# THE IMMUNOTHERAPEUTIC POTENTIAL OF ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

EDITED BY: Andrea Bacigalupo, Charles Craddock and Nicolaus Martin Kröger

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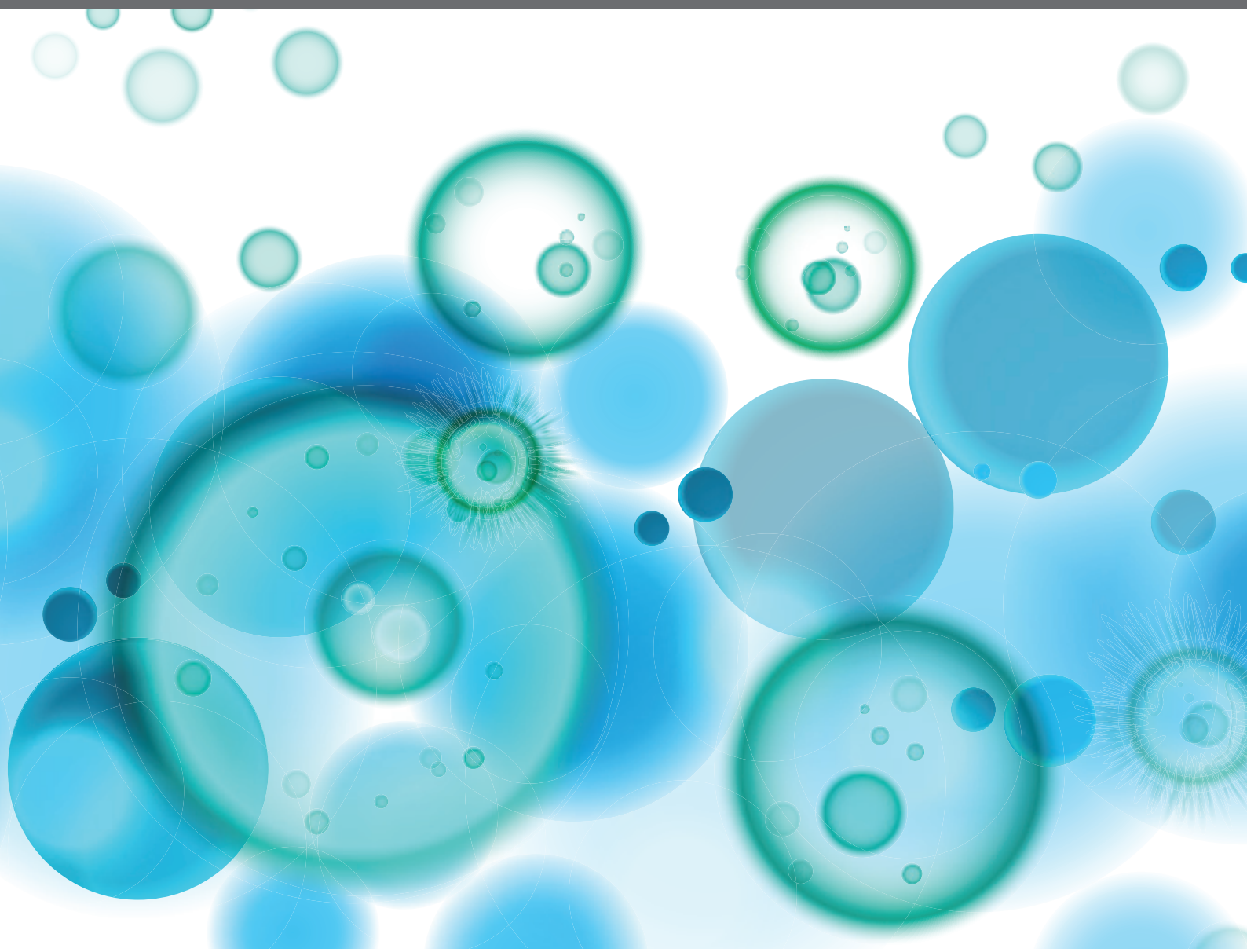

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# THE IMMUNOTHERAPEUTIC POTENTIAL OF ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

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REVIEW
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# Influence of KIR and NK Cell Reconstitution in the Outcomes of Hematopoietic Stem Cell Transplantation

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Natural killer (NK) cells play a significant role in immune tolerance and immune surveillance. Killer immunoglobin-like receptors (KIRs), which recognize human leukocyte antigen (HLA) class I molecules, are particularly important for NK cell functions. Previous studies have suggested that, in the setting of hematopoietic stem cell transplantation (HSCT), alloreactive NK cells from the donor could efficiently eliminate recipient tumor cells and the residual immune cells. Subsequently, several clinical models were established to determine the optimal donors who would exhibit a graft-vs. -leukemia (GVL) effect without developing graft-vs. -host disease (GVHD). In addition, hypotheses about specific beneficial receptor-ligand pairs and KIR genes have been raised and the favorable effects of alloreactive NK cells are being investigated. Moreover, with a deeper understanding of the process of NK cell reconstitution post-HSCT, new factors involved in this process and the defects of previous models have been observed. In this review, we summarize the most relevant literatures about the impact of NK cell alloreactivity on transplant outcomes and the factors affecting NK cell reconstitution.

**Keywords: KIR, NK cell reconstitution, hematopoietic stem cell transplantation, GVHD, infection, relapse**

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective therapy for patients with hematological malignancies. However, relapse, graft-vs. -host disease (GVHD), and infections remain the main causes of treatment failure (1–4). Potential strategies to prevent GVHD and even infections while sparing the graft-vs. -leukemia (GVL) effect have attracted extensive attention. Natural killer (NK) cells, which are a major type of innate lymphocytes, are being researched in this context.

NK cells constitute 5–15% of human peripheral blood lymphocytes (5, 6) and possess the abilities of cytotoxic lysis and rapid cytokine secretion without prior antigen presentation (7, 8). These functions are regulated by various types of receptors expressed on NK cells manifesting multiple functions either activating or inhibitory (9–11) (**Table 1**). Among the NK cell receptors, the killer immunoglobin-like receptor (KIR) is one of the major factors that mediate self-tolerance and anti-tumor/infection responses.

**TABLE 1 |** NK cell receptors and their ligands.

| Inhibitory receptors and their ligands | | Activating receptors and their ligands | | Coreceptors and their ligands | |
|---|---|---|---|---|---|
| KIR2DL1 | HLA-C2 | KIR2DS1 | HLA-C2 | 2B4 | CD48 |
| KIR2DL2 | HLA-C1 | KIR2DS2 | HLA-C1 | NTB-A | NTB-A |
| KIR2DL3 | HLA-C1 | KIR2DS3 | Unknown | CS1 | CS1 |
| KIR2DL4 | HLA-G | KIR2DS4 | HLA-A11 | NKp80 | AICL |
| KIR2DL5 | Unknown | KIR2DS5 | Unknown | TLR | TLRL |
| KIR3DL1 | HLA-Bw4 | KIR3DS1 | HLA-F | DNM-1 | PVR, Netcin-2 |
| KIR3DL2 | HLA-A3/A11 | NKG2C | HLA-E | CD96 | PVR |
| KIR3DL3 | Unknown | NKG2D | MICA, MICB, ULBP1-4 | | |
| NKG2A | HLA-E | NKp30 | B7-H6, BAT3, CMV pp65 | | |
| LIR-1 | HLA class I | NKp44 | Viral hemagglutinins | | |
| | | NKp46 | Viral hemagglutinins | | |
| | | CD16 | IgG-1, 3, 4 | | |

It is well established that KIR genes are located on chromosome 19q13.4 (12). Based on their various structures (the number of extracellular immunoglobulin domains (D) and the long (L) or short (S) tails), 16 KIR genes (including two pseudogenes (P), KIR2DP1 and KIR3DP1) have been classified into four groups (KIR2DL1-5, KIR3DL1-3, KIR2DS1-5, and KIR3DS1). Six genes with short tails are activating KIR genes that encode activating receptors, while the eight genes with long tails are inhibitory KIR genes encoding inhibitory receptors. KIRs could be divided into haplotype A and B according to the activating genes on them. Haplotype A has only one activating gene, KIR2DS4, whereas haplotype B possesses up to five activating KIR genes, including KIR2DS1, 2, 3, 5, and 3DS1 (**Figure 1**). Thus, the A/A genotype is defined as homozygous for A haplotypes, and the B/x genotype consists of at least one B haplotype. Finally, according to the specific KIR gene locus on the chromosome, a centromeric (Cen) and telomeric (Tel) KIR haplotype and genotype are further determined (13–15). Five inhibitory and three activating KIRs recognize specific class I HLA (A, B, or C) ligands, with the inhibitory KIR2DL1 recognizes group 2 HLA-C alleles, KIR2DL2 and KIR2DL3 recognize group 1 HLA-C alleles, KIR3DL1 recognizes HLA-Bw4 alleles, and KIR3DL2 recognizes HLA-A3/-A11 alleles. Moreover, activating KIR2DS1, KIR2DS2, and KIR2DS4 recognize HLA-C2, C1, A11, respectively (15). The ligands of the remaining KIRs remain unknown.

As KIR genes and human leukocyte antigen (HLA) genes are located on different chromosomes, autologous KIR receptor-ligand mismatch may exist (16). Normally, NK cells acquire self-tolerance and functional competence through the education process, in which inhibitory KIRs could be inhibited by self-HLA ligands and activated in a non-self HLA environment. Besides, the decreased responsiveness of activating KIRs in the presence of their cognate ligands also prevents autoimmunity (17–23) (**Figure 2A**). Importantly, infected and/or tumor cells may express inhibitory KIR ligands insufficiently or express activating ligands that may activate NK cells (24–31).

As the first reconstituted lymphocyte subset after transplantation (32, 33), NK cells play a critical role in controlling early relapse and infections. They also possess the ability to eliminate recipient T cells and antigen-presenting cells (APCs), to prevent graft failure and GVHD (34–38) (**Figure 2B**). Three models were established historically in an attempt to optimize donor selection for HSCT based on KIR (**Figure 2A**). The Perugia group in Italy firstly proposed the donor-recipient KIR ligand-ligand model (also known as KIR ligand model) solely based on the HLA phenotype of the donor and recipient. The KIR ligand incompatibility in the GVH direction was defined as the absence in recipients of donor class I allele group(s) recognized by KIRs. Those authors observed that the HLA haplotype-mismatched transplants reduced the rejection and relapse rate and prevented GVHD in patients with acute myeloid leukemia (AML) (36). Subsequently, the second model (named receptor-ligand model or missing ligand model) was raised by Leung et al. based on the compatibilities between the recipient HLA and donor inhibitory KIR. This model focused on donor KIR instead of donor HLA and could, therefore, be used in both HLA-matched and HLA-mismatched transplants. The results of that study suggested that the receptor-ligand model better predicted the risk of primary disease relapse, especially for lymphoid malignancies, compared with the ligand-ligand model (39). Subsequently, with a deeper understanding of KIR haplotypes, the third model analyzed and compared the KIR genotypes of different donors. Cooley et al. showed that unrelated donors with KIR-B haplotypes conferred a significant relapse-free survival (RFS) benefit to patients with AML undergoing T cell-replete HSCT (40). Based on the three models described above, numerous studies have been carried out to explore the impact of NK cell alloreactivity. Clinical results obtained from KIR ligand model, receptor ligand model and KIR haplotype and gene model were summarized in **Tables 2**–**4**, respectively. Nevertheless, the results were controversial, and several key questions remained regarding NK cell biology post-HSCT. What are the exact effects of NK cell alloreactivity on patients after HSCT? How do NK cells reconstitute post-HSCT and which factors may interfere with the reconstitution process? This review summarizes the latest literature on this important topic and offer some instructive hypothesis.

# KIR AND TRANSPLANT OUTCOMES

## NK Cell Alloreactivity and GVHD

GVHD is an important complication of HSCT with high morbidity and mortality in which allogeneic donor immune cells are activated by APCs and then recognize and attack the host tissue (105). Removing donor T cells from grafts reduces the occurrence of GVHD, while it also elevates the risk of graft failure and disease relapse (106–108).

As another component of immune cells, previous murine studies suggested that adoptive transfer of interleukin-2 (IL-2)-activated SCID NK cells with donor bone marrow cells

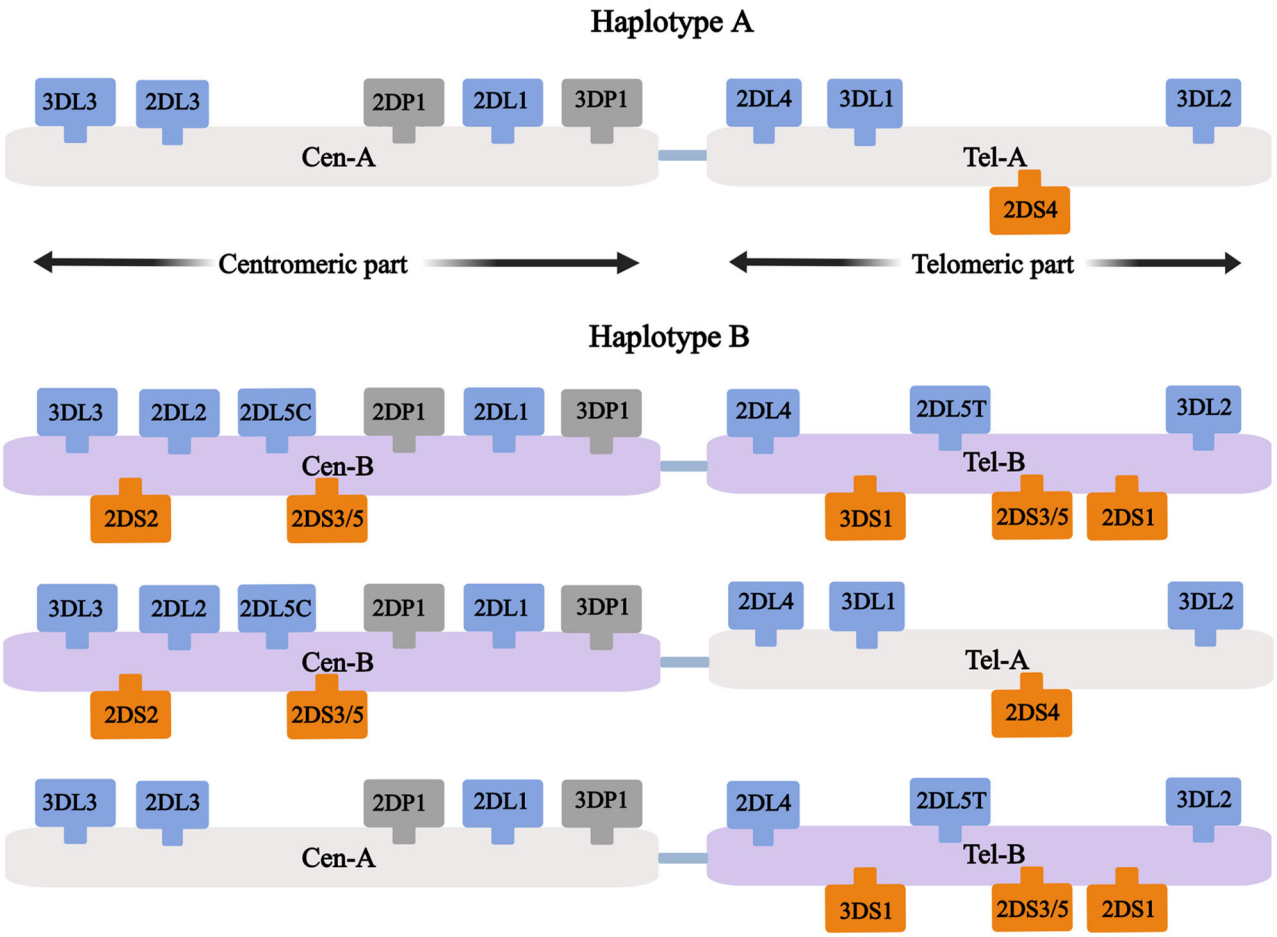


**FIGURE 1 |** Simplified genomic maps of KIR. Inhibitory KIR genes are color-coded in blue, activating KIR genes in orange, and pseudogenes in gray. KIR haplotype A has only one activating KIR gene: KIR2DS4, KIR B haplotype has fixable content of activating KIR genes. KIR haplotype could be further determined as Cen haplotype and Tel haplotype.

promoted engraftment in allogenic hosts with no signs of GVHD (109). Later, Asai et al. reported that hosts receiving MHC-incompatible bone marrow and spleen cells (as a source of T cells) rapidly succumbed to acute GVHD, while hosts who additionally received IL-2-activated donor NK cells on day 0 experienced a significant improvement in survival because of the lower incidence of severe GVHD. They further demonstrated that that the protective effect on GVHD was dependent on the transforming growth factor-beta (TGF-β) and could be abrogated by an anti-TGF-β antibody (35). Moreover, Ruggeri et al. showed that pre-transplant alloreactive Ly49 (Ly49 receptors recognize major histocompatibility complex (MHC) class I molecules in mice, which is analogous to KIR in humans) ligand-mismatched donor NK cell transfusion successfully eliminated host tumor cells and protected against GVHD by depleting host APCs. In contrast, hosts receiving bone marrow grafts without NK cell infusion died of GVHD, and non-alloreactive Ly49 ligand matched NK cell infusion did not provide protection against GVHD (36). Consistently, subsequent studies also found that donor alloreactive NK cells suppressed GVHD by inhibiting T cell proliferation and activation (37, 110). However, the protective role of NK cells in GVHD pathogenesis has also been challenged. Pre-clinical evidence from a xenogeneic model showed that an *in vitro* IL-2-activated human NK cell infusion promoted GVHD in SCID mice via the production of cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) (111, 112). Accordingly, GVHD was inhibited after the administration of anti-IFN-γ and depletion of Poly I:C-activated NK cells in murine studies (113, 114).

In patients with hematological malignancies, a purified (115, 116) or cytokine-induced (117–121) donor NK cell transfusion was also well tolerated and seldom induced severe GVHD (grade III-IV acute GVHD or moderate-to-severe chronic GVHD). More recently, a pilot study suggested that, after haplo-HSCT, patients with refractory AML who received a donor NK cell infusion experienced a significantly lower grade II-IV GVHD than did those without NK cell infusion (122). In contrast, Shah et al. observed that patients who received a donor

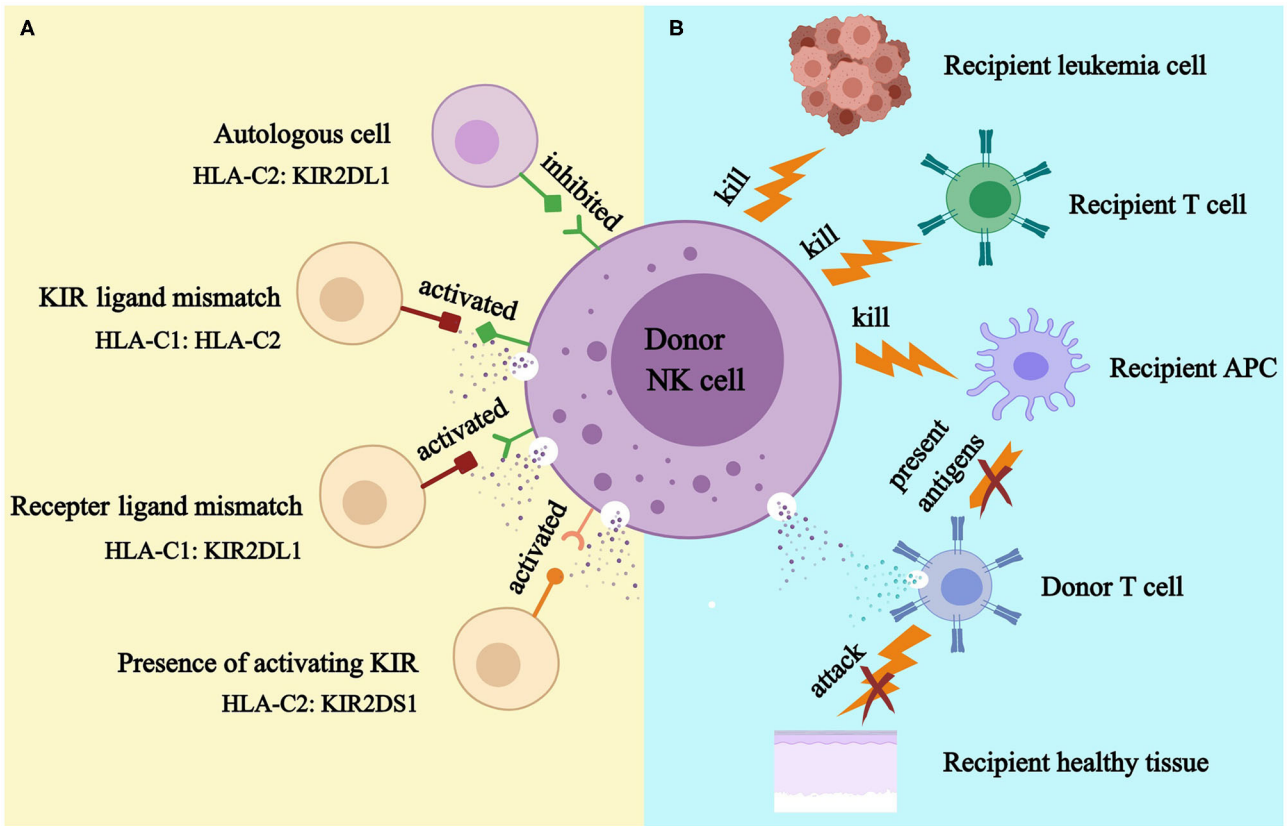


**FIGURE 2 |** KIR models **(A)** and NK cell-mediated killing **(B)**. APC, antigen presenting cell. **(A)**. Donor NK cell is tolerant to self because donor inhibitory KIR is inhibited by its cognate HLA ligand; donor NK cell might kill recipient cell because HLA ligand for donor inhibitory KIR presents in donor but absents in recipient (KIR ligand model); donor NK cell could kill recipient cell because recipient HLA ligand does not inhibit donor inhibitory KIR (receptor ligand model); donor NK cell could kill recipient cell because donor activating KIR is activated by recipient (KIR B haplotypes and KIR B genes). **(B)**. Alloreactive donor NK cell could kill recipient leukemia cell to prevent relapse; it could kill recipient T cell to prevent graft rejection; and it could kill recipient APC to prevent GVHD.

IL-15/4-1BBL-activated NK cell infusion after T cell-depleted (TCD) stem cell transplantation experienced a high risk of GVHD (123).

In addition to the technique of adoptive transfer, many studies have analyzed the effects of innate donor-recipient NK cell alloreactivity on GVHD in a clinical setting. The majority of studies did not report a significant association between these parameters (41–44, 46, 47, 50, 51, 54–56, 59, 65, 66, 79, 81, 83, 87–89, 91–93, 97, 98, 102, 104), while some reported a protective effect (70, 74, 76). Moreover, several studies found that KIR ligand mismatch or receptor-ligand mismatch increased the risk of GVHD (45, 57, 60, 64, 68, 80). Accordingly, two studies performed in China that applied the 'Peking protocol' for HSCT using the granulocyte-colony stimulating factor (G-CSF)-mobilized graft containing a high dose of T cells observed promotive effects of NK cell alloreactivity on GVHD (48, 49).

