Hyun YS, Hyun SJ, Baek IC, et al. Specific Donor HLA Allotypes as Predictors of Cytomegalovirus Disease Risk in Acute Myeloid Leukemia. *HLA* (2020) 96(4):445–55. doi: 10.1111/tan.13966
14. Group A-TL-CS, Lundgren JD, Grund B, Barkauskas CE, Holland TL, Gottlieb RL, et al. A Neutralizing Monoclonal Antibody for Hospitalized

Patients With Covid-19. *N Engl J Med* (2020) 384(10):905–14. doi: 10.1056/NEJMoa2033130
15. Huang AT, Garcia-Carreras B, Hitchings MDT, Yang B, Katzelnick LC, Rattigan SM, et al. A Systematic Review of Antibody Mediated Immunity to Coronaviruses: Kinetics, Correlates of Protection, and Association With Severity. *Nat Commun* (2020) 11(1):4704. doi: 10.1038/s41467-020-18450-4
16. Tzannou I, Watanabe A, Naik S, Daum R, Kuvalekar M, Leung KS, et al. "Mini" Bank of Only 8 Donors Supplies CMV-Directed T Cells to Diverse Recipients. *Blood Adv* (2019) 3(17):2571–80. doi: 10.1182/bloodadvances.2019000371
17. Riddell SR, Greenberg PD. Principles for Adoptive T Cell Therapy of Human Viral Diseases. *Annu Rev Immunol* (1995) 13:545–86. doi: 10.1146/annurev.iy.13.040195.002553
18. Simmons HZ, Bazzell AF, Dains JE. Adverse Effects of Virus-Specific T-Cell Therapy: An Integrative Review. *J Adv Pract Oncol* (2019) 10(2):120–31. doi: 10.6004/jadpro.2019.10.2.3
19. Vasileiou S, Turney AM, Kuvalekar M, Mukhi SS, Watanabe A, Lulla P, et al. Rapid Generation of Multivirus-Specific T Lymphocytes for the Prevention and Treatment of Respiratory Viral Infections. *Haematologica* (2020) 105(1):235–43. doi: 10.3324/haematol.2018.206896
20. O'Reilly RJ, Prockop S, Hasan AN, Koehne G, Doubrovina E. Virus-Specific T-Cell Banks for 'Off the Shelf' Adoptive Therapy of Refractory Infections. *Bone Marrow Transplant* (2016) 51(9):1163–72. doi: 10.1038/bmt.2016.17
21. Papayanni PG, Chasiotis D, Koukoulias K, Georgakopoulou A, Iatrou A, Gavriilaki E, et al. Vaccinated and Convalescent Donor-Derived SARS-CoV-2-Specific T Cells as Adoptive Immunotherapy for High-Risk COVID-19 Patients. *Clin Infect Dis* (2021) 73(11):2073–l82. doi: 10.1093/cid/ciab371
22. Hoppner M, Luhm J, Schlenke P, Koritke P, Frohn C. A Flow-Cytometry Based Cytotoxicity Assay Using Stained Effector Cells in Combination With Native Target Cells. *J Immunol Methods* (2002) 267(2):157–63. doi: 10.1016/S0022-1759(02)00167-9
23. Kaddoura M, AlIbrahim M, Hijazi G, Soudani N, Audi A, Alkalamouni H, et al. COVID-19 Therapeutic Options Under Investigation. *Front Pharmacol* (2020) 11:1196. doi: 10.3389/fphar.2020.01196
24. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and Immunological Features of Severe and Moderate Coronavirus Disease 2019. *J Clin Invest* (2020) 130(5):2620–9. doi: 10.1172/JCI137244
25. Pedersen SF, Ho YC. SARS-CoV-2: A Storm is Raging. *J Clin Invest* (2020) 130(5):2202–5. doi: 10.1172/JCI137647
26. Group RC, Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, et al. Dexamethasone in Hospitalized Patients With Covid-19 - Preliminary Report. *N Engl J Med* (2020) 384(8):693–704. doi: 10.1056/NEJMoa2021436
27. Matthay MA, Thompson BT. Dexamethasone in Hospitalised Patients With COVID-19: Addressing Uncertainties. *Lancet Respir Med* (2020) 8(12):1170–2. doi: 10.1016/S2213-2600(20)30503-8
28. Salama C, Han J, Yau L, Reiss WG, Kramer B, Neidhart JD, et al. Tocilizumab in Patients Hospitalized With Covid-19 Pneumonia. *N Engl J Med* (2021) 384(1):20–30. doi: 10.1056/NEJMoa2030340
29. Guaraldi G, Meschiari M, Cozzi-Lepri A, Milic J, Tonelli R, Menozzi M, et al. Tocilizumab in Patients With Severe COVID-19: A Retrospective Cohort Study. *Lancet Rheumatol* (2020) 2(8):e474–e84. doi: 10.1016/S2665-9913(20)30173-9
30. Weinreich DM, Sivapalasingam S, Norton T, Ali S, Gao H, Bhore R, et al. REGN-COV2, a Neutralizing Antibody Cocktail, in Outpatients With Covid-19. *N Engl J Med* (2021) 384(3):238–51. doi: 10.1056/NEJMoa2035002
31. Mahase E. Covid-19: FDA Authorises Neutralising Antibody Bamlanivimab for non-Admitted Patients. *BMJ* (2020) 371:m4362. doi: 10.1136/bmj.m4362
32. Basar R, Uprety N, Ensley E, Daher M, Klein K, Martinez F, et al. Generation of Glucocorticoid Resistant SARS-CoV-2 T-Cells for Adoptive Cell Therapy. *bioRxiv* (2020) 36(3):109432. doi: 10.1101/2020.09.15.298547
33. Leung W, Soh TG, Linn YC, Low JG, Loh J, Chan M, et al. Rapid Production of Clinical-Grade SARS-CoV-2 Specific T Cells. *Adv Cell Gene Ther* (2020) p1–8. doi: 10.1101/2020.04.24.20077487

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REVIEW
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Recent Advances of Acute Kidney Injury in Hematopoietic Cell Transplantation

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Acute kidney injury (AKI) is a common complication of allogeneic hematopoietic cell transplantation (allo-HCT) and is associated with non-relapse mortality (NRM) and quality of life (QOL). Multiple factors may contribute to AKI during allo-HCT and are often present at the same time making it difficult to determine the cause of AKI in each patient. Nephrotoxic drugs, infections, thrombotic microangiopathy (TMA), and sinusoidal obstruction syndrome (SOS) are well described causes of AKI during allo-HCT. Acute graft-versus-host disease (aGVHD) is a major complication of allo-HCT that mainly targets the intestines, liver, and skin. However, recent studies suggest aGVHD may also attack the kidney and contribute to AKI following allo-HCT. For example, severe aGVHD is associated with AKI, suggesting a link between the two. In addition, animal models have shown donor immune cell infiltration and increased expression of inflammatory cytokines in recipient kidneys after allo-HCT. Therefore, aGVHD may also target the kidney and contribute to AKI following allo-HCT. Herein, we describe the etiology, diagnosis, risk factors, pathophysiology, prevention, and treatment of renal injury after allo-HCT. In addition, we highlight emerging evidence that aGVHD may contribute to the development of AKI after allo-HCT.

Keywords: acute kidney injury, allogeneic hematologic stem cell transplantation, GvHD, experimental BMT, cytokine, calcinurin inhibitors, thrombotic microagiopathy

INTRODUCTION

Hematopoietic cell transplantation (HCT) is a curative therapy for hematologic malignancies and many non-malignant diseases (1). HCT is classified as either autologous (auto-HCT), when recipient hematopoietic stem cells are stored and then infused, or allogeneic (allo-HCT), when the infused hematopoietic stem cells are derived from a related or unrelated donor. Prior to transplantation, conditioning with chemotherapy and/or total body irradiation (TBI) is necessary to eradicate malignant residual tumors and inhibit rejection of donor hematopoietic cells. Myeloablative conditioning with high-dose cyclophosphamide (CY) and TBI or a combination of busulfan (BU) and CY are two

common regimens. Non-myeloablative conditioning or reduced-intensity conditioning with less intense pretreatment is commonly utilized for elderly patients or those with comorbidities (2).

To prevent graft-versus-host disease (GVHD), immunosuppressive prophylaxis is necessary after transplantation (3). GVHD is caused by alloreactive donor T cells, attacking recipient tissues and is a major life-threatening complication of allo-HCT (4). Previously GVHD was classified into acute GVHD (aGVHD) if it developed within 100 days after transplantation or chronic GVHD (cGVHD) if it developed after 100 days. However, GVHD classification is now based on clinical and pathological characteristics (5).

The characteristic symptoms of aGVHD are rash, diarrhea, and jaundice (4). The pathophysiology causing these symptoms begins with tissue damage from conditioning regimens that results in the release of damage-associated molecular patterns (DAMPs). Injury to the intestinal mucosa and skin also causes a breakdown in barrier function. Barrier breakdown allows microbes to invade the body and release pathogen-associated molecular patterns (PAMPs). PAMPs and DAMPs are danger signals that activate the innate immune system to produce proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, which amplify tissue damage and activate antigen-presenting cells (APCs). Activated host APCs then stimulate donor T cells, which in turn produce proinflammatory cytokines, such as interferon (IFN)-γ, that further activate the innate immune system. Finally, tissue damage caused by cytotoxic T lymphocytes and cytotoxic cytokines derived from activated T cells and innate immune cells, results in the development of clinically apparent aGVHD (3, 6).

The most common immunosuppressive regimen used to prevent GVHD after allo-HCT consists of a calcineurin inhibitor (tacrolimus or cyclosporine) in combination with a short-term course of methotrexate (MTX) (4). Systemic high-dose corticosteroids are the first-line treatment for patients who develop aGVHD (7).

The main organs affected by aGVHD are the skin, liver, and gastrointestinal (GI) tract, but a variety of other organs may also be affected (8). Classically, the kidney is not recognized as a main target organ of aGVHD and no renal aGVHD diagnostic criteria have been established (8). However, various factors related to conditioning and GVHD prophylaxis are known to cause renal injury after allo-HCT. In patients with aGVHD and renal dysfunction, it is often difficult to identify the cause of renal dysfunction due to the frequent co-occurrence of multiple possible etiologies. Renal biopsy is the gold-standard examination for deconvoluting multiple possible etiologies of renal injury; however, invasive renal biopsies are rarely safe during the acute phase of GVHD. Recently, animal studies suggest that the kidney may be a target of aGVHD. Here, we describe the pathophysiology and management of acute kidney injury (AKI) after allo-HCT and highlight the emerging association between AKI and aGVHD.

CRITERIA FOR ACUTE KIDNEY INJURY

Although AKI is a common disease, there have been no internationally standardized criteria (9). In 2004, the Acute Dialysis Quality Initiative (ADQI) published the Risk, Injury, Failure, Loss, and End-stage renal disease (RIFLE) criteria (10). The ADQI defined acute renal failure (ARF) as elevated serum creatinine (sCr), decreased glomerular filtration rate (GFR), and decreased urine output. The AQDI also classified the severity of ARF based on the degree to which these parameters were altered (10). Later, the Acute Kidney Injury Network (AKIN) proposed the concept of acute kidney injury (AKI) in order to include early renal injury. The AKIN criteria, published in 2007, modified the RIEFLE criteria by including mild elevations of sCr (11). In 2012, the Kidney Disease Improving Global Outcomes (KDIGO) criteria were proposed, which integrated the RIEFLE and AKIN criteria. The KDIGO criteria for AKI include anyone of the following: 1) an increase in sCr by≧0.3 mg/dl within 48 hours, 2) an increase in sCr to ≧1.5 times baseline within the preceding 7 days, and 3) a urine volume <0.5 ml/kg/h for 6 hours. The severity of AKI is classified by the KDIGO as Stage 1 to 3 based on sCr or urine output (12). The details of each criterion are shown in **Table 1**. Importantly, the latest KDIGO criteria are as or more predictive of life expectancy than either the RIEFLE or AKIN criteria (13–15). The KDIGO criteria are frequently used in recent studies to measure the incidence of AKI after HCT (16–19).

KIDNEY DISEASE AFTER HCT

According to a recently published meta-analysis of reports from 1995-2019, the incidence of AKI after HCT was 55.1%, with Stage 3, the most severe form, occurring in 8.3% of patients (20).

Factors known to contribute to the risk of AKI after HCT include pre-treatment factors such as being female (21), age 55 years or older (22), and underlying conditions such as diabetes (23), hypertension (21), and chronic kidney disease (CKD) (24). Risk factors for AKI associated with HCT include TBI conditioning (22), use of a calcineurin inhibitors (CNIs) for GVHD prevention (23, 25, 26), and use of MTX for GVHD prophylaxis (22, 23). Post-transplant stay in an intensive care unit (21) and the need for mechanical ventilation (27) are also risk factors. Several post-HCT complications also increase the risk for AKI including hepatic sinusoidal obstruction syndrome (SOS) (28), sepsis (28, 29), and cytomegalovirus infection (22). AKI risk can be further increased by agents used to treat post-HCT complications including amphotericin B (30), acyclovir (31), aminoglycosides (32), and the concomitant use of multiple nephrotoxic drugs (33).

AKI is more common in the early phase of HCT due to the risk of conditioning toxicity, sepsis, SOS, and drug-induced renal injury that are more common early post-HCT (23, 34). For these reasons, clinical studies typically assess post-HCT AKI at 100 days post-transplantation (35).

The incidence of AKI varies according to the type of HCT. In auto -HCT recipients, graft failure is less common, and CNIs are not required because there is no risk for GVHD. Less antibiotics are administrated to auto-HCT recipients than to allo-HCT recipients because duration of neutrophilia is shorter. Therefore, the incidence of AKI is less in auto-HCT versus allo-HCT recipients (36–39). AKI incidence is lower following nonmyeloablative compared to myeloablative conditioning due

TABLE 1 | Classification of AKI severity.

	Serum creatinine	Urine output
RIFLE		
Risk	Increase sCr ×1.5 or GFR decrease > 25%	<0.5 ml/kg/h for 6 hours
Injury	Increase sCr ×2 or GFR decrease > 50%	<0.5 ml/kg/h for 12 hours
Failure	Increase sCr ×3 or GFR decrease 75% or sCr > 4 mg/dl	<0.3 ml/kg/h for 24 hours or Anuria for 12 hours
Loss	Complete loss of kidney functions > 4 weeks	
ESKD	End Stage Kidney Disease >3 months	
AKIN		
Stage 1	Increase ≧0.3 mg/dl or 1.5-2 fold from baseline	<0.5 ml/kg/h for 6 hours
Stage 2	2-3 fold from baseline	<0.5 ml/kg/h for 12 hours
Stage 3	>3 fold from baseline or sCr ≧ 4.0 mg/dl with an acute increase of at least 0.5 mg/dl	<0.3 ml/kg/h for 24 hours or Anuria for 12 hours
KDIGO		
Stage 1	1.5–1.9 times or ≧0.3 mg/dl increase	<0.5 ml/kg/h for 6 hours
Stage 2	2.0–2.9 times	<0.5 ml/kg/h for 12 hours
Stage 3	3.0 times or Increase to ≧4.0 mg/dl or initiation of renal replacement therapy or, in patients <18 years, decrease in eGFR to <35 ml/min/1.73 m^2	<0.3 ml/kg/h for 24 hours or Anuria for 12 hours

RIFLE, Risk, Injury, Failure, Loss, and End-stage renal disease; AKIN, Acute Kidney Injury Network; KDIGO, Kidney Disease Improving Global Outcomes; sCr, serum creatinine; GFR, glomerular filtration rate.

to decreased rates of infection, SOS, and organ failure (35, 40). Overall, the greatest risk of AKI is with myeloablative allo-HCT (21-73%), followed by nonmyeloablative allo-HCT (29-56%), and then autologous transplantation (10.4-19%) (36, 41, 42). Reports vary on whether the incidence of AKI is higher with cord blood or HLA mismatched donor transplantation (28, 43–47).

Whether the indication for HCT is for a malignant or nonmalignant disorders does not significantly affect the incidence of AKI (33, 48, 49). However, malignancies are a risk factor for post-transplant CKD (50), and these patients should be monitored carefully for changes in renal function. Multiple myeloma (MM) and immunoglobulin light-chain (AL) amyloidosis, which themselves cause AKI (see the section of "*Etiologies of AKI after HCT*"), have been reported to cause relatively little post-transplant AKI. However, patients with these disorders are typically treated with auto-HCT, which is less nephrotoxic than allo-HCT (19, 24).

AKI after allo-HCT is associated with all-cause (19, 20, 22, 28, 33, 39, 51) and non-relapse mortality (17, 23, 33), and the earlier the onset of AKI, the higher the mortality (52). The severity of AKI decreases overall survival, and mortality worsens to 55-100% with renal failure requiring dialysis (17, 22, 36, 37, 53, 54). AKI after allo-HCT is also a risk factor for CKD (33, 50). Representative studies that focus on the association of AKI with transplant outcomes are shown in **Table 2**. In pediatric HCT recipient, the incidence of AKI is similar to adults (44, 48, 49, 56–58), AKI worsens mortality after HCT (49, 57), and the 1-year survival rate is less than 10% in patients with renal failure requiring renal replacement therapy (56). Fortunately, HCT-related AKI has decreased in recent years due to the increased use of less toxic conditioning regimens, decreased rates of SOS, modified infection prophylaxis, less amphotericin B use, and declining rates of severe aGVHD (59, 60).

ETIOLOGIES OF AKI AFTER HCT

There are various causes of AKI after HCT. An overview is shown in **Figure 1**.

Nephrotoxic Drugs

Most of the renal injury after HCT is thought to be caused by nephrotoxic drugs, particularly CNIs given for GVHD prophylaxis (34). CNIs can cause both AKI and CKD (61); however, CNIs serum concentration does not always correlate with the severity of AKI (26). CNIs cause AKI through a variety of mechanisms. One way is by inhibiting the production of vasodilators and increasing the production of vasoconstrictors, resulting in the contraction of afferent and efferent arterioles. They also cause vacuolation and dysfunction of renal tubules (61), and they increase the levels of oxidative stress that damages the renal endothelium and contributes to the development of thrombotic microangiopathy (TMA) (62). Consistent with the vasoconstrictive effect of CNIs on afferent and efferent arterioles, inhibition of the renin-angiotensin-aldosterone system may be useful for preventing CNI nephrotoxicity (60).

MTX can also be nephrotoxic. The mechanism is thought to relate to direct tubular injury and/or its precipitation in the renal tubules. The risk for MTX-induced nephrotoxicity is increased by high dose intravenous administration, dehydration, and aciduria (63).

Chemotherapeutic agents used in conditioning (cytarabine and fludarabine) can be nephrotoxic and primarily cause acute tubular injury. Vomiting and diarrhea, which are common adverse events of chemotherapy, cause pre-renal AKI due to dehydration (35). CY and BU cause post-renal AKI due to hemorrhagic cystitis (35, 64).

Many antimicrobial agents may induce direct renal injury or acute interstitial nephritis due to allergic reactions leading to AKI (60). For example, aminoglycosides can cause Fanconi syndrome and Bartter-like syndrome (32). The antifungal agent, amphotericin B causes AKI in a dose-dependent manner *via* renal vasoconstriction and direct tubular injury (65). Fortunately, liposomal amphotericin B, which is now more commonly used, is far less nephrotoxic (66). Finally, acyclovir, an antiviral agent, has been associated with crystal-induced tubular injury and obstruction (60, 67).

TABLE 2 | Recent studies on the association of AKI with transplant outcomes.

Study	Year	Type of transplantation	AKI definition	Incidence of AKI	Follow up	Overall mortality (non-AKI vs AKI)	Non-relapse mortality (non-AKI vs AKI)
Mori et al. (54)	2012	allo-HCT	AKIN	62.2%	5 years	25% vs 45%, HR for death; >Stage 3 vs no AKI or stage 1-2; 5.49 (p <0.001)	NA
Sehgal et al. (37)	2017	allo-HCT, auto-HCT (16.9%)	RIFLE	75.4%	3 months	non-AKI 17.6%, risk 40%, injury 36.4%, failure 80% (p=0.027)	NA
Piñana et al. (17)	2017	allo-HCT(RIC)	KDIGO	44%	25 months	non-AKI 22%, grade 1 32%, grade 2 50%, grade 3 70% (p<0.0001)	16% vs 33% (p=0.005)
Liu et al. (55)	2018	haplo-HCT	sCr>1.5-fold rise	43%	2 years	non-AKI 21.1% vs grade 3(sCr>3-fold) 55.4% (p<0.001)	PFS; non-AKI 72.2% vs severe AKI 45.7 (p<0.001)
Khalil et al. (38)	2019	allo-PBSCT, aotu-PBSCT(38.6%)	RIFLE	31.6%	3 months	17% vs 42% survival time; non-AKI 130 vs injury or failure 38 months (p=0.001)	NA
Mima et al. (18)	2019	allo-HCT, auto-HCT (14.8%)	KDIGO	15.7%	100 days	20.2% vs 29.4% (p=0.409)	NA
Andronesi et al. (19)	2019	auto-HCT	KDIGO	10.3%	90 days	0.6% vs 5.3% (p=0.01)	NA
Sakaguchi et al. (33)	2020	allo-HCT	KDIGO	64.9%	5 years	42.7% vs 76.2% (p<0.001)	13.3% vs 59.8% (p<0.001)
Gutiérrez-García et al. (22)	2020	allo-HCT	KDIGO	58%	5 years	AKI 0-1-2, 55% vs AKI-3, 70% (p=0.008)	TRM; AKI 0-1-2, 31% vs AKI-3, 51% (p<0.0001)
Bhasin et al. (39)	2021	auto-HCT(56.1%), allo-HCT	increase in sCr > 0.3 mg/dL	23%	100 days	1.4% vs 15.6% (p<0.001)	NA

AKI, acute kidney injury; HCT, hematopoietic cell transplantation; allo, allogeneic; auto, autologous; RIC, reduced-intensity conditioning; PBSCT, peripheral blood stem cell transplantation; AKIN, Acute Kidney Injury Network; RIFLE, Risk, Injury, Failure, Loss, and End-stage renal disease; KDIGO, Kidney Disease Improving Global Outcomes; sCr, serum creatinine; HR, hazard ratio; PFS, progression free survival; TRM, transplant-related mortality. NA, not applicable.