It is not entirely clear why the reconstituted alloreactive NK cells were unable to prevent GVHD as the adoptively transferred NK cells. Studies have indicated that this discrepancy was probably attributable to the impaired function of early reconstituted NK cells. Shilling et al. first observed that a period of several months or even years was required for the recipient to reconstitute an NK cell repertoire resembling that of the donor (124). Vago et al. also suggested that the NK cells that were reconstituted early after transplantation were immature and exhibited compromised cytotoxicity (125). In addition, NK cell reconstitution is affected by graft composition. Patients receiving more T cells in grafts experience a faster T cell reconstitution (126, 127), while the absolute number of reconstituted NK cells and KIR expression are impaired by the co-grafted T cells (127–130). Other than NK cells, nearly 5% of $CD8^+$ T cells, 0.2% of $CD4^+$ T cells, and 10% of γδ T cells in the peripheral blood also express KIRs (131–133). Therefore, it is possible that the potential beneficial effects of alloreactive NK cells are overwhelmed by the strong alloreactive T cell response. In addition, it was observed that NK cells generated more IFN-γ in the presence of T cells in grafts, leading to a higher occurrence of acute GVHD (aGVHD) (130). Moreover, post-transplant immune suppression also exerted negative effects on NK cell reconstitution (134, 135).

Regarding specific genotypes, some studies have reported that KIR haplotype B donors afforded a significantly reduced risk of GVHD (60, 63, 86, 96). Consistent with these findings,

**TABLE 2 |** Impact of KIR on clinical outcomes in KIR ligand model.

| References | N | Disease | Donor | Graft manipulation | Clinical outcomes |
|---|---|---|---|---|---|
| Ruggeri et al. (36) | 92 | AML, ALL | HRD | TCD* | KIR ligand mismatch: higher EFS and OS, lower relapse (AML)<br>KIR ligand mismatch: lower aGVHD$^{2-4}$ |
| Davies et al. (41) | 175 | Mixed | URD | TCD*, TCR | KIR ligand mismatch: lower OS (myeloid cohort) |
| Giebel et al. (42) | 130 | Mixed | URD | TCD# | KIR ligand mismatch: higher OS and DFS, lower TRM |
| Schaffer et al. (43) | 190 | Mixed | URD | TCD*, TCD# | KIR ligand mismatch: higher IRM and TRM, and lower OS |
| Elmaagacli et al. (44) | 236 | CML | MSD, URD | TCR | KIR ligand mismatch: lower molecular relapse |
| Yabe et al. (45) | 1489 | Mixed | URD | TCD#, TCR | KIR ligand mismatch: higher aGVHD$^{2/3-4}$ and lower OS (HLA-C mismatched transplants) |
| Verneris et al. (46) | 716 | Pediatric AL | URD | TCD#, TCR | KIR ligand mismatch: no significant impact on OS, DFS, relapse, TRM, or aGVHD. |
| Ruggeri et al. (47) | 112 | AML | HRD | TCD* | KIR ligand mismatch: lower relapse (CR group), higher EFS, and lower risk of relapse or death |
| Huang et al. (48) | 116 | Mixed | HRD | TCD# | KIR ligand mismatch: higher aGVHD$^{2-4}$ and relapse, lower OS |
| Zhao et al. (49) | 64 | Mixed | HRD | TCD# | KIR ligand mismatch: higher aGVHD; |
| Michaelis et al. (50) | 57 | Mixed | HRD | TCD* | KIR ligand mismatch: lower EFS (AML) |
| Mancusi et al. (51) | 161 | AML, ALL | HRD | TCD*<br>TCD*+Treg/Tcon | NK-alloreactive donors: lower relapse and higher EFS (AML) |
| Yahng et al. (52) | 100 | AML | HRD | TCD# | KIR ligand mismatch (HVG): higher relapse and CMV reactivation, lower DFS |
| Zhao et al. (53) | 180 | Mixed | HRD | TCD# | KIR ligand match: lower CMV reactivation rate and higher IFN-γ expression |
| Wanquet et al. (54) | 144 | Mixed | HRD | TCD# | KIR ligand mismatch: lower relapse and higher PFS (no CR group) |
| Shimoni et al. (55) | 444 | AML, ALL | HRD | TCD# | KIR ligand mismatch: a trend of higher relapse (AML), lower OS |

*MSD, matched sibling donor; URD, unrelated donor; HRD, haploidentical related donor; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; TCD, T cell depleted; TCR: T cell replete; Treg, regulatory T cells; Tcon, conventional T cells; aGVHD: acute graft vs. host disease; cGVHD: chronic graft vs. host disease; OS, overall survival; RFS, relapse free survival; DFS, disease free survival; EFS, event free survival; IRM: infection related mortality; TRM: transplant related mortality; CMV, cytomegalovirus.*
*TCD*: ex-vivo TCD.*
*TCD#: in-vivo TCD.*

Sivori et al. suggested that donor NK cells expressing KIR2DS1 were efficient in killing allogenic dendric cells in the setting of haplo-HSCT, thus leading to a better GVHD control (136). However, several studies also found that donors with KIR-B/x led to higher GVHD occurrence in recipients compared with donors with A/A, probably because of the more potent production of IFN-γ by alloreactive NK cells (40, 45, 76, 77, 94, 95).

Other factors, such as HLA mismatch, disease type, patient age, GVHD prophylaxis, and graft source, were also reported to interfere with GVHD occurrence in these studies (44, 45, 63, 66, 87, 92, 93, 104). Collectively, the manner in which the reconstituted NK cells affect the risk of GVHD remains largely unknown, and the relationships between NK and T cells during the initiation and process of GVHD warrant further investigation.

## NK Cell Alloreactivity and Infection

Infections are especially challenging for patients after HSCT because of the immunological derangement caused by multiple factors, including an intensive conditioning regimen, immunosuppressive agents, and other complications, such as GVHD (137, 138).

Several studies have reported that patients receiving KIR ligand-mismatched transplants are more vulnerable to infections. Schaffer et al. first reported that KIR ligand mismatch was associated with an increased infection-related mortality (43). Similarly, results from Zhao et al. showed that recipients from the KIR ligand-mismatched group experienced a significantly higher cytomegalovirus (CMV) reactivation rate. Moreover, the percentage of interferon-gamma (IFN-γ)-expressing NK cells in the peripheral blood was significantly higher in the KIR ligand matched group 30 and 100 days post-HSCT compared with the KIR ligand-mismatched group (53). The higher level of IFN-γ secretion from the NK cells might trigger Th1 immune responses, antigen presentation cell activation, and macrophage killing (7, 8), leading to lower infection rate. While, KIR ligand mismatch may increase the risk of infection by eliminating recipient APCs by donor alloreactive NK cells (36).

Many studies have found that KIR-B genes protect patients with HSCT against infections and most of them were predominantly T cell replete (TCR) transplants (81, 84, 87, 96, 139, 140). Cook et al. first observed that KIR haplotype B donors exhibited a significant reduction in the rate of CMV reactivation in sibling allo-HSCT (139). Wu et al. and Zaia et al. reported that donors expressing higher numbers of activating KIRs were associated with a lower CMV reactivation rate (62, 84). Specifically, activating KIR2DS2 and KIR2DS4 may play a major protective role (84, 140). Importantly, transplantations from donors with KIR2DS1 correlated with better infectious control (51, 96). Mancusi et al. further demonstrated that the binding

**TABLE 3 |** Impact of KIR on clinical outcomes in receptor ligand model.

| References | N | Disease | Donor | Graft manipulation | Clinical outcomes |
|---|---|---|---|---|---|
| Leung et al. (39) | 36 | Mixed | HRD | TCD* | Receptor ligand mismatch: lower relapse |
| Cook et al. (56) | 220 | Mixed | MSD | / | HLA-C2C2 patients vs. HLA-C1/x patients: lower OS (myeloid cohort) |
| Verheyden et al. (57) | 65 | Mixed | MSD | TCD*, TCR | HLA-C1C2 patients vs. HLA-C1C1 or C2C2 patients: lower aGVHD |
| Hsu et al. (58) | 1770 | Mixed | URD | TCR | Missing ligand for donor iKIR: lower relapse (HLA mismatched transplants) |
| Clausen et al. (59) | 43 | Mixed | MSD | TCR | Ligand missing to KIR3DL2 plus one other iKIR vs. others: lower relapse and higher OS |
| Ludajic et al. (60) | 124 | Mixed | URD | TCD#, TCR | Missing ligand for donor KIR2DL1: higher aGVHD$^{2-4}$; |
| Linn et al. (61) | 151 | Mixed | MSD | TCR | Missing ligand for donor iKIR: no impact on OS and RFS |
| Wu et al. (62) | 48 | Mixed | URD | TCD# | HLA group C1 vs. C2: higher CMV reactivation rate |
| Gagne et al. (63) | 264 | Mixed | URD | TCR | Missing HLA-C1 ligand: lower OS (myeloid cohort) |
| Clausen et al. (64) | 100 | Mixed | MSD | TCR | HLA-C1C2 patients vs. HLA-C1C1 or C2C2 patients: lower relapse and aGVHD$^{2-4}$, higher RFS |
| Björklund et al. (65) | 105 | AML, MDS | MSD | TCD#, TCR | Receptor ligand mismatch: no significant impact on OS, relapse and GVHD |
| Wu et al. (66) | 116 | Mixed | URD | TCD#, TCR | Missing ligand for donor iKIR: lower relapse, higher OS and DFS (myeloid cohort); |
| Zhou et al. (67) | 219 | Mixed | MSD | / | HLA-C1C1 patients vs. HLA-C2/x patients: lower aGVHD$^{2-4}$ |
| Sobecks et al. (68) | 909 | AML, MDS | URD | TCD#, TCR | Missing ligand for donor iKIR: higher aGVHD$^{3-4}$ and TRM (AML); Missing HLA-C2 for donor KIR2DL1: higher aGVHD$^{2/3-4}$ (AML) |
| Park et al. (69) | 59 | Mixed | MSD, URD | TCD#, TCR | Receptor ligand mismatch: higher OS, DFS and lower relapse |
| Cardozo et al. (70) | 50 | Mixed | MSD | TCR | Patients with all ligands present vs. missing ligand for donor iKIR: higher aGVHD; Missing ligand for donor iKIR: higher OS (myeloid cohort) |
| Faridi et al. (71) | 281 | Mixed | MSD, URD | TCD# | Missing ligand for donor iKIR: lower relapse and better RFS (URD) |
| Neuchel et al. (72) | 1446 | Mixed | URD | TCR | HLA-C2C2 vs. HLA-C1/x patients: lower OS, DFS, higher relapse (myeloid cohort) |
| Arima et al. (73) | 10638 | Mixed | MSD, URD | TCD*, TCD# TCR | HLA-C1C1 patients vs. HLA-C1C2 patients: lower relapse and higher RFS (AML and CML); HLA-C1C1 patients vs. HLA-C1C2 patients: higher relapse (ALL) |
| Gaafar et al. (74) | 87 | Mixed | MSD | TCR | KIR2DL1: HLA-C2 match: higher aGVHD$^{2-4}$ (AML) |
| Arima et al. (75) | 2884 | ALL | MSD, URD | TCD, TCR | HLA-C1C1 patients vs. HLA-C1C2 patients: higher relapse |
| Chen et al. (76) | 84 | Mixed | HRD | TCD# | Missing HLA-C2 ligand for donor KIR2DL1: higher OS and lower RRM (myeloid cohort); Missing HLA-C for donor iKIR: lower aGVHD$^{2-4}$ (lymphoid cohort); |
| Zhao et al. (77) | 97 | CML | HRD | TCD# | Receptor ligand match: lower relapse |
| Zhao et al. (78) | 188 | Mixed | HRD | TCD# | Receptor ligand match: lower relapse and higher LFS |
| Solomon et al. (79) | 208 | Mixed | HRD | TCD# | Receptor ligand mismatch: higher OS and DFS, lower relapse |
| Willem et al. (80) | 51 | Mixed | HRD | TCD# | KIR2DL/HLA mismatch: higher GVHD and lower relapse |

*AL, acute leukemia; MDS, myelodysplastic syndromes; iKIR, inhibitory KIR; LFS, leukemia-free survival.*

of KIR2DS1 to HLA-C2 triggered pro-inflammatory cytokine production by alloreactive NK cells (51). Moreover, without a cognate ligand (HLA-C1) in recipients, donor KIR2DS2 was associated with a higher CMV reactivation rate after HLA-identical sibling HSCTs (81). Apart from CMV reactivation, the incidence of bacterial infections was also reduced when patients had KIR-B/x donors (87). In contrast with previous results, KIR2DS2 gene and Cen-B/x donors related to a higher incidence of CMV reactivation and infection-related mortality in TCD transplants (53, 100). The reasons for these differing results may be due to the different graft composition. As previously described, NK cells generate more IFN-γ in TCR transplants, which may benefit the infection control (130). Of notice, the activating KIR targets outside of HLA are largely unknown, and these clinical observations still need to be confirmed by definitive functional analysis in the future.

## NK Cell Alloreactivity and Relapse/Survival

Primary disease relapse remains the main obstacle that hampers the long-term survival of patients with hematological malignancies. Previous experience showed that adoptive transfer of autologous NK cell for patients with tumors was safe but inefficient (141–145), probably because autologous NK cells could not overcome the inhibition mediated by

**TABLE 4 |** Impact of KIR on clinical outcomes in KIR haplotype and gene model.

| References | N | Disease | Donor | Graft manipulation | Clinical outcomes |
|---|---|---|---|---|---|
| Cooley et al. (40) | 448 | AML | URD | TCR | KIR B/x donor: higher RFS and cGVHD |
| Cook et al. (56) | 220 | Mixed | MSD | / | KIR2DS2: lower OS (HLA-C2C2 patients with myeloid diseases) |
| Verheyden et al. (57) | 65 | Mixed | MSD | TCD*, TCR | Donor co-presenting KIR2DS1 and 2DS2: lower relapse |
| Chen et al. (81) | 131 | Mixed | MSD | TCR | KIR2DS2: higher CMV reactivation (HLA-C2C2 patients);<br>Additional activating KIR genes in donor: higher OS and lower CMV reactivation |
| Yabe et al. (45) | 1489 | Mixed | URD | TCD#, TCR | KIR2DS2: higher aGVHD$^{3-4}$ (HLA-C mismatched transplants) |
| Schellekens et al. (82) | 83 | Mixed | MSD | TCR | KIR2DS1: higher OS (HLA-C1C1 patients);<br>More activating KIRs in donor or patients: higher relapse;<br>KIR2DS5 in patients or both in donor and patients: higher relapse |
| van der Meer et al. (83) | 70 | Mixed | MSD | TCD* | KIR2DS5: higher LFS and lower relapse (HLA-C1C1 or HLA-C2C2 patients);<br>KIR2DS5: lower LFS and higher relapse (HLA-C1C2 patients) |
| Ludajic et al. (60) | 124 | Mixed | URD | TCD#, TCR | KIR2DS2: lower aGVHD$^{2-4}$ (HLA-C1C2 patients) |
| Zaia et al. (84) | 211 | Mixed | MSD, URD | TCR | Donor co-presenting KIR 2DS2 and 2DS4: lower CMV reactivation;<br>Donor aKIR gene content $\geq$5: lower CMV reactivation |
| Wu et al. (62) | 48 | Mixed | URD | TCD# | High aKIRs group: lower CMV reactivation rate |
| Gagne et al. (63) | 264 | Mixed | URD | TCR | KIR B/x donor: lower aGVHD$^{3-4}$ (HLA identical pairs with myeloid disease) |
| Bao et al. (85) | 75 | Mixed | URD | TCD# | KIR B/x donor: higher OS |
| Venstrom et al. (86) | 1087 | Mixed | URD | TCD*, TCR | KIR3DS1: lower aGVHD$^{2-4}$;<br>KIR3DS1: lower aGVHD$^{2-4}$, TRM and mortality (AML, CML and ALL) |
| Wu et al. (66) | 116 | Mixed | URD | TCD#, TCR | KIR2DS3: higher relapse, lower OS and DFS (myeloid cohort);<br>More numbers of activating KIR genes in donor: higher relapse |
| Tomblyn et al. (87) | 116 | Mixed | URD | TCD*, TCR | KIR B/x donor: lower bacterial infections by day 180 |
| Cooley et al. (88) | 1409 | AML, ALL | URD | TCR | KIR B/x donor: lower relapse and higher DFS (AML);<br>Cen-BB vs. Cen-BA or AA: lower relapse and higher DFS (AML);<br>Tel-B/x vs. Tel-AA: lower relapse (AML);<br>B content $\geq$ 2: lower relapse (AML) |
| Venstrom et al. (89) | 1277 | AML | URD | TCD*, TCR | Donor KIR2DS1 with HLA-C1/x patients vs. with HLA-C2C2 patients: lower relapse;<br>KIR3DS1: higher OS |
| Zhou et al. (67) | 219 | Mixed | MSD | / | Cen-B/x donor: higher OS, RFS and lower relapse |
| Impola et al. (90) | 134 | Mixed | MSD | / | KIR 2DL2 or KIR 2DS2: better RFS (AML) |
| Bao et al. (91) | 210 | Mixed | URD | TCD# | KIR B/x donor: higher OS, RFS and lower NRM (AML and MDS);<br>Cen-B/x donor: higher OS, RFS (AML and MDS at standard risk) |
| Cardozo et al. (70) | 50 | Mixed | MSD | TCR | KIR2DS2: lower OS and EFS |
| Bachanova et al. (92) | 614 | NHL | URD | TCD#, TCR | KIR B/x donor: lower relapse and better PFS (HLA matched transplants) |
| Kamenaric et al. (93) | 111 | Mixed | MSD, URD | TCD# | KIR2DS4 (neg vs. pos): no impact on GVHD (MSD) |
| Hosokai et al. (94) | 106 | Mixed | MSD, URD | TCR | KIR B/x donor: higher aGVHD$^{3-4}$ (more evdient in HLA mismatched transplants) |
| Neuchel et al. (72) | 1446 | Mixed | URD | TCR | KIR2DS2: higher OS and DFS (HLA-C2C2 patients);<br>KIR2DS1: lower relapse but higher TRM (HLA-C2C2 patients);<br>KIR2DS5: lower relapse (HLA-C2C2 patients) |
| Gaafar et al. (74) | 87 | Mixed | MSD | TCR | KIR2DS2: HLA-C1 match: higher aGVHD$^{2-4}$ (AML);<br>KIR2DS1: HLA-C2 match: higher cGVHD (AML);<br>Donor presenting KIR2DL1 or 2DS2: higher cGVHD (AML) |
| Sahin et al. (95) | 96 | AML, CML | MSD | TCR | KIR B/x donor: higher cGVHD |
| Heatley et al. (96). | 152 | Mixed | MSD | TCR | KIR2DS2: higher OS (AML);<br>Cen-B/x donor: higher OS (AML) and lower aGVHD$^{2-4}$ (AML);<br>Tel B/x donor: lower CMV reactivation |
| Babor et al. (97) | 317 | Pediatric ALL | MSD, URD | TCD#, TCR | Higher ct-KIR score: lower relapse |
| Tordai et al. (98) | 314 | Mixed | MSD, URD | / | The combination of KIR2DS1 donor with HLA-C2 pos patients: higher OS |

*(Continued)*

**TABLE 4 |** Continued

| References | N | Disease | Donor | Graft manipulation | Clinical outcomes |
|---|---|---|---|---|---|
| Nakamura et al. (99) | 288 | AML | MSD, URD | TCD*, TCD# | CMV reactivation: lower relapse and higher NRM (more evident in KIR B/x donor or when donor presenting KIR2DS1) |
| Bultitude et al. (100) | 119 | AML | URD | TCD, TCR | Cen-B/x donor: lower OS and NRM, higher IRM |
| Weisdorf et al. (101) | 2662 | AML | URD | TCD#, TCR | KIR B/x donor: lower relapse and higher LFS (RIC) |
| Verneris et al. (46) | 716 | Pediatric AL | URD | TCD#, TCR | KIR gene content: no significant impact on OS, DFS, relapse, TRM, or aGVHD |
| Zhao et al. (49) | 64 | Mixed | HRD | TCD# | KIR2DS3: higher aGVHD and cGVHD;<br>KIR2DS5: higher aGVHD |
| Symons et al. (102) | 86 | Mixed | HRD | TCD# | KIR B/x donor: lower NRM and higher OS, EFS (KIR AA patients) |
| Chen et al. (76) | 84 | Mixed | HRD | TCD# | KIR2DS2: higher OS (lymphoid cohort);<br>KIR2DS1: higher GVHD (lymphoid cohort) |
| Michaelis et al. (50) | 57 | Mixed | HRD | TCD* | KIR B/x donor: lower relapse |
| Zhao et al. (77) | 97 | CML | HRD | TCD# | KIR2DS3: lower EFS and OS, higher TRM;<br>KIR2DS5: higher EFS and OS, lower TRM;<br>KIR B/x donor: higher aGVHD$^{3-4}$ |
| Oevermann et al. (103) | 85 | Pediatric ALL | HRD | TCD* | KIR B/x donor: lower relapse and better EFS;<br>High donor KIR-B content: lower relapse and better EFS |
| Mancusi et al. (51) | 161 | AML, ALL | HRD | TCD*<br>TCD*+Treg/Tcon | Tel B/x vs. Tel AA: lower NRM and higher EFS (NK-alloreactive donors)<br>KIR2DS1/3DS1: lower NRM and higher EFS (NK-alloreactive donors)<br>KIR 2DS1 binding to HLA C2: increased inflammatory cytokine |
| Zhao et al. (53) | 180 | Mixed | HRD | TCD# | KIR2DS2: higher CMV reactivation |
| Solomon et al. (79) | 208 | Mixed | HRD | TCD# | KIR B/x donor with 2DS2 vs. KIR B/x donor without 2DS2: higher OS and DFS, lower relapse and NRM;<br>KIR B/x donor with 2DS2 vs. KIR A/A donor: higher OS and DFS, lower NRM |
| Perez-Martinez et al. (104) | 192 | Pediatric mixed | HRD | TCD*, TCD# | KIR AA donor: higher relapse and lower DFS |

*pos: positive; neg: negative; NHL, non-Hodgkin lymphoma; PFS, progression-free survival; NRM: non-relapse mortality. TCD*: ex-vivo TCD; TCD#: in-vivo TCD.*

tumor cells expressing self-HLA. In contrast, allogenic (117), especially haploidentical, donor NK cell infusion demonstrated wide prospects in the salvage treatment (115, 120, 121) and prophylactic treatment (118, 119) of patients with hematological malignancies. In allo-HSCT, whether the reconstituted alloreactive NK cells prevent the disease relapse remains controversial.

In HLA-mismatched transplants, the Perugia group first observed that, in the context of T cell depletion, high stem cell dose, and absence of post-transplant immune suppression, KIR ligand mismatch reduced the risk of relapse and markedly improved survival in patients with AML, but not in those with acute lymphoblast leukemia (ALL) (36). This protective effect on relapse or survival was supported by many clinical studies (42, 44, 47, 51, 54), especially in myeloid disease (44, 47, 51) and transplants with TCD grafts (42, 47, 51, 54). However, conflicting results stemmed from many studies that failed to replicate these results (39, 46, 58, 102), and some even reached the opposite conclusions (41, 43, 45, 48, 50, 55).

Studies using the receptor-ligand model including HLA-matched donor-recipient pairs also reported conflicting results. Leung et al. first reported that the receptor-ligand model was more accurate than the KIR ligand model when predicting the risk of relapse, especially for lymphoid malignancies. Moreover, the potency of the relapse protection positively correlated with the number of receptor-ligand mismatch pairs (39). Subsequently, the protective effect of receptor-ligand mismatch has been confirmed by many investigations (58, 59, 66, 69, 71, 73, 76, 79, 80). Moreover, a survival advantage was also observed in patients with receptor-ligand mismatch compared with receptor-ligand matched pairs (59, 66, 69–71, 73, 76, 79). However, several other studies described opposite results (63, 64, 75, 77, 78). Of notice, two studies from Japan observed that the lack of the HLA-C2 ligand for donor inhibitory KIR afforded relapse protection in patients with AML and chronic myeloid leukemia, but increased the relapse rate in patients with ALL (73, 75). To date, no plausible explanation has been put forward for this disparity in relapse.

In contrast to the controversial results described above, transplantations from KIR haplotype B donors achieved greater agreement. Cooley et al. observed that patients with AML with KIR-B/x donors experienced a 30% improvement in RFS compared with those with A/A donors (40). Subsequently, many further investigations confirmed this beneficial effect of the KIR-B haplotype on relapse and survival in patients with hematological malignancies (50, 51, 57, 67, 72, 76, 79, 81, 85, 88–92, 96, 98, 101–104). Five of these studies reported that the protection effects mainly existed in the KIR Cen-B locus (67,

88, 91, 92, 96). Babor et al. further suggested that the presence of Cen-B with absence of Tel-B improved leukemia control in pediatric patients with ALL (97). At the genetic level, the KIR2DS2 gene, which is located on the Cen-B motif (72, 76, 79, 90, 92, 96), and the KIR2DS1 gene, located on the Tel-B motif (51, 72, 82, 98), were found to be related to a decreased relapse rate or an improved survival. However, several studies found that Cen-B donors indicated a lower OS (56, 70, 100). Meanwhile, Verneris et al. did not find any association between transplant outcomes and NK cell alloreactivity or KIR gene content in pediatric patients with acute leukemia (46).

Recently, Krieger et al. developed a scoring system, in which interactions of multiple KIR genes and HLA ligands were quantitatively analyzed. This comprehensive method raised an improved strategy to select a donor and exhibited great potential in the future (146).

Collectively, it is still controversial to determine an optimal donor who exhibits the best NK cell function using the three established KIR models. A better knowledge of NK cell reconstitution after HSCT may promote a better understanding of how NK cells affect the transplant outcomes in these patients. More in-depth studies focusing on "functional changes in NK cells" rather than "match or mismatch" may help us get closer to an optimal donor.

# NK CELL RECONSTITUTION AFTER TRANSPLANTATION

## Maturation and Differentiation of NK Cells

NK cells are derived from the $CD34^+$ hematopoietic stem and precursor cells in the bone marrow, which then migrate to the periphery (147). Recent evidence suggested that not only the bone marrow, but also secondary lymphoid tissues contribute to the development of NK cells (148). According to the surface expression of CD56, NK cells could be divided in two main subtypes: $CD56^{bright}$ and $CD56^{dim}$ NK cells. $CD56^{bright}$ NK cells exist mainly in lymph nodes and tonsils, while $CD56^{dim}$ NK cells, the more mature subset transformed from $CD56^{bright}$ NK cells, are dominant in the peripheral blood (7, 147, 149, 150). $CD56^{bright}$ and $CD56^{dim}$ NK cells are equipped with distinct functions. The former population responds rapidly to interleukin-mediated stimulation with proliferation and cytokine secretion, while the latter population displays higher cytolytic capacity and lower proliferation (7, 8, 149). During the process of maturation, CD94/NKG2A is the first receptor that is expressed on immature NK cells. Together with the downregulation of CD56 expression, NK cells upregulate CD16 expression, lose NKG2A, and acquire KIR receptors. Finally, a subset of $CD56^{dim}$ cells continue to differentiate and express CD57, together with an increased KIR expression and a completely abolished proliferative ability (150, 151).

In HSCTs with post-transplant cyclophosphamide (PT-Cy) as GVHD prophylaxis, NK cells experience two waves of expansion. After graft infusion, peripheral NK cells and T cells (mainly mature cells from the donor) were detectable at very low levels. PT-Cy administration results in a further decrease in T cells and NK cells, and NK cells are barely detectable in the peripheral blood. Subsequently, the reconstituted NK cells gradually recover and express high levels of CD56 and NKG2A. Around 60 days after transplantation, the KIR expression returns to normal. The expression of CD56 and NKG2A gradually decreases and becomes stable at 9–12 months post-transplantation. Other receptors expressed on NK cells, such as DNAM-1and 2B4, also require several months to return to normal (152). In summary, post-transplantation NK cell reconstitution is a long-term process (124, 125, 152).

## KIR Education: From Anergic to Responsive

As described earlier, the random combination of KIR receptor and HLA ligand can exist in healthy individuals. However, the autoimmune attack is inhibited because each NK cell expresses at least one self-inhibitory receptor. To avoid autoreactivity, NK cells must undergo an education process: NK cells expressing inhibitory KIR for self-HLA ligand (self-KIR) are educated, which means that these cells can be inhibited by self-inhibitory signals and become alloreactive against self-HLA-deficient targets. In contrast, NK cells expressing an inhibitory KIR that lacks a self-HLA ligand (non-self KIR) are uneducated, which means that they are tolerant to the self but also to infected or malignant cells (19, 21).

In the last decades, studies on KIR education have much extended our knowledge of NK cell function. After transplantation, most reconstituted NK cells express a donor-like KIR repertoire that is significantly different from that of recipient NK cells prior to transplantation (124, 151). Therefore, reconstituted NK cells expressing donor KIR may exert alloreactivity in recipients, or become anergic, as recipients may not present the cognate HLA (**Figure 3**). Foley et al. and Björklund et al. observed that reconstituted NK cells with non-self KIR remained tolerant, while those with self KIR acquired better functions after transplantation (65, 153). However, Yu et al. reached the opposite conclusion that alloreactive NK cells broke the self-tolerance and displayed functional capacities in the first 3 months, then gradually acquired self-tolerance by day 100 post-transplantation (154). Rathmann et al. also suggested that alloreactive NK cells were increased in the peripheral blood and exhibited a GVL effect in the early period after transplantation (155). One possible explanation for this observation is that the infusion of a megadose of donor $CD34^+$ cells may create a transient donor dominant HLA environment in recipient bone marrow, and the early reconstituted NK cells expressing non-self KIR for the recipient may become educated by donor HLA and acquire functions (156). After migration to a recipient-dominant environment, reconstituted NK cells may gradually lose their responsiveness.

In murine studies, it was observed that mature NK cells from major histocompatibility complex (MHC) class I-sufficient mice become hyporesponsive after transfusion into MHC class I-deficient mice. Conversely, anergic NK cells from MHC class I-deficient mice acquired functions after exposure to the MHC class I-sufficient environment (157, 158). Using a murine

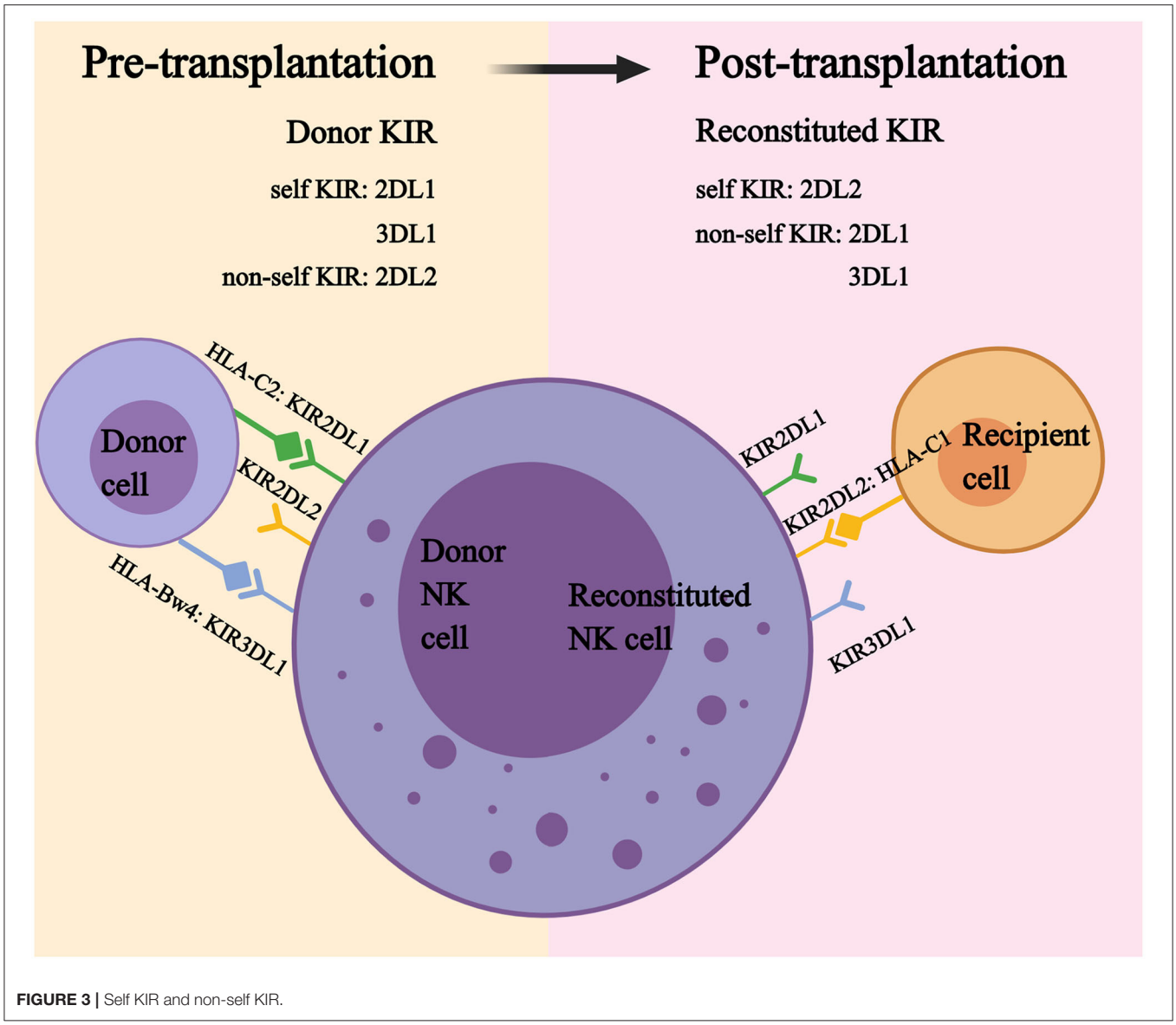


**FIGURE 3 |** Self KIR and non-self KIR.

transgenic model of HLA-B*27:05 exhibiting the Bw4 ligand for KIR3DL1, Boudreau et al. observed similar results in stem cell transplantation. $CD34^+$ cells from $KIR3DL1^+$ donors were transfused to B27 $Tg^+$ and $Tg^-$ mice, respectively. A functional analysis suggested that the most cytotoxic responsive cells were $KIR3DL1^+$ NK cells from $Bw4^+$ donors and developed in B27 $Tg^+$ mice ($Bw4^+$ donors and $Tg^+$ mice), while the least-responsive cells were $KIR3DL1^+$ NK cells from $Bw4^-$ donors and developed in $Tg^-$ mice ($Bw4^-$ donors and $Tg^-$ mice). Recipients with the other two combinations ($Bw4^+$ donors and $Tg^-$ mice and $Bw4^-$ donors and $Tg^+$ mice) displayed a medium level of responsiveness. The stepwise escalation of NK cell responsiveness suggested that both the donor and recipient MHC environments are critical for the maintenance and adjustment of NK cell education (159).

Recently, the Nowak team proposed that inhibitory KIR (iKIR)-HLA pairs could predict the post-HSCT NK cell education status, i.e., donors presenting cognate HLA for donor iKIR and recipients lacking it predict a downward education level; in contrast, recipients presenting cognate HLA for donor iKIR and donors lacking it predict an upward education level. Those authors found that the decrease in iKIR–HLA pairs post-transplantation is associated with a higher relapse and poorer survival (160–162), indicating that reconstituted NK cells acquire better functions after interaction with more cognate HLA class I ligands in recipients. Zhao et al. also observed that, when the donors and recipients expressed three major HLA ligands (HLA-C1, C2, Bw4), patients with AML and myelodysplastic syndrome (MDS) experienced the lowest relapse rate, and NK cells expressing three inhibitory receptors exhibited the greatest cytotoxicity and cytokine responsiveness against K562 targets (163).

Based on the findings described above, it is likely that three factors (donor KIR, donor HLA, and recipient HLA) all

contribute to the variation in NK cell function. Therefore, the KIR ligand and receptor-ligand models, which only take two factors into account, may not accurately predict donors that exhibit the greatest NK cell function post-transplantation.

## Factors That Affect NK Cell Reconstitution

Although CMV reactivation suggests an immune-compromised state, patients who experienced CMV reactivation had a lower relapse rate or better survival (70, 98, 99, 164). This protective effect might be attributed to the rapid maturation of NK cells. During CMV reactivation, NK cells that express NKG2C rapidly expand and continue to increase for 1 year (165). The number of $CD56^{dim}$ NK cells in the peripheral blood, their KIR expression, and IFN-$\gamma$ production in response to K562 cells were also elevated in patients who developed CMV reactivation (165–173). Furthermore, nearly 60% of $NKG2C^+$ NK cells achieved complete differentiation and expressed CD57 after CMV reactivation. These cells were termed memory-like NK cells and could be detected long after the primary CMV infection, offering a long-lasting protection (147, 166). In contrast, for non-CMV-infected patients, a higher proportion of $NKG2A^+$ $NKG2C^-$ $KIR^-$ NK cells in the peripheral blood indicates a slow NK cell maturation. Interestingly, CMV antigen exposure to recipients also leads to an increased frequency of $NKG2C^+$ NK cells, accompanied by increased KIR expression and decreased NKG2A expression (174).

As mentioned above, T cells in the graft impair the recovery of NK cells and KIR reconstitution (127–130). A possible explanation for this observation is that T cells compete with NK cells for IL-15, a cytokine that regulates immune cell survival and development (175, 176). Unlike *ex-vivo* TCD grafts, pre-transplant anti-thymocyte globulin (ATG) administration results in partial T cell depletion. Two recent studies found that ATG administration promoted NK cell recovery and delayed the reconstitution of $CD4^+$ and $CD8^+$ T cells, while sparing the effector memory T and regulatory T cells (Tregs) (177, 178). Compared with ATG, PT-Cy is more efficient in eliminating NK cells, with a higher residual ratio of $CD4^+$ T cells and Tregs (179). Of note, several studies showed that T cells in the graft may contribute to a better NK cell function (153, 180). Several studies reported that $CD56^{bright}$ NK cells in lymph nodes could be stimulated by IL-2-producing T cells, resulting in NK cell maturation with higher IFN-$\gamma$ secretion and cytotoxic functions (181, 182).

The relationship between GVHD and NK cell reconstitution remains controversial. Previous studies demonstrated that GVHD correlated with an impaired NK cell reconstitution and KIR expression (183–185). Ullrich et al. found that $CD56^{bright}$ NK cells were dramatically decreased in patients with GVHD, while $CD56^{dim}$ NK cells, the more mature subtype, did not show significant changes (185). In addition, Hu et al. found that the NKG2A subset of $CD56^{dim}$ NK cells was significantly decreased in patients with GVHD. Remarkably, a functional analysis showed that $NKG2A^+$ NK cells from GVHD and non-GVHD patients exhibited a comparable GVL effect. Furthermore, the co-culture of donor T cells with $NKG2A^+$ cells from non-GVHD patients suggested that $NKG2A^+$ NK cells inhibit T cell proliferation and activation, indicating that the decreased number of $NKG2A^+$ NK cells might be a cause, rather than a consequence, of GVHD (186). In addition, the administration of immunosuppressive agents could also affect immune recovery. Both Ullrich et al. and Giebel et al. suggested that steroid treatment, rather than GVHD, was related to the delayed NK cell reconstitution (184, 187).

# FUTURE DIRECTIONS

Numerous studies have found that alloreactive NK cells affect treatment outcomes. Although great progress has been made through both pre-clinical and clinical investigations based on the three KIR models, the controversy remains, especially regarding the benefits of KIR alloreactivity on relapse control. Recent findings showed that donor KIR, donor HLA, and recipient HLA environment all contribute to the variation of NK cell function. The newly proposed iKIR-HLA pair model needs to be further examined in the future.

NK cells, the lymphocytes that are reconstituted first after transplantation, could be negatively affected by the T cells in the graft. However, NK cell function could also be promoted through T-cell-mediated activation. The exact interactions between NK and T cells, as well as the strategy to trigger a potential synergistic NK and T cell effect remains to be investigated.

It is noteworthy that the protective role of NK cell alloreactivity in relapse protection mostly exists in myeloid disease; in fact, some studies even found that NK cell alloreactivity increased the risk of relapse for patients with lymphoid disease. The discrepancy between expressing ligands among different diseases and their binding affinity to KIR should raise more attention. In this way, we might identify which patients would benefit from the KIR-based donor selection.

# CONCLUSION

In the early period after transplantation, reconstituted alloreactive NK cell may not directly influence GVHD occurrence, as it is immature and it could be affected by T cells and immunosuppressive agents. The compatibility between donor KIR and the recipient HLA ligand may protect patients from infection. In the late period after transplantation, the iKIR-HLA pair model may reflect the variation in NK cell function, and quantitative analysis of KIR-HLA interactions may provide more convincing results regarding relapse and survival.

# AUTHOR CONTRIBUTIONS

YZ and HH designed. FG and YY wrote this paper. All authors revised and approved the final manuscript.

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# Efficacy and Safety of Eculizumab in the Treatment of Transplant-Associated Thrombotic Microangiopathy: A Systematic Review and Meta-Analysis

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**Background:** Transplant-associated thrombotic microangiopathy (TA-TMA) is a dangerous and life-threatening complication in patients undergoing hematopoietic stem cell transplantation (HSCT). Eculizumab has been used in the treatment of TA-TMA, and several studies have confirmed the benefit of Eculizumab in patients with TA-TMA. However, the results remain controversial. We conducted a systematic review and meta-analysis to evaluate the efficacy and safety of Eculizumab for TA-TMA.

**Materials and Methods:** We searched PubMed and Embase for studies on the efficacy and safety of Eculizumab in TA-TMA patients. Efficacy outcomes consisted of overall response rate (ORR), complete response rate (CRR), and survival rate at the last follow-up (SR). Safety outcomes were adverse events (AEs), including infection, sepsis, impaired liver function, infusion reactions, and death.

**Results:** A total of 116 patients from six studies were subjected to meta-analysis. The pooled estimates of ORR, CRR, and SR for TA-TMA patients were 71% (95% CI: 58–82%), 32% (95% CI: 11–56%), and 52% (95% CI: 40–65%), respectively. Only one patient presented with a severe rash, and infection was the most common AEs. The main causes of death were infection and GvHD.

**Conclusion:** Current evidence suggests that Eculizumab improves SR and ORR in patients with TA-TMA and that Eculizumab is well tolerated. However, the number of studies is limited, and the findings are based mainly on data from observational studies. Higher quality randomized controlled trials and more extensive prospective cohort studies are needed.

**Keywords: Eculizumab, terminal complement inhibitor, transplant-associated thrombotic microangiopathy, hematopoietic stem cell transplantation, efficacy, safety, meta-analysis**

## INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a recognized treatment for both malignant and non-malignant diseases. While this treatment has increased cure rates and reduced disease mortality, its complications remain life-threatening and of concern. Transplant-associated thrombotic microangiopathy (TA-TMA) is one of the most devastating complications of hematopoietic stem cell transplantation. A recent study reported a 3-year cumulative incidence rate of 3% for TA-TMA, and TA-TMA was associated with high mortality (HR = 3.1, 95% CI: 2.8–16.3%) (1). Treatment intensity, use of calcitonin inhibitors (CNIs), graft-*versus*-host disease (GvHD), and viral infection are risk factors for TA-TMA (2, 3). Patients with TA-TMA are characterized by microangiopathic hemolytic anemia, unexplained thrombocytopenia, elevated lactate dehydrogenase (LDH), and endothelial injury-related organ failure, such as hypertension, chronic kidney disease (CKD), pulmonary hypertension, gastrointestinal or central nervous system disease (4). TA-TMA is mainly defined with two standard diagnostic criteria. One is the International Working Group (IWG) (thrombocytopenia in the blood; new-onset, prolonged or progressive thrombocytopenia; sudden and persistent elevation of LDH; decreased hemoglobin or increased transfusion requirements, decreased serum hemoglobin) (5), and the other is overall-TMA (O-TMA) as demonstrated by Cho et al. (Elevated LDH; new-onset thrombocytopenia with a platelet count $<50 \times 10^9$/L or thrombocytopenia >50%; new-onset anemia with hemoglobin below the lower limit of normal or requiring transfusion support; the presence of typed cells in peripheral blood or histological evidence of microangiopathy in tissue specimens; no coagulation dysfunction, negative Coombs test) (6). The mechanism of how systemic microvascular endothelial injury leads to TA-TMA remains unclear. Due to its similar histomorphology to atypical hemolytic uremic syndrome (aHUS), most studies suggest that it is dysregulation of the complement system that causes TA-TMA to occur (7–9). Subsequently, C5 is cleaved to C5a and C5b, forming a cell membrane attack complex (MAC, C5b-9) on the surface of the endothelial cells, resulting in further endothelial cell damage (8). Significantly elevated plasma C3b, sC5b-9 levels were observed in TA-TMA patients (10).