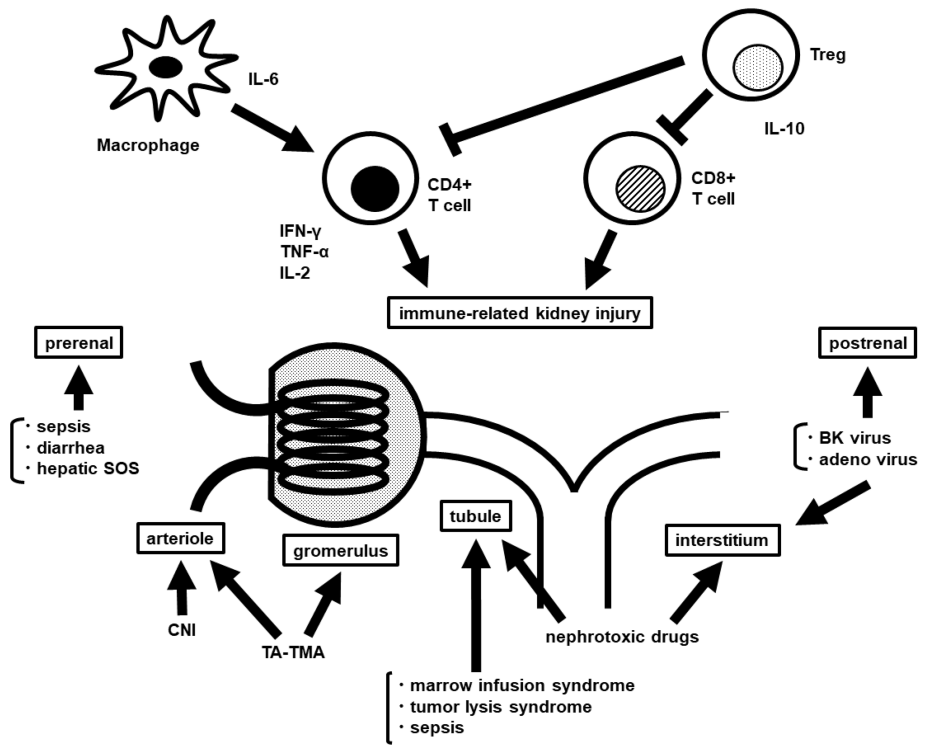

FIGURE 1 | Overview of the pathophysiology of AKI after HCT. AKI, acute kidney injury; HCT, hematopoietic cell transplantation; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; Treg, regulatory T cell; SOS, sinusoidal obstruction syndrome; CNI, calcineurin inhibitor; TA-TMA, transplantation associated-thrombotic microangiopathy.

Hematological Disease-Associated AKI

Hematologic diseases themselves can cause renal injury. For example, AKI occurs in 20-50% of patients with multiple myeloma (68). The most common cause of AKI in patients with multiple myeloma is cast nephropathy, in which large amounts of light chains bind to Tamm-Horsfall protein in the tubules and form insoluble casts, resulting in tubular obstruction and injury. Other causes include light chain deposition disease, AL amyloidosis, and hypercalcemia (69). Direct invasion of the kidney by lymphoma and leukemia can also cause AKI (70).

Complications of HCT

Recipients are immunocompromised and prone to sepsis after HCT (60). Gram-negative bacteria is more common in cord blood transplantation than in bone marrow transplantation (71), likely due to a longer period of neutropenia. Sepsis causes systemic vasodilatation, hypotension, and cytokine-induced endothelial damage, leading to AKI (72). As mentioned above, some of the antimicrobial agents used in the treatment of sepsis are nephrotoxic.

Adenovirus and BK virus infections are also common opportunistic infections following allo-HCT and often lead to AKI (35, 60). Adenoviruses may cause hemorrhagic cystitis and, rarely, necrotizing tubulointerstitial nephritis (73). Adenovirus infections are more common in transplants from unrelated donors and in pediatric patients. Severe adenovirus infection can cause hepatitis, pneumonitis, and encephalitis, and multi-organ failure (74). Reactivation of BK virus may lead to hemorrhagic cystitis, ureteral stricture, and tubulointerstitial nephritis (75). Acute GVHD, allo-HCT, and BK viremia are still associated with increased risk for hemorrhagic cystitis (76, 77).

Nephrotic syndrome, while rare (0.4-6.0%), may also develop following allo-HCT (34). Membranous glomerulonephritis (MGN) and minimal change disease (MCD) account for about two-thirds and one-quarter of nephrotic syndrome cases following allo-HCT, respectively (78). Intriguingly, the onset of nephrotic syndrome following allo-HCT has been associated with recent reduction in the dose of immunosuppressive drugs (78) and the onset of GVHD (79). Most cases occur more than 6 months after transplantation and are considered a rare manifestation of cGVHD (34, 60). However, *de novo* nephrotic syndrome without GVHD also occurs (80).

Marrow infusion syndrome is caused by hemolysis of erythrocytes that release hemoproteins into the recipient. These hemoproteins cause symptoms of hemolysis, such as fever and vomiting. They can also cause acute tubular injury by forming casts in the tubules. Hemolysis resulting in marrow infusion syndrome often occurs during the preservation of stem cells or upon infusion of grafts containing the cryoprotectant dimethyl sulfoxide (DMSO), which can cause hemolysis in recipients of DMSO-containing grafts. Marrow infusion syndrome is mitigated and treated by intravenous hydration and by rinsing or red blood cell-depleting the graft (81).

Tumor lysis syndrome (TLS) occurs when a large number of tumor cells lyse and release toxic cellular contents. It is characterized by hyperkalemia, hypocalcemia, hyperphosphatemia, hyperuricemia, and crystal-induced kidney injury (82). While more common during induction chemotherapy for leukemia, TLS is relatively rare following HCT because most patients come to transplant following multiple treatment courses that dramatically reduce tumor burden (82).

Hepatic sinusoidal obstruction syndrome (SOS) is characterized by painful hepatomegaly, jaundice, and weight gain due to fluid retention (83). AKI often co-occurs with SOS (84, 85), which is more frequently seen after allo- than auto-HCT (81), and severe SOS may lead to multiple organ failure (86). SOS develops following sinusoidal endothelial damage from conditioning therapy, resulting in hepatic portal hypertension, ascites, and increased abdominal pressure. While the exact cause of renal injury in SOS is uncertain and likely multifactorial, decreased renal blood flow due to elevated abdominal pressure likely contributes to tubular injury, which further exacerbates fluid retention and multiorgan failure (86, 87).

Thrombotic Microangiopathy

Transplantation-associated-thrombotic microangiopathy (TA-TMA) is another complication of HCT associated with a substantial risk of mortality (88). It typically develops subacute or chronically (89), and can also lead to AKI (60). Vascular endothelial damage associated with transplantation results in thrombus formation and fibrin deposition in the capillaries and small arteries, microangiopathic hemolytic anemia, and consumptive thrombocytopenia (88). TBI, high-dose BU, CNIs, aGVHD Grade II-IV, infections (BK virus, cytomegalovirus, parvovirus B19, aspergillus species, adenovirus), peripheral blood stem cell transplantation, and use of unrelated donors are all risk factors for TA-TMA (62).

Diagnostic criteria for TA-TMA have been developed by the Blood and Marrow Transplant Clinical Trial Network (BMT-CTN) (90) and the European Group for Blood and Marrow Transplantation (EBMT) (91). Both sets of criteria require the presence of schistocytes and elevated lactate dehydrogenase (LDH). The BMT-CTN criteria also requires worsening renal function (90).

The Kidney is the most vulnerable organ to TA-TMA (62, 89). Renal TA-TMA presents as both AKI and CKD (62, 89) and is often accompanied by hypertension, proteinuria, and a decreased GFR (89). The histopathology of renal TA-TMA is characterized by fibrin deposition in the glomeruli, narrowing of the capillary lumen, presence of fragmented red blood cells, basement membrane duplication, mesangiolysis, and edema of the endothelium (89, 92).

Although endothelial damage plays a major role in the pathogenesis of TA-TMA (62), it is unclear whether it is a direct complication of transplantation or a manifestation of GVHD, infection, or drug toxicity (60). Factors known to cause endothelial damage include CNIs, mammalian target of rapamycin (mTOR) inhibitors, chemotherapy, and TBI (35). Recent studies have also suggested the involvement of complement activation (62, 88).

Several clinical studies have shown an association between TA-TMA and aGVHD (93, 94), but these were retrospective studies confounded by the use of CNIs (88). Nevertheless, clinical studies

have suggested that vascular endothelial cells are targeted by donor T cells (95), and some studies suggest that TA-TMA may be caused by GVHD of the vascular endothelium (89, 96, 97).

Kidney Disease Associated With GVHD

Renal injury after allo-HCT is generally attributed to the etiologies describes above. However, aGVHD is a risk factor for AKI (23, 28, 53, 54, 98), and recent studies have suggested that the kidney may be a direct target of aGVHD (34). Traditionally, the kidney was not considered a target of aGVHD (35). However, diarrhea associated with severe GVHD can indirectly cause dehydration leading to AKI, and CNIs used for GVHD prophylaxis can also cause AKI. Hence, the association between kidney injury and aGVHD is controversial. In the following section, we review studies investigating the relationship between aGVHD and AKI.

Clinical Studies

Hingorani et al. (99) measured cytokines in the urine of patients who underwent allogeneic or autologous transplantation. Increased urine IL-6 and IL-15 levels after HCT were associated with an increased risk of developing proteinuria, and an increased urine MCP-1 level after HCT was associated with chronic kidney disease at 1 year. Thus, these data suggested kidney inflammation occurs after HCT.

Inflammatory cytokines are involved in the pathogenesis of GVHD, but they are not unique to GVHD and are elevated by other HCT-related complications and inflammatory disorders (100). In studies exploring GVHD-specific biomarkers, elafin was identified as a biomarker for cutaneous GVHD (101). Elafin is an elastase-specific protease inhibitor expressed mainly in epithelial tissues, is secreted in response to IL-1 and TNF-α, and has functions such as antibacterial activity, inflammatory cell recruitment, and dendritic cell activation (102, 103). In a study that measured urine elafin levels in patients after HCT (98), it was found that patients with AKI had higher urine elafin levels than those without AKI, and patients with albuminuria also had higher urine elafin levels than those without albuminuria. In addition, elafin was associated with increased risk of CKD and death. These data suggest that inflammation similar to cutaneous aGVHD may occur in the kidney.

Histological diagnosis of renal dysfunction shortly after HCT is rare, and pathological diagnostic criteria have not been established (35). Nonetheless, several renal histopathology studies using tissue obtained by biopsy or autopsy have been reported. For example, Girsberger et al. (104) reported that renal biopsy pathology was consistent with TA-TMA in 29%, CNI toxicity in 24%, and membranous glomerulonephritis in 18% of patients who presented with deterioration of kidney function or proteinuria after HCT (12 allo-HCT, 5 auto-HCT). In 137 autopsies (114 allo-HCT, 23 autologous HCT), the most common renal pathology was acute tubular damage (40%), followed by chronic vascular and interstitial change (11%), and TMA (10%). A small number of cases of membranous glomerulonephritis (1%) and acute interstitial nephritis (1%) were also observed. The median time from transplantation was 497 days for biopsies and 91 days for autopsies; therefore, cGVHD may have a greater association with kidney injury than aGVHD. Mii et al. (97) studied renal biopsy (two cases) and autopsy (5 cases) tissue from patients who developed renal TA-TMA. The median interval between HCT and renal biopsy or autopsy was 7 months. Five of the 7 patients underwent allo-HCT, all 7 patients underwent conditioning that included TBI, and all but one patient received a CNI for GVHD prophylaxis. In addition to TA-TMA changes, all patients had glomerulitis, tubulitis, and peritubular capillaritis with T cell infiltration. Based on these results, the authors concluded that the kidney is a potential target for GVHD.

Studies With Animal Models

Animal models have been important tools for studying the pathophysiology of HCT complications, most notably GVHD, and for developing new therapies to treat these complications (105). In addition to the above-described clinical studies, kidney injury associated with aGVHD has also been studied in animal models. These models revealed important insights into the relationship between kidney injury post allo-HCT and GVHD.

Two studies that measured renal function in mice after allo-HCT reported that sCr did not increase (106, 107). Because creatinine is a waste product of muscle metabolism (108), the lack of sCr elevation may have been due to loss of muscle mass after allo-HCT. By contrast, blood urea nitrogen (BUN), another marker of renal function, was elevated in a rat model (107).

In addition to markers of renal function, elevated markers of renal injury have also been observed in mouse models. These markers include urine protein, albumin (106), N-acetyl-beta-D-glucosaminidase (NAG) (106, 107), and neutrophil gelatinase-associated lipocalin (NGAL) (109), which mainly reflects tubular injury (110, 111). The expression of αKlotho, which is down-regulated in AKI and CKD (112), was also decreased in allo-HCT mice (109).

Higo et al. (107) evaluated renal lesions in a rat bone marrow transplantation (BMT) model. The kidneys in the allo-HCT group were infiltrated with donor leukocytes. Areas with mild inflammation were characterized by CD4+ T cell, CD8+ T cell, and CD68+ macrophage infiltration of the interstitium around small arteries. Whereas in lesions with moderate to severe inflammation, the cellular infiltrate extended into the interstitium surrounding the tubules. Peritubular capillaritis, tubulitis, acute glomerulitis, and endarteritis were also observed in lesions with moderate to severe inflammation. There was no renal deposition of immunoglobulin or complement. In a study using a mouse BMT model (106), similar results were reported. Specifically, allo-HCT recipient mice developed aGVHD within 4 weeks; renal tissue was infiltrated with CD4+ T cells, CD8+ T cells, FoxP3+ T cells, and macrophages; and endarteritis, interstitial nephritis, tubulitis, and glomerulitis were observed.

Ma et al. (113) observed the presence of renal TA-TMA in a TBI-conditioned murine BMT model. In the kidneys of allo-HCT recipients, in addition to tubulitis and interstitial nephritis, mesengiolysis, mesangial proliferation, mesangial edema, subendothelial thickening, endothelial thickening, lumen narrowing, fibrinoid necrosis of afferent arterioles, and microthrombi were observed. All of which are similar to patients with renal TA-TMA. Immunostaining showed C3

complement deposition in the glomeruli, and these glomerular lesions were attenuated in C3-deficient mice, suggesting that complement activation may also be involved in renal injury.

In the kidneys of mice following allo-HCT, increased expression of messenger RNAs for TNF-α, IFN-γ, IL-1α, IL-2, IL-6, and IL-10, as well as the adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) have been reported (106). Sadeghi et al. (114) performed BMT in mice using chemotherapy conditioning and compared kidney transcript expression patterns to those in the liver. Genes that were upregulated in the kidneys of allogeneic recipients, compared to syngeneic recipients and muscle without GVHD, included genes involved in antigen presentation, immune response, and leukocyte migration. These patterns were similar to those in the liver.

Collectively, these pre-clinical studies suggest that infiltration of donor-derived immune cells, changes in cytokines and chemokines, and activation of the complement system may be responsible for renal injury after allo-HCT.

PREVENTION AND TREATMENT OF AKI AFTER HCT

Principles of Prevention and Treatment of AKI

AKI is triggered by a variety of factors related to HCT. Therefore, reducing these HCT-related complications is key to preventing AKI. One important strategy to reduce HCT-related complications is to tailor the choice of conditioning regimen and donor source according to each patient's disease status and comorbidities. The largest contributor to AKI after HCT is drug-induced kidney injury, which can be mitigated by administrating appropriate doses of nephrotoxic agents, and using less nephrotoxic agents when possible. For example, limiting exposure to nephrotoxic antimicrobials decreases the incidence and severity of AKI (115).

Treatment of AKI depends on whether it is pre-renal, renal, or post-renal. Pre-renal AKI is caused by inadequate renal blood flow and responds to hydration. Hydration is also used to prevent renal injury from nephrotoxic agents such as IV contrast for imaging studies. However, care must be taken to avoid fluid overload and pulmonary edema in fluid-sensitive patients including those with decreased cardiac function. Renal AKI is unresponsive to hydration, and oliguria or anuria may persist for several weeks. Blood pressure and fluid balance should be tightly controlled, and nephrotoxic drugs should be discontinued. Depending on the cause of renal AKI, pharmacotherapy with furosemide, atrial natriuretic peptide, and low-dose dopamine may be used, but there is a lack of evidence for their efficacy in preventing or treating renal AKI. In the case of post-renal AKI, obstruction and hydronephrosis are diagnosed by imaging studies, and the main treatment is relief of the obstruction (12, 116).

Renal replacement therapy (RRT) is required for severe renal dysfunction. Patients with prolonged oliguria or anuria, for which RRT is essential for life support, are absolute indications for RRT. There is no consensus on whether earlier initiation of RRT improves the prognosis of severe AKI (117–120).

Disease-Specific Treatment

When the cause of AKI is determined, treatment should focus on correcting it. For example, AKI related to hepatic SOS should be treated with defibrotide, aggressive attempts to maintain fluid balance, and possibly methylprednisolone (121, 122). Prophylactic use of ursodeoxycholic acid (123), defibrotide (124), and fresh frozen plasma (125) should be considered in those at high risk of SOS.

There is no established treatment for TA-TMA, but potential contributing factors should be eliminated when possible. For example, if an infection is thought to contribute, then treatment should be directed toward the pathogen, and every effort should be made to avoid further kidney injury. If CNI therapy is thought to contribute, then CNI withdrawal or dose reduction should be considered (62). Plasma exchange may be performed for the treatment of severe TA-TMA, but the response is usually poor (126, 127). Other potentially efficacious treatments include recombinant thrombomodulin (128), defibrotide (129), rituximab (a monoclonal antibody against CD20) (130), and eculizumab (a monoclonal antibody against complement C5) (131). However, none of these have been investigated in large-scale prospective studies.

Hemorrhagic cystitis (caused by adenovirus or BK virus infection) may require surgical decompression with a nephrostomy tube if urinary tract obstruction cannot be relieved by bladder irrigation from a urinary catheter. The antiviral drug cidofovir is effective for hemorrhagic cystitis caused by adenovirus (132, 133) and may be effective for hemorrhagic cystitis caused by BK virus (134). Ganciclovir (135) and valganciclovir (136) have also been reported to be effective against hemorrhagic cystitis caused by adenovirus.

CONCLUSION

AKI is a common complication of HCT and an important determinant of HCT-related mortality. As described above, AKI after HCT can be caused by a variety of HCT complications and by many drugs commonly used before, during, and after HCT. Furthermore, the agents used to prevent and treat many HCT-related complications can contribute to kidney injury. In individual patients, it is common for several etiologies of AKI to be present at once. In fact, it is likely that these multiple etiologies act in combination. Due to the presence of multiple etiologies for AKI, it is often difficult to quantify the contribution of any one factor in individual patients. In addition, uncharacterized factors may also contribute to renal injury after HCT. For instance, the kidneys are not considered a primary aGVHD target organ, but recent data suggests that renal aGVHD may cause AKI.

Additional research is needed to identify the factors that cause AKI in HCT recipients. This research will hopefully improve the clinical ability to pinpoint specific causes of AKI in individual patients, and lead to therapies targeting each underlying pathologic etiology. Such advances in the diagnosis,

prevention, and treatment of AKI in HCT recipients will improve the safety of HCT.

AUTHOR CONTRIBUTIONS

MM, KI, and TT conceived of the concept and important topics to include in the article. All authors contributed to writing this review and critical appraisal and review of the final version.

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REFERENCES

1. Copelan EA. Hematopoietic Stem-Cell Transplantation. *N Engl J Med* (2006) 354(17):1813–26. doi: 10.1056/NEJMra052638
2. Singh AK, McGuirk JP. Allogeneic Stem Cell Transplantation: A Historical and Scientific Overview. *Cancer Res* (2016) 76(22):6445–51. doi: 10.1158/0008-5472.CAN-16-1311
3. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-Versus-Host Disease. *Lancet* (2009) 373(9674):1550–61. doi: 10.1016/S0140-6736(09)60237-3
4. Zeiser R, Blazar BR. Acute Graft-Versus-Host Disease - Biologic Process, Prevention, and Therapy. *N Engl J Med* (2017) 377(22):2167–79. doi: 10.1056/NEJMra1609337
5. Toubai T, Sun Y, Reddy P. GVHD Pathophysiology: Is Acute Different From Chronic? *Best Pract Res Clin Haematol* (2008) 21(2):101–17. doi: 10.1016/j.beha.2008.02.005
6. Ramadan A, Paczesny S. Various Forms of Tissue Damage and Danger Signals Following Hematopoietic Stem-Cell Transplantation. *Front Immunol* (2015) 6:14. doi: 10.3389/fimmu.2015.00014
7. Toubai T, Magenau J. Immunopathology and Biology-Based Treatment of Steroid-Refractory Graft-Versus-Host Disease. *Blood* (2020) 136(4):429–40. doi: 10.1182/blood.2019000953
8. Mariotti J, Penack O, Castagna L. Acute Graft-Versus-Host-Disease Other Than Typical Targets: Between Myths and Facts. *Transplant Cell Ther* (2021) 27(2):115–24. doi: 10.1016/j.bbmt.2020.09.033
9. Thomas ME, Blaine C, Dawnay A, Devonald MA, Ftouh S, Laing C, et al. The Definition of Acute Kidney Injury and Its Use in Practice. *Kidney Int* (2015) 87(1):62–73. doi: 10.1038/ki.2014.328
10. Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky Pworkgroup ADQI. Acute Renal Failure - Definition, Outcome Measures, Animal Models, Fluid Therapy and Information Technology Needs: The Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. *Crit Care* (2004) 8(4):R204–12. doi: 10.1186/cc2872
11. Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DG, et al. Acute Kidney Injury Network: Report of an Initiative to Improve Outcomes in Acute Kidney Injury. *Crit Care* (2007) 11(2):R31. doi: 10.1186/cc5713
12. Kidney Disease. Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group. KDIGO Clinical Practice Guideline for Acute Kidney Injury. *Kidney Int Suppl* (2012) 2:1–138. doi: 10.1038/kisup.2012.1
13. Luo X, Jiang L, Du B, Wen Y, Wang M, Xi X, et al. A Comparison of Different Diagnostic Criteria of Acute Kidney Injury in Critically Ill Patients. *Crit Care* (2014) 18(4):R144. doi: 10.1186/cc13977
14. Fujii T, Uchino S, Takinami M, Bellomo R. Validation of the Kidney Disease Improving Global Outcomes Criteria for AKI and Comparison of Three Criteria in Hospitalized Patients. *Clin J Am Soc Nephrol* (2014) 9(5):848–54. doi: 10.2215/CJN.09530913
15. Zeng X, McMahon GM, Brunelli SM, Bates DW, Waikar SS. Incidence, Outcomes, and Comparisons Across Definitions of AKI in Hospitalized Individuals. *Clin J Am Soc Nephrol* (2014) 9(1):12–20. doi: 10.2215/CJN.02730313
16. Canet E, Lengline E, Zafrani L, Peraldi MN, Socié G, Azoulay E. Acute Kidney Injury in Critically Ill Allo-HSCT Recipients. *Bone Marrow Transplant* (2014) 49(8):1121–2. doi: 10.1038/bmt.2014.100
17. Piñana JL, Perez-Pitarch A, Garcia-Cadenas I, Barba P, Hernandez-Boluda JC, Esquirol A, et al. A Time-to-Event Model for Acute Kidney Injury After Reduced-Intensity Conditioning Stem Cell Transplantation Using a Tacrolimus- and Sirolimus-Based Graft-Versus-Host Disease Prophylaxis. *Biol Blood Marrow Transplant* (2017) 23(7):1177–85. doi: 10.1016/j.bbmt.2017.03.035
18. Mima A, Tansho K, Nagahara D, Tsubaki K. Incidence of Acute Kidney Disease After Receiving Hematopoietic Stem Cell Transplantation: A Single-Center Retrospective Study. *PeerJ* (2019) 7:e6467. doi: 10.7717/peerj.6467
19. Andronesi AG, Tanase AD, Sorohan BM, Craciun OG, Stefan L, Varady Z, et al. Incidence and Risk Factors for Acute Kidney Injury Following Autologous Stem Cell Transplantation for Multiple Myeloma. *Cancer Med* (2019) 8(6):3278–85. doi: 10.1002/cam4.2187
20. Kanduri SR, Cheungpasitporn W, Thongprayoon C, Bathini T, Kovvuru K, Garla V, et al. Incidence and Mortality of Acute Kidney Injury in Patients Undergoing Hematopoietic Stem Cell Transplantation: A Systematic Review and Meta-Analysis. *QJM* (2020) 113(9):621–32. doi: 10.1093/qjmed/hcaa072
21. Kersting S, Koomans HA, Hené RJ, Verdonck LF. Acute Renal Failure After Allogeneic Myeloablative Stem Cell Transplantation: Retrospective Analysis of Incidence, Risk Factors and Survival. *Bone Marrow Transplant* (2007) 39(6):359–65. doi: 10.1038/sj.bmt.1705599
22. Gutiérrez-García G, Villarreal J, Garrote M, Rovira M, Blasco M, Suárez-Lledó M, et al. Impact of Severe Acute Kidney Injury and Chronic Kidney Disease on Allogeneic Hematopoietic Cell Transplant Recipients: A Retrospective Single Center Analysis. *Bone Marrow Transplant* (2020) 55(7):1264–71. doi: 10.1038/s41409-020-0843-3
23. Piñana JL, Valcárcel D, Martino R, Barba P, Moreno E, Sureda A, et al. Study of Kidney Function Impairment After Reduced-Intensity Conditioning Allogeneic Hematopoietic Stem Cell Transplantation. A Single-Center Experience. *Biol Blood Marrow Transplant* (2009) 15(1):21–9. doi: 10.1016/j.bbmt.2008.10.011
24. Fadia A, Casserly LF, Sanchorawala V, Seldin DC, Wright DG, Skinner M, et al. Incidence and Outcome of Acute Renal Failure Complicating Autologous Stem Cell Transplantation for AL Amyloidosis. *Kidney Int* (2003) 63(5):1868–73. doi: 10.1046/j.1523-1755.2003.00936.x
25. Wolff D, Wilhelm S, Hahn J, Gentilini C, Hilgendorf I, Steiner B, et al. Replacement of Calcineurin Inhibitors With Daclizumab in Patients With Transplantation-Associated Microangiopathy or Renal Insufficiency Associated With Graft-Versus-Host Disease. *Bone Marrow Transplant* (2006) 38(6):445–51. doi: 10.1038/sj.bmt.1705454
26. Abramson MH, Gutgarts V, Zheng J, Maloy MA, Ruiz JD, Scordo M, et al. Acute Kidney Injury in the Modern Era of Allogeneic Hematopoietic Stem Cell Transplantation. *Clin J Am Soc Nephrol* (2021) 16(9):1318–27. doi: 10.2215/CJN.19801220
27. Liu H, Ding JH, Liu BC, Zhao G, Chen BA. Early Renal Injury After Nonmyeloablative Allogeneic Peripheral Blood Stem Cell Transplantation in Patients With Chronic Myelocytic Leukemia. *Am J Nephrol* (2007) 27(4):336–41. doi: 10.1159/000103213
28. Liu H, Li YF, Liu BC, Ding JH, Chen BA, Xu WL, et al. A Multicenter, Retrospective Study of Acute Kidney Injury in Adult Patients With Nonmyeloablative Hematopoietic SCT. *Bone Marrow Transplant* (2010) 45(1):153–8. doi: 10.1038/bmt.2009.99
29. Wanchoo R, Stotter BR, Bayer RL, Jhaveri KD. Acute Kidney Injury in Hematopoietic Stem Cell Transplantation. *Curr Opin Crit Care* (2019) 25(6):531–8. doi: 10.1097/MCC.0000000000000657
30. Hingorani SR, Guthrie K, Batchelder A, Schoch G, Aboulhosn N, Manchion J, et al. Acute Renal Failure After Myeloablative Hematopoietic Cell

Transplant: Incidence and Risk Factors. *Kidney Int* (2005) 67(1):272–7. doi: 10.1111/j.1523-1755.2005.00078.x
31. Izzedine H, Launay-Vacher V, Deray G. Antiviral Drug-Induced Nephrotoxicity. *Am J Kidney Dis* (2005) 45(5):804–17. doi: 10.1053/j.ajkd.2005.02.010
32. Lopez-Novoa JM, Quiros Y, Vicente L, Morales AI, Lopez-Hernandez FJ. New Insights Into the Mechanism of Aminoglycoside Nephrotoxicity: An Integrative Point of View. *Kidney Int* (2011) 79(1):33–45. doi: 10.1038/ki.2010.337
33. Sakaguchi M, Nakayama K, Yamaguchi H, Mii A, Shimizu A, Inai K, et al. Risk Factors for Acute Kidney Injury and Chronic Kidney Disease Following Allogeneic Hematopoietic Stem Cell Transplantation for Hematopoietic Malignancies. *Acta Haematol* (2020) 143(5):452–64. doi: 10.1159/000504354
34. Hingorani S. Renal Complications of Hematopoietic-Cell Transplantation. *N Engl J Med* (2016) 374(23):2256–67. doi: 10.1056/NEJMra1404711
35. Lopes JA, Jorge S, Neves M. Acute Kidney Injury in HCT: An Update. *Bone Marrow Transplant* (2016) 51(6):755–62. doi: 10.1038/bmt.2015.357
36. Parikh CR, Coca SG. Acute Renal Failure in Hematopoietic Cell Transplantation. *Kidney Int* (2006) 69(3):430–5. doi: 10.1038/sj.ki.5000055
37. Sehgal B, George P, John MJ, Samuel C. Acute Kidney Injury and Mortality in Hematopoietic Stem Cell Transplantation: A Single-Center Experience. *Indian J Nephrol* (2017) 27(1):13–9. doi: 10.4103/0971-4065.177138
38. Khalil AA, Khalil LT, Awidi A. Incidence, Risk Factors and Prognosis of Acute Kidney Injury Following Hematopoietic Stem Cell Transplant: A Pilot Study. *Int J Stem Cells* (2019) 12(1):43–50. doi: 10.15283/ijsc18056
39. Bhasin B, Ber Ce P, Szabo A, Chhabra S, D'Souza A. Correlates and Outcomes of Early Acute Kidney Injury After Hematopoietic Cell Transplantation. *Am J Med Sci* (2021) 362(1):72–7. doi: 10.1016/j.amjms.2021.03.013
40. Parikh CR, Schrier RW, Storer B, Diaconescu R, Sorror ML, Maris MB, et al. Comparison of ARF After Myeloablative and Nonmyeloablative Hematopoietic Cell Transplantation. *Am J Kidney Dis* (2005) 45(3):502–9. doi: 10.1053/j.ajkd.2004.11.013
41. Lopes JA, Jorge S. Acute Kidney Injury Following HCT: Incidence, Risk Factors and Outcome. *Bone Marrow Transplant* (2011) 46(11):1399–408. doi: 10.1038/bmt.2011.46
42. Kogon A, Hingorani S. Acute Kidney Injury in Hematopoietic Cell Transplantation. *Semin Nephrol* (2010) 30(6):615–26. doi: 10.1016/j.semnephrol.2010.09.009
43. Yu ZP, Ding JH, Chen BA, Liu BC, Liu H, Li YF, et al. Risk Factors for Acute Kidney Injury in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. *Chin J Cancer* (2010) 29(11):946–51. doi: 10.5732/cjc.010.10293
44. Hirano D, Kakegawa D, Miwa S, Umeda C, Takemasa Y, Tokunaga A, et al. Independent Risk Factors and Long-Term Outcomes for Acute Kidney Injury in Pediatric Patients Undergoing Hematopoietic Stem Cell Transplantation: A Retrospective Cohort Study. *BMC Nephrol* (2020) 21(1):373. doi: 10.1186/s12882-020-02045-8
45. Mae H, Ooi J, Takahashi S, Tomonari A, Tsukada N, Konuma T, et al. Early Renal Injury After Myeloablative Cord Blood Transplantation in Adults. *Leuk Lymphoma* (2008) 49(3):538–42. doi: 10.1080/10428190701824577
46. Mae H, Ooi J, Takahashi S, Kato S, Kawakita T, Ebihara Y, et al. Acute Kidney Injury After Myeloablative Cord Blood Transplantation in Adults: The Efficacy of Strict Monitoring of Vancomycin Serum Trough Concentrations. *Transpl Infect Dis* (2013) 15(2):181–6. doi: 10.1111/tid.12038
47. Gutgarts V, Sathick IJ, Zheng J, Politikos I, Devlin SM, Maloy MA, et al. Incidence and Risk Factors for Acute and Chronic Kidney Injury After Adult Cord Blood Transplantation. *Biol Blood Marrow Transplant* (2020) 26(4):758–63. doi: 10.1016/j.bbmt.2019.12.768
48. Musiał K, Kałwak K, Zwolińska D. The Impact of Allogeneic Hematopoietic Stem Cell Transplantation on Kidney Function in Children-A Single Center Experience. *J Clin Med* (2021) 10(5):1113. doi: 10.3390/jcm10051113
49. Matsuoka D, Hirabayashi K, Murase T, Saito S, Nakazawa Y. Impact of Acute Kidney Injury on Overall Survival in Children and Young Adults Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. *Pediatr Blood Cancer* (2021) 68(9):e29167. doi: 10.1002/pbc.29167
50. Lugthart G, Jordans CCE, de Pagter APJ, Bresters D, Jol-van der Zijde CM, Bense JE, et al. Chronic Kidney Disease Ten Years After Pediatric Allogeneic Hematopoietic Stem Cell Transplantation. *Kidney Int* (2021) 100(4):906–14. doi: 10.1016/j.kint.2021.05.030
51. Coca SG, Yusuf B, Shlipak MG, Garg AX, Parikh CR. Long-Term Risk of Mortality and Other Adverse Outcomes After Acute Kidney Injury: A Systematic Review and Meta-Analysis. *Am J Kidney Dis* (2009) 53(6):961–73. doi: 10.1053/j.ajkd.2008.11.034
52. Shingai N, Morito T, Najima Y, Kobayashi T, Doki N, Kakihana K, et al. Early-Onset Acute Kidney Injury Is a Poor Prognostic Sign for Allogeneic SCT Recipients. *Bone Marrow Transplant* (2015) 50(12):1557–62. doi: 10.1038/bmt.2015.188
53. Kersting S, Dorp SV, Theobald M, Verdonck LF. Acute Renal Failure After Nonmyeloablative Stem Cell Transplantation in Adults. *Biol Blood Marrow Transplant* (2008) 14(1):125–31. doi: 10.1016/j.bbmt.2007.09.016
54. Mori J, Ohashi K, Yamaguchi T, Ando M, Hirashima Y, Kobayashi T, et al. Risk Assessment for Acute Kidney Injury After Allogeneic Hematopoietic Stem Cell Transplantation Based on Acute Kidney Injury Network Criteria. *Intern Med* (2012) 51(16):2105–10. doi: 10.2169/internalmedicine.51.7418
55. Liu Y, Xu L, Zhang X, Wang Y, Liu K, Chen H, et al. Acute Kidney Injury Following Haplo Stem Cell Transplantation: Incidence, Risk Factors and Outcome. *Bone Marrow Transplant* (2018) 53(4):483–6. doi: 10.1038/s41409-017-0030-3
56. Koh KN, Sunkara A, Kang G, Sooter A, Mulrooney DA, Triplett B, et al. Acute Kidney Injury in Pediatric Patients Receiving Allogeneic Hematopoietic Cell Transplantation: Incidence, Risk Factors, and Outcomes. *Biol Blood Marrow Transplant* (2018) 24(4):758–64. doi: 10.1016/j.bbmt.2017.11.021
57. Daraskevicius J, Azukaitis K, Dziugeviciute-Tupko J, Peciulyte M, Planciunaite R, Vaitkeviciene G, et al. Phenotypes and Baseline Risk Factors of Acute Kidney Injury in Children After Allogeneic Hematopoietic Stem Cell Transplantation. *Front Pediatr* (2020) 8:499. doi: 10.3389/fped.2020.00499
58. Prasad M, Jain NG, Radhakrishnan J, Jin Z, Satwani P. Risk Factors for Chronic Kidney Disease Following Acute Kidney Injury in Pediatric Allogeneic Hematopoietic Cell Transplantation. *Bone Marrow Transplant* (2021) 56(7):1665–73. doi: 10.1038/s41409-021-01228-7
59. Gooley TA, Chien JW, Pergam SA, Hingorani S, Sorror ML, Boeckh M, et al. Reduced Mortality After Allogeneic Hematopoietic-Cell Transplantation. *N Engl J Med* (2010) 363(22):2091–101. doi: 10.1056/NEJMoa1004383
60. Renaghan AD, Jaimes EA, Malyszko J, Perazella MA, Sprangers B, Rosner MH. Acute Kidney Injury and CKD Associated With Hematopoietic Stem Cell Transplantation. *Clin J Am Soc Nephrol* (2020) 15(2):289–97. doi: 10.2215/CJN.08580719
61. Naesens M, Kuypers DR, Sarwal M. Calcineurin Inhibitor Nephrotoxicity. *Clin J Am Soc Nephrol* (2009) 4(2):481–508. doi: 10.2215/CJN.04800908
62. Wanchoo R, Bayer RL, Bassil C, Jhaveri KD. Emerging Concepts in Hematopoietic Stem Cell Transplantation-Associated Renal Thrombotic Microangiopathy and Prospects for New Treatments. *Am J Kidney Dis* (2018) 72(6):857–65. doi: 10.1053/j.ajkd.2018.06.013
63. Abelson HT, Fosburg MT, Beardsley GP, Goorin AM, Gorka C, Link M, et al. Methotrexate-Induced Renal Impairment: Clinical Studies and Rescue From Systemic Toxicity With High-Dose Leucovorin and Thymidine. *J Clin Oncol* (1983) 1(3):208–16. doi: 10.1200/JCO.1983.1.3.208
64. Perazella MA. Pharmacology Behind Common Drug Nephrotoxicities. *Clin J Am Soc Nephrol* (2018) 13(12):1897–908. doi: 10.2215/CJN.00150118
65. Botero Aguirre JP, Restrepo Hamid AM. Amphotericin B Deoxycholate Versus Liposomal Amphotericin B: Effects on Kidney Function. *Cochrane Database Syst Rev* (2015) 11:CD010481. doi: 10.1002/14651858.CD010481.pub2
66. Mistro S, Maciel IEM, de Menezes RG, Maia ZP, Schooley RT, Badaró R. Does Lipid Emulsion Reduce Amphotericin B Nephrotoxicity? A Systematic Review and Meta-Analysis. *Clin Infect Dis* (2012) 54(12):1774–7. doi: 10.1093/cid/cis290
67. Perazella MA. Crystal-Induced Acute Renal Failure. *Am J Med* (1999) 106(4):459–65. doi: 10.1016/s0002-9343(99)00041-8
68. Finkel KW, Cohen EP, Shirali A, Abudayyeh AForum ASoNO-N. Paraprotein-Related Kidney Disease: Evaluation and Treatment of Myeloma Cast Nephropathy. *Clin J Am Soc Nephrol* (2016) 11(12):2273–9. doi: 10.2215/CJN.01640216

69. Hutchison CA, Batuman V, Behrens J, Bridoux F, Sirac C, Dispenzieri A, et al. The Pathogenesis and Diagnosis of Acute Kidney Injury in Multiple Myeloma. *Nat Rev Nephrol* (2011) 8(1):43–51. doi: 10.1038/nrneph.2011.168
70. Rosner MH, Perazella MA. Acute Kidney Injury in Patients With Cancer. *N Engl J Med* (2017) 376(18):1770–81. doi: 10.1056/NEJMra1613984
71. Averbuch D, Tridello G, Hoek J, Mikulska M, Pabst T, Yañez San Segundo L, et al. Intercontinental Study on Pre-Engraftment and Post-Engraftment Gram-Negative Rods Bacteremia in Hematopoietic Stem Cell Transplantation Patients: Risk Factors and Association With Mortality. *J Infect* (2020) 81(6):882–94. doi: 10.1016/j.jinf.2020.11.002
72. Schrier RW, Wang W. Acute Renal Failure and Sepsis. *N Engl J Med* (2004) 351(2):159–69. doi: 10.1056/NEJMra032401
73. Ito M, Hirabayashi N, Uno Y, Nakayama A, Asai J. Necrotizing Tubulointerstitial Nephritis Associated With Adenovirus Infection. *Hum Pathol* (1991) 22(12):1225–31. doi: 10.1016/0046-8177(91)90104-w
74. Baldwin A, Kingman H, Darville M, Foot AB, Grier D, Cornish JM, et al. Outcome and Clinical Course of 100 Patients With Adenovirus Infection Following Bone Marrow Transplantation. *Bone Marrow Transplant* (2000) 26(12):1333–8. doi: 10.1038/sj.bmt.1702716
75. Hirsch HH, Steiger J. Polyomavirus Bk. *Lancet Infect Dis* (2003) 3(10):611–23. doi: 10.1016/s1473-3099(03)00770-9
76. Leung AY, Mak R, Lie AK, Yuen KY, Cheng VC, Liang R, et al. Clinicopathological Features and Risk Factors of Clinically Overt Haemorrhagic Cystitis Complicating Bone Marrow Transplantation. *Bone Marrow Transplant* (2002) 29(6):509–13. doi: 10.1038/sj.bmt.1703415
77. Erard V, Kim HW, Corey L, Limaye A, Huang ML, Myerson D, et al. BK DNA Viral Load in Plasma: Evidence for an Association With Hemorrhagic Cystitis in Allogeneic Hematopoietic Cell Transplant Recipients. *Blood* (2005) 106(3):1130–2. doi: 10.1182/blood-2004-12-4988
78. Brukamp K, Doyle AM, Bloom RD, Bunin N, Tomaszewski JE, Cizman B. Nephrotic Syndrome After Hematopoietic Cell Transplantation: Do Glomerular Lesions Represent Renal Graft-Versus-Host Disease? *Clin J Am Soc Nephrol* (2006) 1(4):685–94. doi: 10.2215/CJN.00380705
79. Beyar-Katz O, Davila EK, Zuckerman T, Fineman R, Haddad N, Okasha D, et al. Adult Nephrotic Syndrome After Hematopoietic Stem Cell Transplantation: Renal Pathology Is the Best Predictor of Response to Therapy. *Biol Blood Marrow Transplant* (2016) 22(6):975–81. doi: 10.1016/j.bbmt.2015.12.014
80. Numata A, Morishita Y, Mori M, Saito O, Takemoto F, Ando Y, et al. *De Novo* Postallogeneic Hematopoietic Stem Cell Transplant Membranous Nephropathy. *Exp Clin Transplant* (2013) 11(1):75–8. doi: 10.6002/ect.2012.0078
81. Zager RA. Acute Renal Failure in the Setting of Bone Marrow Transplantation. *Kidney Int* (1994) 46(5):1443–58. doi: 10.1038/ki.1994.417
82. Mughal TI, Ejaz AA, Foringer JR, Coiffier B. An Integrated Clinical Approach for the Identification, Prevention, and Treatment of Tumor Lysis Syndrome. *Cancer Treat Rev* (2010) 36(2):164–76. doi: 10.1016/j.ctrv.2009.11.001
83. McDonald GB. Hepatobiliary Complications of Hematopoietic Cell Transplantation, 40 Years on. *Hepatology* (2010) 51(4):1450–60. doi: 10.1002/hep.23533
84. Gruss E, Bernis C, Tomas JF, Garcia-Canton C, Figuera A, Motellón JL, et al. Acute Renal Failure in Patients Following Bone Marrow Transplantation: Prevalence, Risk Factors and Outcome. *Am J Nephrol* (1995) 15(6):473–9. doi: 10.1159/000168889
85. Merouani A, Shpall EJ, Jones RB, Archer PG, Schrier RW. Renal Function in High Dose Chemotherapy and Autologous Hematopoietic Cell Support Treatment for Breast Cancer. *Kidney Int* (1996) 50(3):1026–31. doi: 10.1038/ki.1996.405
86. Bearman SI. The Syndrome of Hepatic Veno-Occlusive Disease After Marrow Transplantation. *Blood* (1995) 85(11):3005–20. doi: 10.1182/blood.V85.11.3005.bloodjournal85113005
87. Coppell JA, Richardson PG, Soiffer R, Martin PL, Kernan NA, Chen A, et al. Hepatic Veno-Occlusive Disease Following Stem Cell Transplantation: Incidence, Clinical Course, and Outcome. *Biol Blood Marrow Transplant* (2010) 16(2):157–68. doi: 10.1016/j.bbmt.2009.08.024
88. Jodele S, Laskin BL, Dandoy CE, Myers KC, El-Bietar J, Davies SM, et al. A New Paradigm: Diagnosis and Management of HSCT-Associated Thrombotic Microangiopathy as Multi-System Endothelial Injury. *Blood Rev* (2015) 29(3):191–204. doi: 10.1016/j.blre.2014.11.001
89. Mii A, Shimizu A, Yamaguchi H, Tsuruoka S. Renal Complications After Hematopoietic Stem Cell Transplantation: Role of Graft-Versus-Host Disease in Renal Thrombotic Microangiopathy. *J Nippon Med Sch* (2020) 87(1):7–12. doi: 10.1272/jnms.JNMS.2020_87-102
90. Ho VT, Cutler C, Carter S, Martin P, Adams R, Horowitz M, et al. Blood and Marrow Transplant Clinical Trials Network Toxicity Committee Consensus Summary: Thrombotic Microangiopathy After Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2005) 11(8):571–5. doi: 10.1016/j.bbmt.2005.06.001
91. Ruutu T, Barosi G, Benjamin RJ, Clark RE, George JN, Gratwohl A, et al. Diagnostic Criteria for Hematopoietic Stem Cell Transplant-Associated Microangiopathy: Results of a Consensus Process by an International Working Group. *Haematologica* (2007) 92(1):95–100. doi: 10.3324/haematol.10699
92. Siami K, Kojouri K, Swisher KK, Selby GB, George JN, Laszik ZG. Thrombotic Microangiopathy After Allogeneic Hematopoietic Stem Cell Transplantation: An Autopsy Study. *Transplantation* (2008) 85(1):22–8. doi: 10.1097/01.tp.0000297998.33418.7e
93. Changsirikulchai S, Myerson D, Guthrie KA, McDonald GB, Alpers CE, Hingorani SR. Renal Thrombotic Microangiopathy After Hematopoietic Cell Transplant: Role of GVHD in Pathogenesis. *Clin J Am Soc Nephrol* (2009) 4(2):345–53. doi: 10.2215/CJN.02070508
94. Epperla N, Li A, Logan B, Fretham C, Chhabra S, Aljurf M, et al. Incidence, Risk Factors for and Outcomes of Transplant-Associated Thrombotic Microangiopathy. *Br J Haematol* (2020) 189(6):1171–81. doi: 10.1111/bjh.16457
95. Luft T, Dietrich S, Falk C, Conzelmann M, Hess M, Benner A, et al. Steroid-Refractory GVHD: T-Cell Attack Within a Vulnerable Endothelial System. *Blood* (2011) 118(6):1685–92. doi: 10.1182/blood-2011-02-334821
96. Tichelli A, Gratwohl A. Vascular Endothelium as 'Novel' Target of Graft-Versus-Host Disease. *Best Pract Res Clin Haematol* (2008) 21(2):139–48. doi: 10.1016/j.beha.2008.02.002
97. Mii A, Shimizu A, Kaneko T, Fujita E, Fukui M, Fujino T, et al. Renal Thrombotic Microangiopathy Associated With Chronic Graft-Versus-Host Disease After Allogeneic Hematopoietic Stem Cell Transplantation. *Pathol Int* (2011) 61(9):518–27. doi: 10.1111/j.1440-1827.2011.02704.x
98. Hingorani S, Finn LS, Pao E, Lawler R, Schoch G, McDonald GB, et al. Urinary Elafin and Kidney Injury in Hematopoietic Cell Transplant Recipients. *Clin J Am Soc Nephrol* (2015) 10(1):12–20. doi: 10.2215/CJN.01840214
99. Hingorani S, Gooley T, Pao E, Sandmaier B, McDonald G. Urinary Cytokines After HCT: Evidence for Renal Inflammation in the Pathogenesis of Proteinuria and Kidney Disease. *Bone Marrow Transplant* (2014) 49(3):403–9. doi: 10.1038/bmt.2013.197
100. Chen YB, Cutler CS. Biomarkers for Acute GVHD: Can We Predict the Unpredictable? *Bone Marrow Transplant* (2013) 48(6):755–60. doi: 10.1038/bmt.2012.143
101. Paczesny S, Braun TM, Levine JE, Hogan J, Crawford J, Coffing B, et al. Elafin Is a Biomarker of Graft-Versus-Host Disease of the Skin. *Sci Transl Med* (2010) 2(13):13ra2. doi: 10.1126/scitranslmed.3000406
102. Williams SE, Brown TI, Roghanian A, Sallenave JM. SLPI and Elafin: One Glove, Many Fingers. *Clin Sci (Lond)* (2006) 110(1):21–35. doi: 10.1042/CS20050115
103. Shaw L, Wiedow O. Therapeutic Potential of Human Elafin. *Biochem Soc Trans* (2011) 39(5):1450–4. doi: 10.1042/BST0391450
104. Girsberger M, Halter JP, Hopfer H, Dickenmann M, Menter T. Kidney Pathology After Hematologic Cell Transplantation-A Single-Center Observation Study of Indication Biopsies and Autopsies. *Biol Blood Marrow Transplant* (2018) 24(3):571–80. doi: 10.1016/j.bbmt.2017.11.008
105. Boieri M, Shah P, Dressel R, Inngjerdingen M. The Role of Animal Models in the Study of Hematopoietic Stem Cell Transplantation and GvHD: A Historical Overview. *Front Immunol* (2016) 7:333. doi: 10.3389/fimmu.2016.00333
106. Schmid PM, Bouazzaoui A, Schmid K, Birner C, Schach C, Maier LS, et al. Acute Renal Graft-Versus-Host Disease in a Murine Model of Allogeneic Bone Marrow Transplantation. *Cell Transplant* (2017) 26(8):1428–40. doi: 10.1177/0963689717720295