There is no consensus on care strategies for TA-TMA. Conventional treatments, including supportive care measures, withdrawal of CNIs, therapeutic plasma exchange (TPE) and pharmacological treatments such as rituximab, defibrotide, and daclizumab have been used in the treatment of TA-TMA (8). Recently, the use of Eculizumab for the treatment of TA-TMA has raised concerns. With more and more case reports reaching remission (11–13), Eculizumab has shown its benefits in the treatment of patients with TA-TMA. Eculizumab is a terminal complement inhibitor that works by inhibiting the cleavage of C5 to C5a and C5b. C5b-9 is then blocked from forming on the surface of endothelial cells (14). Since the FDA approved Eculizumab for the treatment of atypical hemolytic uremic syndrome (aHUS) (15), TA-TMA patients treated with Eculizumab were treated according to the standard regimen of aHUS (16). Patients received induction therapy with Eculizumab 900mg once a week for four weeks. When hematological signs of TA-TMA resolved, maintenance therapy was continued with 1,200 mg given every two weeks (17, 18). Some researchers have paid attention to evaluate the therapeutic benefits of Eculizumab in patients with TA-TMA. A study by Joslyn et al. showed a hematological response rate of 70% in patients with TA-TMA after Eculizumab treatment (19), similar to the 67% reported by Michelle et al. (20), but much worse compared to the 93% reported by Stephan et al. (21). In addition, the survival rate of 60% (19) studied by Joslyn et al. was similar to that of Michelle et al.'s 67% (20) and more favorable than that of Stephan et al. (33%) (21). The efficacy results vary from study to study.

To determine which factors may contribute to diversity in response rates and survival, we systematically reviewed relevant studies of Eculizumab in patients with TA-TMA and performed a meta-analysis to better understand the efficacy and safety of Eculizumab.

## MATERIALS AND METHODS

### Search Strategy

PubMed and Embase databases were searched from their inception up to February 15, 2020, for relevant studies, and publication language was restricted as English. The search strategy was based on the following combined MeSH terms: ((((“Transplantation”[Mesh]) OR Transplantations[Title/Abstract])) AND ((“Thrombotic Microangiopathies”[Mesh]) OR (((Microangiopathies, Thrombotic[Title/Abstract]) OR Microangiopathy, Thrombotic[Title/Abstract]) OR Thrombotic Microangiopathy[Title/Abstract]))) AND (((((((Eculizumab) OR Alexion) OR Soliris) OR 5G1.1) OR H5G1.1VHC +H5G1.1VLC) OR H5G1.1) OR H5G1-1) OR H5G11). The systematic review and meta-analysis were conducted and reported in compliance with the PRISMA statement (22).

### Selection Criteria

Studies eligible in the meta-analysis met the following criteria: (1) Patients developed TMA after hematopoietic stem cell transplantation; (2) Eculizumab was regarded as first-line therapy or second-line therapy; (3) studies are cohort studies and data from case, letter, review, conference abstract were not taken into consideration. (4) Outcomes of this meta-analysis will include complete response, overall response, survival rate, and adverse events (AEs). To minimize bias in the selected pieces of literature, each paper with a title and a general meeting, our inclusion criteria were checked by two reviewers independently. Then full texts were identified and reevaluated carefully. Any disagreements were further discussed and resolved by consulting a senior investigator to reach a consensus.

### Outcome Measures

The diagnosis of TA-TMA was identified according to IWG (5) or O-TMA (6). Hematological response (HR) was defined as disappearance of schistocytes, normalization of LDH and

haptoglobin, and dependence of transfusion. Complete response (CR) was defined as hematological response with resolution of organ dysregulation caused by TMA. Among outcomes of patients undergoing Eculizumab therapy, which included hematological response (HR), complete response (CR) and no response (NR), an effective overall response (OR) was composed of CR and HR. The survival rate (SR) was evaluated at the last follow-up of each study. Adverse events were reported at baseline and a follow-up visit with a focus on meningococcal infections, serious infections, sepsis, hepatic impairment, infusion reaction, and death.

## Data Extraction

We extracted general characteristics, including the surname of the first author, year of publication, setting, sample size from each included study. Pretreatment patient data collected included age, gender, primary disease, type of transplant, diagnostic criteria, the level of serum sC5b-9, time from HSCT to TA-TMA diagnosis, time from TA-TMA diagnosis to Eculizumab use. Treatment variables included median days of Eculizumab therapy, median Eculizumab dose, outcomes and prognosis.

## Quality Assessment

The Newcastle–Ottawa Quality Assessment Scale for Cohort Studies was applied to evaluate the quality and risk of bias of included studies (23).

## Statistical Analysis

Efficacy was evaluated by the overall response rate (ORR), complete response rate (CRR), survival rate (SR). Safety of Eculizumab was evaluated by adverse events (AEs) including treatment-emergent adverse events (TEAEs), treatment-related adverse events (TRAEs), serious adverse events (SAEs), and cause of death. All the raw data extracted from the studies were transformed with the Freeman–Tukey double arcsine method. Estimated proportions (ES) with 95% confidence intervals (CIs) were calculated for ratio outcomes. The presence of heterogeneity was assessed by using the chi-square test of heterogeneity and the $I^2$ measure of inconsistency. Higher $I^2$ value and lower P-value indicate a greater degree of heterogeneity, and $I^2$ values ≤25%, between 25 and 50%, and ≥50% were equal to low, median, and substantial heterogeneity, respectively. A random-effects model was used regardless of heterogeneity. Considering some significant factors might affect clinical response, survival and prognosis, subgroup analyses and meta-regression for the overall response rate (ORR) and survival rate (SR) were performed based on publication year, setting, sample size, age, primary disease, median days between transplant to TA-TMA, Eculizumab as first-line therapy, overall median therapy duration, the median number of Eculizumab doses if relevant data were available. The p-value of meta-regression of publication <0.05 accounted for the existence of heterogeneity. Funnel plots were inappropriate to perform as the total number of included studies were six (<10). Sensitivity analyses were conducted further to decide the stability and reliability of the results we performed by deletion of every single investigation. All statistical analyses were conducted using R (version 3.6.2). A two-tailed P value of less than 0.05 was considered statistically significant.

# RESULTS

## Data Sources

In all, 592 publications were initially identified based on literature search parameters (**Figure 1**). A total of 98 were discarded for duplicates, and 482 records were removed by inspecting the titles and abstracts based on prospective search criteria. After full-text evaluations of the remaining 12 articles, six were considered to be eligible for the systematic review and meta-analysis according to the selection criteria (19–21, 24–26). The necessary information of the included six articles was summarized in **Table 1**. These articles were published, ranging from 2015 to 2020. TA-TMA was diagnosed mainly by adopting O-TMA proposed by Cho et al. Treatment with Eculizumab was primarily administered following the recommended dose for aHUS. And for pediatric patients, the dose of Eculizumab followed the protocol of Jodele et al. Baseline characteristics of patients including age, gender, primary disease, type of transplant and Eculizumab treatment, and outcomes of efficacy and safety endpoints were described in **Table 2**. TA-TMA was diagnosed at a median age of 23 years (range1.2–66) post-transplantation. Most patients had transplant performed for hematological malignancy, neuroblastoma, as well as immune deficiency. Of patients with available information, 82.8% received allogeneic hematopoietic cell transplantation, 69.9% received CNI treatment at the time of diagnosis, aGvHD occurred in 39.7%, and viral infection was 26.7%.

## Efficacy Outcomes

A total of six articles, including 116 patients were eligible for the analysis of overall response rate (ORR) (19–21, 24–26). It showed that the heterogeneity among the included studies was median ($I^2$ = 30%, P = 0.21). Pooled result of ORR in TA-TMA patients treated with Eculizumab was 71% (95%CI: 58–82%) (**Figure 2**). Subgroup analysis and meta-regression were conducted to evaluate the potential effects of setting, sample size, age, primary disease, median days between transplant to TA-TMA, Eculizumab as first-line or second-line, therapy duration, the median number of Eculizumab doses (**Table S1**). Subgroup analysis of setting showed that the pooled ORR of single-center (ORR = 74%, 95%CI:57–88%) was numerically higher than that of multicenter (ORR = 63%, 95%CI:35–87%). Subgroup analysis of the number of Eculizumab dose showed that the pooled ORR of dose ≥8 was 75% (95%CI:58–89%), which is higher than that <8 (ORR = 59%, 95%CI:37–80%). The therapy duration <60 days achieved a higher survival rate (ORR = 84%, 95%CI:57–100%) than that ≥60 days (ORR = 64%, 95%CI:54–75%). However, the p-value of meta-regression of variables were all >0.05, which did not account for the existence of heterogeneity. The application of sensitivity analysis showed that the study by Stephan et al. (21) impacted

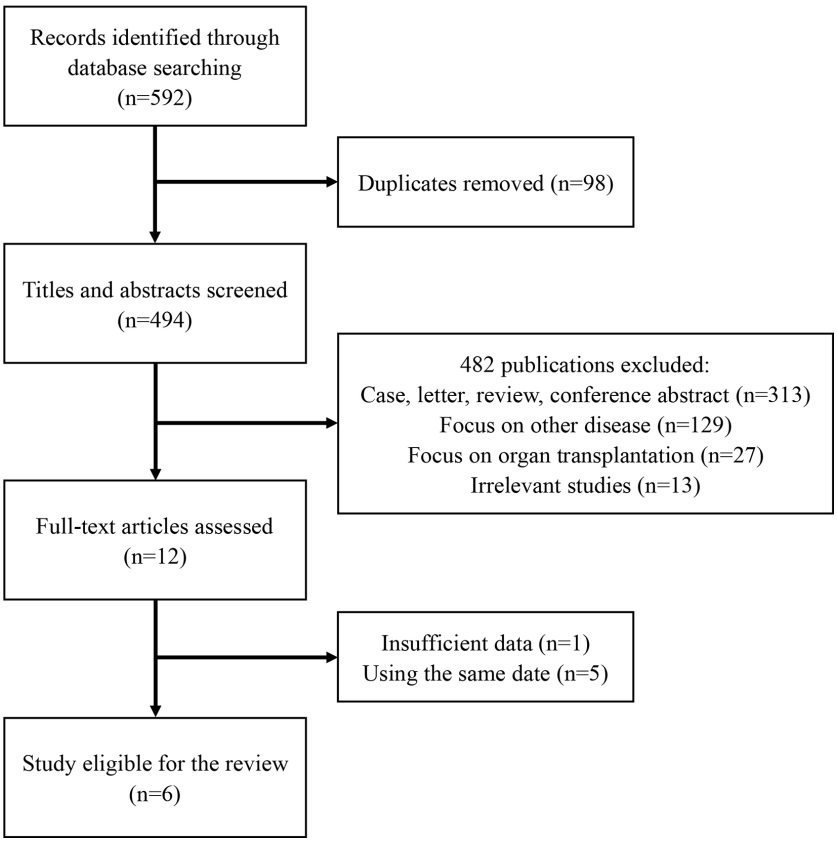


**FIGURE 1** | Preferred Reporting Items for Systematic Reviews and Meta-Analyses analysis.

the overall results (**Figure S1**), which was a historically controlled, single-center study as opposed to the other observational studies.

Among these included studies, five studies reported the complete response rate (CRR) for TA-TMA patients receiving Eculizumab treatment (19, 20, 24–26). The heterogeneity among included studies was substantial ($I^2$ = 73%, P < 0.01) and the pooled estimate of CRR was 32% (95%CI: 11–56%), which was much lower than overall response (**Figure 3**). As the number of studies was limited, the source of heterogeneity could not be analyzed by meta-regression. Sensitivity analysis of CRR in TA-TMA patients treated with Eculizumab informed that Sonata et al. (26) might be the source of heterogeneity (**Figure S2**). The CRR of the study was 88% which was much higher than other studies, and it is an observational study consisting of 64 pediatric patients diagnosed as high-risk TA-TMA. All the patients were offered Eculizumab as first-line therapy, and the median number of Eculizumab doses given was 11, which is more than that of other studies.

The survival rate (SR) of TA-TMA patients treated with Eculizumab was analyzed in six articles (19–21, 24–26). The heterogeneity among the six included studies was low ($I^2$ = 24%, P = 0.25). Pooled estimate of SR was 52% (95%CI: 40–65%) (**Figure 4**). Subgroup analysis and meta-regression were conducted to evaluate the potential effects of publication year, setting, sample size, age, primary disease, median days between transplant to TA-TMA, therapy duration, the median number of Eculizumab doses (**Table S2**). Subgroup analysis of sample size showed that the pooled SR of the size ≤10 (SR = 68%, 95%CI:47–86%) was numerically higher than that >10 (SR = 45%, 95% CI:29–61%). Subgroup analysis of age showed that the pooled SR of pediatric TA-TMA patients (SR = 59%, 95%CI:47–70%) was numerically higher than that of adult patients (SR = 40%, 95% CI:24–57%). Subgroup analysis of primary disease indicated that the pooled SR of hematological disease was 40% (95%CI:24–57%), which is lower than that of primary disease containing the hematological disease and others (SR = 59%, 95%CI:47–70%). The pooled SR of TA-TMA diagnosed during first 100 days after transplant was 59% (95%CI:48–70%), which was significantly higher than that of TA-TMA diagnosed more than 100 days after transplant (SR = 33%, 95%CI:16–53%). The therapy duration of more than 65 days achieved a higher survival rate (SR = 59%, 95%CI:47–70%) than that less than 65 days (SR = 40%, 95% CI:24–57%). P-value of median days between transplant and TA-TMA was 0.0266, which can explain the source of heterogeneity. The result of sensitivity analysis indicated that omitting the study of Prajwal et al. (25) and Stephan et al. (21) may influence the pooled results (**Figure S3**).

**TABLE 1** | Study from which patient-level data were provided and included in meta-analysis.

| Study | Year | setting | diagnostic criteria | Patients with TMA after HSCT in the study | TA-TMA patients with Eculizumab treatment in the study | Eculizumab regime |
|---|---|---|---|---|---|---|
| Flore et al. (24) | 2015 | Multicenter | O-TMA criteria | 12 | 12 | Induction therapy: 900 mg weekly for 4 weeks<br>Maintenance therapy: 1,200 mg every 2 weeks |
| Prajwal et al. (25) | 2016 | Multicenter | O-TMA criteria | 9 | 9 | Adult patients: induction therapy was 900 mg weekly for 4 weeks, followed by 1,200 mg every 2 weeks for maintenance therapy.<br>Pediatric patients: the first dose was based on weight, subsequent dose according to CH50. |
| Stephan et al. (21) | 2017 | Single-center | O-TMA criteria | 39 | 15 | Induction therapy: 900 mg weekly for 4 weeks<br>Maintenance therapy: 1,200 mg every 2 weeks |
| Joslyn et al. (19) | 2018 | Single-center | O-TMA criteria | 10 | 10 | Induction therapy: 900 mg weekly for 4 weeks<br>Maintenance therapy: 1,200 mg every 2 weeks |
| Michelle et al. (20) | 2019 | Single-center | Before 2010, IWG criteria After 2010, O-TMA criteria | 9 | 6 | Adult patients: induction therapy was 900 mg weekly for 4 weeks, followed by 1,200 mg every 2 weeks for maintenance therapy.<br>Pediatric patients: the first dose was based on weight, subsequent dose according to CH50. |
| Sonata et al. (26) | 2020 | Single-center | O-TMA criteria | 177 | 64 | The first dose was based on weight, subsequent dose according to CH50. |

*CH50, a total hemolytic complement activity level; HSCT, Hematopoietic stem cell transplantation; IWG, International Working Group; O-TMA, overall-TMA; TA-TMA, Transplant associated thrombotic microangiopathy.*

## Safety Outcomes

All six studies were reported that treatment of TA-TMA with Eculizumab was well tolerated (19–21, 24–26). Among 116 patients, only one case was reported to get severe skin rash leading to drug discontinuation during Eculizumab therapy (19). Three studies were reported that some patients developed the infection after starting Eculizumab therapy for TA-TMA (21, 24, 26), and no meningococcal infections were reported. After the Eculizumab therapy, many survivors suffered from CKD and HTN, and much of them were still depend on dialysis (**Table 2**). As the data from the studies were limited, further studies need to analyze the prognosis.

From a total of 55 subjects who died, cause of death can be divided into four risk factors: GvHD, infection, TA-TMA related organ failure, relapse of disease, which were presented in **Figure 5**. Among these risk factors, the proportion of infection was 31% (95%CI:6–61%), which is much higher than the other three factors. And the proportion of GvHD was 26% (95%CI:2–59%). TA-TMA related death was occupied 23% (95%CI:10–38%). Death related to relapse of primary disease was the least. No study was reported that death is related to the use of Eculizumab.

## Risk of Bias

The Newcastle-Ottawa scale was used to assess the risk of bias. The NOS scores of every study ranged from 7 to 9, with an average of 7.7. The detailed information of NOS scores is shown in **Table S3**.

# DISCUSSION

In all six observational studies, including 116 patients, were included in a systematic review and meta-analysis to investigate the efficacy and safety of Eculizumab in patients with TA-TMA (19–21, 24–26). After Eculizumab treatment, almost 71% of patients responded to the therapy. However, the number of patients who reached full response was significantly lower (32%). Patients with TA-TMA treated with Eculizumab had a 52% survival rate at a median follow-up of 13.5 months after HSCT. Previous treatment strategies for TA-TMA after HSCT have focused on conventional therapies, including withdrawal of CNIs, plasmapheresis, defibrillation, rituximab, and combinations of several therapies (8). A retrospective study showed that 24% of TA-TMA patients (n = 33) underwent plasmapheresis and achieved a clinical response with an SR of 45% at 100 days after diagnosis (27). Corti et al. reported a total of 12 TA-TMA patients undergoing defibrillation in two centers with an ORR and SR of 67 and 50%, respectively (28). Another study by Au et al. showed an ORR of 80% (n = 4/5) for rituximab treatment and a study SR of 60% at a median follow-up of 305 (250–440) d (29). Due to the small sample size reported above, it is difficult to perform a systematic and comprehensive comparison between conventional therapy and Eculizumab. Nonetheless, the summary ORR and SR of Eculizumab from our meta-analysis appeared higher than traditional treatment.

A retrospective study by Prajwal et al. provided an evaluation of efficacy for TA-TMA patients treated with Eculizumab (25). Although they also demonstrated higher response rates and survival in TA-TMA patients treated with Eculizumab, the articles they included were mostly cases (11–13). Cases usually report successful treatment rather than unsuccessful treatment. The benefit of Eculizumab may be significantly overestimated, and it is difficult to explain the heterogeneity between cases. In addition, this retrospective analysis focused on patients with

**TABLE 2 |** Baseline characteristics for patients included in meta-analysis.

| Variable | Flore et al. (n = 12) | Prajwal et al. (n = 9) | Stephan et al. (n = 15) | Joslyn et al. (n = 10) | Michelle et al. (n = 6) | Sonata et al. (n = 64) |
|---|---|---|---|---|---|---|
| Age, median (range) | 39(1.2–66) | 7(2–61) | 48(23–66) | 44(17–59) | 5.2(2.5–25) | 5.5(2.7–11.7) |
| Gender, male (%) | 7(58) | 6(67) | 7(47) | 4(40) | 4(67) | 40(63) |
| Primary disease | Hematological disease | Hematological disease/others | Hematological disease | Hematological disease | Hematological disease/others | Hematological disease/others |
| Type of transplant | Allo/UCB | Allo/Auto | Allo | Allo/UCB | Auto | Allo/Auto/UCB |
| Conditioning regimen | MAC/RIC | MAC/RIC | MAC/RIC | MAC/RIC | MAC | MAC/RIC |
| Other risk factors at diagnosis, number (%) | | | | | | |
| CNI used | 8(67) | 7(78) | 9(60) | 10(100) | NA | 49(77) |
| aGvHD | 8(67) | 5(56) | 12(80) | 7(70) | 0(0) | 14(22) |
| Affection | 6(50) | 2(22) | 8(67) | 8(80) | 1(17) | 6(9) |
| Interval between HSCT and diagnosis, median days | 121 | 68 | 264 | 93 | 35 | <100[a] |
| sC5b-9 | NA | NA | 456(127-810) | NA | 151.5(100-460) | 398(282-544) |
| Interval between diagnosis and Eculizumab therapy, median days | 31 | 24 | 10 | 4 | 18 | NA |
| Eculizumab therapy, median days | 65 | 178 | 52.5 | 48.5 | 110 | 66 |
| First-line therapy, number (%)/second-line therapy, number | 5(42)/7 | 2(22)/7 | 11(73)/4 | 7(70)/3 | 6(100)/0 | 64(100)/0 |
| Eculizumab dose, median dose | 6 | 8 | 9 | 6 | 9.5 | 11 |
| Overall response, number (%) | 6(50) | 7(78) | 13(93)[b] | 7(70) | 4(67) | 41(64) |
| Complete response, number (%) | 2(17) | 5(56) | NA | 1(10) | 1(17) | 36(56) |
| Survivals, number (%) | 4(33) | 7(78) | 5(33) | 6(60) | 4(67) | 35(55) |
| Median follow-up months | 14 | 12 | 8 | 13 | 30 | 15 |
| AEs during Eculizumab therapy | Infection | No | Infection | Skin rash | NA | Infection |
| Cause of death, numbers (%) | | | | | | |
| TA-TMA related | 4(50) | 0 | 2(20) | 0 | 1(50) | 8(28) |
| Infection | 2(25) | 0 | 8(80) | 2(50) | 0 | 6(21) |
| GvHD | 2(25) | 2(100) | 0 | 1(25) | 0 | 14(48) |
| Relapse of the primary disease | 0 | 0 | 0 | 1(25) | 1(50) | 1(3) |
| Prognosis | CKD | CKD | CKD | CKD | CKD/HTN | CKD/HTN |

*Allo,; Auto, Autologous HSCT; CNI, calcineurin inhibitors; HTN hypertension; MAC, myeloablative regimen; RIC, reduced intensity regimen; UCB, umbilical cord blood.*
[a]*As 92% patients were diagnosed TA-TMA at a median of 23 days (IQR 3–48), and five had TA-TMA between 118 and 221 days after transplant, we regarded that the median days of interval between HSCT and diagnosis was less than 100 days.*
[b]*one unknown response due to early death.*

refractory discontinuation of calcineurin inhibitors and plasma exchange in patients with TA-TMA. Eculizumab was considered second-line therapy, whereas patients receiving Eculizumab as first-line therapy were not considered. What's more, adverse events during Eculizumab treatment were not analyzed. Our current meta-analysis incorporates the largest number of observational studies to date. Among these included studies, we joined the largest cohort to date, which included the terminal complement blocker Eculizumab as first-line treatment in patients with TA-TMA, which provides a higher weighting in the meta-data. Subgroup analysis and meta-regression were performed to detect heterogeneous sources. A sensitivity analysis was performed to demonstrate the stability and reliability of the findings. More importantly, our study provides not only a pooled assessment of efficacy but also of AEs and cause of death. Therefore, the current meta-analysis is a more comprehensive and credible analysis of the effectiveness and safety of Eculizumab for TA-TMA.

Because efficacy outcomes vary widely across centers, our current study analyzed which factors may contribute to differences in response rates and survival rates. Since the p-values of meta-regression were all greater than 0.05, the subgroup analysis of ORR could not explain the source of the difference. Flore et al. (24) studied the lowest ORR compared to four other studies (19–21, 26). Due to the multicenter and retrospective nature of the study, there was heterogeneity in patient inclusion criteria, primary disease, stem cell transplantation characteristics, and Eculizumab regimens. It seems that this setup may be the source of the ORR discrepancy. More importantly, the overall response in the study results was good, but the complete response rate was meagre. This indicates that the kidney damage is advanced by the time of Eculizumab treatment. As a result, most patients do not get organ recovery. From this, it appears that the delay in Eculizumab treatment may prevent patients from achieving optimal response and maximum recovery of organ function. However, in the study by Joslyn et al., the median duration between diagnosis and Eculizumab therapy was shorter, at four days, compared to other studies. Only one patient achieved a complete response and organ function was restored (19). This suggests that earlier initiation of Eculizumab may have no significant effect on restoring organ function. How to improve the full response rate remains a big question. Our subgroup analysis of TA-TMA patient survival revealed that the time between HSCT and TA-TMA diagnosis is a potential source of SR heterogeneity, as the p value for meta-regression for days

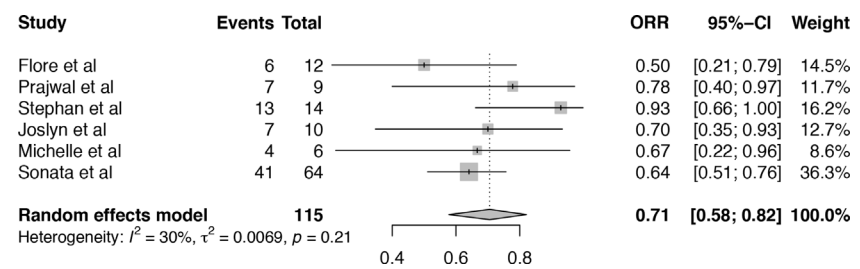


**FIGURE 2** | Forest plot of the estimated proportions (95% CI) for overall response rate (ORR) of the TA-TMA patients after Eculizumab treatment.

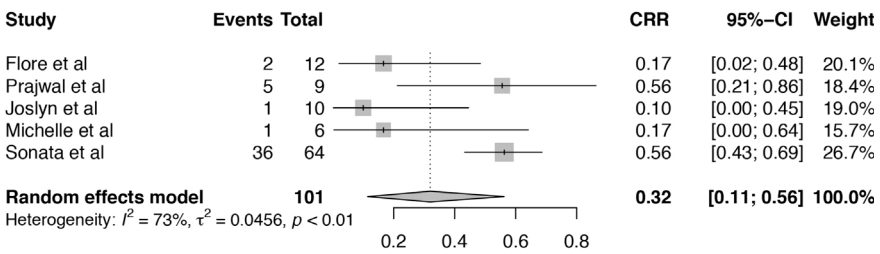


**FIGURE 3** | Forest plot of the estimated proportions (95% CI) for complete response rate (CRR) of the TA-TMA patients after Eculizumab treatment.

between transplantation and TA-TMA is 0.0266. TA-TMA diagnosed within the first 100 days after transplantation is more susceptible to publication bias than TA-TMA more than 100 days after transplantation. Therefore, early diagnosis of TA-TMA is the key to successful treatment of TA-TMA. However, the early diagnosis of TA-TMA faces challenges due to overlapping clinical features and the lack of standard diagnostic criteria as most studies support the idea that unregulated complement activation leads to the development of TA-TMA occurrence (7–9). The search for sensitivity and specificity of complement activation monitoring biomarkers should be of interest. Recently, Orsolya et al. showed that early elevation of sC5b-9 is a predictor of late development of TA-TMA (30). In this study, sC5b-9 levels increased from baseline levels to day 28 in patients with TA-TMA (n = 10), while the same trend was observed in only nine patients (p = 0.031) without TA-TMA (n = 23). In our meta-analysis, sC5b-9 levels were documented in three articles, and elevated sC5b-9 was observed in TA-TMA patients in two studies. However, the timing of the detection of sC5b-9 levels was not elaborated. Further studies are needed to determine whether terminal pathway activation is an independent predictor of TA-TMA after HSCT. Median age and primary disease may be another two factors contributing to significant differences between studies though their p-values of meta-regression are both 0.0827. Children seem to achieve higher SR than adults. In addition, SR of TA-TMA patients under treatment of Eculizumab reported in the studies which focused on hematological disease is lower than that of other studies. Sensitivity analysis of ORR and SR showed that the investigation by Stephan et al. (21) was a source of heterogeneity in SR of this meta-analysis. The SR (33%) in this single-center analysis was significantly lower compared to other studies. Patients in this study were diagnosed as hematological disease and the median age was 48 years, which was older than that of other five reports included. Additionally, the median days from hematopoietic stem cell transplantation to TA-TMA diagnosis were 264 days, the longest in all the studies.

The safety profile seems to indicate that Eculizumab is well tolerated. A more substantial observational data set covering a 5-year registry of patients with aHUS reported that no new safety issues were identified in patients treated with adult or pediatric Eculizumab (31). In our meta-analysis, only one case of TRAEs, *i.e.* one patient with a severe rash, was reported, resulting in discontinuation of Eculizumab therapy. The most commonly reported AEs are infections. Eculizumab is a monoclonal antibody that inhibits C5 cleavage and prevents terminal complement activation (4). Patients treated with Eculizumab have an increased risk of infection, especially meningococcal infections, due to the lack of adequate functional complement (17). Whereas a study by Sonata et al. reported no cases of meningococcal infections in patients who had not received the meningococcal vaccine (32). And in our present study, no meningococcal infections were reported, which corresponds to the findings of Sonata et al. However, among patients treated with Eculizumab, the highest number of deaths due to infection was seen in the study by Stephan et al. Based on their report, an

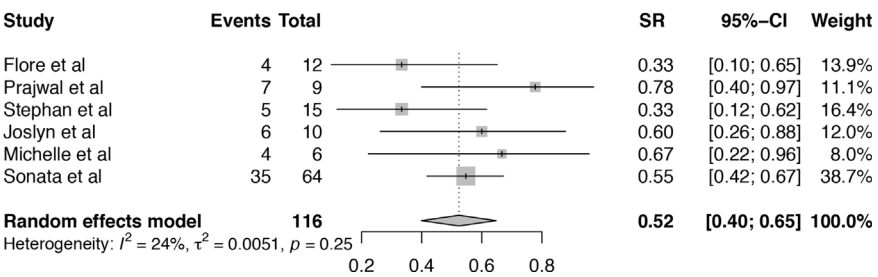


**FIGURE 4** | Forest plot of the estimated proportions (95% CI) for survival rate (SR) of the TA-TMA patients after Eculizumab treatment.

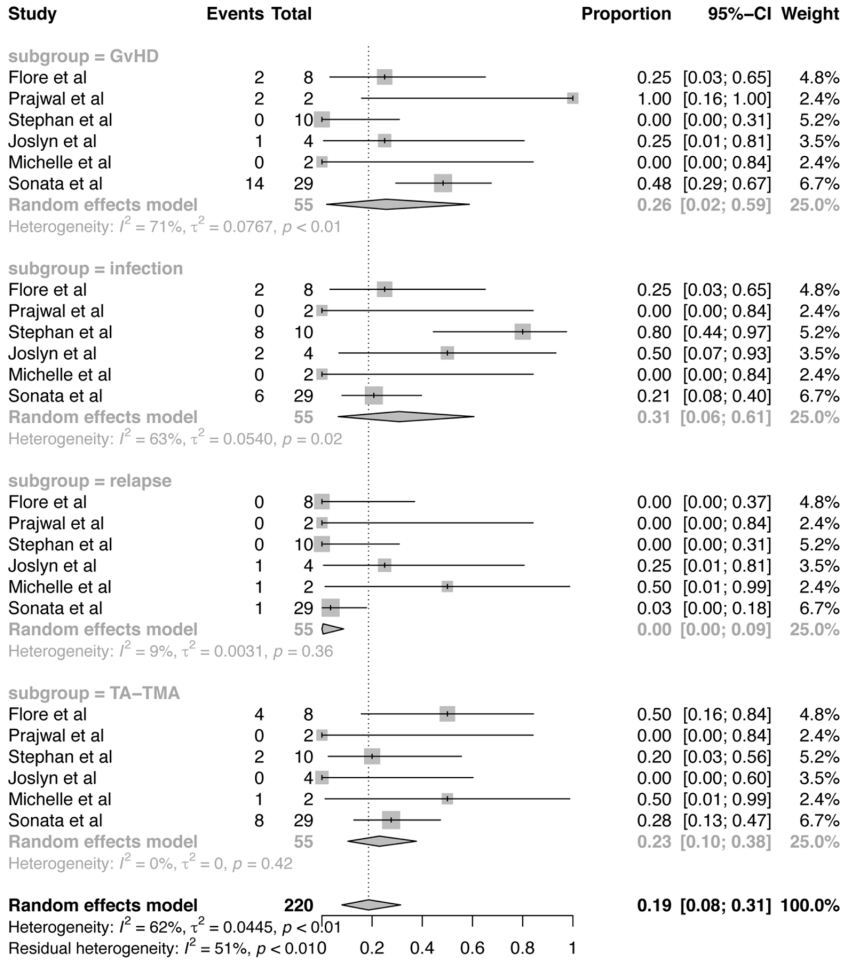


**FIGURE 5** | Forest plot of the estimated proportions (95% CI) for cause of death after Eculizumab treatment.

increase in mortality due to infection in the group treated with Eculizumab was found compared to the conventional treatment group (21). Therefore, precaution and treatment of infection are equally urgent during the treatment of TA-TMA. GvHD is a risk factor that leads not only to TA-TMA but also to death during treatment. It has been shown that GvHD almost always precedes the diagnosis of TA-TMA, and there may be a mechanical link between TA-TMA, GvHD, and endothelial injury (33). Another study reported that the occurrence of TA-TMA was associated with risk factors such as aGvHD grade ≥2, steroid-refractory aGvHD, and CMV reactivation/end-organ disease, but not with conditioning regimen (RIC or MAC), TBI use or TBI dose, primary condition, donor type, age, or gender. More importantly, patients diagnosed with TA-TMA combined with

aGvHD had significantly lower overall survival compared to patients with TA-TMA alone or GvHD (median 5.6 *vs.* 7.6 *vs.* 55.4 months; $p < 0.0001$) (34). The relationship between TA-TMA and GvHD is unclear. Future studies should provide information on the relationship between GvHD and Evidence of the TA-TMA link. TA-TMA itself is another major cause of death because of endothelial injury-related organ failure. It is necessary to explore how endothelial cells are damaged. One study from our center has reported that heme oxygenase-1 (HO-1) was significantly decreased in patients with TA-TMA and suppressed oxidative stress could attenuate complement deposition in TMA plasma-challenged HUVECs (35). The nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor, initiates transcription of the HO-1 gene to protect cells from oxidative stress (36). Further experimental study about Nrf2 and endothelial injury is undertaken in our center.

Another area that needs to be discussed is how much Eculizumab needs to be given to achieve a hematological response and the duration of Eculizumab treatment for TA-TMA. For adult patients, the dose of induction therapy was 900 mg per week for four weeks. If the patient responds to induction therapy, the treatment is maintained at 1,200 mg administered every two weeks. For pediatric patients, the initial dose is based on body weight, and subsequent dose adjustments are based on maintaining total hemolytic complement activity (CH50) levels. Patients weighing less than 40 kg started at 600 mg and others at 900 mg. Induction therapy is also administered weekly for four weeks, and CH50 should be maintained at complement activator enzyme (CAE) levels of 0 to 3 (18, 37). The treatment then transitions to maintenance therapy, which adequately suppresses CH50 to a scale of three CAE (4) and then to maintenance therapy. Regarding the question of when Eculizumab can be safely discontinued, the study by Prajwal et al. proposed that ECU can be suspended after determining clinical symptoms and laboratory manifestations (38).

There are still some potential limitations to our study. First, there is a complete lack of randomized controlled trials and a limited study population size, and investigators have conducted limited studies on the efficacy of Eculizumab for TA-TMA. Second, although there is a great deal of heterogeneity among the included studies, the limited number of included studies prevents us from analyzing the sources of heterogeneity. Third, AEs are generalized in the article, so we do not have access to security data for AEs. Despite these limitations, our review is the first comprehensive meta-analysis of all eligible studies that analyzed the efficacy and safety of Eculizumab in patients with TA-TMA.

## CONCLUSION

This systematic review and meta-analysis suggest that Eculizumab improves SR and ORR in patients with TA-TMA. Furthermore, patients with TA-TMA diagnosed within the first 100 days after HSCT are more likely to achieve better outcomes with Eculizumab compared to patients with TA-TMA diagnosed more than 100 days after HSCT. In addition, Eculizumab is well-tolerated, but the prevention and treatment of infection still require attention. Further RCTs and extensive prospective cohort studies are needed to evaluate efficacy and safety, particularly for Eculizumab for TA-TMA.

## 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 in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

RZ, MZ, and JQ contributed to the study conception and design, and writing the manuscript. RZ, WM, and ZZ performed data collection and analysis. DW and YH commented on the research design, data analysis, writing the manuscript, and supervision of the study. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.564647/full#supplementary-material

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SYSTEMATIC REVIEW
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# TKI Maintenance After Stem-Cell Transplantation for *FLT3*-ITD Positive Acute Myeloid Leukemia: A Systematic Review and Meta-Analysis

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This analysis aimed to systematically review and synthesize the existing evidence regarding the outcome of tyrosine kinase inhibitor (TKI) maintenance therapy after allogeneic stem-cell transplantation for patients with *FLT3*-ITD-mutated acute myeloid leukemia (AML). We searched publicly available databases, references lists of relevant reviews, registered trials, and relevant conference proceedings. A total of 7 studies comprising 680 patients were included. Five studies evaluated sorafenib and 2 studies evaluated midostaurin, compared with control. The incidence of relapse was significantly reduced after TKI therapy, showing an overall pooled risk ratio (RR) of 0.35 (95% confidence interval [CI], 0.23-0.51; $P < 0.001$), with a marked 65% reduced risk for relapse. The overall pooled RR for relapse-free survival and overall survival showed significantly improved outcome after TKI maintenance therapy, being 0.48 (95% CI, 0.37–0.61; $P < 0.001$) and 0.48 (95% CI, 0.36–0.64; $P < 0.001$). The risk for relapse or death from any cause was reduced by 52% using TKI. No difference in outcome was seen for non-relapse mortality, and the risk for chronic or acute graft-vs. -host disease appeared to be increased, at least for sorafenib. In conclusion, post-transplant maintenance therapy with TKI was associated with significantly improved outcome in relapse and survival in patients with *FLT3*-ITD positive AML.

**Keywords: sorafenib, midostaurin, maintenance, allogeneic stem cell transplantation, FLT3-internal tandem duplication, acute myeloid leukemia, graft-vs.-host disease**

## INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy derived from hematopoietic stem cells with a series of abnormalities on the level of cytogenetics, genetics, and epigenetics (1, 2). Prognosis of this disease varies widely according to mutation profile, patient age, and comorbidities (2, 3). The duplication in Fms-like tyrosine kinase 3-internal tandem (*FLT3*-ITD) occurs in about 25% of adult AML patients (4–7). Patients harboring *FLT3*-ITD, particularly those with a high allelic ratio, show increased relapse rates and inferior survival, despite undergoing allogeneic stem-cell transplantation (6, 8).

In the front-line setting of *FLT3*-mutated AML, combining conventional chemotherapy with a multi-targeted tyrosine kinase inhibitor (TKI), namely midostaurin, resulted in improved overall survival (9). Another multi-targeted TKI, sorafenib, has been approved for solid tumors such as hepatocellular and renal cell cancer (10, 11), but it has also shown efficacy in terms of prolonged progression-free survival in younger AML patients in combination with upfront chemotherapy (12), but not in the elderly population (13). In the relapsed/refractory setting, patients with *FLT3*-ITD-positive AML receiving TKI monotherapy showed promising outcomes (14–16), while this approach may remain a palliative strategy which is furthermore limited by emerging TKI resistance (17, 18). In contrast, when patients with *FLT3*-ITD-mutated AML relapsing after allogeneic stem-cell transplantation received sorafenib, the outcome may differ profoundly, as suggested by long-term remissions in selected patients (19, 20).

To reflect the increasing interest within clinical and basic research, we aimed to systematically review the current body of literature and to synthesize the existing evidence regarding the outcome of TKI maintenance therapy after allogeneic stem-cell transplantation for patients with *FLT3*-ITD-mutated AML.

# METHODS

The methodology of this systematic review with meta-analysis was undergone in accordance with the Cochrane handbook. Further, dimensions of reporting were assessed with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines and the meta-analysis of observational studies in epidemiology (MOOSE) checklist and adhered accordingly (21, 22). The research question was defined using the PICOS framework: population, FLT3-ITD mutated AML; intervention, stem-cell transplantation with TKI maintenance; comparator, placebo, or no maintenance; outcome, survival and relapse; study design, retrospective and prospective comparative studies.

## Search Strategy

Medline and the Cochrane Library were searched (until August 11, 2020, respectively). Additionally, meeting abstracts archived between 2017 and 2020 from hematology/oncology meetings were screened. Review of clinicaltrials.gov was performed until August 11, 2020. The search strategy consisted of keywords specific to each database and considered all trial designs of human subjects and was not restricted by language. Search terms included all subject headings and associated keywords for "sorafenib or midostaurin or gilteritinib" and "leukemia or leukemia." Reference lists of relevant reports were reviewed in addition.

## Study Selection, Data Extraction, and End Points

Two reviewers (NG and NK) independently screened titles, abstracts, and the full text of relevant articles. Disagreements were resolved by consensus. Studies were included if they fulfilled the following criteria: adult patients with *FLT3*-ITD AML; prospective or retrospective studies reporting on patients receiving TKI therapy after stem-cell transplantation; evaluating a comparison with a control; reporting at least on relapse-free survival and/or cumulative incidence of relapse.

The following information was extracted from the included studies: the name of the first author, year of publication, study design, TKI treatment, control, number of participants, conditioning intensity for stem-cell transplantation, frequency of high-risk cytogenetics within the studied population, length of follow-up, and primary, and secondary outcomes. Primary end points for data synthesis were relapse-free survival and cumulative incidence of relapse. Secondary end points were overall survival, non-relapse mortality, chronic and acute graft-vs. -host disease (GVHD). Relapse-free survival was defined as time from randomization to first event of either AML relapse or death from any cause in prospective studies or as defined in retrospective studies. Definition of relapse was used in accordance with the included studies.

## Risk of Bias and Quality Assessment

Risk of bias for prospective trials was addressed in accordance with tools developed by the Cochrane Collaboration, and the risk of bias for retrospective comparisons was assessed using the ROBINS-I tool (23). The certainty of the evidence for each outcome was assessed using the grading of recommendations assessment, development, and evaluation (GRADE) approach (24), including considerations of risk of bias, inconsistency, indirectness, imprecision, and publication bias. Retrospective studies were judged a priori as having serious risk of bias, in accordance with the GRADE approach. The resulting overall certainty of the evidence was assessed as high, moderate, low, or very low. All end points within the quality assessment were considered as being of critical importance.

## Data Synthesis and Analysis

Risk ratios (RRs) and 95% confidence intervals (CIs) were calculated for primary and secondary end points by pooling the results from studies using the Mantel-Haenszel method and the random-effects model. Heterogeneity was assessed using $I^2$ and was categorized from moderate to high (25). Prespecified subgroups were different TKIs (midostaurin and sorafenib). All values with $P < 0.05$ were considered statistically significant. Means were calculated for the end point of safety. Analyses were performed using R statistical software version 3.6.1 using the meta and metafor packages (R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/)(26).

# RESULTS

## Search Results

A total of 1050 citations were identified from the electronic database search and from other sources including meeting abstracts. After duplicates were removed, 800 unique citations remained. Based on title and abstract screening, 752 citations were excluded. Forty-one citations were excluded on the basis

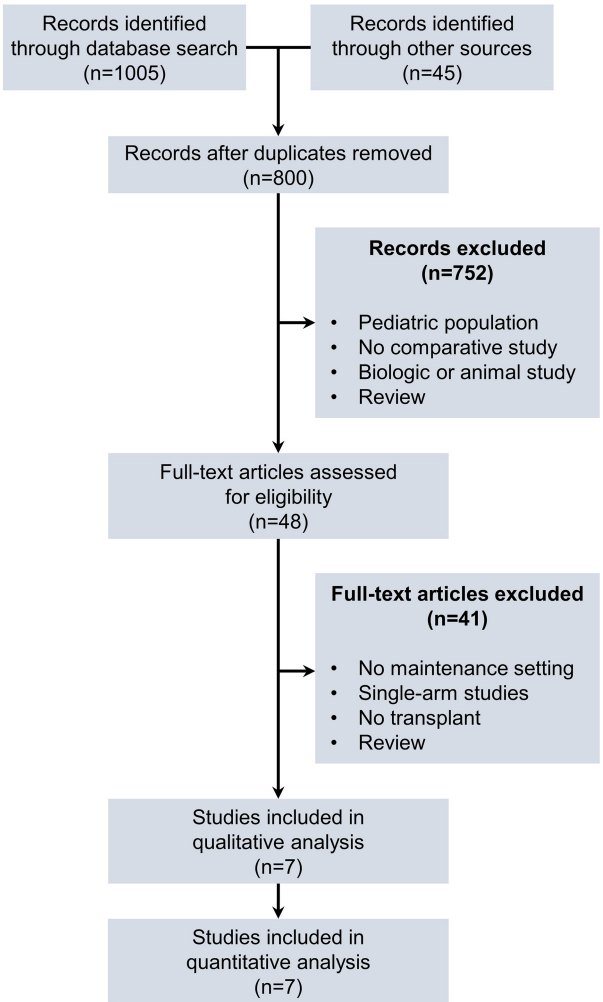


**FIGURE 1 |** Study selection process.

of screening full-text articles. Reasons for exclusion were: studies with no maintenance setting; lack of direct comparison results; no patients undergoing allogeneic stem-cell transplantation; and review articles. Seven studies (27–33) were included in qualitative and quantitative analyses (**Figure 1**).

## Study Characteristics and Risk of Bias

A total of 680 patients were included in the 7 studies. Three studies (28, 31, 32) were prospective randomized studies and 1 study (29) was a prospective study that compared TKI intervention with historical controls using propensity score matching. One prospective study was an abstract, and fully published data were not accessible during finalization of the present manuscript (32). The remaining 3 studies (27, 30, 33) were of retrospective design. Five studies evaluated the efficacy of sorafenib comprising 504 patients while the remaining 2 studies evaluated the TKI midostaurin and comprised 176 patients. Median age in the TKI group ranged from 24 to 55 years and frequency of patients having complete remission at time of transplantation in the TKI group ranged from 61 to 100%. Four studies only used myeloablative conditioning transplantation. Median time of follow-up ranged from 18 to 59 months. The remaining characteristics are summarized in **Table 1**.