107. Higo S, Shimizu A, Masuda Y, Nagasaka S, Kajimoto Y, Kanzaki G, et al. Acute Graft-Versus-Host Disease of the Kidney in Allogeneic Rat Bone Marrow Transplantation. *PloS One* (2014) 9(12):e115399. doi: 10.1371/journal.pone.0115399
108. Pasala S, Carmody JB. How to Use… Serum Creatinine, Cystatin C and GFR. *Arch Dis Child Educ Pract Ed* (2017) 102(1):37–43. doi: 10.1136/archdischild-2016-311062
109. Amin R, He R, Gupta D, Zheng W, Burmakin M, Mohammad DK, et al. The Kidney Injury Caused by the Onset of Acute Graft-Versus-Host Disease Is Associated With Down-Regulation of αklotho. *Int Immunopharmacol* (2020) 78:106042. doi: 10.1016/j.intimp.2019.106042
110. Bazzi C, Petrini C, Rizza V, Arrigo G, Napodano P, Paparella M, et al. Urinary N-Acetyl-Beta-Glucosaminidase Excretion Is a Marker of Tubular Cell Dysfunction and a Predictor of Outcome in Primary Glomerulonephritis. *Nephrol Dial Transplant* (2002) 17(11):1890–6. doi: 10.1093/ndt/17.11.1890
111. Paragas N, Qiu A, Zhang Q, Samstein B, Deng SX, Schmidt-Ott KM, et al. The Ngal Reporter Mouse Detects the Response of the Kidney to Injury in Real Time. *Nat Med* (2011) 17(2):216–22. doi: 10.1038/nm.2290
112. Hu MC, Moe OW. Klotho as a Potential Biomarker and Therapy for Acute Kidney Injury. *Nat Rev Nephrol* (2012) 8(7):423–9. doi: 10.1038/nrneph.2012.92
113. Ma Q, Li D, Vasquez HG, You MJ, Afshar-Kharghan V. Kidney Injury in Murine Models of Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2019) 25(10):1920–4. doi: 10.1016/j.bbmt.2019.06.027
114. Sadeghi B, Al-Chaqmaqchi H, Al-Hashmi S, Brodin D, Hassan Z, Abedi-Valugerdi M, et al. Early-Phase GVHD Gene Expression Profile in Target Versus Non-Target Tissues: Kidney, A Possible Target? *Bone Marrow Transplant* (2013) 48(2):284–93. doi: 10.1038/bmt.2012.120
115. Benoit SW, Goldstein SL, Dahale DS, Haslam DB, Nelson A, Truono K, et al. Reduction in Nephrotoxic Antimicrobial Exposure Decreases Associated Acute Kidney Injury in Pediatric Hematopoietic Stem Cell Transplant Patients. *Biol Blood Marrow Transplant* (2019) 25(8):1654–8. doi: 10.1016/j.bbmt.2019.04.022
116. Ostermann M, Bellomo R, Burdmann EA, Doi K, Endre ZH, Goldstein SL, et al. Controversies in Acute Kidney Injury: Conclusions From a Kidney Disease: Improving Global Outcomes (KDIGO) Conference. *Kidney Int* (2020) 98(2):294–309. doi: 10.1016/j.kint.2020.04.020
117. Gaudry S, Hajage D, Schortgen F, Martin-Lefevre L, Pons B, Boulet E, et al. Initiation Strategies for Renal-Replacement Therapy in the Intensive Care Unit. *N Engl J Med* (2016) 375(2):122–33. doi: 10.1056/NEJMoa1603017
118. Zarbock A, Kellum JA, Schmidt C, Van Aken H, Wempe C, Pavenstädt H, et al. Effect of Early vs Delayed Initiation of Renal Replacement Therapy on Mortality in Critically Ill Patients With Acute Kidney Injury: The ELAIN Randomized Clinical Trial. *JAMA* (2016) 315(20):2190–9. doi: 10.1001/jama.2016.5828
119. Barbar SD, Clere-Jehl R, Bourredjem A, Hernu R, Montini F, Bruyère R, et al. Timing of Renal-Replacement Therapy in Patients With Acute Kidney Injury and Sepsis. *N Engl J Med* (2018) 379(15):1431–42. doi: 10.1056/NEJMoa1803213
120. Bagshaw SM, Wald R, Adhikari NKJ, Bellomo R, da Costa BR, Dreyfuss D, et al. Timing of Initiation of Renal-Replacement Therapy in Acute Kidney Injury. *N Engl J Med* (2020) 383(3):240–51. doi: 10.1056/NEJMoa2000741
121. Richardson PG, Riches ML, Kernan NA, Brochstein JA, Mineishi S, Termuhlen AM, et al. Phase 3 Trial of Defibrotide for the Treatment of Severe Veno-Occlusive Disease and Multi-Organ Failure. *Blood* (2016) 127(13):1656–65. doi: 10.1182/blood-2015-10-676924
122. Al Beihany A, Al Omar H, Sahovic E, Chaudhri N, Al Mohareb F, Al Sharif F, et al. Successful Treatment of Hepatic Veno-Occlusive Disease After Myeloablative Allogeneic Hematopoietic Stem Cell Transplantation by Early Administration of a Short Course of Methylprednisolone. *Bone Marrow Transplant* (2008) 41(3):287–91. doi: 10.1038/sj.bmt.1705896
123. Tay J, Tinmouth A, Fergusson D, Huebsch L, Allan DS. Systematic Review of Controlled Clinical Trials on the Use of Ursodeoxycholic Acid for the Prevention of Hepatic Veno-Occlusive Disease in Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2007) 13(2):206–17. doi: 10.1016/j.bbmt.2006.09.012
124. Corbacioglu S, Cesaro S, Faraci M, Valteau-Couanet D, Gruhn B, Rovelli A, et al. Defibrotide for Prophylaxis of Hepatic Veno-Occlusive Disease in Paediatric Haemopoietic Stem-Cell Transplantation: An Open-Label, Phase 3, Randomised Controlled Trial. *Lancet* (2012) 379(9823):1301–9. doi: 10.1016/S0140-6736(11)61938-7
125. Matsumoto M, Kawa K, Uemura M, Kato S, Ishizashi H, Isonishi A, et al. Prophylactic Fresh Frozen Plasma may Prevent Development of Hepatic VOD After Stem Cell Transplantation *via* ADAMTS13-Mediated Restoration of Von Willebrand Factor Plasma Levels. *Bone Marrow Transplant* (2007) 40(3):251–9. doi: 10.1038/sj.bmt.1705724
126. Laskin BL, Goebel J, Davies SM, Jodele S. Small Vessels, Big Trouble in the Kidneys and Beyond: Hematopoietic Stem Cell Transplantation-Associated Thrombotic Microangiopathy. *Blood* (2011) 118(6):1452–62. doi: 10.1182/blood-2011-02-321315
127. Li A, Makar RS, Hurwitz S, Uhl L, Kaufman RM, Stowell CP, et al. Treatment With or Without Plasma Exchange for Patients With Acquired Thrombotic Microangiopathy Not Associated With Severe ADAMTS13 Deficiency: A Propensity Score-Matched Study. *Transfusion* (2016) 56(8):2069–77. doi: 10.1111/trf.13654
128. Fujiwara H, Maeda Y, Sando Y, Nakamura M, Tani K, Ishikawa T, et al. Treatment of Thrombotic Microangiopathy After Hematopoietic Stem Cell Transplantation With Recombinant Human Soluble Thrombomodulin. *Transfusion* (2016) 56(4):886–92. doi: 10.1111/trf.13437
129. Corti P, Uderzo C, Tagliabue A, Della Volpe A, Annaloro C, Tagliaferri E, et al. Defibrotide as a Promising Treatment for Thrombotic Thrombocytopenic Purpura in Patients Undergoing Bone Marrow Transplantation. *Bone Marrow Transplant* (2002) 29(6):542–3. doi: 10.1038/sj.bmt.1703414
130. Ostronoff M, Ostronoff F, Calixto R, Florêncio R, Florêncio M, Domingues MC, et al. Life-Threatening Hemolytic-Uremic Syndrome Treated With Rituximab in an Allogeneic Bone Marrow Transplant Recipient. *Bone Marrow Transplant* (2007) 39(10):649–51. doi: 10.1038/sj.bmt.1705657
131. Jodele S, Fukuda T, Mizuno K, Vinks AA, Laskin BL, Goebel J, et al. Variable Eculizumab Clearance Requires Pharmacodynamic Monitoring to Optimize Therapy for Thrombotic Microangiopathy After Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2016) 22(2):307–15. doi: 10.1016/j.bbmt.2015.10.002
132. Ljungman P, Ribaud P, Eyrich M, Matthes-Martin S, Einsele H, Bleakley M, et al. Cidofovir for Adenovirus Infections After Allogeneic Hematopoietic Stem Cell Transplantation: A Survey by the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant* (2003) 31(6):481–6. doi: 10.1038/sj.bmt.1703798
133. Nagafuji K, Aoki K, Henzan H, Kato K, Miyamoto T, Eto T, et al. Cidofovir for Treating Adenoviral Hemorrhagic Cystitis in Hematopoietic Stem Cell Transplant Recipients. *Bone Marrow Transplant* (2004) 34(10):909–14. doi: 10.1038/sj.bmt.1704682
134. Philippe M, Ranchon F, Gilis L, Schwiertz V, Vantard N, Ader F, et al. Cidofovir in the Treatment of BK Virus-Associated Hemorrhagic Cystitis After Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2016) 22(4):723–30. doi: 10.1016/j.bbmt.2015.12.009
135. Nakazawa Y, Suzuki T, Fukuyama T, Katsuyama Y, Tanaka M, Yanagisawa R, et al. Urinary Excretion of Ganciclovir Contributes to Improvement of Adenovirus-Associated Hemorrhagic Cystitis After Allogeneic Bone Marrow Transplantation. *Pediatr Transplant* (2009) 13(5):632–5. doi: 10.1111/j.1399-3046.2008.01027.x
136. Yanagisawa T, Saito S, Katsuyama Y, Hirabayashi K, Shigemura T, Tanaka M, et al. Successful Induction of Therapeutic Urinary Concentration by Intravenous Ganciclovir and Oral Valganciclovir With Remission of Adenoviral Hemorrhagic Cystitis After Cord Blood Transplantation. *Pediatr Transplant* (2018) e13241. doi: 10.1111/petr.13241

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Targeting Glycolysis in Alloreactive T Cells to Prevent Acute Graft-*Versus*-Host Disease While Preserving Graft-Versus-Leukemia Effect

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Alloreactive donor T cells undergo extensive metabolic reprogramming to become activated and induce graft-versus-host disease (GVHD) upon alloantigen encounter. It is generally thought that glycolysis, which promotes T cell growth and clonal expansion, is employed in this process. However, conflicting data have been reported regarding the requirement of glycolysis to induce T cell-mediated GVHD due to the lack of T cell-specific treatments using glycolysis inhibitors. Importantly, previous studies have not evaluated whether graft-versus-leukemia (GVL) activity is preserved in donor T cells deficient for glycolysis. As a critical component affecting the clinical outcome, it is necessary to assess the anti-tumor activity following treatment with metabolic modulators in preclinical models. In the present study, we utilized T cells selectively deficient for glucose transporter 1 ($Glut1^{T\text{-}KO}$), to examine the role of glycolysis exclusively in alloreactive T cells without off-targeting effects from antigen presenting cells and other cell types that are dependent on glycolysis. We demonstrated that transfer of $Glut1^{T\text{-}KO}$ T cells significantly improved acute GVHD outcomes through increased apoptotic rates, impaired expansion, and decreased proinflammatory cytokine production. In addition to impaired GVHD development, donor $Glut1^{T\text{-}KO}$ T cells mediated sufficient GVL activity to protect recipients from tumor development. A clinically relevant approach using donor T cells treated with a small

molecule inhibitor of glycolysis, 2-Deoxy-D-glucose ex vivo, further demonstrated protection from tumor development. These findings indicate that treatment with glycolysis inhibitors prior to transplantation selectively eliminates alloreactive T cells, but spares non-alloreactive T cells including those that protect against tumor growth. The present study has established a definitive role for glycolysis in acute GVHD and demonstrated that acute GVHD can be selectively prevented through targeting glycolysis.

Keywords: allogeneic hematopoietic cell transplantation, GvHD, T cells, glycolysis, GVL effects, metabolism

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT), which provides donor T cell-mediated protection known as graft-versus-leukemia (GVL) effect, is a critical curative option for many types of hematologic malignancies (1, 2). However, donor T cells that recognize recipient alloantigens can also contribute to graft-versus-host disease (GVHD), the primary cause of non-relapse mortality (3). Nonspecific treatments such as T cell depletion therapies and broad immunosuppressants are linked to elevated rates of relapse and opportunistic infections (4–6). Novel approaches are therefore necessary for selectively targeting alloreactive T cells to preserve non-alloreactive T cells that mediate anti-tumor immunity.

The current understanding in T cell function is tightly linked to the metabolic state, prompting the use of metabolic modulation to dampen harmful inflammatory responses. Naïve, memory T cells and effector T cells adopt distinct metabolic profiles to support survival and functional requirements. Previous studies explored manipulation of T cell bioenergetics to dampen the inflammatory response as a result of increased dependence on oxidative phosphorylation (OXPHOS) (7). In the current study, we investigate the potential of targeting a different metabolic pathway, glycolysis, in acute GVHD mediated by donor T cells. In contrast to naïve and memory T cells, activated effector T cells become highly dependent on aerobic glycolysis to fulfill biosynthetic demands for cell growth and division (8, 9), cytokine production (10, 11), which promote pathogenic T cell responses in various inflammatory conditions (12–16). Despite the large body of studies, the role of glycolysis in the pathogenicity of alloreactive T cells and the sparing of GVL activity with glycolysis blockade remain poorly understood (7, 17–20). Previous studies indicated that alloreactive T cells activated *in vivo* are primarily dependent on OXPHOS and fatty acid oxidation (FAO) (17, 18). In contrast, *in vivo* studies by Nguyen et al. showed that alloreactive T cells preferentially utilize glycolysis through phenotypic analysis (20). However, these studies could not exclude the dependence of antigen presenting cells (APCs) on glycolysis due to the systemic treatment with glycolysis inhibitors (20–23). Non-specific treatments using metabolic inhibitors can affect the function and survival of other cell types and cannot be assumed to accurately reflect the biology of alloreactive T cells. Other groups also demonstrated indirect connections between glycolysis and T cell-mediated GVHD (24, 25). More importantly, whether glycolysis inhibition is capable of preserving anti-tumor effects of non-alloreactive T cells is unknown. It is imperative to evaluate GVL effects in preclinical studies to prevent tumor relapse prior to the introduction of glycolysis inhibitors to the clinical setting.

A model limiting the utilization of glycolysis exclusively in T cells is necessary to address its role in T cell-driven GVHD and the preservation of GVL effects. Glucose uptake in T cells can be facilitated through glucose transporter (Glut) family members Gluts 1, 3, 6, and 8 (15). Glut1, the primary glucose transporter in T cells, is upregulated as soon as 2 hours following activation (15). Transgenic animals that constitutively express Glut1 are susceptible to the development of systemic inflammatory diseases (26, 27). Given the discordant findings (7, 17, 18, 20), we previously utilized animals harboring a T cell-specific genetic deletion for Glut1 ($Glut1^{T\text{-}KO}$) to address the role of glycolysis in GVHD (15). However, whether the effect on disease progression is a strain-specific phenomenon, the mechanisms leading to the differences in GVHD development, and impacts on GVL effects were not examined. Donor T cells derived from these animals are functionally deficient for glycolysis, allowing for the examination of glycolysis in T cell-mediated GVHD. In the current study, we examined the molecular pathways by which glycolysis modified the pathogenic phenotype of alloreactive T cells through proliferative response and cell death mechanisms, demonstrating a key role for glycolysis without confounding factors from other glycolysis-dependent cell types (21–23). We also evaluated for the first time the therapeutic potential and feasibility for the separation of GVL from GVHD through ex vivo glycolysis inhibition using the small molecule inhibitor, 2-Deoxy-d-glucose (2-DG).

METHODS

Mice

C57BL/6 (H-2^b, CD45.2), C3H/HeJ (H-2^k, CD45.2), BALB/c (H-2^d, CD45.2), B6.SJL (H-2^b, CD45.1) mice were purchased from Jackson laboratories (Bar Harbor, ME). $Glut1^{T\text{-}KO}$ ($Glut1^{fl/fl}$ x $CD4^{Cre}$) mice, $Glut1^{fl/fl}$ mice, and TCR-tg 4C mice are in the C57BL/6 background as described previously (15, 28–30). Wild-type (WT) animals include both C57BL/6 and littermate controls. All mice were maintained in a specific pathogen-free facility at Duke University. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Duke University Medical Center.

Tumor Cell Lines

Luciferase (Luc)- and the enhanced green fluorescent protein (EGFP)-expressing (Luc-EGFP) BCL1 cells, a B-cell leukemia/lymphoma cell line of BALB/c origin, were a generous gift from Dr. Defu Zeng (City of Hope, Duarte, CA). A20 cells, another B-cell leukemia/lymphoma cell line of BALB/c origin, were initially purchased from ATCC (Manassas, VA). A20 cells expressing the Luc-EGFP gene were made by lentivirus-mediated gene transduction. Briefly, 293T cells cultured in Dulbecco's Modified Eagle Medium (DMEM) media (Sigma-Aldrich, St. Louis, MO, USA) were co-transfected with pLEX (ThermoFisher)-EF1a-luciferase-EGFP together with the packaging plasmids, pMD2.G (a gift from Didier Trono (Addgene plasmid # 12259) and psPAX2 (A gift from Didier Trono (Addgene plasmid # 12260)), by calcium phosphate precipitation. After 24 hours, the DMEM media was replaced with fresh medium. At 48 hours after transfection, medium containing lentivirus was harvested and filtered through a 0.45 μM syringe filter. Viral infection was carried out in a 12-well plate using 5×10^5 A20 cells with 0.5 ml of lentiviral medium containing 10 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO). At 24 hours after infection, cells were selected with 1 μg/mL puromycin for 7 days and clonal Luc-EGFP positive cells were then selected by FACS sorting. Periodically, cells were treated with puromycin to weed out cells which had silenced reporter gene expression.

Murine Cell Preparation and T Cell Stimulation

Murine total, $CD4^+$, or $CD8^+$ T cells were isolated from splenocytes by negative selection using mouse Pan T Cell Isolation Kit II (Miltenyi, Germany). Dendritic cells (DCs) were isolated from splenocytes using CD11c Microbeads UltraPure (Miltenyi). Bone marrow cells were collected from femurs and tibia by flushing using a syringe and passing through a strainer. To prepare T cell depleted bone marrow (TCDBM), bone marrow cells were first incubated with anti-CD90.2 antibody (clone 30H12; BD Pharmingen, CA) on ice for 1 hour. Subsequently, cells were treated with Low Tox-M Rabbit Complement (Cedarlane, Burlington, Canada) for 1 hour at 37°C and washed twice for injection. For *in vitro* T cell stimulation, 7.5 x 10^5 T cells isolated from donor spleens were incubated in 12 wells, flat-bottomed plates with 1.5 x 10^5 BALB/c irradiated DCs (20 Gy) at 37°C in 5% CO_2 for 16 hours; irradiated BALB/c splenocytes (20 Gy) were used when indicated. T cells isolated from the recipient spleens were utilized for *in vivo* expansion analyses. For antibody stimulation *in vitro*, 12 wells, flat-bottomed plates were coated with goat anti-hamster IgG antibody (Invitrogen) at 20ug/ml overnight, followed by wash with PBS prior to stimulation with anti-CD3 at 1ug/ml (BD Pharmingen, clone 145-2C11) and anti-CD28 at 0.3ug/ml (Invitrogen, clone 37.51) antibodies. For metabolic assays, T cells were co-cultured with BALB/c irradiated DCs or IL-7 (0.3 ng/ml) for 120 hours. For intracellular staining of TNFα, WT or $Glut1^{T\text{-}KO}$ T cells were stimulated with purified BALB/c DCs for 72 hours, with the addition of PMA (Sigma, 20ng/ml), ionomycin (Sigma, 1uM), and monensin (Thermofisher) 4 hours prior to collection. For ex vivo inhibition assays, 1 x 10^6 T cells were first stimulated with irradiated BALB/c splenocytes (20 Gy) for 16 hours in complete RPMI with 10% fetal bovine serum. Following 16 hours, T cells were washed and stimulated with freshly isolated BALB/c splenocytes (irradiated) for an additional 24 hours, 48 hours, 72 hours, or 96 hours in the presence of media control or 2-DG at a final concentration of 8mM.

Human Cell Preparation and T Cell Stimulation

Human T cells were purified using RosetteSep human T cell enrichment cocktail (STEMCELLTechnologies, Vancouver, Canada) from donor peripheral blood mononuclear cells (PBMCs). T cells (1.25 x 10^5 cells) were co-cultured with irradiated PBMCs (20 Gy) from unrelated donors (5 x 10^5 cells) for 16 hours, followed by 24-hour incubation with 2-DG, washed and incubated with PBMC stimulators or Dynabeads human T-Activator CD3/CD28 for 72 hours (Thermo Fisher, Waltham, MA). Human samples from de-identified healthy donors were obtained from American Red Cross under an approved protocol.

GVHD Model

Recipient mice were lethally irradiated at 9.5 Gy for C3H/HeJ, 8.5 Gy for BALB/c, or 10.5 Gy for C57BL/6 mice using a Mark I-68A ^{137}Cs irradiator (JL Shepherd and Associates, San Fernando, CA) and transplanted *via* tail vein injection within 4 hours following irradiation. Recipients were transplanted with 1 x 10^7 TCDBM cells/mouse from C57BL/6 donors with or without 1 x 10^6 T cells from WT or $Glut1^{T\text{-}KO}$ mice. Survival, weight change, skin changes (hair loss and ruffling, erythema), hunching posture, diarrhea, and activity were monitored daily for clinical grading. Mice that met humane endpoints were sacrificed according to Duke University IACUC protocols.

GVL Model

Recipient BALB/c mice were lethally irradiated at 8.5 Gy, followed by transplantation with 1 x 10^7 TCDBM cells/mouse from C57BL/6 donors with or without T cells from WT or $Glut1^{T\text{-}KO}$ mice, along with 5 x 10^5 Luc-EGFP BCL1 cells or 1 x 10^5 Luc-EGFP A20 cells. Survival and weight loss were recorded daily. Recipients were further monitored for tumor growth by bioluminescent imaging (BLI) and GVHD evidence by skin changes, activity, posture, and diarrhea. Biopsies were taken from spleen and liver for evidence of tumor growth. Mortality due to GVHD or tumor was distinguished by BLI, necropsy, and histology. In the absence of tumor detection, the cause of death was ruled as GVHD.

Bioluminescent Imaging

Mice were anesthetized using isofluorane, followed by D-Luciferin injection (50 mg/kg, PerkinElmer, CT) 10 minutes prior to imaging. Imaging was performed using a Xenogen IVIS 100 imaging system (Xenogen Corporation, Alameda, CA) for maximal signal intensity at 5-minute exposure time. Living

Image 2.5 software (Caliper, Newton, MA) was used for imaging analyses.

Histologic Analysis

Biopsy samples were taken from skin, small and large intestines, liver, and spleen and were stored in neutral buffered formalin. Specimens were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin-eosin (H&E). Coded slides were assessed by D.C. single blinded to the GVHD status. Histological GVHD was graded using a semi-quantitative system based on histologic changes in the small intestine, colon, skin, and liver. Histological characteristics used for scoring included inflammatory infiltrates, apoptosis of keratinocytes, separation of dermal-epidermal junction, and formation of cleft, follicular dropout, and fibrosis in the skin; inflammation, apoptosis of bile duct epithelial cells, apoptosis of hepatocytes, cholestasis, fibrosis, and parenchyma in the liver; and lamina propria inflammatory cell infiltrate, crypt regeneration, crypt epithelial cell apoptosis, crypt loss, mucosal ulceration, and fibrosis in the intestine (31).

Mixed Lymphocyte Reaction

Purified T cells (2.5 x 10^5 cells) were incubated in 96-wells, flat-bottomed plates with 5 x 10^5 irradiated (20 Gy) BALB/c splenocytes for indicated periods at 37°C in 5% CO_2. Cells were pulsed with ^{3}H-thymidine (1μCi [0.037MBq]/well) 16 hours before being counted by a MicroBeta Trilux liquid scintillation counter (EG&G Wallac, Turku, Finland).

Metabolic Assays

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) assays were performed using the XF24 extracellular flux analyzer (Seahorse Bioscience) as previously described (32). ECAR was measured at indicated time points following sequential compound injections (10 mM glucose, 1 μM oligomycin, and 20 mM 2-DG). Basal OCR was measured prior to compound injection. Glucose uptake assays were described previously (Wieman et al., 2007). 2-Deoxy-d-[H^3] glucose (2 μCi/reaction) was added to T cell cultures and quenched by 200 μM phloretin (Calbiochem, San Diego, CA). Radioactivity of solubilized cell pellets was measured using a scintillation counter.

Enzyme-Linked Immunosorbent Assay

Supernatants from T cell cultures were collected and assessed by enzyme-linked immunosorbent assay (ELISA) using antibodies against interferon γ (IFNγ) and interleukin-2 (IL-2) (BD Pharmingen, San Jose, CA) as described previously (26).

Flow Cytometry

The following antibodies were used to detect surface protein expression: anti-CD4-PE (clone H129.19), anti-CD4-APC (clone RM4-5), anti-CD8-PE-Cy7 (clone 53-6.7) were purchased from BD Pharmingen (San Diego, CA) and BD Biosciences (Franklin Lakes, New Jersey); anti-CD69-PerCP-Cy5.5 (clone H1.2F3) was purchased from Biolegend (San Diego, CA). Fixable Viability Dye eFluor 780 (catalog 65-0865) was used to distinguish viable cells (eBioscience, San Diego, CA). For intracellular staining, anti-pS6-PE (eBioscience, clone cupk43k) and anti-Bim-PE (CST, Danvers, MA, clone C34C5) were used; anti-Mcl-1 (clone Y37) and anti-Noxa (clone 114C307) primary antibodies were purchased from Abcam (Cambridge, UK). Secondary antibodies, anti-rabbit IgG Fab2-AF647 (catalog ab181347) and anti-mouse IgG Fab2-AF647 (catalog ab169358) were purchased from Abcam. For intracellular staining, cells were first stained with antibodies for surface proteins, fixed with 4% PFA, then permeabilized using 0.5% Tween 20 in PBS. For staining of TNFα, eBioscience Foxp3/Transcription Factor Staining Buffer Set was used (ThermoFisher). Cells were fixed and permeabilized at 4 degrees Celsius for 30 minutes, followed by washing with 1x permeabilization buffer, intracellular staining for 20 minutes. Following staining, cells were washed twice before running. For secondary staining, secondary antibodies were added following addition of unconjugated primary antibodies. For isotype controls, Rabbit IgG XP (R)-PE (CST, catalog 5742S), mouse IgG1k-PE (BD Pharmingen, clone MOPC-21), Rabbit IgG (Abcam, ab37415), mouse IgG1k (Abcam, clone B11/6) were used. For apoptosis assay, the Apoptosis Detection Kit (BD Pharmingen), which includes Annexin V-PE and 7-Amino-Actinomycin D (7AAD), was used. Stained samples were analyzed using FACSCanto Flow Cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Western Blotting

Cells were lysed with Pierce™ IP Lysis Buffer (ThermoFischer), which contains 25mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, supplemented with protease inhibitor (Thermo Scientific) and phosphatase inhibitor (Thermo Scientific). Cell debris was then removed by spinning for 5 minutes at 4°C. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Whole cell extracts (50μg of proteins) were fractionated by SDS-PAGE and transferred to a nitro cellular membrane using a transfer apparatus according to manufacturer's instructions (Bio-Rad). Membranes were blocked with LICOR blocking buffer, washed and incubated with primary antibodies (1:1000 in blocking buffer) at 4°C for 12 hours. After washing, membranes were incubated with a 1:10000 dilution (in blocking buffer) of fluorescent 700 or 800 anti-rabbit or anti-mouse antibodies for 1 hour at room temperature. Blots were washed with TBST five times and scanned using LICOR machine. Anti-Puma antibody (ab9643), anti-Noxa antibody (clone 114C307, ab13654), and anti-Mcl-1 antibody (clone Y37, ab32087) were purchased from Abcam. Anti-Mdm2 antibody (clone D-7, sc-13161) was purchased from Santa Cruz Biotechnology Inc. (Dallas. TX).

Statistics

Data were analyzed using Prism Graphpad (Version 6, San Diego, CA). Error bars represent mean ± SEM. Unpaired two-tailed student's *t* tests, one-way ANOVA with Tukey's multiple comparisons test, were utilized for group comparisons. Survival curve comparisons were performed using Log-Rank (Mantel-Cox) test. P-values < 0.05 were considered statistically significant.

RESULTS

Glut1 Is Required for Donor T Cells to Induce GVHD

We previously demonstrated the role of Glut1 in T cell-mediated acute GVHD using the C57BL/6 → BALB/c major histocompatibility complex (MHC)-mismatched bone marrow transplant (BMT) model (15). To confirm that the observation is not strain-specific, C3H/HeJ recipients were also utilized for the transfer of Glut1$^{T\text{-}KO}$ or wild-type (WT) C57BL/6 T cells to induce acute GVHD. All WT recipients died from GVHD within 20 days while eight out of ten Glut1$^{T\text{-}KO}$ T cell recipients survived long-term (**Figure 1A**). In addition, Glut1$^{T\text{-}KO}$ T cell recipients showed comparable body weight and clinical scores with TCDBM recipients (**Figures 1B, C**). Therefore, consistent with the previous study, these findings further support a key role for Glut1 to promote donor cell pathogenicity.

We further examined whether Glut1 is required for CD4$^+$ and CD8$^+$ T cells to induce acute GVHD, respectively. In contrast to WT recipients, both CD4$^+$ Glut1$^{T\text{-}KO}$ and CD8$^+$ Glut1$^{T\text{-}KO}$ T cell recipients survived long-term (**Figures 1D, G**). However, the kinetics of GVHD development and target organs affected differed. Both body weights and clinical scores in CD4$^+$ Glut1$^{T\text{-}KO}$ T cell recipients significantly improved early following BMT (**Figures 1D–F**). In contrast, the kinetics of GVHD development in CD8$^+$ T cell recipients is relatively delayed, leading to improvement in Glut1$^{T\text{-}KO}$ T cell recipients later during disease progression compared to the control group (**Figures 1G–I**). Target organ damage was also assessed by histology (**Figures S1, S2**). Both small intestine and colon exhibited reduced damage in CD4$^+$ Glut1$^{T\text{-}KO}$ compared to WT recipients (**Figures S1A, S2A**). In contrast, skin damage was significantly reduced in CD8$^+$ Glut1$^{T\text{-}KO}$ recipients (**Figures S1B, S2B**). Overall, transfer of either CD4$^+$ or CD8$^+$ Glut1$^{T\text{-}KO}$ T cells significantly improved long-term survival and ameliorated acute GVHD.

Glut1 Is Required for the Metabolic Reprogramming and Expansion of Alloreactive T Cells

T cells rapidly undergo metabolic reprogramming following activation, prioritizing glucose metabolism to promote growth

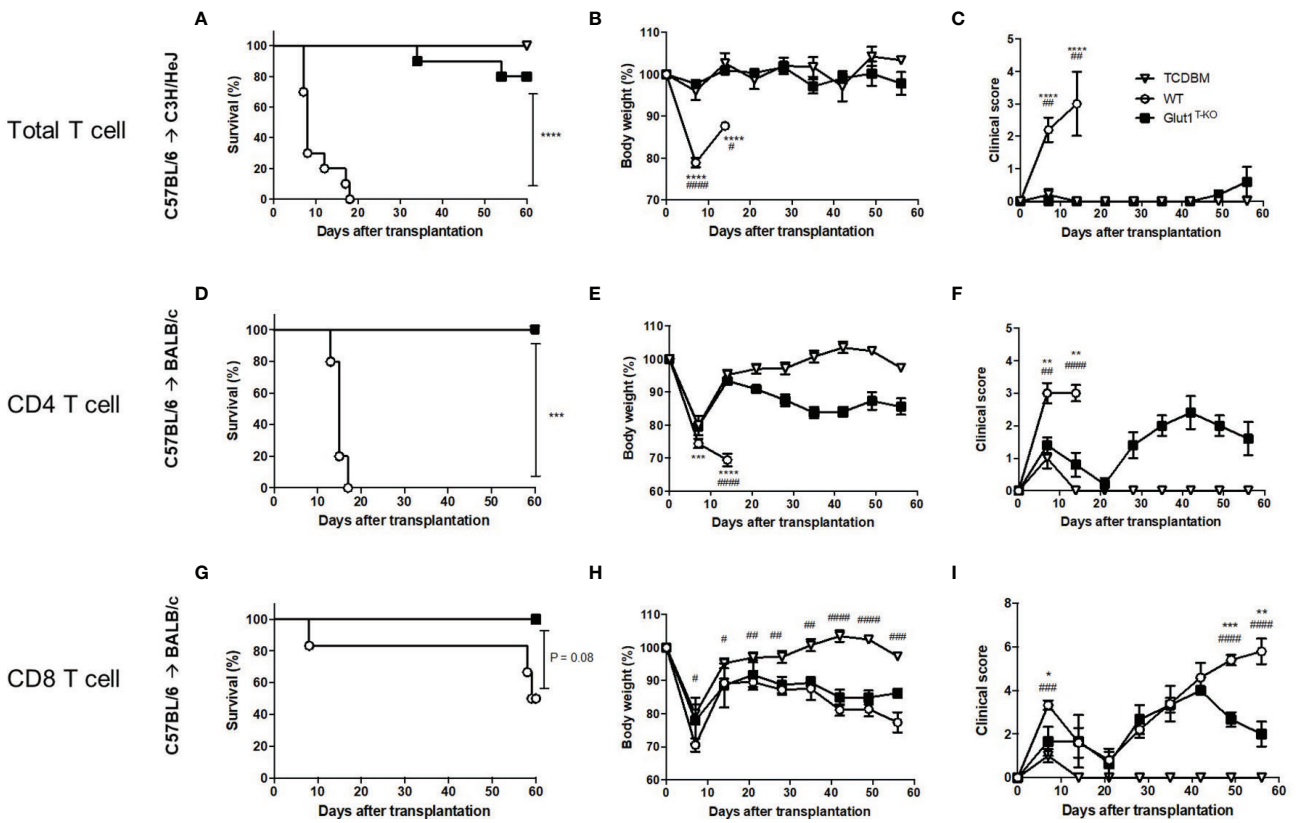

FIGURE 1 | Glut1 is required for donor T cells to induce acute GVHD. Acute GVHD was induced by transplantation of C57BL/6-derived 1 x 10^7 TCDBM or along with 1 x 10^6 WT or Glut1$^{T\text{-}KO}$ total T cells into lethally irradiated (9.5 Gy) C3H/HeJ recipients **(A–C)**. Lethally irradiated (8.5 Gy) BALB/c recipients were transplanted with 1 x 10^6 WT or Glut1$^{T\text{-}KO}$ CD4$^+$ **(D–F)** or CD8$^+$ T cells **(G–I)**, along with 1 x 10^7 TCDBM from C57BL/6 donors. Recipients were monitored for survival **(A, D, G)**, body weight **(B, E, H)**, clinical score **(C, F, I)** up to 56 days after transplantation. Data were representative of three experiments. ****P < 0.0001, ***P < 0.001, log-rank test [n = 10 per group, A; n = 5 per group, (**D, G**)]; data are shown as mean ± SEM **(B, C, E, F, H, I)**, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (Glut1$^{T\text{-}KO}$ vs. WT); $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001, $^{\#\#\#\#}$P < 0.0001(TCDBM vs. WT), 2-tailed Student t test.

and proliferation (33). We first assessed whether alloreactive Glut1$^{T\text{-}KO}$ T cells were able to initiate metabolic reprogramming. Glut1$^{T\text{-}KO}$ T cells had significantly decreased glucose uptake following alloantigen stimulation (**Figure 2A**). Alloreactive Glut1$^{T\text{-}KO}$ T cells were unable to utilize glycolysis, indicated by ECAR compared to WT T cells during glycolysis stress test (**Figure 2B**). Metabolic assays further confirmed deficient glycolysis (**Figure S2A**), glycolytic capacity (**Figure S2B**), and glycolytic reserve (**Figure S2C**) of alloreactive Glut1$^{T\text{-}KO}$ T cells compared to control. These results suggest that Glut1$^{T\text{-}KO}$ T cells display overall significant defects in glucose uptake and glycolytic metabolism upon alloantigen challenge.

Mammalian target of rapamycin complex 1 (mTORC1) regulates glucose metabolism through HIF1α and c-Myc to support biosynthesis and proliferation (33, 34). Although rapamycin has been shown to dampen GVHD by inhibiting glycolysis (20), it is unclear whether glucose availability modulates mTORC1 activity to regulate alloreactive T cell response. We hypothesize that mTORC1, a nutrient sensor (34), responds to glucose availability to modulate donor cell pathogenicity. Alloreactive T cells positive for CD69 expression (**Figures S3A** and **S3B**) were assessed for the phosphorylation status of the small ribosomal subunit S6 (pS6), a downstream target for mTORC1 signaling. Phosphorylation of S6 (Ser235/236) in resting Glut1$^{T\text{-}KO}$ T cells was significantly lower than WT T cells (**Figure 2C**). Following stimulation, Glut1$^{T\text{-}KO}$ T cells demonstrated profoundly decreased phospho-S6 levels (**Figure 2C**). Glucose availability therefore leads to sustained mTORC1 activation in alloreactive T cells.

To determine the requirement for T cell expansion, tritium thymidine uptake was assessed in MLR. Glut1$^{T\text{-}KO}$ T cells displayed drastically impaired thymidine uptake as early as 64 hours following stimulation (**Figure 2D**). To test whether glycolysis is required for *in vivo* expansion upon alloantigen encounter, T cells were transferred into irradiated allogeneic or syngeneic recipients. While expansion in syngeneic recipients did not differ, Glut1$^{T\text{-}KO}$ T cells exhibited significantly impaired capacity to undergo expansion compared to WT T cells in allogenic recipients (**Figure 2E**). Furthermore, Glut1$^{T\text{-}KO}$ T cells failed to undergo robust proliferation, indicated by the lack of subsequent generations following divisions (**Figure 2F**). Similar defects were observed in CD69^{+} alloreactive Glut1$^{T\text{-}KO}$ T cells (**Figures S4A, S4B**). Glycolysis has also been linked to cytokine production through the sequestration of cytokine transcripts (10). Expression of inflammatory cytokines IFNγ and IL-2 was measured in alloreactive T cells. Glut1$^{T\text{-}KO}$ T cells displayed significantly reduced capacity to produce both cytokines compared to WT T cells (**Figure 2G**). TNFα expression was also assessed in WT and Glut1$^{T\text{-}KO}$ T cells following 72 hours of stimulation with purified BALB/c DCs. While Glut1$^{T\text{-}KO}$ CD4 T cells exhibited slightly reduced expression compared to WT group, Glut1$^{T\text{-}KO}$ CD8 T cells demonstrated increased TNFα expression relative to WT T cells (**Figure 2H**). In summary, we demonstrated that glycolysis is indispensable for alloreactive T cell expansion and effector cytokine production, which cannot be rescued by OXPHOS in Glut1$^{T\text{-}KO}$ T cells (**Figure S2D**).

Glut1 Is Required for the Survival of Alloreactive T Cells

In addition to proliferation, alteration of survival signals is a potential modulator of pathogenicity. Glut1 expression has been shown to support resting T cell survival through the stabilization of pro-survival factors (35). To determine whether the apoptotic pathway is involved in regulating viability in response to glucose metabolism, we assessed the expression of various candidate proteins 16 hours following activation by anti-CD3 and anti-CD28 antibodies. Proteins linked to the apoptotic pathway, including Mdm2, Puma, and Noxa, were drastically increased in activated Glut1$^{T\text{-}KO}$ T cells relative to WT control (**Figure 3A**). By contrast, the anti-apoptotic protein Mcl-1 was significantly upregulated compared to WT T cells (**Figure 3A**).

We further assessed whether glycolysis is also involved in regulating cell survival in alloreactive T cells. Viability analysis demonstrated significantly less live Glut1$^{T\text{-}KO}$ T cells compared to WT T cells following alloantigen stimulation *in vitro* (**Figure S5A**). Apoptosis was subsequently assessed using Annexin V and 7AAD staining. While both CD4^{+} and CD8^{+} alloreactive Glut1$^{T\text{-}KO}$ T cells underwent increased apoptosis compared to WT T cells, the apoptosis kinetics differed. CD8^{+} CD69^{+} Glut1$^{T\text{-}KO}$ T cells appeared to undergo apoptosis earlier than CD4^{+} CD69^{+} T cells inferred from percentages of Annexin V^{+} cells (**Figure 3B**).

Regulation of pro-apoptotic and anti-apoptotic protein expression can alter the survival outcome in response to cellular stress. Impaired glucose metabolism can lead to apoptosis in response to endoplasmic reticulum (ER) stress mediated by Bim, a pro-apoptotic Bcl-2-family protein (36). Bim expression is higher in Glut1$^{T\text{-}KO}$ T cells compared to WT T cells in both freshly isolated state and activated state (**Figures S5B** and **3C**). Though the demand for glycolysis is lower in resting T cells compared to activated T cells, a minimal rate of glycolysis is still required to meet basal energy demands (26), potentially contributing to the difference in baseline Bim expression. Regulation of anti-apoptotic Bcl-2 proteins such as Mcl-1 can also regulate survival (35, 37) Alloantigen-stimulated Glut1$^{T\text{-}KO}$ T cells failed to provide adequate survival signal through Mcl-1 compared to WT T cells (**Figure 3C**). Differences in Mcl-1 expression were readily detected in both anti-CD3 and anti-CD28 antibody activated T cells and alloreactive T cells (**Figures 3A, D**). Baseline differences in Mcl-1 expression between WT and Glut1$^{T\text{-}KO}$ T cells were detectable using Western blots (**Figure 3A**) but not flow cytometry (**Figure S5C**), which can be attributed to variation in detection sensitivity between methods of detection. In addition, the balance of Mcl-1 and Noxa, a BH3-only pro-apoptotic factor and a binding partner for Mcl-1, can be regulated by glucose availability (38). Since Noxa expression was reduced in the presence of both anti-CD3 + anti-CD28 antibodies as well as alloantigens (**Figures 3A** and **S5D**), the skewed Noxa/Mcl-1 ratio may render Glut1$^{T\text{-}KO}$ T cells more prone to apoptosis.