The duration of maintenance treatment differed between the studies. Maintenance was administered for 24 months or until occurrence of relapse, or limiting toxicity in Burchert et al. (31) In both studies from Xuan et al. (27, 28) TKI was given until day 180 after transplantation or until intolerable adverse events occurred. Maziarz et al. (32) applied TKI for twelve 4-week cycles. Patients in the study from Shi et al. (33) received TKI maintenance at a median of 238 days (range, 21–385 days). In Brunner et al. (30) TKI therapy was planned for 12–24 months, leaving continuation or early withdrawal to the discretion of the treating physician. Schlenk et al. (29) gave TKI therapy for 365 days.

Low risk of bias was assessed in 2 prospective randomized studies (28, 31), 4 studies showed moderate risk of bias (16, 27, 30, 33), and 2 studies conferred high risk of bias (29, 32). Overall, the risk of bias of the included studies according to each end point was judged to be serious. Publication bias could not be assessed due to the number of <10 studies included in the analysis, which is in accordance with the Cochrane handbook recommendations. **Supplementary Tables 1**, **2** depict the summary of the risk of bias profile for each dimension within each study and **Supplementary Table 3** summarizes the quality of evidence for each end point.

## Relapse-Free Survival and Incidence of Relapse

The primary end point of relapse-free survival was assessed in all 7 studies at 18–59 months follow-up. The overall pooled RR showed significantly better relapse-free survival after TKI therapy, being 0.48 (95% CI, 0.37–0.61; $P < 0.001$) with no relevant heterogeneity ($I^2 = 0\%$, **Figure 2A**). The quality of the evidence was high. Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.21$). However, the pooled RR for midostaurin was 0.60 (95% CI, 0.39–0.94; $I^2 = 0\%$) while a larger effect was seen for sorafenib, being 0.43 (95% CI, 0.32–0.58; $I^2$=0%), compared with control.

Incidence of relapse was assessed in six studies. The overall pooled RR showed significantly reduced incidence of relapse, being 0.35 (95% CI, 0.23–0.51; $P < 0.001$) in favor of the TKI therapy with no relevant heterogeneity ($I^2 = 0\%$, **Figure 3A**). The quality of the evidence was high. Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.72$). One study evaluated midostaurin, with a pooled RR of 0.43 (95% CI, 0.12–1.50). Sorafenib showed significantly reduced incidence of relapse showing a RR 0.34 (95% CI, 0.22–0.51; $I^2 = 0\%$), compared with control.

## Overall Survival and Non-relapse Mortality

Significantly improved outcome for TKI therapy was also seen in overall survival, which was assessed in 6 studies. The overall pooled RR was 0.48 (95% CI, 0.36-0.64; $P < 0.001$) in favor of the TKI therapy with no relevant heterogeneity ($I^2 = 0\%$, **Figure 2B**). The quality of the evidence was high. Subgroup analyses showed no significant difference in outcome between midostaurin and

**TABLE 1 |** Study characteristics.

| Study | Design | N | Age in TKI group (range) | TKI | Comparator | Myeloablative conditioning | CR at transplant[b] | High-risk cytogenetics | Length of follow-up |
|---|---|---|---|---|---|---|---|---|---|
| Burchert et al. (31) | Randomized phase 2 | 83 | 54 (23–74) | Sorafenib | Placebo | TKI: 42%, Placebo: 47% | TKI: 63%, placebo: 48% | TKI: 2%, Placebo: 8% | 42 months |
| Brunner et al. (30) | Retrospective | 81 | 55 (20–74) | Sorafenib | No TKI | TKI: 54%, No: 49% | 100% (CR1) | 8% | 27 months |
| Schlenk et al. (29) | Prospective phase 2, propensity score matching with historical controls | 116[a] | 54 (18–70) | Midostaurin | Historical control | NR | TKI: 61%, control: 43% | NR | 24 months |
| Xuan et al. (28) | Randomized phase 3 | 202 | 35 (26–42) | Sorafenib | No TKI | 100% | TKI: 73%, no: 77% | TKI: 7%, no: 5% | 21 months |
| Xuan et al. (27) | Retrospective | 82 | 37 (15–55) | Sorafenib | No TKI | 100% | 77% | TKI: 6%, no: 1% | 59 months |
| Maziarz et al. (32) | Randomized phase 2 | 60 | 18–70[c] | Midostaurin | No TKI | 100% | NR | NR | 18 months |
| Shi et al. (33) | Retrospective | 56 | 24 (14–62) | Sorafenib | No TKI | 100% | 100% | 17% | 24 months |

*N, number; TKI, tyrosine kinase inhibitor; HSCT, hematopoietic stem-cell transplantation; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; CR, complete remission; NR, not reported.*

[a] *The original number of patients in the study was 284, here we report on the subgroup analyses of patients that actually underwent midostaurin maintenance after stem-cell transplantation or not.*

[b] *As reported in the patient characteristics of the trials.*

[c] *Inclusion criteria, age distribution not given.*

sorafenib ($P = 0.30$). The pooled RR for midostaurin, which was evaluated only in 1 study, was 0.60 (95% CI, 0.36–1.00). A larger effect was seen for sorafenib after synthesis of the remaining 7 studies, with a RR 0.48 (95% CI, 0.36–0.64; $I^2 = 0\%$), compared with control.

Non-relapse mortality was assessed in 5 studies, which evaluated the efficacy of sorafenib. No significant difference between sorafenib and the control was seen, showing an overall pooled RR of 0.87 (95% CI, 0.51–1.47; $P = 0.60$) with no relevant heterogeneity ($I^2 = 0\%$, **Figure 3B**). The quality of the evidence was low.

## Graft-vs.-Host Disease and Safety

Chronic GVHD was assessed in 6 studies. No significant difference in the incidence was seen, with a trend toward higher incidence after TKI therapy showing an overall pooled RR of 1.14 (95% CI, 0.93–1.41; $P = 0.21$) with no relevant heterogeneity ($I^2 = 0\%$, **Figure 4A**). The quality of the evidence was low. Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.19$). However, the pooled RR for midostaurin was 0.79 (95% CI, 0.43–1.44) while results for sorafenib suggested higher risk for chronic GVHD showing a RR of 0.43 (95% CI, 0.32–0.57; $I^2 = 0\%$), compared with control.

Similar results were yielded for acute GVHD, which was assessed in six studies. The overall pooled RR was 1.22 (95% CI, 0.96–1.55; $P = 0.10$) with no relevant heterogeneity ($I^2 = 0\%$, **Figure 4B**). The quality of the evidence was high. No difference was seen between the TKIs ($P = 0.48$). One study which evaluated midostaurin showed a RR of 1.06 (95% CI, 0.67–1.68), while risk for acute GVHD appeared to be increased after sorafenib therapy showing a RR of 1.29 (95% CI, 0.98–1.70; $I^2 = 0\%$), when compared with control.

The safety profile could be assessed in the two randomized controlled trials on sorafenib (28, 31), for which means were calculated (**Table 2**). Frequency of adverse events were mostly comparable while skin toxicity was seen more frequently in the sorafenib group (19.5%) in comparison with the control group (6.3%), and hematologic toxicities such as neutropenia and thrombocytopenia, albeit in low absolute numbers, were more frequently observed in the sorafenib group (8.7 and 8.9%) compared with the control group (4.8 and 4.3%).

# DISCUSSION

Patients with *FLT3*-ITD mutated AML undergoing allogeneic stem-cell transplantation have a high risk of relapse (34). Because oncogenic addiction is caused by *FLT3*-ITD (35), it was reasonable to hypothesize that it could be a potential therapeutic target in *FLT3*-ITD mutated patients (36). While evidence accumulated that the multi-targeted TKI midostaurin can improve outcome in the front-line setting (9), whether specifically targeting *FLT3*-ITD using TKI therapy after allogeneic stem-cell transplantation can improve outcome was long unknown (6, 37, 38).

This first evidence synthesis for TKI therapy after allogeneic stem-cell transplantation in *FLT3*-ITD mutated AML found TKI therapy using midostaurin or sorafenib in comparison with control was significantly associated with better outcome in relapse and relapse-free survival. The risk for relapse was reduced by marked 65% and the risk for relapse or death from any cause

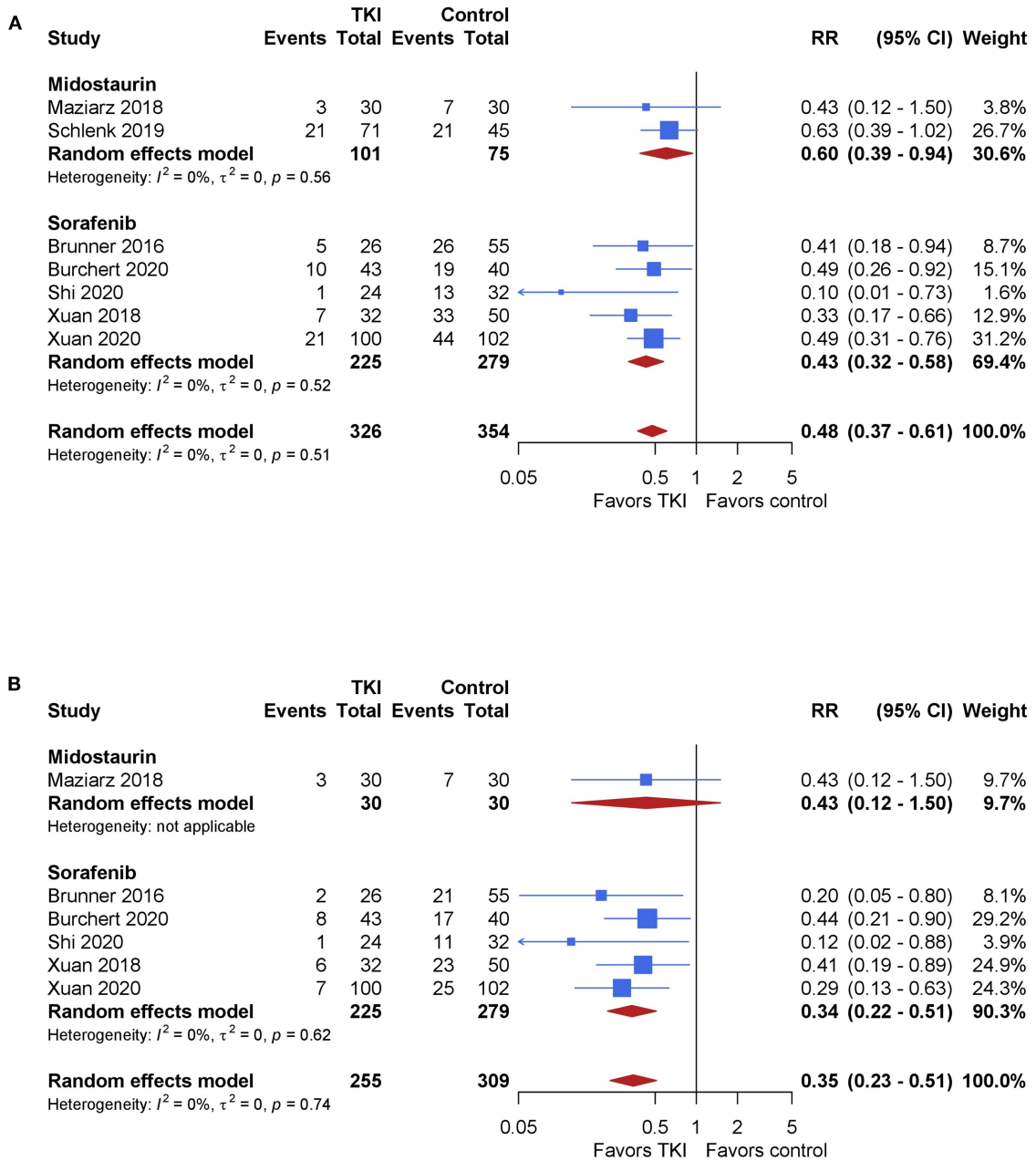


**FIGURE 2 |** The impact of TKI therapy on primary end points of relapse-free survival and cumulative incidence of relapse. Relapse-free survival **(A)** was assessed in all 7 studies at 18–59 months follow-up. The overall pooled RR showed significantly better relapse-free survival after TKI therapy, being 0.48 (95% CI, 0.37–0.61; $P < 0.001$) with no relevant heterogeneity ($I^2 = 0\%$). Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.21$). Incidence of relapse **(B)** was assessed in six studies. The overall pooled RR showed significantly reduced incidence of relapse, being 0.35 (95% CI, 0.23–0.51; $P < 0.001$) in favor of the TKI therapy with no relevant heterogeneity ($I^2 = 0\%$). Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.72$).

was reduced by 53% using TKI. Furthermore, overall survival was significantly improved after TKIs with a risk reduction for death from any cause by 52%. No significant difference for non-relapse mortality was noted, which was only assessed in studies on sorafenib. The risk for GVHD appeared to be increased for TKI therapy.

Although the results of this analysis did not seem to be influenced by different TKIs, more studies evaluated the role of sorafenib (6). Two studies used midostaurin, of which 1 is a still ongoing phase 2 randomized study and 1 a priori studied the effects of midostaurin throughout the therapeutic course, with a subgroup analysis of

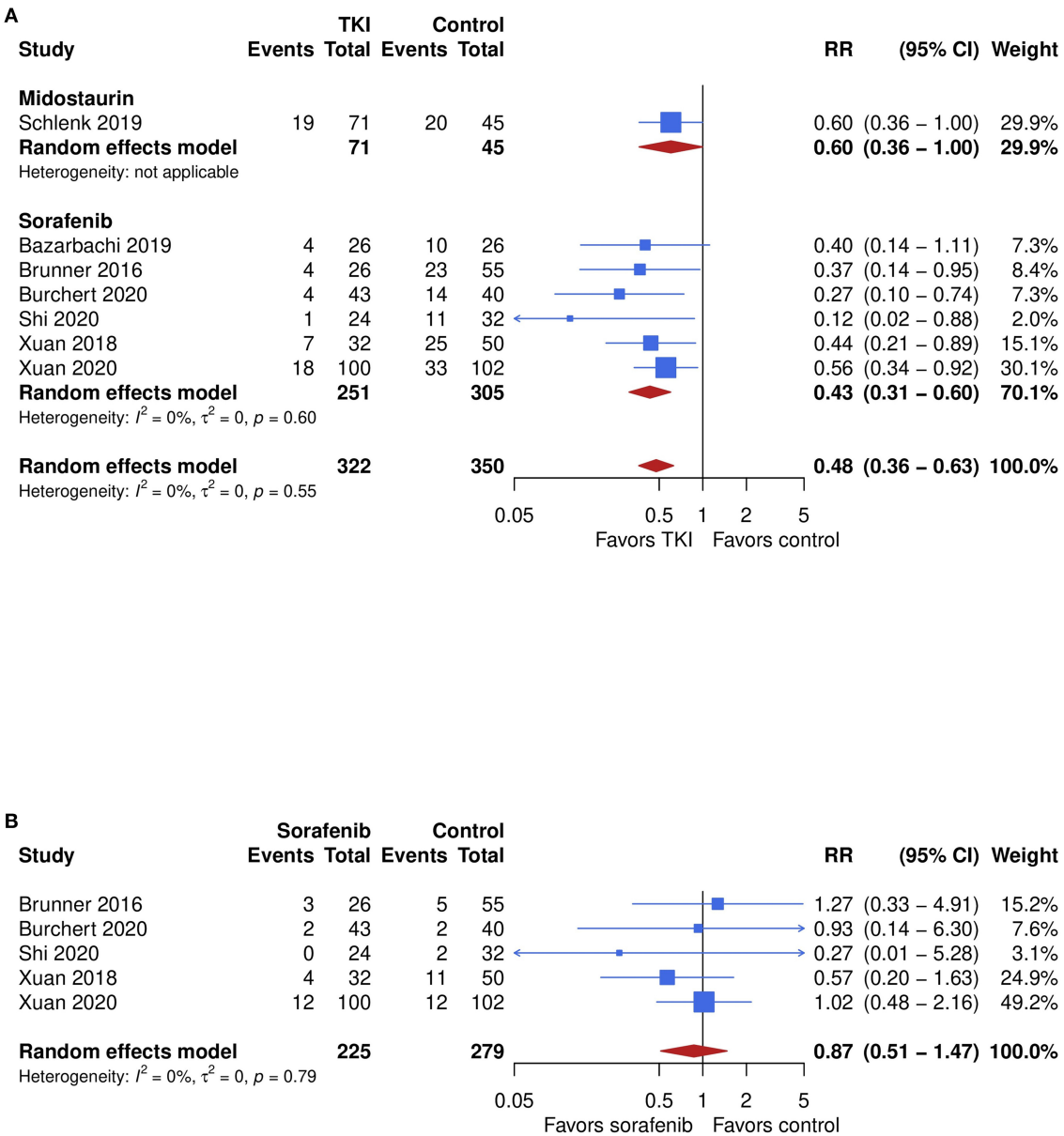


**FIGURE 3 |** The impact of TKI therapy on secondary end points of overall survival and non-relapse mortality. Significantly improved outcome for TKI therapy was also seen in overall survival **(A)**, which was assessed in 6 studies. The overall pooled RR was 0.48 (95% CI, 0.36–0.64; $P < 0.001$) in favor of the TKI therapy with no relevant heterogeneity ($I^2 = 0\%$). Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.30$). Non-relapse mortality **(B)** was assessed in 5 studies, which evaluated the efficacy of sorafenib. No significant difference between sorafenib and the control was seen, showing an overall pooled RR of 0.87 (95% CI, 0.51–1.47; $P = 0.60$) with no relevant heterogeneity ($I^2 = 0\%$).

post-transplant therapy compared with no post-transplant therapy. Other TKIs, for example, quizartinib and gilteritinib, which inhibit FLT3 more specifically and potently in comparison with midostaurin (39), showed improvement in overall survival in relapsed/refractory patients (18, 40). Gilteritinib is also being investigated for post-transplantation maintenance in AML patients with *FLT3*-ITD in a phase 3 randomized study (NCT02997202). Further research is needed to ascertain the comparative efficacy and safety of different TKIs post-transplantation therapy in *FLT3*-ITD mutated AML.

Given the well-described impact of minimal residual disease (MRD) on the outcomes after allogeneic stem-cell transplantation for AML (41, 42), and with the availability of a commercially available, next-generation sequencing-based MRD test for such patients, demonstration of a benefit of TKI therapy (or control) is critical to develop and incorporate TKIs into risk-based maintenance approaches (43). Both

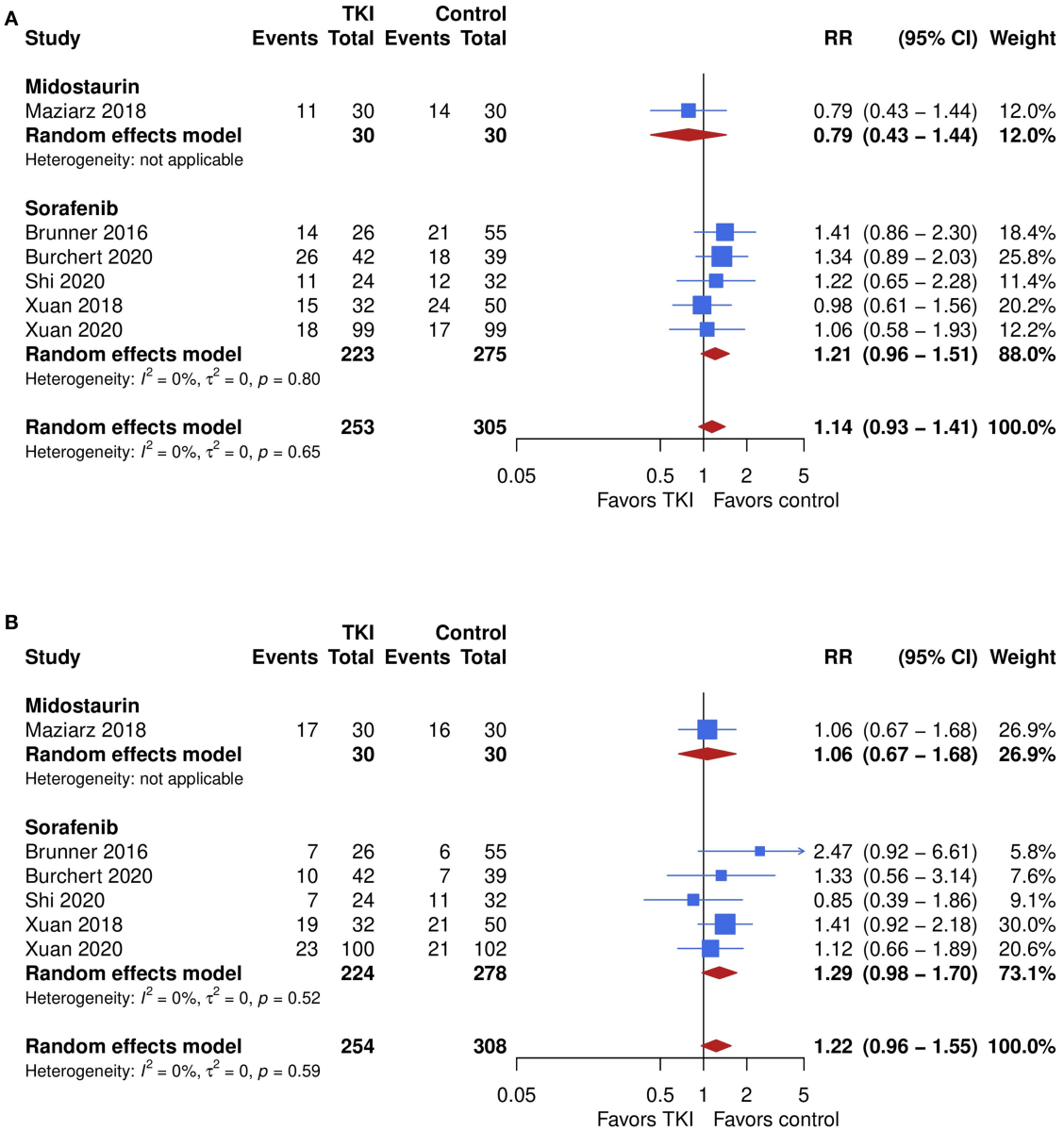


**FIGURE 4 |** The impact of TKI therapy on secondary end points of acute and chronic GVHD. Chronic GVHD **(A)** was assessed in six studies. No significant difference in the incidence was seen, with a trend toward higher incidence after TKI therapy showing an overall pooled RR of 1.14 (95% CI, 0.93–1.41; $P = 0.21$) with no relevant heterogeneity ($I^2 = 0\%$). Subgroup analyses showed no significant difference in outcome between midostaurin and sorafenib ($P = 0.19$). However, the pooled RR for midostaurin was 0.79 (95% CI, 0.43–1.44) while results for sorafenib suggested higher risk for chronic GVHD showing a RR of 0.43 (95% CI, 0.32–0.57; $I^2 = 0\%$), compared with control. Similar results were yielded for acute GVHD **(B)**, which was assessed in six studies. The overall pooled RR was 1.22 (95% CI, 0.96–1.55; $P = 0.10$) with no relevant heterogeneity ($I^2 = 0\%$). No difference was seen between the TKIs ($P = 0.48$). One study which evaluated midostaurin showed a RR of 1.06 (95% CI, 0.67–1.68), while risk for acute GVHD appeared to be increased after sorafenib therapy showing a RR of 1.29 (95% CI, 0.98–1.70; $I^2 = 0\%$), when compared with control.

prospective randomized studies on sorafenib showed subgroup results according to the MRD status at time of randomization (28, 31). While the Chinese study group showed significantly reduced incidence of relapse after sorafenib with hazard ratios of 0.28 for patients with undetectable MRD and 0.25 for detectable MRD (28), patients with undetectable MRD appeared to have better relapse-free survival in the German study group, but this comparison was not statistically significant (31). In the German study group, patients with detectable MRD had significantly improved relapse-free survival, while the results need to be interpreted with caution owing to the relatively low numbers of patients in each group. The ongoing BMT CTN 1506 study on gilteritinib includes the critical objective to better understand the

**TABLE 2 |** Safety of sorafenib in 2 randomized controlled trials.

| | Sorafenib ($n = 143$) | Control ($n = 142$) |
|---|---|---|
| Neutropenia | 8.7% | 4.8% |
| Thrombocytopenia | 8.9% | 4.3% |
| Skin toxicity | 19.5% | 6.3% |
| Infections | 29.6% | 28.0% |
| Gastrointestinal toxicity | 25.2% | 21.7% |
| Cardiac and renal insufficiency | 11.8% | 7.8% |

impact of MRD on outcomes with post-transplantation TKI maintenance.