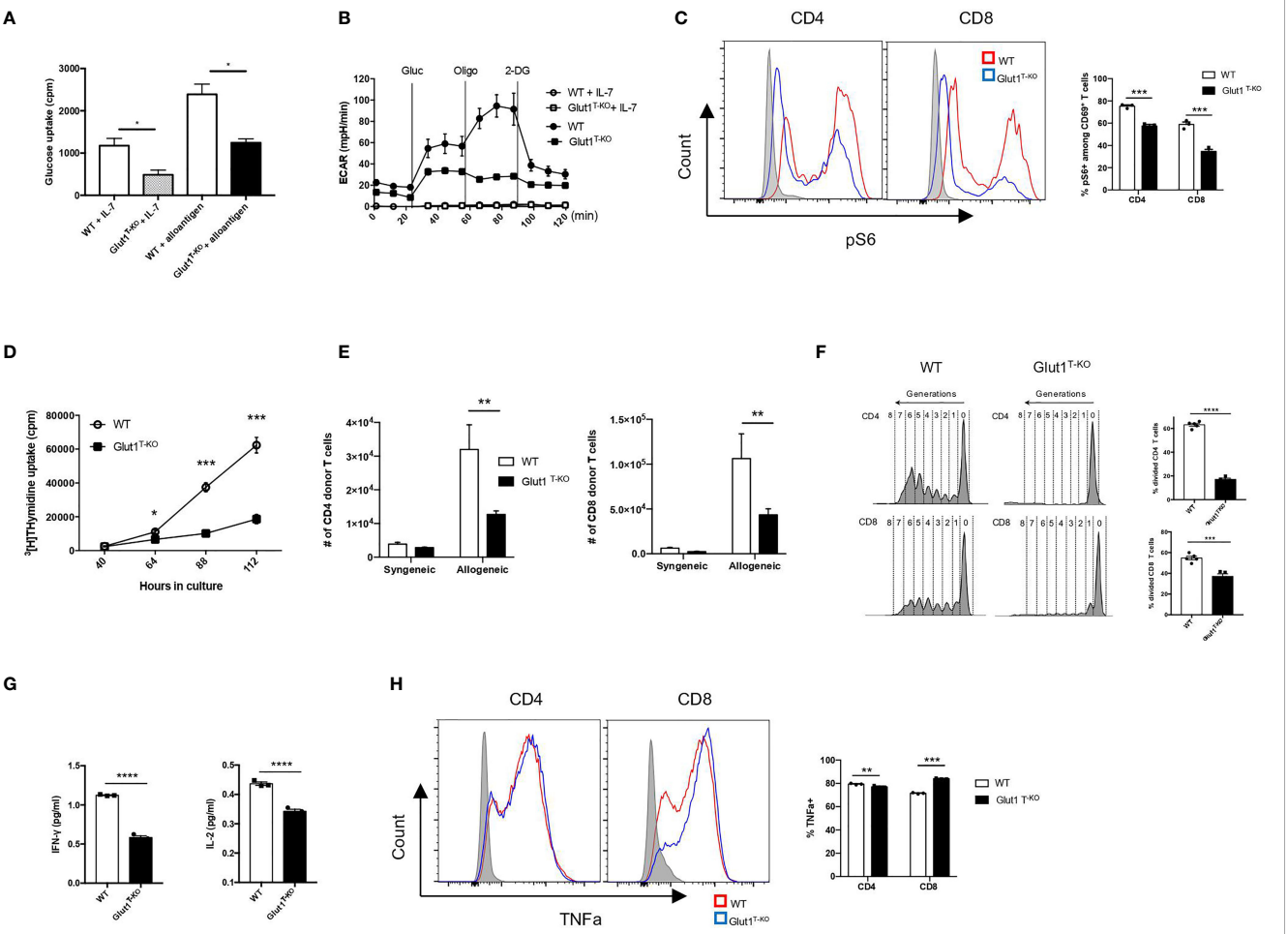

FIGURE 2 | Glut1 mediates the expansion of alloreactive T cells. WT or Glut1$^{T\text{-}KO}$ T cells were stimulated with BALB/c dendritic cells. After 5 days in culture, glucose uptake was measured **(A)**. ECAR was assessed with the addition of glucose (gluc), oligomycin (olig), and 2-DG **(B)**. WT or Glut1$^{T\text{-}KO}$ T cells were stimulated for 16 hours using BALB/c DCs and analyzed by flow cytometry for phospho-S6 levels in CD69^{+} T cells **(C)**. Expansion *in vitro* was measured by ^{3}H-TdR incorporation assay at indicated time points after culture **(D)**. Expansion *in vivo* was measured in T cells isolated from the spleen seven days after transplantation with 1 x 10^{7} TCDBM from B6.SJL donors and 1 x 10^{6} WT or Glut1$^{T\text{-}KO}$ T cells on the C57BL/6 background into BALB/c recipients **(E)**. T cell proliferation following isolation from the spleen was analyzed (left panel) and measured by frequency (right panel) 58 hours following transfer of CellTrace Violet (CTV)-labelled T cells along with B6.SJL TCDBM into BALB/c recipients **(F)**. IFNγ and IL-2 production were assayed by ELISA using supernatants from T cell cultures **(G)**. **(H)** WT or Glut1$^{T\text{-}KO}$ T cells were stimulated with purified BALB/c DCs for 72 hours, with the addition of PMA (20ng/ml), ionomycin (1uM), and monensin 4 hours prior to intracellular staining for TNFα. Data are representative of two (F, n = 3) or three experiments (n = 3, A-D, and G; n = 5, E and F) and are shown as mean ± SEM **(B–F)**. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (Glut1$^{T\text{-}KO}$ vs. WT), 2-tailed Student t test.

Glut1 Deficiency in Donor T Cells Ameliorates GVHD While Preserving GVL Effects

It is crucial for GVHD treatments to selectively inhibit alloreactive T cells without compromising the GVL effect. To test the effect of glycolysis inhibition on GVL preservation, lethally irradiated BALB/c recipients were engrafted with WT or Glut1$^{T\text{-}KO}$ T cells, TCDBM, accompanied by challenge with BCL1 cells, a BALB/c-derived leukemia/lymphoma cell line.

TCDBM + BCL1 group succumbed to tumor challenge within 31 days following transplantation (**Figures 4A, B**), indicated by BLI (**Figure 4C**). Histology analysis further confirmed metastatic invasion of the liver parenchyma, indicated by enlarged and hyperchromatic nuclei of neoplastic cells (**Figure 4D**). While Glut1 deficiency did not completely protect recipients from GVHD as evidenced by gradual weight loss (**Figure 4B**), analyses of target organ histology indicated lower pathological scores in the skin and large intestine (**Figures S6**). Furthermore, transfer of 1 x 10^{6} Glut1$^{T\text{-}KO}$ T cells significantly improved long term survival in majority of recipients compared to both TCDBM + BCL1 and WT T cell recipients, which all died from tumor or GVHD (**Figures 4A–E**). BLI analysis and necropsy revealed that Glut1$^{T\text{-}KO}$ recipients remained tumor-free, demonstrating the preservation of GVL effects (**Figures 4C–E**). In contrast, all WT T cell recipients succumbed to GVHD within 100 days (**Figure 4E**). To confirm that the GVL effect of Glut1$^{T\text{-}KO}$ T cells is not restricted to a specific tumor model, a second lymphoma cell line of BALB/c origin (A20) was used to evaluate

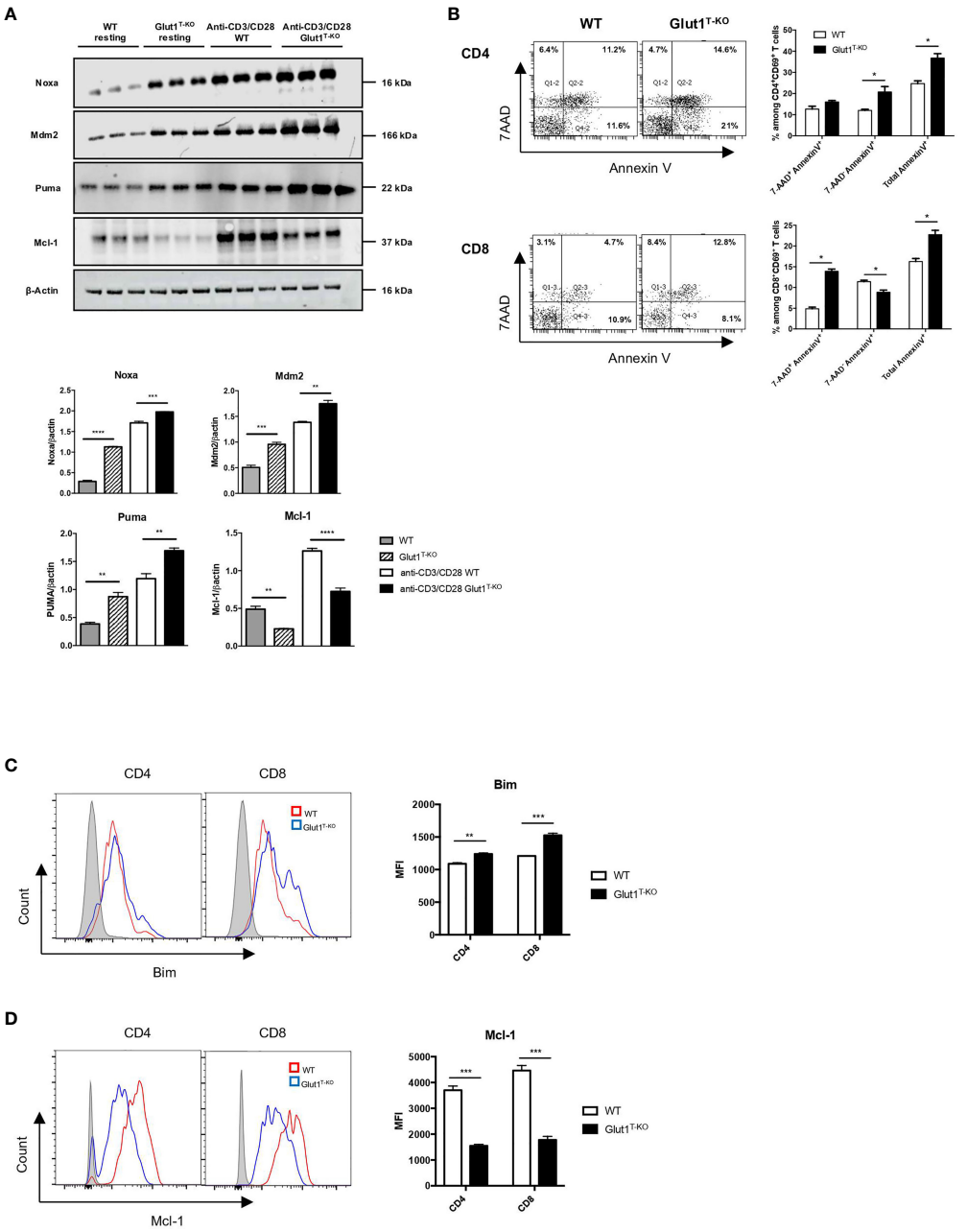

FIGURE 3 | Glut1 modulates alloreactive T cell survival. **(A)** Freshly isolated WT or Glut1$^{T\text{-}KO}$ T cells or those stimulated with anti-CD3 (1ug/ml) and anti-CD28 (0.3ug/ml) antibodies for 16 hours were assessed for the expression of Mdm2, Puma, Noxa, and Mcl-1 using Western blotting (upper panel). Results were quantified for fresh T cells and antibody-stimulated T cells (lower panel) (n = 3, one-way ANOVA with a Tukey's multiple comparisons test). **(B)** WT or Glut1$^{T\text{-}KO}$ T cells were stimulated for 16 hours by irradiated (20 Gy) BALB/c DCs and analyzed by flow cytometry for Annexin V and 7AAD. T cells were gated on CD69^{+} CD4^{+} or CD8^{+} T cells. Bim **(C)** and Mcl-1 **(D)** expression were evaluated. Data are representative of three experiments (n = 3) and are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, 2-tailed Student t test.

protection against tumor development. Transfer of Glut1$^{T\text{-}KO}$ T cells significantly improved survival compared to TCDBM + A20 and WT recipients (**Figure 4F**). While body weights of Glut1$^{T\text{-}KO}$ recipients were lower than that of TCDBM control groups due to GVHD, they were significantly higher than WT recipients, demonstrating ameliorated GVHD development (**Figure 4G**). BLI analyses and necropsy showed that tumor growth was absent in all Glut1$^{T\text{-}KO}$ recipients (**Figures 4H, I**), recapitulating protection against tumor using a different tumor model. A low dose of Glut1$^{T\text{-}KO}$ T cells was also tested using the BCL1 tumor model and provided limited protection against tumor development (**Figure S7**), suggesting a role for glycolysis in

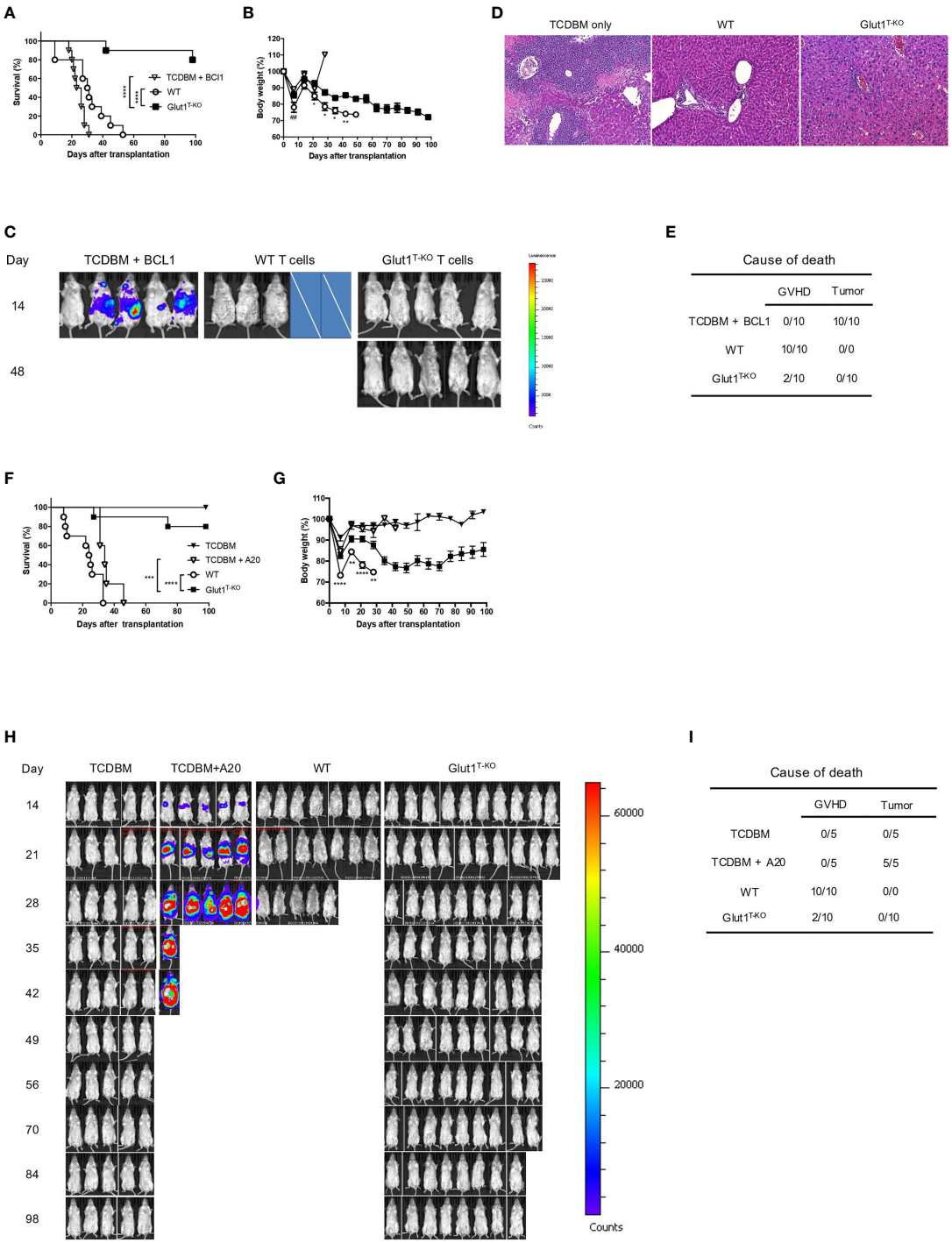

FIGURE 4 | Transfer of Glut1$^{T\text{-}KO}$ T cells inhibits GVHD development and spares GVL activity. Lethally irradiated (8.5 Gy) BALB/c recipients were transplanted with 1 x 10^6 WT or Glut1$^{T\text{-}KO}$ T cells, along with 1 x 10^7 TCDBM and 5 x 10^5 BCL1 cells. Recipients were monitored for survival **(A)** and body weight **(B)** up to 100 days after transplantation. Development of leukemia/lymphoma **(C)** was monitored by BLI. Cross symbols indicate death prior to BLI. H&E histology (10x & 40x) of liver **(D)** from recipients was assessed (time at sample collection: TCDBM + BCL1, day 21; WT, day 27; Glut1$^{T\text{-}KO}$, day 105). Cause of death due to GVHD or tumor development was summarized **(E)**. **(F)** Lethally irradiated (8.5 Gy) BALB/c recipients were transplanted with 1 x 10^6 WT or Glut1$^{T\text{-}KO}$ T cells, along with 1 x 10^7 TCDBM and 1 x 10^5 A20 cells. Recipients were monitored for survival **(F)** and body weight **(G)** up to 100 days after transplantation. Development of leukemia/lymphoma **(H)** was monitored by BLI. Cause of death due to GVHD or tumor development was summarized **(I)**. ***P < 0.001, ****P < 0.0001, log-rank test **(A, F)**; *P < 0.05, **P < 0.01, ****P < 0.0001 (Glut1T-KO vs. WT); $^{\#\#}$P < 0.01 (TCDBM vs. WT), 2-tailed Student t test **(B, G)**. Data are representative of three experiments (n = 10 per group, 1 x 10^6 T cell recipients).

GVL. However, this limitation can be overcome by increased dose of Glut1$^{T\text{-}KO}$ T cells, indicating that glycolysis is not absolutely necessary for donor T cells to exert anti-tumor effects. Collectively, these data indicate that transfer of Glut1$^{T\text{-}KO}$ T cells at sufficient concentrations is capable of preventing tumor growth and mortalities caused by GVHD, supporting the hypothesis that glycolysis targeting selectively inhibits alloreactive T cells.

Inhibition of Glycolysis by 2-DG Selectively Targets Murine and Human Alloreactive T Cells *In Vitro*

A clinically-relevant approach for glycolysis inhibition to ameliorate GVHD has been previously explored, though systemic treatments (20) can induce toxicity in the brain and skeletal muscles (39–41). To improve treatment specificity, donor T cells can be treated ex vivo in the presence of recipient alloantigens. A panel of small molecule inhibitors were evaluated for inhibition of alloreactive T cell proliferation. Both glucose analogs, fludeoxyglucose (FDG) and 2-DG, remarkably suppressed donor T cell response following stimulation (**Figure 5A**). The same effect was observed for 2-DG when the T cell response was measured by the number of CD69-expressing activated T cells (**Figure S8A, S8B**). 2-DG also showed potent inhibitory effect on 4C CD4$^+$ T cells bearing transgenic T cell receptors (TCR-tg) specific for BALB/c alloantigens (**Figures S8C, S8D**). The Glut1 inhibitor, WZB117, also inhibited alloresponse (**Figure 5A**). As 2-DG has been shown to dampen inflammatory T cell response and given its wide accessibility in clinical trials, 2-DG was selected for subsequent assays (42–45).

Incubation of recipient antigen-stimulated donor T cells with glycolysis inhibitors prior to BMT can spare non-alloreactive T cells, reducing toxicity to graft recipients and selectively suppressing alloresponse (**Figure 5B**). Incubation with 2-DG selectively triggered apoptosis in activated alloreactive T cells, indicated by a profound and consistent increase in AnnexinV$^+$ populations and a corresponding decrease in the absolute number of alloreactive CD4 T cells (**Figures 5C, D**, upper panels). In contrast, 2-DG did not impact cell death outcomes in non-activated T cells treated with IL-7 (**Figures 5C, D**, lower panels). Similar findings were observed in alloantigen-stimulated versus IL-7-treated CD8 T cells (**Figure S9**). Following 2-DG incubation, secondary challenge with either alloantigens or anti-CD3- and anti-CD28-coated beads demonstrated that only alloresponse was significantly inhibited (**Figure 5E**). Importantly, as inhibition occurs exclusively during the ex vivo stimulation process, suppression of alloresponse by 2-DG prevents toxicity due to non-specific systemic treatments. Similarly, to test the efficacy in human T cells, purified donor T cells were first primed with irradiated PBMCs from irrelevant allogeneic donors, incubated with 2-DG, and followed by PBMC rechallenge or anti-CD3 and anti-CD28 antibody stimulation. Alloreactive responses underwent a dose-dependent reduction compared to non-specific stimulation, indicating that the proliferative capacity of alloreactive T cells is highly dependent on the ability of T cells to perform glycolysis (**Figure 5E**). Therefore, the optimal concentration of 2-DG at 8mM was utilized for subsequent *in vivo* assays.

2-DG-Mediated Inhibition of Glycolysis *Ex Vivo* Significantly Reduces GVHD While Preserving GVL Effects

Given that Glut1$^{T\text{-}KO}$ T cells preserved GVL effect and the promising *in vitro* data, we next tested the therapeutic potential of T cell-specific glycolysis inhibition using a clinically relevant model. Alloantigen-activated T cells were treated with 2-DG as shown in **Figure 5B** for 24-96 hours, followed by transfer into recipients along with TCDBM and BCL1 cells. Ex vivo inhibition for 24 hours demonstrated limited potency in GVHD prevention, while longer incubation periods (48-96 hours) with 2-DG significantly limited GVHD development without impairing GVL activity as demonstrated by survival, body weight, BLI tumor screening, and clinical scores (**Figures 6A–D** and **S10A, B**). Body weights of 72-hour-treated T cell recipients were significantly higher than those receiving media control T cells later following transplantation (**Figure 6B**). Interestingly, recipients for donor T cells treated with 2-DG for 48 hours gained an optimal survival advantage compared to those receiving uncultured and untreated T cells and those incubated with media control, resulting in the least amount of deaths by proportions caused by GVHD (**Figures 6A, D**). Protection against both GVHD and tumor development conferred by 48-hour-treated T cells was further assessed in a second tumor model using the A20 cell line. Ex vivo 2-DG treatment significantly improved survival compared to TCDBM + A20, WT T cell, and media control recipients (**Figure 6E**). Furthermore, transfer of 2-DG-treated T cells improved body weight compared to WT T cell recipients, as well as exhibiting reduced GVHD severity relative to both WT and media control recipients (**Figures 6F** and **S10C**). Together with BLI analyses and necropsy results (**Figures 6G, H**), we demonstrated that GVHD and tumor development can be attenuated using ex vivo 2-DG treatment. The results from these experiments provide further evidence that targeting glycolysis in alloantigen-specific T cells ex vivo preserves T cell response against irrelevant antigens, potentially providing protection against malignancies and opportunistic pathogens.