Recent basic research findings indicate that the synergism of T-cells and sorafenib may metabolically reprogram AML-reactive T-cells, providing potential to contribute to immune-mediated curative treatment of *FLT3*-ITD mutated AML relapse (44). Furthermore and in general, a graft-vs.-leukemia effect is considered to be associated with the occurrence of GVHD (45). The findings of the present data synthesis suggest that at least sorafenib might increase the incidence of GVHD. Whether other mechanisms are involved in this effect requires further investigation.

In terms of safety, multi-targeted TKIs such as midostaurin and sorafenib are relatively non-specific and exert off-target activities. The prospective study on front-line midostaurin showed no unexpected adverse events (9). Higher grade 3–4 adverse events were seen for anemia (92.7 vs. 87.8%), rash (14.1 vs. 7.6%), and nausea (9.6 vs. 5.6%) in comparison with placebo, with no necessary dose modification for hematologic toxicity. With respect to sorafenib, small-sample studies have shown that the most common adverse events were related to hematological, skin, and gastrointestinal toxicities. In the present analysis, safety of post-transplantation TKI therapy could only be assessed for both prospective studies on sorafenib which showed no unexpected and comparable rates of adverse events when compared with control (**Table 2**). Only skin toxicity appeared to be slightly increased, but the overlap in skin rashes between an adverse event caused by sorafenib and graft-vs.-host disease of the skin represents a difficulty for the differential diagnosis (27, 46). Furthermore, 60 and 50% of patients in the Chinese and German study needed a dose modification (interruption or reduction) because of adverse events. Dose reductions did not seem to limit sorafenib efficacy but more attention in view of TKI-specific toxicities and dose intensities is needed.

As with any meta-analysis, the present evidence synthesis regarding TKIs after stem-cell transplantation has several limitations. The conditioning intensity for transplantation was not homogenous. Four studies only used myeloablative conditioning transplantation (27, 28, 32, 33). Comparative analyses on the superiority of one conditioning over another are inconclusive and may be interpreted on the subgroup level (42, 47–50), and the evidence on the impact of conditioning on outcome after TKIs is immature (51). Furthermore, the time of initiation of TKI was not homogeneous between studies and this meta-analysis could not account for differences in dosage schemes nor duration of treatment or treatment interruptions. Additionally, the present analysis may not provide any evidence for favoring one TKI over another. Further, RRs had to be calculated at different time of follow-up in the included studies, ranging from 18 to 59 months. This issue can be controlled for only when patient-level data are available. The risk of selection bias in meta-analyses of different donor stem-cell transplantation studies or due to the incorporation of findings from retrospective and prospective studies cannot be completely ruled out (52, 53). One prospective study on midostaurin was not adequately powered to identify a statistical difference between the groups (32), and on prospective study on sorafenib was prematurely terminated owing to slow patient recruitment (31). However, upfront exclusion of certain studies may even increase heterogeneity. And last, associations of allele ratios or TKD mutations cannot be addressed by analyses as presented here and further prospective evaluations are warranted.

In sum, this analysis identified a significant improvement in relapse-free survival, overall survival, and relapse incidence after post-transplant TKI therapy in *FLT3*-ITD mutated AML. These effects are irrespective of the TKI, while there is more consistent evidence for sorafenib so far. Ongoing studies could further help to better dissect patient subgroups that may benefit the most and identify refined relation of FLT3 selectivity vs. immune-stimulatory off-target activities governing TKI therapy after stem-cell transplantation in *FLT3*-ITD mutated AML.

## 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/s.

## AUTHOR CONTRIBUTIONS

NG and NK had full access to all the data in the study, designed the study, retrieved, analyzed and interpreted the data, and wrote the first draft of the manuscript. All authors interpreted the data, wrote the manuscript, and approved the final version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.630429/full#supplementary-material

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REVIEW
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# Interplay Between the Intestinal Microbiota and Acute Graft-Versus-Host Disease: Experimental Evidence and Clinical Significance

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for many hematological disorders and autoimmune diseases, but acute graft-versus-host disease (aGVHD) has remained a major obstacle that limits allo-HSCT and exhibits a daunting mortality rate. The gastrointestinal system is among the most common sites affected by aGVHD. Experimental advances in the field of intestinal microbiota research enhanced our understanding - not only of the quantity and diversity of intestinal microbiota - but also their association with homeostasis of the immune system and disease pathogenesis, including that of aGVHD. Meanwhile, ever-growing clinical evidence suggest that the intestinal microbiota is dysregulated in patients who develop aGVHD and that the imbalance may affect clinical outcomes, indicating a potential predictive role for microbiota dysregulation in aGVHD severity and prognosis. The current animal and human studies investigating the intestinal microbiota in aGVHD and the understanding of the influence and management of the microbiota in the clinic are reviewed herein. Taken together, monitoring and remodeling the intestinal microecology following allo-HSCT may provide us with promising avenues for diagnosing, preventing or treating aGVHD in the clinic.

**Keywords: hematopoietic stem cell transplantation, acute graft-versus-host disease, intestinal microbiota, diversity, strategies**

## INTRODUCTION

Malignancies of the hematopoietic system and therapy-refractory autoimmune diseases are frequently associated with high mortality and hence represent the most common indications to perform allogeneic hematopoietic stem cell transplantation (allo-HSCT) (1, 2). However, graft-versus-host disease (GVHD), in which donor-derived T cells recognize host tissues as foreign, causing inappropriate and aberrant immune attacks, remains one of the major limitations to HSCT. Approximately 40-60% of patients receiving allo-HSCT may suffer from GVHD (3–5) with a

mortality rate of 15% to 20% (3, 4, 6–9). Clinically, prophylaxis of acute GVHD (aGVHD) involves immunosuppression of donor cells, but there is no standard approach, and it often varies by institution (3). Treatment protocols can also be challenging because therapeutic options are limited, response rates for corticosteroids are only approximately 50%, and response durations are typically brief (6, 7). In addition, several drugs are reported to be effective in patients not responding to corticosteroids, but most data are unconvincing, and combination therapies tried to date have yielded modest or no benefit over corticosteroids alone (10–12). Because of the small number of results from well-designed, large-scale, clinical studies, there is considerable variability in dealing with aGVHD worldwide, which leads to updated consensus recommendations that still have problems (13).

Much work has been done to research the biological mechanisms participating in the pathogenesis of aGVHD, but the specific nature of these interactions has not been fully elucidated, especially the relationship between aGVHD and the intestinal microbiota. The intestinal microbiota has been proven to be critical for maintaining healthy tissues and stimulating immunity (14), and increasing evidence has revealed that dysbiosis in intestinal microbial populations is linked to human disease and defects in immunity (15–18). Recent studies have notably widened our understanding of the interactions between the loss of intestinal bacterial diversity and aGVHD following allo-HSCT. This review provides an update of current knowledge on the cross-talk between them, with the purpose of determining improved prophylactics and therapies for aGVHD based on the role of the intestinal microbiota.

## THE INTESTINAL TRACT AND INTESTINAL MICROBIOTA

The gastrointestinal tract consists of the mucous layer, submucous layer, muscular layer and serosa from the inner to outer layers. The intestinal mucosa, the innermost layer of the gastrointestinal tract, can be further divided into the epithelium, lamina propria and muscularis mucosae (19). The epithelium is a single-cell layer that contains unique secretory cells and stem cells, and many immune cells in the lamina propria help to monitor pathogens and maintain immune tolerance to food and commensal antigens (20). The mucosal surface maintains an intact biological barrier that prevents substantial bacterial and other detrimental invasion into the host tissue and blood circulation under steady-state homeostasis; this function is implemented by epithelial tissues, gut-associated lymphoid tissue (GALT) and important secretory components (21). The commensal intestinal microbiota also contributes to the maintenance of intestinal ecological balance. An overview of homeostasis between the microbiota and the host intestinal mucosa is shown in **Figure 1A**.

The human intestinal tract hosts $10^{13}$ to $10^{14}$ microbial organisms of approximately 1000 species (**Table 1**) (22, 23). Although viruses and fungi are also present in considerable amounts and diversity, the vast majority of these organisms are bacteria collectively termed the gut microbiota (24–26), which play an important role in the synthesis of a variety of vitamins and amino acids, participating in the metabolism of carbohydrates and proteins and promoting the absorption of various mineral elements (27). The balance and diversity of the gut microbiota is of great importance for the human body, as researchers have found close connections between changes in the gut microbiota and human diseases, such as obesity (28), diabetes (29, 30), functional bowel syndrome (31), autism (32), and autoimmune diseases (e.g., rheumatoid arthritis (33)). For decades, the analysis of the intestinal microbiota has been largely dependent on ex vivo cultivation of bacteria, which yields only 10–30% of the population, limiting knowledge of the bacterial composition (34). In recent years, the development of next-generation sequencing technologies, such as 16S rDNA sequencing and metagenomics, has allowed for further identification of microorganisms, which clarifies the detailed and specific role of the intestinal microbiota in aGVHD, leading to a new era of research (35, 36).

## INTESTINAL MICROBIOTA IN THE MECHANISM OF AGVHD

### Pathophysiology

Development of aGVHD is considered a three-step process. The microbiota-linked pathogenesis of gastrointestinal aGVHD is summarized herein.

In the first step, when the conditioning regimen of allo-HSCT damages the intestinal epithelium, homeostasis between the host and intestinal commensals is disturbed (**Figure 1B**). Total-body irradiation (TBI) induces dose-dependent damage to the gut lining including killing intestinal stem cells (ISCs), depleting or inhibiting non-epithelial cells, injuring intestinal crypts and causing gastrointestinal tract syndrome (37–39), by mechanisms such as increasing p53-mediated epithelial apoptosis (40) and plasminogen activator inhibitor-type 1 (PAI-1)-mediated enteritis (41). The intestinal mucosa is the major target tissue, and histological evidence has shown villous shortening, increased lymphocytic cell infiltration, crypt destruction and epithelial apoptosis. Crypt cell degeneration has been suggested to be the initial lesion of gastrointestinal aGVHD (42–44), and loss of goblet cells and Paneth cells has been shown to lead to translocation of dominant luminal pathogens and pathogen-associated molecular patterns (PAMPs) as well as intestinal dysbiosis, which further accelerates gastrointestinal aGVHD and infections (45). Moreover, some studies have provided evidence that these toxic effects are partially mediated by the intestinal microbiota. Lai et al. found that mice treated with antibiotics or deficient in myeloid differentiation primary response gene 88 (MyD88), a crucial adaptor for recognition of microbial molecules, showed less crypt loss and less damage to progenitor and stem

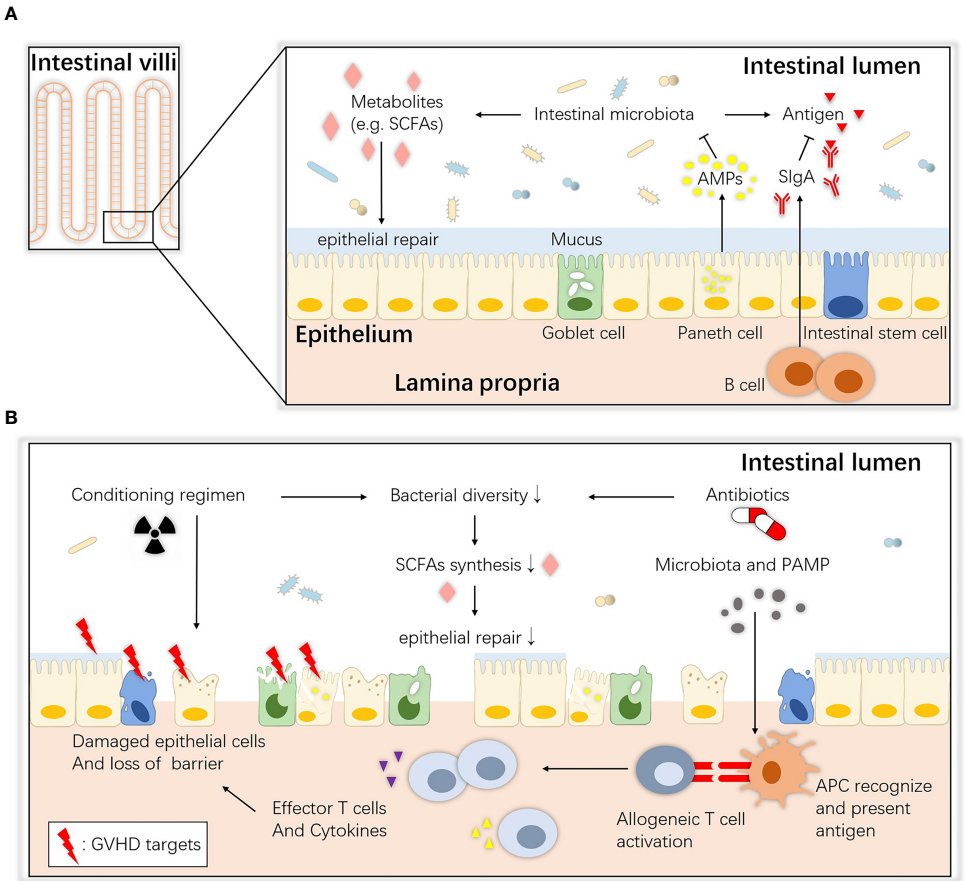


**FIGURE 1** | Overview of intestinal ecology and gastrointestinal aGVHD. **(A)** Homeostasis between the commensals and host intestinal epithelium. At steady state, host intestinal epithelial cells live with commensals, and their interaction maintains immune and biological homeostasis. ISCs maintain the regeneration of the epithelium, Paneth cells secrete AMPs that create a sterility gradient, goblet cells produce mucus to separate the microbiota from host epithelial tissue, and immune cells such as B lymphocytes secrete SIgA to neutralize biologically active microbial antigens. Together, they maintain an intact barrier on the mucosa surface. SCFAs (e.g., butyrate) are bacterial fermentation products that can be used as an energy source and regulate the differentiation, recruitment and activation of immune cells. **(B)** Pathogenesis of gastrointestinal aGVHD. During allo-HSCT, the antibiotics and altered diet and cell damage caused by the conditioning regimen all lead to dysbiosis and metabolic disorders. Then, the depletion of SCFAs may also contribute to epithelial defects, allowing translocation of pathogenic bacteria and PAMPs. APCs (e.g., DCs) recognize them and elicit Th1 and Th17 responses and the release of proinflammatory factors that enhance tissue damage. ISCs, intestinal stem cells; AMPs, antimicrobial peptides; SCFAs, short-chain fatty acids; SIgA, secretory immunoglobulin A; APC, antigen-presenting cell; PAMP, pathogen-associated molecular pattern.

**TABLE 1** | Bacterial taxonomy of some important microbiota constituents in the literature.

| Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|
| **Verrucomicrobia** | Verrucomicrobiae | Verrucomicrobiales | | Akkermansia | |
| **Proteobacteria** | γ-proteobacteria | Enterobacteriales | Enterobacteriaceae | Escherichia | *E. coli* |
| **Bacteroidetes** | Bacteroidia | Bacteroidales | Bacteroidaceae | Bacteroide | |
| **Firmicutes** | Clostridia | Clostridiales | Lachnospiraceae | Blautia | |
| | | | | Eubacterium | |
| | | | Ruminococcaceae | | |
| | Erysipelotrichia | Erysipelotrichiales | | Erysipelatoclostridium | |
| | Bacilli | Lactobacillales | Lactobacteriaceae | Enterococcus | Enterococci |
| | | | | Lactobacillus | |

cells after radiation (46). Seth et al. showed that the microbiota protects against dextran sodium sulfate–induced intestinal damage after radiation (47). It is possible that the microbiota affects the initiation of damage.

In terms of the alloreactive cells at the second step, it is well known that potential pathogens and their antigen molecules can activate T cells and affect differentiation. A study showed that cohousing laboratory mice with feral mice produced mice with

immune systems closer to those of adult humans, with a preference toward effector and memory T cell populations, suggesting that 'dirtier' mice have more intrinsic activation leading to more GVHD (48). After observing their existence, studies have shown that activated T cells access the epithelium *via* gut-specific homing molecules on the T cells and adhesion ligands on the vasculature, such as MAdCAM-1; the intestinal crypts are the primary location invaded by T cells, wherein they directly interact with ISCs (49–54). For T cell differentiation, the induction of both T helper (Th) and regulatory T (Treg) cells is influenced by the microbiota. Breaching the intestinal barrier or penetration by the microbiota in aGVHD activates the IL-23 pathway by JAK-STAT, stimulating epithelial cells to produce serum amyloid A proteins, which leads to Th17 differentiation, an important subset in aggravating aGVHD (55–57). Interestingly, some species, such as *Bacteroides fragilis*, prevent IL-17 production by releasing polysaccharide A (PSA) and promoting Foxp3$^+$ Tregs, and Tregs maintain gastrointestinal homeostasis *via* the release of IL-10 (58), which could alleviate aGVHD. Other species, i.e., Clostridiales, produce SCFAs (e.g., butyrate and propionate), which block histone deacetylases (HDACs) through the G-protein receptor (GPR) to promote acetylation of histone H3 in Tregs at the Foxp3 locus, which also induces Treg differentiation (59). In addition, innate lymphoid cells 3 (ILC3s), an activated population with expression of the natural cytotoxicity receptor (NCR, such as NKp44) and nuclear hormone receptor RORγt were found mainly in mucosal tissues and emerged as modulators of conditioning-induced tissue damage in the context of aGVHD, secreting IL-22 and IL-17, which are necessary in defense against bacterial pathogens (60, 61). NCR$^+$ ILC3-derived IL-22 has already been found to be crucial in epithelial recovery and protect ISCs from damage by activating STAT3 and downstream regulators of cellular proliferation and survival which finally attenuates aGVHD (62, 63). But there is no direct evidence that NCR$^-$ ILC3s-derived IL-17 is involved in the pathology of aGVHD (64). Granulocyte macrophage colony-stimulating factor (GM-CSF) produced by ILC3s is also essential for the normal development of intestinal dendritic cells (DCs) involved in Treg induction (65). These interactions are summarized in **Figure 2**.

The third step links cytokine storms and inflammatory amplification, which induce direct damage and establish typical aGVHD injury. Damage to the intestine plays a central role in amplifying systemic GVHD by propagating a proinflammatory cytokine milieu (66). Moreover, the many combinations of cytokines (e.g., TNF, INF-γ, IL-1, IL-2, and IL-17) and costimulatory networks at the T cell surface are definitely complex, in addition to numerous products produced by the intestinal microbiota. The role of the intestinal microbiota in regulating cytokines has been elucidated in some previous studies. Atarashi et al. showed that on the basis of high potency in enhancing Treg abundance and inducing anti-inflammatory molecules, 17 rationally selected strains of Clostridia result the increase of IL-10 in the gut (67). Another study reported that increased abundance of Enterobacteriaceae is positively correlated with IL-17A aggravating aGVHD (68).

In addition to the abovementioned mechanisms, the pathogenesis of aGVHD involves many other specific mechanisms that require deeper investigation. However, it is increasingly clear that the intestinal microbiota indeed participates in the initiation and development of aGVHD.

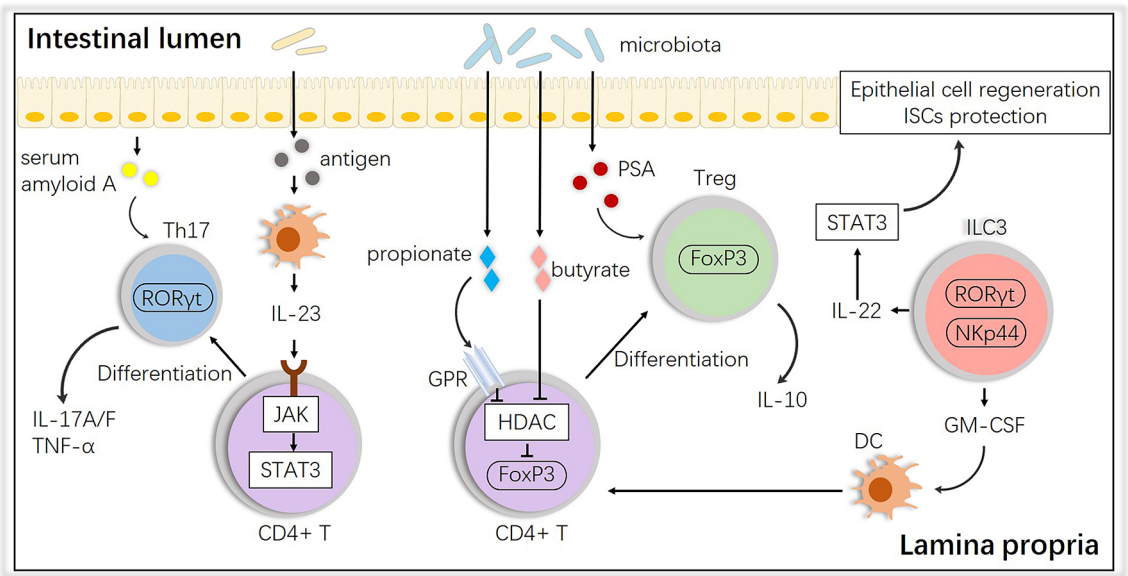


**FIGURE 2** | Impact of the intestinal microbiota on T cell subsets and interactions with ILC3s. The intestinal microbiota influences the differentiation of T cells into anti-inflammatory Tregs or proinflammatory Th17 cells, and ILCs play an important role in this process. PSA, polysaccharide A; GM-CSF, granulocyte macrophage colony-stimulating factor; HDAC, histone deacetylase; GPR, G protein receptor.