DISCUSSION

Activated T cells are dependent on aerobic glycolysis to support growth, division, and effector functions (9, 19, 46). Previous studies revealed conflicting results regarding the role of glycolysis in the pathogenesis of T cell-mediated GVHD (7, 15, 17, 18, 20). In the current study, we utilized T cells genetically deficient for Glut1 to directly demonstrate the requirement for glycolysis in donor T cell-mediated acute GVHD without affecting glycolysis in antigen presenting cells. We established that glycolysis modulates the magnitude of T cell response through proliferation and survival. We further demonstrated ex vivo glycolysis inhibition that specifically targets alloreactive T cells

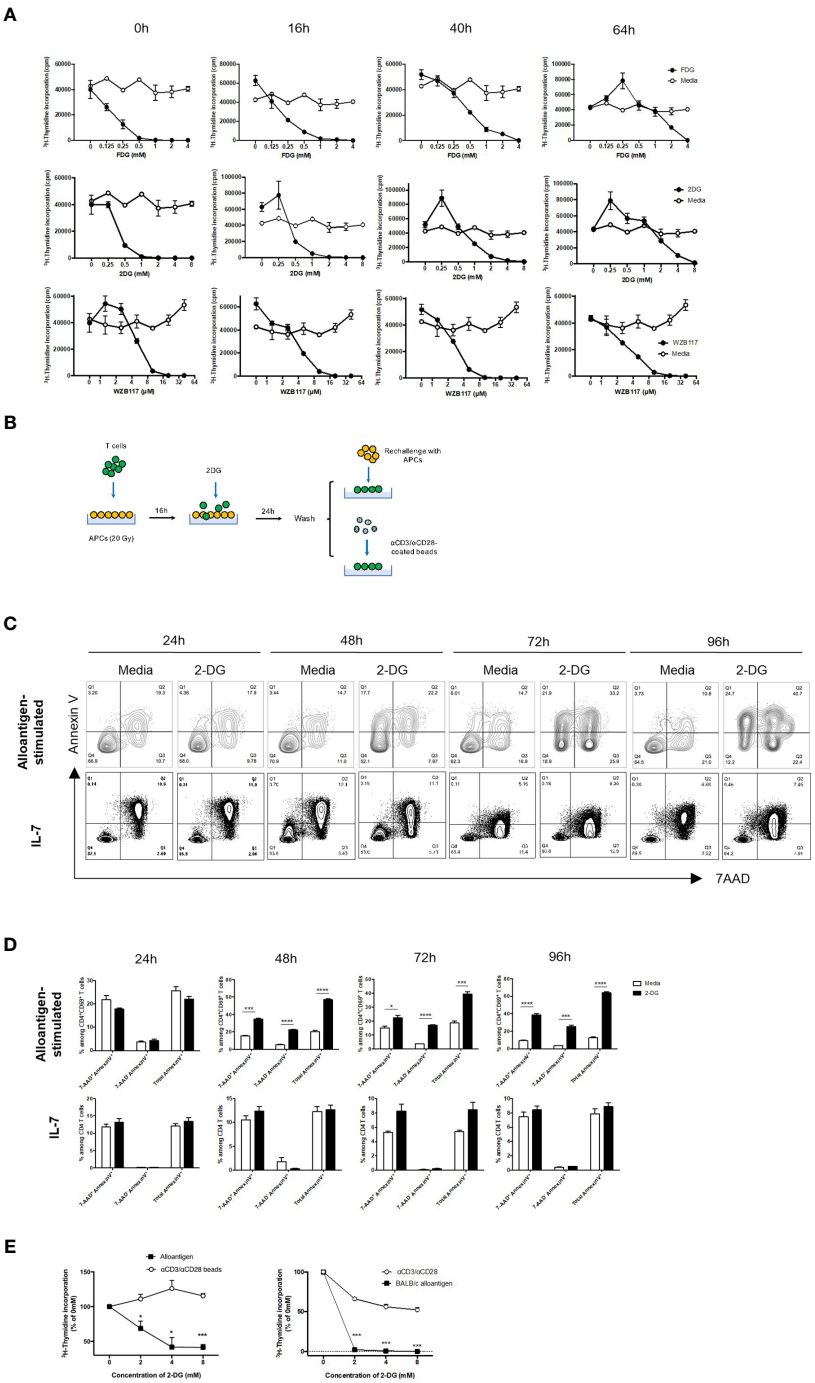

FIGURE 5 | 2-DG treatment selectively suppresses alloreactive T cells. WT or Glut1$^{T\text{-}KO}$ T cells were stimulated for 0 hour, 16 hours, 40 hours, or 64 hours using irradiated (20 Gy) BALB/c splenocytes, followed by the addition of various concentrations of small molecule inhibitors and cultured for a total of 112 hours for the assessment of thymidine incorporation. T cell response was determined following the addition of FDG or media only (H_2O), 2-DG or media only (H_2O), WZB117 or media only (EtOH) **(A)**. Schematic diagram of T cells stimulated with irradiated MHC-mismatched APCs for 16 hours, followed by the addition of various concentrations of 2-DG, washed, then rechallenged with alloantigens or anti-CD3 and anti-CD28 antibodies **(B)**. WT T cells were first stimulated with irradiated (20 Gy) BALB/c DCs for 16 hours, followed by incubation with freshly isolated irradiated DCs in the presence of media control or 8mM 2-DG for 24-96 hours; WT T cells were cultured in IL-7 (10ng/ml) for 16 hours plus 24-96 hours, and analyzed for Annexin V and 7AAD **(C, D)**. T cells were gated on CD4$^+$ CD69$^+$ for alloantigen-stimulated samples and CD4$^+$ for IL-7-treated samples. **(E)** The proliferative response of mouse (left panel) and human T cells (right panel) cultured according to **(B)** was measured by ^{3}H-TdR incorporation assay. *P < 0.05, ***P < 0.001, ****P < 0.0001, 2-tailed Student t test; data are representative of two experiments (n = 3 per group).

to prevent acute GVHD while sparing GVL effect as a preventive approach.

Several studies reported that elevated glucose metabolism is strongly associated with donor T cell-mediated GVHD (47, 48). Positron emission tomography studies revealed that glucose analog uptake is correlated with donor cell infiltration and GVHD symptoms (48). A retrospective single-cell RNA sequencing study identified upregulated genes encoding glycolytic enzymes in GVHD patients (47). Despite the phenotypic analyses and the existing paradigm for the dependence of activated T cells on glycolysis, other groups reported that alloreactive T cells *in vivo* are less glycolytic and are primarily dependent on oxidative metabolism, namely FAO (7, 17, 18). However, the absence of conditioning-associated tissue damage in these models contributes to limited release of inflammatory cytokines during the priming phase of T cells, subsequently altering the regulation of metabolic reprogramming and inflammatory response. Moreover, the evaluation of key glycolysis parameters and contribution of homeostatic expansion were not addressed. Contrary to this report, Nguyen et al. used a comprehensive metabolite analysis to demonstrate that alloreactive T cells derived from an irradiated model highly upregulate glycolysis compared to syngeneic recipients. The administration of glycolysis inhibitors in recipients alleviated disease progression. However, the systemic treatment led to off-target effects that compromised efficacy that could be attributed to organ toxicity (20). Functional studies that specifically target glycolysis in T cells are therefore necessary to address the role of glycolysis in GVHD pathogenesis. To this end, we utilized donor T cells genetically deficient for Glut1, a major glucose transporter in activated T cells. We showed that in contrast to WT T cells, Glut1$^{T\text{-}KO}$ alloreactive T cells showed significantly impaired capacity to adopt aerobic glycolysis (**Figures 2A, B** and **S2A–S2C**). Transplant experiments using Glut1$^{T\text{-}KO}$ T cells remarkably prevented acute GVHD-associated clinical traits and improved survival (**Figure 1**). Glycolysis is required for both CD4$^+$ and CD8$^+$ T cell-mediated pathogenesis as transfer of Glut1$^{T\text{-}KO}$ CD4$^+$ or CD8$^+$ T cells showed significant increase in body weight, reduced clinical score and target organ damage compared to controls (**Figure 1**). However, disease kinetics and target organ damage differed due to difference in natural disease progression associated with the transfer of CD4$^+$ versus CD8$^+$ T cells, with rapid improvement of disease progression and alleviated gastrointestinal tract damage in CD4$^+$ T cell recipients compared to delayed disease resolution and reduced skin damage in CD8$^+$ T cell recipients. It was observed that the Glut1$^{T\text{-}KO}$ T cell recipients in the C57BL/6 → BALB/c setting (**Figures 1D–I**, **4**) had not completely recovered their body weights when they were sacrificed after day +100. Even though we did not observe obvious signs of chronic GVHD at necropsy (data not shown) and histological analysis (**Figure S1, S6**), these observations do raise a question whether this strategy has any impact on chronic GVHD. More comprehensive analyses using chronic GVHD models will be needed to answer this question. Taken together, the above experiments demonstrated dramatically improved long-term survival compared to recipients of donor cells with intact glycolysis, providing solid evidence that glycolysis is selectively required for acute GVHD development.

mTORC1 activation has been shown to support cell growth, proliferation, and T_{eff} functions by promoting translation and anabolic metabolism (19, 49–51). Nguyen et al. showed that mTORC1-deficient T cells have reduced ability to induce GVHD, accompanied by a less glycolytic phenotype (20). However, it remains unclear whether the utilization of glycolysis directly promotes mTORC1 signaling in alloreactive T cells. As a sensor for metabolic cues, mTORC1 activity can be modulated by glucose availability (8). Consistently, the current study (**Figure 2C**) demonstrated impaired mTORC1 activation as a direct consequence of impaired glycolysis. Additionally, mTORC1 activity is negatively regulated by AMPK, an energy stress sensor that promotes catabolic pathways including FAO and OXPHOS (52).

Glycolysis has been implicated to support survival as well as antigen-specific expansion (15). We first assessed the impact on T cell expansion. *In vitro* experiments indicated a profound defect in clonal expansion of alloreactive T cells upon antigen stimulation (**Figure 2D**). This observation was recapitulated *in vivo* by the numbers of donor T cells recovered (**Figure 2E**) and percentages of divided donor cells using proliferation dye (**Figure 2F**). The difference in response was exclusively seen in allogeneic recipients but not syngeneic controls (**Figure 2E**), indicating that alloreactive T cells are dependent on glycolysis for proliferation. Modulation of inflammatory cytokine secretion is also a critical determinant of T cell pathogenicity. Previous studies demonstrated a role for glycolytic enzymes in the translational regulation of inflammatory cytokines by engaging/disengaging glycolysis upon TCR crosslinking (10, 11). Glycolysis inhibition has also been linked to diminished cytokine production in previous studies, where 2-DG was systemically delivered to BMT recipients (20). However, glycolysis is also required for DC maturation and migration (21–23). Proliferative T cell response can be severely impaired when activated by DCs previously treated with 2-DG (23). In the currently study, we directly demonstrated defects in both proliferation and inflammatory cytokine production in Glut1$^{T\text{-}KO}$ alloreactive T cells without simultaneously targeting non-T cells.

Glut1$^{T\text{-}KO}$ T cells previously demonstrated reduced viability compared to WT control after stimulation with plate-bound antibodies (15). To determine whether apoptosis is involved in increased cell death of Glut1$^{T\text{-}KO}$ T cells, we assessed the expression of proteins involved in the regulation of apoptotic pathway, including Mdm2, Puma, Noxa, and Mcl-1. Proteins linked to the induction of apoptotic pathway were significantly upregulated as opposed to downregulation of the anti-apoptotic Mcl-1 in activated Glut1$^{T\text{-}KO}$ T cells compared to WT control (**Figure 3A**). Specifically, Puma, which is sensitive to rapid upregulation in response to glucose deprivation to promote apoptosis (53), was drastically induced in Glut1$^{T\text{-}KO}$ T cells, suggesting that glycolysis plays a critical role in regulating apoptosis in activated T cells. We also assessed Mdm2 expression due to its role in regulating cellular stress response

and apoptosis. We demonstrated that Mdm2 is significantly increased expression in Glut1$^{T\text{-}KO}$ T cells (**Figure 3A**). We further evaluated whether alloreactive T cells are susceptible to apoptosis due to nutrient availability and cellular stress in the context of glycolysis. Annexin V and 7AAD staining confirmed that activated alloreactive Glut1$^{T\text{-}KO}$ T cells are prone to undergo apoptosis (**Figure 3B**). BH3-only Bcl-1 family members, including Bim, has been implicated in lymphocyte cell death during prolonged glucose deprivation (53). We showed that alloreactive Glut1$^{T\text{-}KO}$ T cells upregulate Bim expression (**Figure 3C**), an indicator for ER stress and disruption of glucose metabolism (36, 53, 54). Mcl-1, a prosurvival factor, is also linked to glycolysis and metabolic stress (35, 38, 55). Regulated post-translationally, Mcl-1 is rapidly stabilized following TCR crosslinking (56, 57) and couples with Noxa to modulate the apoptosis threshold (38). With a short half-life of 30 min, Mcl-1 has a rapid turnover rate and is highly sensitive to changes in global translation downstream of mTORC1 (58). Indeed, Glut1$^{T\text{-}KO}$ T cells are incapable of sustaining mTORC1 activation (**Figure 2C**) and Mcl-1 expression (**Figure 3D**) during alloantigen challenge. It is possible that Mcl-1 expression is regulated by mTORC1 in response to glucose utilization to regulate T cell survival. Overall, the above findings demonstrate increased apoptosis induction in activated Glut1$^{T\text{-}KO}$ T cells compared to WT control. Interestingly, a previous study showed that viability was only slightly reduced in T cells following stimulation in the presence of 2-DG (59). While these findings appear to be contradictory to the current study, this is potentially attributed to the difference in 2-DG concentration as a higher concentration was used in the current study. Importantly, the timing of 2-DG addition is different. Whereas 2-DG was added at the beginning of stimulation in the previous study, the current assays involved 2-DG addition 16 hours following stimulation, which preferentially affects already activated T cells that are highly sensitive to glycolysis usage.

Since allo-HSCT is the primary curative option for malignant leukemia and lymphomas, it is critical to assess the impact of glycolysis inhibition on GVL activity. Our results demonstrated for the first time that Glut1$^{T\text{-}KO}$ T cells provide superior protection in recipients against tumor growth compared to TCDBM + tumor recipients (**Figures 4C, H**). Although GVHD was not completely eliminated with transfer of Glut1$^{T\text{-}KO}$ T cells, both survival and body weight are significantly improved in comparison to WT T cell recipients (**Figures 4A, B, F, G**). The reduced but detectable GVHD development (**Figures 4B** and **S6**) in Glut1$^{T\text{-}KO}$ T cell recipients may be contributed by metabolic processes other than glycolysis, including glutaminolysis and pentose phosphate pathway (20), hence simultaneous targeting of the above pathways is likely to further improve the abrogation of GVHD. However, the data support that Glut1$^{T\text{-}KO}$ T cell retain the capacity to eliminate tumor development (**Figures 4C, H**). Previous studies suggest that expression of cytotoxic granules, such as granzyme B and perforin in CD8$^+$ T cells, are not regulated by aerobic glycolysis (11), potentially mediating GVL effect in Glut1$^{T\text{-}KO}$ T cells. Despite the earlier onset of apoptosis in glycolysis-inhibited alloreactive CD8$^+$ T cells compared to CD4$^+$ T cells (**Figure S9B**), ex vivo stimulated T cells were capable of controlling tumor development and improving survival outcome compared to TCDBM + tumor and untreated donor T cell recipients (**Figure 6**).

Furthermore, Glut1$^{T\text{-}KO}$ CD8$^+$ T cells, which exhibited higher TNFα expression compared to control T cells (**Figure 2H**), suggesting a potential contribution to tumor killing mediated by Glut1$^{T\text{-}KO}$ CD8$^+$ T cells. Glut1$^{T\text{-}KO}$ T cells may also be able to facilitate GVL without meeting the threshold for GVHD induction, as the T cell dose to induce GVHD appears to be 10-fold higher than GVL in clinical studies (60–63). IFN-γ production (**Figure 2G**) and OXPHOS-dependent memory T cells can also contribute to GVL activity (64–66). Collectively, we showed that T cells with impaired glycolysis retained the capacity to prevent tumor development in allogeneic recipients. Interestingly, studies by Uhl demonstrated that leukemia-derived lactic acid interferes with both glycolysis and OXPHOS in T cells, leading to reduced protection against tumor, which appears to be contradictory to the current finding (67). However, Glut1$^{T\text{-}KO}$ T cells demonstrate comparable basal OCR level to WT control (**Figure S2D**), suggesting that the preservation of OXPHOS may be critical for GVL preservation in Glut1$^{T\text{-}KO}$ T cell recipients. In regard to the metabolic flexibility of T cells, previous studies showed that glucose deprivation in activated T cells can be partially compensated by increased respiration (59). In addition, a recent study demonstrated that CD8$^+$ T cells can utilize inosine as an alternative carbon source when glucose utilization is restricted to mediate tumor-killing in xenograft models (68). Hence it is possible that the metabolic plasticity of CD8$^+$ T cells contributes to GVL preservation when glycolysis is impaired.

To evaluate the therapeutic potential in a clinically-relevant setting, we sought to assess selective inhibition of alloreactive T cells to remedy off-target effects on other cell types that utilize glycolysis. In line with this approach, we and others previously examined ex vivo treatments to eliminate T cells activated by recipient antigens (69–71). In the currently study, 2-DG strongly induced apoptosis in activated alloreactive T cells (**Figure 5C**). We further validated this approach using both murine and human models, where alloresponse was subdued upon alloantigen rechallenge while response against nonspecific stimulation remained intact (**Figures 5D, E**). The ex vivo assay using 2-DG also demonstrated the translational value of targeted glycolysis inhibition prior to transplantation (**Figures 6A–D**). As expected, recipients of control T cells treated with media only demonstrated reduced survival with increased incubation time. In contrast, optimal 2-DG inhibition for 48 hours yielded significantly improved survival without losing GVL effect compared to recipients of untreated T cells or those treated with media control. Protection against both tumor and GVHD development through 2-DG inhibition for 48 hours was further evaluated using a second tumor model with the A20 cell line, highlighting the therapeutic potential of ex vivo glycolysis inhibition (**Figures 6E–H**). Overall, we observed that the desired efficacy for acute GVHD suppression and GVL can be achieved through selective inhibition of alloreactive T cells ex vivo (**Figure 6**).

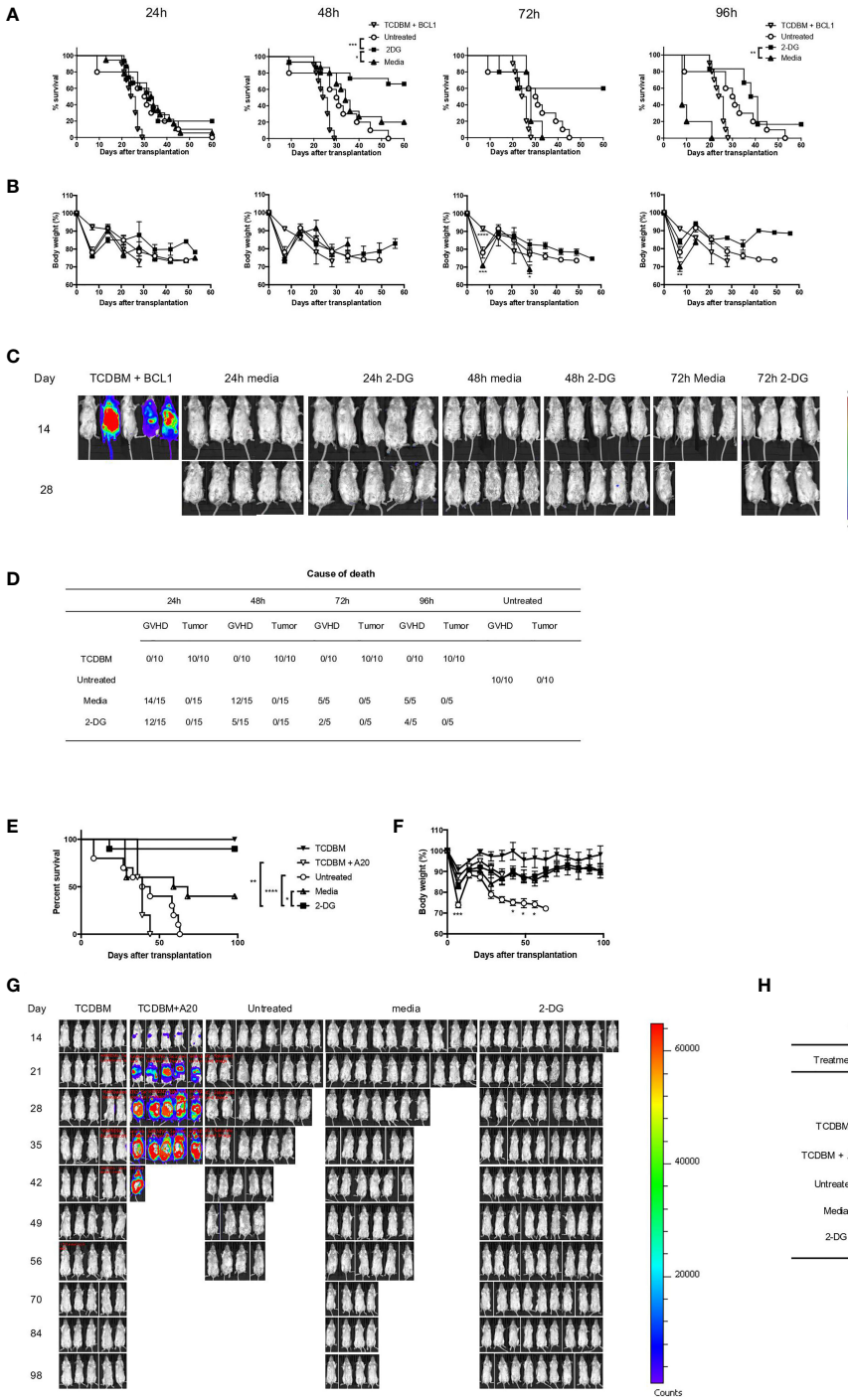

FIGURE 6 | 2-DG treatment ameliorates GVHD and preserves GVL effect. 1 x 10^6 T cells from C57BL/6 donor spleens were first stimulated for 16 hours with irradiated BALB/c splenocytes, followed by addition of 8mM 2-DG or media control for indicated periods. T cells treated ex vivo or untreated control T cells at the same dose were transplanted into BALB/c recipients, along with 1 x 10^7 TCDBM from C57BL/6 donors and 5 x 10^5 BCL1 cells. Recipients were monitored for survival **(A)**, body weight **(B)**, and tumor growth in TCDBM and TCDBM + BCL1, 24-hour, 48-hour and 72-hour media control or 2-DG-treated groups. Tumor development was detected using BLI imaging on day 14 and day 28 following transplantation **(C)**. Cause of death due to GVHD or tumor development was summarized for different groups **(D)**. 1 x 10^6 T cells treated as shown in **(A)** for 48 hours or untreated control T cells at the same dose were transplanted into BALB/c recipients, along with 1 x 10^7 TCDBM from C57BL/6 donors and 1 x 10^5 A20 cells. Recipients were monitored for survival **(E)**, body weight **(F)**, and tumor growth **(G)**. Cause of death was summarized for various groups **(H)**. *P < 0.05, **P < 0.01, ***P < 0.001, log-rank test; data are representative of two experiments (n = 15 per group, recipients for 24-hour and 48-hour ex vivo cultured T cells; n = 5, recipients for 72-hour and 96-hour ex vivo cultured T cells).

In summary, by using T cells genetically incapable of utilizing glycolysis, we demonstrated that glycolysis is definitively required for alloreactive T cells to induce acute GVHD. We further established a role for glycolysis in promoting donor T cell pathogenicity through regulating proliferation, cell death, and proinflammatory cytokine production. One potential limitation of 2-DG glycolysis inhibition in the clinical setting is its application as a preventative procedure but not as a curative treatment due to toxicity if administered systemically. However, reagents better tolerated for allogeneic BMT with low toxicity can be used as a curative treatment, including 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, which has been assessed in a murine GVHD model (20). Overall, the current study demonstrated that we can target glycolysis in alloreactive T cells to prevent acute GVHD without losing the GVL activity.

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 author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the Duke University Medical Center. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YH, YZ, and BJC designed the research studies, analyzed the data. YH, YZ, YJ, PS, XN, WH, CX, and AN participated in conducting experiments and acquiring data. YZ and BJC participated in writing the manuscript. YH, YZ, BJC, ANM, NJM, DMC, TVB, ZL, NJC, MC, CH participated in editing the manuscript. YZ, BJC, and NJC participated in revising the manuscript. DMC performed pathological analysis. FL and CL generated A20 cells expressing Luc-EGFP gene. 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.2022.751296/full#supplementary-material

REFERENCES

1. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-Versus-Host Disease. *Lancet (Lond Engl)* (2009) 373:1550–61. doi: 10.1016/S0140-6736(09)60237-3
2. Jagasia M, Arora M, Flowers MED, Chao NJ, McCarthy PL, Cutler CS, et al. Risk Factors for Acute GVHD and Survival After Hematopoietic Cell Transplantation. *Blood* (2012) 119:296–307. doi: 10.1182/blood-2011-06-364265
3. Zeiser R, Blazar BR. Acute Graft-Versus-Host Disease - Biologic Process, Prevention, and Therapy. *N Engl J Med* (2017) 377:2167–79. doi: 10.1056/NEJMra1609337
4. Bacigalupo A, Van Lint MT, Occhini D, Gualandi F, Lamparelli T, Sogno G, et al. Increased Risk of Leukemia Relapse With High-Dose Cyclosporine A After Allogeneic Marrow Transplantation for Acute Leukemia. *Blood* (1991) 77:1423–8. doi: 10.1182/blood.V77.7.1423.1423
5. Locatelli F, Zecca M, Rondelli R, Bonetti F, Dini G, Prete A, et al. Graft Versus Host Disease Prophylaxis With Low-Dose Cyclosporine-A Reduces the Risk of Relapse in Children With Acute Leukemia Given HLA-Identical Sibling Bone Marrow Transplantation: Results of a Randomized Trial. *Blood* (2000) 95:1572–9. doi: 10.1182/blood.V95.5.1572.005k12_1572_1579
6. Weaver CH, Clift RA, Deeg HJ, Storb R, Appelbaum FR, Bensinger W, et al. Effect of Graft-Versus-Host Disease Prophylaxis on Relapse in Patients Transplanted for Acute Myeloid Leukemia. *Bone Marrow Transplant* (1994) 14:885–93.
7. Gatza E, Wahl DR, Opipari AW, Sundberg TB, Reddy P, Liu C, et al. Manipulating the Bioenergetics of Alloreactive T Cells Causes Their Selective Apoptosis and Arrests Graft-Versus-Host Disease. *Sci Trans Med* (2011) 3:67ra8. doi: 10.1126/scitranslmed.3001975
8. Chapman NM, Boothby MR, Chi H. Metabolic Coordination of T Cell Quiescence and Activation. *Nat Rev Immunol* (2020) 20:55–70. doi: 10.1038/s41577-019-0203-y
9. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling Immunity: Insights Into Metabolism and Lymphocyte Function. *Sci (New York NY)* (2013) 342:1242454. doi: 10.1126/science.1242454
10. Chang C-H, Curtis JD, Maggi LB Jr, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. *Cell* (2013) 153:1239–51. doi: 10.1016/j.cell.2013.05.016
11. Menk AV, Scharping NE, Moreci RS, Zeng X, Guy C, Salvatore S, et al. Early TCR Signaling Induces Rapid Aerobic Glycolysis Enabling Distinct Acute T Cell Effector Functions. *Cell Rep* (2018) 22:1509–21. doi: 10.1016/j.celrep.2018.01.040
12. Araujo L, Khim P, Mkhikian H, Mortales CL, Demetriou M. Glycolysis and Glutaminolysis Cooperatively Control T Cell Function by Limiting Metabolite Supply to N-Glycosylation. *eLife* (2017) 6. doi: 10.7554/eLife.21330
13. Li W, Qu G, Choi SC, Cornaby C, Titov A, Kanda N, et al. Targeting T Cell Activation and Lupus Autoimmune Phenotypes by Inhibiting Glucose Transporters. *Front Immunol* (2019) 10:833. doi: 10.3389/fimmu.2019.00833
14. Liu RT, Zhang M, Yang CL, Zhang P, Zhang N, Du T, et al. Enhanced Glycolysis Contributes to the Pathogenesis of Experimental Autoimmune Neuritis. *J Neuroinflamm* (2018) 15:51. doi: 10.1186/s12974-018-1095-7
15. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, et al. The Glucose Transporter Glut1 Is Selectively Essential for CD4 T Cell Activation and Effector Function. *Cell Metab* (2014) 20:61–72. doi: 10.1016/j.cmet.2014.05.004
16. Stimpson SE, Chen J, Newby BN, Khattri RB, Chapman H, Angelini T, et al. Human CD8+ T-Cells Require Glycolysis to Elicit Effector Function. *bioRxiv* (2020). doi: 10.1101/2020.02.05.935627

17. Byersdorfer CA, Tkachev V, Opipari AW, Goodell S, Swanson J, Sandquist S, et al. Effector T Cells Require Fatty Acid Metabolism During Murine Graft-Versus-Host Disease. *Blood* (2013) 122:3230–7. doi: 10.1182/blood-2013-04-495515
18. Glick GD, Rossignol R, Lyssiotis CA, Wahl D, Lesch C, Sanchez B, et al. Anaplerotic Metabolism of Alloreactive T Cells Provides a Metabolic Approach to Treat Graft-Versus-Host Disease. *J Pharmacol Exp Ther* (2014) 351:298–307. doi: 10.1124/jpet.114.218099
19. Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC. Glucose Metabolism in Lymphocytes is a Regulated Process With Significant Effects on Immune Cell Function and Survival. *J leukocyte Biol* (2008) 84:949–57. doi: 10.1189/jlb.0108024
20. Nguyen HD, Chatterjee S, Haarberg KMK, Wu Y, Bastian D, Heinrichs J, et al. Metabolic Reprogramming of Alloantigen-Activated T Cells After Hematopoietic Cell Transplantation. *J Clin Invest* (2016) 126:1337–52. doi: 10.1172/JCI82587
21. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-Driven Early Glycolytic Reprogramming *via* the Kinases TBK1-IKKε Supports the Anabolic Demands of Dendritic Cell Activation. *Nat Immunol* (2014) 15:323–32. doi: 10.1038/ni.2833
22. Everts B, Amiel E, van der Windt GJ, Freitas TC, Chott R, Yarasheski KE, et al. Commitment to Glycolysis Sustains Survival of NO-Producing Inflammatory Dendritic Cells. *Blood* (2012) 120:1422–31. doi: 10.1182/blood-2012-03-419747
23. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-Like Receptor–Induced Changes in Glycolytic Metabolism Regulate Dendritic Cell Activation. *Blood* (2010) 115:4742–9. doi: 10.1182/blood-2009-10-249540
24. Fujiwara H, Maeda Y, Kobayashi K, Nishimori H, Matsuoka K, Fujii N, et al. Programmed Death-1 Pathway in Host Tissues Ameliorates Th17/Th1-Mediated Experimental Chronic Graft-Versus-Host Disease. *J Immunol (Baltimore Md: 1950)* (2014) 193:2565–73. doi: 10.4049/jimmunol.1400954
25. Saha A, Aoyama K, Taylor PA, Koehn BH, Veenstra RG, Panoskaltsis-Mortari A, et al. Host Programmed Death Ligand 1 is Dominant Over Programmed Death Ligand 2 Expression in Regulating Graft-Versus-Host Disease Lethality. *Blood* (2013) 122:3062–73. doi: 10.1182/blood-2013-05-500801
26. Jacobs SR, Herman CE, Maciver NJ, Wofford JA, Wieman HL, Hammen JJ, et al. Glucose Uptake is Limiting in T Cell Activation and Requires CD28-Mediated Akt-Dependent and Independent Pathways. *J Immunol (Baltimore Md: 1950)* (2008) 180:4476–86. doi: 10.4049/jimmunol.180.7.4476
27. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. *J Immunol (Baltimore Md: 1950)* (2011) 186:3299–303. doi: 10.4049/jimmunol.1003613
28. Brennan TV, Hoang V, Garrod KR, Liu FC, Hayden T, Kim J, et al. A New T-Cell Receptor Transgenic Model of the CD4+ Direct Pathway: Level of Priming Determines Acute Versus Chronic Rejection. *Transplantation* (2008) 85:247–55. doi: 10.1097/TP.0b013e31815e883e
29. Brennan TV, Jaigirdar A, Hoang V, Hayden T, Liu FC, Zaid H, et al. Preferential Priming of Alloreactive T Cells With Indirect Reactivity. *Am J Transplant* (2009) 9:709–18. doi: 10.1111/j.1600-6143.2009.02578.x
30. Young CD, Lewis AS, Rudolph MC, Ruehle MD, Jackman MR, Yun UJ, et al. Modulation of Glucose Transporter 1 (GLUT1) Expression Levels Alters Mouse Mammary Tumor Cell Growth *In Vitro* and *In Vivo*. *PloS One* (2011) 6:e23205. doi: 10.1371/journal.pone.0023205
31. Chen BJ, Deoliveira D, Cui X, Le NT, Son J, Whitesides JF, et al. Inability of Memory T Cells to Induce Graft-Versus-Host Disease Is a Result of an Abortive Alloresponse. *Blood* (2007) 109:3115–23. doi: 10.1182/blood-2006-04-016410
32. Caro-Maldonado A, Wang R, Nichols AG, Kuraoka M, Milasta S, Sun LD, et al. Metabolic Reprogramming is Required for Antibody Production That Is Suppressed in Anergic But Exaggerated in Chronically BAFF-Exposed B Cells. *J Immunol (Baltimore Md: 1950)* (2014) 192:3626–36. doi: 10.4049/jimmunol.1302062
33. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The Transcription Factor Myc Controls Metabolic Reprogramming Upon T Lymphocyte Activation. *Immunity* (2011) 35:871–82. doi: 10.1016/j.immuni.2011.09.021
34. Chi H. Regulation and Function of mTOR Signalling in T Cell Fate Decisions. *Nat Rev Immunol* (2012) 12:325–38. doi: 10.1038/nri3198
35. Zhao Y, Altman BJ, Coloff JL, Herman CE, Jacobs SR, Wieman HL, et al. Glycogen Synthase Kinase 3alpha and 3beta Mediate a Glucose-Sensitive Antiapoptotic Signaling Pathway to Stabilize Mcl-1. *Mol Cell Biol* (2007) 27:4328–39. doi: 10.1128/MCB.00153-07
36. Altman BJ, Rathmell JC. Metabolic Stress in Autophagy and Cell Death Pathways. *Cold Spring Harbor Perspect Biol* (2012) 4:a008763. doi: 10.1101/cshperspect.a008763
37. Wensveen FM, van Gisbergen KP, Derks IA, Gerlach C, Schumacher TN, van Lier RA, et al. Apoptosis Threshold Set by Noxa and Mcl-1 After T Cell Activation Regulates Competitive Selection of High-Affinity Clones. *Immunity* (2010) 32:754–65. doi: 10.1016/j.immuni.2010.06.005
38. Wensveen FM, Alves NL, Derks IA, Reedquist KA, Eldering E. Apoptosis Induced by Overall Metabolic Stress Converges on the Bcl-2 Family Proteins Noxa and Mcl-1. *Apoptosis: Int J Programmed Cell Death* (2011) 16:708–21. doi: 10.1007/s10495-011-0599-8
39. Granchi C, Minutolo F. Anticancer Agents That Counteract Tumor Glycolysis. *ChemMedChem* (2012) 7:1318–50. doi: 10.1002/cmdc.201200176
40. Lampidis TJ, Kurtoglu M, Maher JC, Liu H, Krishan A, Sheft V, et al. Efficacy of 2-Halogen Substituted D-Glucose Analogs in Blocking Glycolysis and Killing "Hypoxic Tumor Cells". *Cancer Chemother Pharmacol* (2006) 58:725–34. doi: 10.1007/s00280-006-0207-8
41. Mohanti BK, Rath GK, Anantha N, Kannan V, Das BS, Chandramouli BA, et al. Improving Cancer Radiotherapy With 2-Deoxy-D-Glucose: Phase I/II Clinical Trials on Human Cerebral Gliomas. *Int J Radiat Oncol Biol Phys* (1996) 35:103–11. doi: 10.1016/S0360-3016(96)85017-6
42. Choi S-C, Titov AA, Abboud G, Seay HR, Brusko TM, Roopenian DC, et al. Inhibition of Glucose Metabolism Selectively Targets Autoreactive Follicular Helper T Cells. *Nat Commun* (2018) 9:4369. doi: 10.1038/s41467-018-06686-0
43. Raez LE, Papadopoulos K, Ricart AD, Chiorean EG, Dipaola RS, Stein MN, et al. A Phase I Dose-Escalation Trial of 2-Deoxy-D-Glucose Alone or Combined With Docetaxel in Patients With Advanced Solid Tumors. *Cancer Chemother Pharmacol* (2013) 71:523–30. doi: 10.1007/s00280-012-2045-1
44. Stein M, Lin H, Jeyamohan C, Dvorzhinski D, Gounder M, Bray K, et al. Targeting Tumor Metabolism With 2-Deoxyglucose in Patients With Castrate-Resistant Prostate Cancer and Advanced Malignancies. *Prostate* (2010) 70:1388–94. doi: 10.1002/pros.21172
45. Yin Y, Choi SC, Xu Z, Perry DJ, Seay H, Croker BP, et al. Normalization of CD4+ T Cell Metabolism Reverses Lupus. *Sci Trans Med* (2015) 7:274ra18. doi: 10.1126/scitranslmed.aaa0835
46. Buck MD, O'Sullivan D, Pearce EL. T Cell Metabolism Drives Immunity. *J Exp Med* (2015) 212:1345–60. doi: 10.1084/jem.20151159
47. Assmann JC, Farthing DE, Saito K, Maglakelidze N, Oliver B, Warrick KA, et al. Glycolytic Metabolism of Pathogenic T Cells Enables Early Detection of GVHD by 13C-MRI. *Blood* (2021) 137:126–37. doi: 10.1182/blood.2020005770
48. Stelljes M, Hermann S, Albring J, Köhler G, Löffler M, Franzius C, et al. Clinical Molecular Imaging in Intestinal Graft-Versus-Host Disease: Mapping of Disease Activity, Prediction, and Monitoring of Treatment Efficiency by Positron Emission Tomography. *Blood* (2008) 111:2909–18. doi: 10.1182/blood-2007-10-119164
49. Düvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, et al. Activation of a Metabolic Gene Regulatory Network Downstream of mTOR Complex 1. *Mol Cell* (2010) 39:171–83. doi: 10.1016/j.molcel.2010.06.022
50. Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, et al. SREBP Activity Is Regulated by Mtorc1 and Contributes to Akt-Dependent Cell Growth. *Cell Metab* (2008) 8:224–36. doi: 10.1016/j.cmet.2008.07.007
51. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of Immune Responses by mTOR. *Annu Rev Immunol* (2012) 30:39–68. doi: 10.1146/annurev-immunol-020711-075024
52. Herzig S, Shaw RJ. AMPK: Guardian of Metabolism and Mitochondrial Homeostasis. *Nat Rev Mol Cell Biol* (2018) 19:121–35. doi: 10.1038/nrm.2017.95

53. Coloff JL, Mason EF, Altman BJ, Gerriets VA, Liu T, Nichols AN, et al. Akt Requires Glucose Metabolism to Suppress Puma Expression and Prevent Apoptosis of Leukemic T Cells. *J Biol Chem* (2011) 286:5921–33. doi: 10.1074/jbc.M110.179101
54. Zhao Y, Coloff JL, Ferguson EC, Jacobs SR, Cui K, Rathmell JC. Glucose Metabolism Attenuates P53 and Puma-Dependent Cell Death Upon Growth Factor Deprivation. *J Biol Chem* (2008) 283:36344–53. doi: 10.1074/jbc.M803580200
55. Pradelli LA, Bénéteau M, Chauvin C, Jacquin MA, Marchetti S, Muñoz-Pinedo C, et al. Glycolysis Inhibition Sensitizes Tumor Cells to Death Receptors-Induced Apoptosis by AMP Kinase Activation Leading to Mcl-1 Block in Translation. *Oncogene* (2010) 29:1641–52. doi: 10.1038/onc.2009.448
56. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen Synthase Kinase-3 Regulates Mitochondrial Outer Membrane Permeabilization and Apoptosis by Destabilization of MCL-1. *Mol Cell* (2006) 21:749–60. doi: 10.1016/j.molcel.2006.02.009
57. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-Only E3 Ubiquitin Ligase, Catalyzes the Polyubiquitination of Mcl-1 and Regulates Apoptosis. *Cell* (2005) 121:1085–95. doi: 10.1016/j.cell.2005.06.009
58. Adams KW, Cooper GM. Rapid Turnover of Mcl-1 Couples Translation to Cell Survival and Apoptosis. *J Biol Chem* (2007) 282:6192–200. doi: 10.1074/jbc.M610643200
59. Renner K, Geiselhöringer AL, Fante M, Bruss C, Färber S, Schönhammer G, et al. Metabolic Plasticity of Human T Cells: Preserved Cytokine Production Under Glucose Deprivation or Mitochondrial Restriction, But 2-Deoxy-Glucose Affects Effector Functions. *Eur J Immunol* (2015) 45:2504–16. doi: 10.1002/eji.201545473
60. Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, Falzetti F, et al. Treatment of High-Risk Acute Leukemia With T-Cell-Depleted Stem Cells From Related Donors With One Fully Mismatched HLA Haplotype. *New Engl J Med* (1998) 339:1186–93. doi: 10.1056/NEJM199810223391702
61. Urbano-Ispizua A, Rozman C, Pimentel P, Solano C, de la Rubia J, Brunet S, et al. The Number of Donor CD3(+) Cells Is the Most Important Factor for Graft Failure After Allogeneic Transplantation of CD34(+) Selected Cells From Peripheral Blood From HLA-Identical Siblings. *Blood* (2001) 97:383–7. doi: 10.1182/blood.V97.2.383
62. Urbano-Ispizua A, Rozman C, Pimentel P, Solano C, de la Rubia J, Brunet S, et al. Risk Factors for Acute Graft-Versus-Host Disease in Patients Undergoing Transplantation With CD34+ Selected Blood Cells From HLA-Identical Siblings. *Blood* (2002) 100:724–7. doi: 10.1182/blood-2001-11-0057
63. Zhang P, Chen BJ, Chao NJ. Prevention of GVHD Without Losing GVL Effect: Windows of Opportunity. *Immunol Res* (2011) 49:49–55. doi: 10.1007/s12026-010-8193-7
64. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang C-H, Sanin DE, et al. Mitochondrial Dynamics Controls T Cell Fate Through Metabolic Programming. *Cell* (2016) 166:63–76. doi: 10.1016/j.cell.2016.05.035
65. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, et al. Inhibiting Glycolytic Metabolism Enhances CD8+ T Cell Memory and Antitumor Function. *J Clin Invest* (2013) 123:4479–88. doi: 10.1172/JCI69589
66. van der Windt GJ, Pearce EL. Metabolic Switching and Fuel Choice During T-Cell Differentiation and Memory Development. *Immunol Rev* (2012) 249:27–42. doi: 10.1111/j.1600-065X.2012.01150.x
67. Uhl FM, Chen S, O'Sullivan D, Edwards-Hicks J, Richter G, Haring E, et al. Metabolic Reprogramming of Donor T Cells Enhances Graft-Versus-Leukemia Effects in Mice and Humans. *Sci Trans Med* (2020) 12(567): eabb8969. doi: 10.1126/scitranslmed.abb8969
68. Wang T, Gnanaprakasam JNR, Chen X, Kang S, Xu X, Sun H, et al. Inosine Is an Alternative Carbon Source for CD8(+)-T-Cell Function Under Glucose Restriction. *Nat Metab* (2020) 2:635–47. doi: 10.1038/s42255-020-0219-4
69. Cavazzana-Calvo M, Stephan JL, Sarnacki S, Chevret S, Fromont C, de Coene C, et al. Attenuation of Graft-Versus-Host Disease and Graft Rejection by Ex Vivo Immunotoxin Elimination of Alloreactive T Cells in an H-2 Haplotype Disparate Mouse Combination. *Blood* (1994) 83:288–98. doi: 10.1182/blood.V83.1.288.288
70. Chen BJ, Cui X, Liu C, Chao NJ. Prevention of Graft-Versus-Host Disease While Preserving Graft-Versus-Leukemia Effect After Selective Depletion of Host-Reactive T Cells by Photodynamic Cell Purging Process. *Blood* (2002) 99:3083–8. doi: 10.1182/blood.V99.9.3083
71. Guinan EC, Boussiotis VA, Neuberg D, Brennan LL, Hirano N, Nadler LM, et al. Transplantation of Anergic Histoincompatible Bone Marrow Allografts. *N Engl J Med* (1999) 340:1704–14. doi: 10.1056/NEJM199906033402202

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