## Metabolites

The intestinal microbiota generates a wide range of bioactive metabolites serving as mediators and has pervasive consequences in aGVHD; modifications in bacteria-derived metabolites may be a new perspective regarding this disease (69).

Short-chain fatty acids (SCFAs) are one of the major microbial-derived metabolites found exclusively in the intestinal tract, which function in maintaining the epithelial barrier and colonocyte survival as well as play a diverse array of immune regulatory roles (70, 71). It has been reported that butyrate, one of the three main SCFAs, has a protective effect against aGVHD in murine models; butyrate restoration improved histone acetylation and IEC junctional integrity and decreased IEC apoptosis, ultimately mitigating aGVHD (72–74).

3-Indoxyl sulfate (3-IS), another promising metabolite analyzed in aGVHD, is a tryptophan metabolite of commensal colonic bacteria that has been identified as an indirect marker of a balanced microbiota and predicts the outcome of allo-HSCT (75, 76). Moreover, studies have also shown that gut tryptophan-produced indole metabolites reduce GVHD severity *via* type I interferon (IFN I) (77).

Respiratory metabolites may hold potential as surrogate markers for aGVHD (78–80). Various microbiota constituents are known to produce volatile metabolites, and volatile organic compounds (VOCs) generated during pathologic processes have been reported monitored in diseases such as obesity (81), hepatitis (82) and IBD (83). More recently, Hamilton et al. analyzed the VOCs of patients with and without aGVHD and correctly classified 89% (17 of 19) and 90% (9 of 10) of them, respectively, showing that breath analysis is a feasible and promising noninvasive method to detect and potentially monitor aGVHD (84).

Choline, phosphatidylcholine and carnitine-containing dietary ingredients can be metabolized into trimethylamine (TMA) and subsequently converted into trimethylamine N-oxide (TMAO), which could induce vascular inflammation and endothelial dysfunction (85). Dietary habit such as high-choline diet producing high level of TMAO alters distinct quality and quantity of gut microbiota which might affect microbial metabolites and GVHD severity. The latest research further explored TMAO in aGVHD and found that TMAO enhanced the allogenic GVH reaction. In an animal model, the group stimulated with TMAO showed a worse survival rate, higher GVHD scores and more damage to target tissues, which resulted from Th1 and Th17 differentiation (86).

These studies innovatively provide the link between microbiota-derived metabolites and aGVHD, which sheds light on alleviating aGVHD by controlling metabolism.

# LOSS OF MICROBIOTA DIVERSITY IN AGVHD

Views about the role of the microbiota in aGVHD are separated into two roles: a direct role and an indirect role. The former considers that the intestinal microbiota directly promotes tissue injury or inflammation after allo-HSCT, and animals possess normal immunocompetence but abnormal microbiota community compositions, so eliminating this proinflammatory factor by antimicrobial treatment should protect the recipient from aGVHD (87–89). The latter considers the intestinal microbiota to play an indirect role, as animals raised in germ-free conditions have abnormal development with highly aberrant immunity, and this kind of animal does not possess a prerequisite immune ability to develop aGVHD (90–92). Some researchers even support that both direct and indirect effects may occur simultaneously (93). Furthermore, we can identify some common alterations or differences from animal experiments and clinical studies.

## Animal Studies

Earlier transplantation studies on animals in the 1970s focused on manifestations such as better survival and lower mortality with the management of the intestinal microbiota (94, 95). Subsequently, protection from GVHD in germ-free animals was confirmed in the 1980s (87), including xenogeneic transplantation that typically abrogates severe GVHD (88, 89). Antibiotic-mediated gut decontamination in mice (96) and dogs (97) showed that both microbes and aberrant immune development most proximately affect the development of GVHD. However, few animal studies have measured the impact of antibiotic treatment on microbiota composition or linked specific bacterial species to GVHD at this time.

In the early 21st century, advances in technology advanced research on the alterations (**Table 2**). Shono et al. found that piperacillin-tazobactam and imipenem-cilastatin therapy led to distinct patterns of gut microbiota composition in GVHD mice, with an increasing abundance of an Akkermansia strain, a bacterium with mucus-degrading capabilities, which raises the possibility that mucus degradation may contribute to murine GVHD (102). At the phylum level, the proportions of Firmicutes and Bacteroidetes, two of the major enteric commensals, were found to be decreased in GVHD mice, while the abundance of *E. coli* at the genus level was higher, which could be due to the development of systemic infection (100, 101). Furthermore, for Clostridiales and Lactobacillales, at the order level of Firmicutes, diversity analysis showed loss of the former but expansion of the latter, while eliminating Lactobacillales from mice before transplantation aggravated GVHD, and reintroducing the predominant Lactobacillus species showed a significant protective effect (99). Similar to these results, a report showed that the acute phase of GVHD was characterized by a shift toward the Enterobacteriaceae family. Bacteroides and Enterococcus abundances increased during GVHD, whereas Clostridia, Bifidobacteria and Bacillus were less abundant, but in this report, Lactobacillus abundance was decreased in GVHD (98), Moreover, subsequent studies also presented contrary results that the abundances of Lactobacillus and another uncultured bacterium from Firmicutes increased while the abundances of *E. coli* and another uncultured bacterium from Bacteroidetes decreased in allogeneic immunoglobulin yolk (IgY)-treated GVHD animals (103). However, the exact mechanisms of these alterations remain unclear, although several mechanisms have

**TABLE 2 |** Summary of nearly 10 years of experimental studies investigating the association between alterations in the intestinal microbiota and aGVHD.

| Year | Mouse model | Relative abundance alteration of microbiota | Relationship with aGVHD | Ref |
|---|---|---|---|---|
| 2010 | (Age- and sex-matched) Balb/c→C57B6(TLR$^{-/-}$, MyD88$^{-/-}$, TRIF$^{-/-}$ and WT) | ● *Bacteroides*, Enterococcus ↑<br>● Clostridia, Bifidobacteria, Bacillus, Lactobacilli↓ | GVHD development is accompanied by shift towards proinflammatory bacterial species(enterobacteria, enterococci and *Bacteroides*/Prevotella). | (98) |
| 2012 | B10.BR→B6<br>Balb/c→B10.BR<br>B6→BM12 | ● Clostridiales↓<br>● Lactobacillales↑ | Increased microbial chaos early after allo-HSCT is a potential risk factor for subsequent GVHD. | (99) |
| 2012 | (F) B6→B6D2F1<br>(F) C3H.Sw→B6<br>(F) B6-Ly5.1→B6D2F1 | ● Firmicutes, Bacteroidetes↓<br>● *E. coli*↑ | Diversity of the microbial community was significantly reduced in mice with GVHD. | (100) |
| 2015 | (F) B6→BALB.B<br>(F) B6/SJL→BALB.B | ● *E. coli*↑ | Diversity of the microbial community was significantly reduced in mice with GVHD. | (101) |
| 2016 | (F) C57BL/6→129S1 | ● Erysipelotrichia, Enterococcus, Akkermansia↑<br>● Clostridiales↓ | Aggravated GVHD mortality was associated with imipenem-cilastatin or piperacillin-tazobactam treatment mice which lead to an increase in Akkermansia muciniphila. | (102) |
| 2017 | C57BL/6→B6D2F1<br>B6D2F1→B6D2F1 | ● *Lactobacillus* and another uncultured bacterium from Firmicutes↑<br>● *E. coli* and another uncultured bacterium from Bacteroidetes↓ | Improvement of aGVHD in animals treated with immunoglobulin may be mediated by reducing pathogenic bacteria such as *E. coli* and increasing probiotic bacteria such as *Lactobacillus*. | (103) |
| 2017 | BALB/c(WT, IL-17A $^{-/-}$, IL-17RA $^{-/-}$)→B6(WT, IL-17A $^{-/-}$, IL-17RA $^{-/-}$)<br>BALB/c(WT)→B6(WT, IL-17RA/B/C$^{-/-}$) | ● Microbiome of WT mice shifted toward that of the IL-17RA/C–deficient mice during cohousing prior to transplant | IL-17–sensitive microbiota controls susceptibility to aGVHD with increased susceptibility to aGVHD transferred to WT mice *via* cohousing with IL-17RA or IL-17RC–deficient mice. | (57) |
| 2019 | (M) C57BL/6→BALB/c<br>(M) C57BL/6→129S1/Sv<br>(M) LP/J→C57BL/6 | ● Enterococcus↑ | Enterococcus expands in mouse after allo-HSCT and exacerbates GVHD severity which is dependent on the lactose. | (104) |
| 2019 | (F) BALB/c→B6(WT and Nlrp6$^{-/-}$)<br>(F) C3H.sw→B6(WT and Nlrp6$^{-/-}$) | ● Verrucomicrobia, Proteobacteria, Bacteroidetes↑<br>● Firmicutes↓ | Host NLRP6 play a pathogenic role in aggravating GVHD which was independent of indigenous microbiota changes. | (105) |

been proposed in earlier publications, including agglutination, opsonization, and toxin neutralization (106, 107).

Collectively, it is evident that not all bacteria have the same effect on GVHD, the composition of bacterial species and their function matter. The perturbations in the diversity and function of bacterial species may be caused by animal strains or research environment. However, one special bacterial species seems to be adverse in GVHD. Enterococci, gram-positive, facultative, anaerobic bacteria, occur in low abundance in the healthy host gut, with reports showing that Enterococcus expansion is associated with increased bloodstream infection and mortality in HSCT (108, 109). The data mentioned above (98) also revealed an increase in enterococci in the development of GVHD. In a recent study (104), in line with previous suspicion, enterococci similarly expanded and dominated the microbiota after allo-HSCT in GVHD mice; lactose drove this growth, and a lactose-free diet attenuated enterococcal expansion and T cell–driven inflammation in GVHD. Indeed, fecal domination by specific translocation of pathogenic bacteria, such as Enterococcus, is a significant risk factor for the development of aGVHD and increased overall GVHD-related mortality. Some easily overlooked factors, such as dietary elements, may play important roles in the progression, which should be closely managed.

The development of biogenetics has led to a focus on specific gene sites. For example, host NOD-like receptor family pyrin domain-containing 6 (NLRP6) regulates microbiota-dependent protection in intestinal colitis and tumorigenesis (110–113), but Tomomi et al. found an inverse effect in GVHD, in which host NLRP6 played a pathogenic role in aggravating intestinal damage (105). Interestingly, this influence was independent of indigenous microbiota changes. Toll-like receptors (TLRs), which sense bacterial lipoproteins and LPS and DNA, are suspected to modulate innate immune responses, and a major role of TLR9-mediated sensing of bacterial DNA in the aggravation of GVHD has been reported (114–116). With gene knockout, Markus et al. similarly proved that bacterial innate immune receptor TLR$^{-/-}$ mice showed significantly reduced GVHD mortality, which was further confirmed by less pronounced GVHD scores over time (98).

Furthermore, some groups have demonstrated substantial differences between the gut microbiota of mice purchased from different commercial vendors or repositories (117). There are clear differences in microbiota composition and diversity between sexes in previous studies (118, 119). In addition, Floris et al. showed that BALB/c mice had higher abundance and diversity of immunoglobulin A (IgAs) than C57BL/6 mice which is correlated with increased microbiota diversity (120). And aging alters the gut microbiota in mice, in which aged microbiome leads to an exaggerated systemic inflammatory response and reduced levels of SCFAs in young mice (121). In conclusion, gut microbiota composition can be influenced by housing, gender, host genetic,

age, et al, and further studies are needed to determine the impact of different murine models and strains on aGVHD.

## Human Studies

Early in the 1980s, in a clinical report of 130 patients with aplastic anemia undergoing allo-HSCT, gut decontamination and laminar airflow isolation were shown to lower the incidence of aGVHD (122). However, subsequent clinical research of human aGVHD has demonstrated that loss of intestinal microbiota diversity is associated with aGVHD, as microbiota disruption characterized by expansions of potentially pathogenic bacteria and reduction in alpha diversity (a variable that reflects the number of unique bacterial taxa present and their relative frequencies) have been reported. Some advanced clinical studies performed in recent years may further provide us with a better understanding of these alterations (**Table 3**).

In 2012, Jenq et al. studied changes in the microbiota of patients undergoing allo-HSCT and found that only the GVHD group had decreased microbial diversity with increased Lactobacillales abundance and loss of Clostridiales, suggesting that shifts in diversity were a result of GVHD rather than allo-HSCT or antibiotic exposure (99). In 2014, Taur Y et al. found that loss of bacterial diversity in stool specimens was associated with increased mortality from GVHD, and survival at 3 years after allo-HSCT was 36%, 60%, and 67% for patients with low, intermediate, and high microbiota diversity, respectively (123). However, patients in this study received T cell-depleted grafts, which could also reduce GVHD. Jenq et al. further investigated 64 allo-HSCT recipients of T cell–replete grafts and found that a higher abundance of Blautia was associated with a reduced risk of GVHD-related mortality and increased overall survival (124). Blautia is a genus that belongs to the class Clostridia. As reported in previous studies, Clostridiales rescue intestinal epithelial cell damage by upregulating Treg cells through the production of the SCFA butyrate (59, 67, 130), and alteration of the indigenous microbiota with 17 rationally selected strains of high butyrate-producing Clostridia led to decreased GVHD (74). Consistent with these findings, in 2017, Simms et al. reported a significant

**TABLE 3** | Summary of human studies investigating the association between the microbiota and GVHD in the past 10 years.

| Year | Patients | Relative abundance alteration of microbiota | Outcomes | Ref |
|---|---|---|---|---|
| 2012 | 18 adult patients (8 GVHD vs. 10 non-GVHD) | ● In GVHD patients: Lactobacillales↑ Clostridiales↓ | GVHD group had decreased stool microbial diversity, microbial chaos early after transplantation is a potential risk factor for subsequent GVHD. | (99) |
| 2014 | 80 adult patients | ● In GVHD patients: Enterococcus, Streptococcus, *Lactobacillus*↑ | Increased mortality from GVHD was associated with lower diversity of microbiota at engraftment, which showed a strong predictive effect on mortality. | (123) |
| 2015 | 115 adult patients | ● In GVHD patients: Blautia↓ | Increased abundance of commensal bacteria belonging to the Blautia genus is associated with reduced lethal GVHD and improved OS. | (124) |
| 2017 | 29 pediatric patients | ● In GVHD patients: Enterobacteriaceae, Enterococcus↑ ● In non-GVHD patients: anti-inflammatory Clostridia(AIC), *Bacteroidetes*, Bifidobacterium ↑ | Exposure to antianaerobic antibiotics clindamycin lead to depletion of Clostridia species which is associated with GVHD in pediatric HSCT patients. | (125) |
| 2017 | 66 adult patients (52 GVHD vs. 14 non-GVHD) | ● In GVHD patients: oral Actinobacteria, oral Firmicutes↑ Lachnospiraceae↓ | The stool microbiota at neutrophil recovery post-HSCT is predictive of subsequent development of aGVHD. | (126) |
| 2018 | 81 adult patients (32 GVHD vs. 49 non-GVHD) | ● In GVHD patients: Enterobacteriaceae↑ Lachnospiraceae, Ruminococcaceae↓ | Intestinal microbiota might induce aGVHD by influencing the Treg/Th17 balance. | (68) |
| 2019 | 141 adult patients (83 grade 0-I aGVHD vs. 58 grade II-IV aGVHD) | ● In GVHD patients: Proteobacteria, Gammaproteobacteria, Enterobacteriaceae↑ Firmicutes, Clostridia, Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, Blautia, Lachnoclostridium, Erysipelatoclostridium, Eubacterium↓ | GVHD group had lower diversity of microbiota. The AIM score defined as microbiota diversity of 4 bacterials (Lachnospiraceae, Peptostreptococcaceae, Erysipelotrichaceae, Enterobacteriaceae) was positively correlated with aGVHD grade and could be predictive of the development of aGVHD. | (127) |
| 2019 | 1325 adult male patients | ● In GVHD patients: enterococcal↑ | Expansion of enterococcal was associated with GVHD and mortality which can be driven by lactose. | (104) |
| 2020 | 70 patients (35GVHD vs. 35 non-GVHD) | ● In GVHD patients: Lachnospiraceae, Blautia, Ruminococcaceae↓ | Microbiota alterations were highly specific of GI aGVHD severity with lower bacterial biomass, a-diversity and decreased butyrate. | (128) |
| 2020 | 1362 adult patients from 4 centers | ● In GVHD patients: Enterococcus, Klebsiella, Escherichia, Staphylococcus, Streptococcus↑ | Patterns of microbiota disruption during allo-HSCT were similar across transplantation centers and geographic locations which were characterized by loss of diversity and domination by single taxa, lower diversity was associated with higher risks of TRM and death attributable to GVHD. | (129) |

*OS, overall survival; AIM score, accumulated intestinal microbiota score; TRM, transplant-related mortality.*

decline in anti-inflammatory Clostridia in pediatric patients with aGVHD (125). Therefore, it can be speculated that some microbial taxa, such as Blautia, are beneficial for the outcomes of HSCT and mitigation of aGVHD, as they behave as drivers in this process, which should be protected and used in a probiotic approach. By contrast, the Enterococcus genus from Lactobacillales contributes to inflammation, whose role in human aGVHD is the same as that in animals, as expansion of Enterococcus association with increased GVHD in humans has been reported (104, 131). Lactobacillus, another genus of Lactobacillales, showed a possible protective effect in human GVHD (99). In 2018, Lijie Han et al. found that GVHD patients showed a higher abundance of Proteobacteria and a lower abundance of Clostridia, which was correlated with the Treg/Th17 ratio and H3 acetylation, indicating an interaction among alterations in the microbiota, allogenic T cell activation and histone acetylation (68). One year later, this team (127) proved again that in aGVHD, the diversity of the microbiota was significantly lower, with decreases in Clostridia, Lachnospiraceae, Blautia, Eubacterium, and Erysipelatoclostridium abundance and increases in Enterobacteriaceae abundance. This finding was consistent with a study in 2017 showing a persistent lack of Lachnospiraceae and Bacteroidaceae species in GVHD patients, whereas Lachnospiraceae was negatively correlated with neutrophil recovery (126). In addition, the specific actors in the intestinal ecosystem involved in the pathologic process of aGVHD have been explored more recently (128). Shown in stool samples, microbiota alterations were highly specific to gastrointestinal aGVHD severity, and a negative correlation was observed with the Lachnospiraceae, especially the Blautia genus, and Ruminococcaceae families. On the other hand, geographic variations matter, while a recent study analyzing 8767 fecal samples from 1362 patients with allo-HSCT at 4 different centers likewise showed a similar association between lower intestinal diversity and higher risks of transplantation-related death and death attributable to GVHD (129).

From these data, we conclude that restoring intestinal microbiota diversity after allo-HSCT is beneficial in the clinic, protective indigenous probiotics should be preserved to balance the alteration of intestinal microbiota community for patients.

# CLINICAL INTERVENTIONS AND VALUE OF THE INTESTINAL MICROBIOTA IN AGVHD

## Diet

Certain diets may contribute to the development of GVHD given that food is one of the most important factors affecting the composition of the intestinal microbiota (132). One example is a choline diet, and a murine model has shown that a high-choline diet enhances the allogenic GVH reaction, which leads to more aGVHD (86). The effect of parenteral or enteral nutrition on the intestinal ecosystem during HSCT has also been evaluated, with preference toward the latter choice, as enteral feeding has been shown to protect against GVHD in several studies (133–135), while parenteral nutrition was associated with poor outcomes and other complications (136, 137). The type of oral nutrition may be another important factor. Recently, elaborate foods known as a neutropenic diet for allo-HSCT patients have been reexamined, and advanced evidence has shown limited benefit and even potentially harm of supplying aGVHD patients with neutropenic diets (138, 139).

## Prebiotics and Probiotics

Intervention in the intestinal microbiota with a nutritional approach including prebiotics and probiotics may be another promising treatment option for aGVHD.

Prebiotics are indigestible compounds, usually indigestible carbohydrates, that bacteria have an advantage in metabolizing, resulting in the production of SCFAs and metabolites with a potential immunomodulatory role (140, 141). Strategies have been studied in the setting of aGVHD to modulate the intestinal microbiota by supplementation with inulin, oligosaccharides, galacto-oligosaccharides, and potato starch, showing beneficial results. In a recent study, Yoshifuji et al. found that intake of resistant starch and GFO (glutamine, fiber, and oligosaccharide) shortened the duration of oral mucositis and diarrhea and reduced the incidence and severity of aGVHD (142). Other clinical trials focused on fructooligosaccharide, potato-based starch, and gluten-free diets are currently being studied for potential benefit (143).

Probiotics are ingestible formulations of live bacteria that can modulate intestinal homeostasis. A probiotic strategy achieved by FMT consists of introducing one strain or selected strains of microorganisms that confer a benefit. An early report showed that a probiotic-rich diet prior to HSCT is associated with earlier neutrophil engraftment and a shorter duration of febrile neutropenia (144). Some probiotics were found to be safe to administer during aGVHD, such as *Lactobacillus plantarum* reported in pediatric patients (145) and *Lactobacillus rhamnosus* GG in murine models (146). However, controversies exist. Recently, there have been some concerns regarding the safety of administering living microorganisms to immunocompromised patients with altered gut permeability, as some clinical cases have demonstrated sepsis (147), bacteremia (148) and meningitis (149) after treatment of pediatric patients.

## Antibiotics

Given that the intestinal microbiota critically affects transplant outcomes, correctly managing the influence of the microbiota in GVHD—antibiotics has been encouraged. The advent of techniques to generate and maintain germ-free rodents since the 1940s made it possible to examine the microbiota in animals (123), and subsequent studies demonstrated the benefits of using antibiotics. Bekkum and Jones et al. found that germ-free mice housed in sterile conditions or mice treated with antibiotics developed less severe aGVHD (94, 95). Vossen et al. showed that in a cohort of 112 pediatric patients, recipients treated with total gastrointestinal decontamination (GID) using high doses of nonabsorbable antibiotics prevented moderate-to-severe aGVHD, suggesting that the translocation of luminal bacteria and their cell wall-derived compounds might be inhibited during

total GID (150). Weber et al. analyzed 394 patients receiving allo-HSCT and found that the treatment of rifaximin correlated with lower enterococcal positivity and higher urinary 3-indoxyl sulfate concentrations. Patients on rifaximin showed lower 1-year transplant-related mortality and higher overall survival. Infectious complications with systemic antibiotic use did not abrogate the beneficial effects of rifaximin on intestinal microbiota composition in the early course of allo-HSCT or outcome (151).

However, the use of gut-decontamination prophylactic antibiotics seems to be a doubled-edged sword. A retrospective analysis in 2018 mentioned above (68) proved that β-lactam antibiotic administration was an independent risk factor for aGVHD according to the Cox regression model for multivariate analysis of aGVHD. Although showing no significant adverse effect, in an allo-BMT murine model, treatment of both donor mice with broad-spectrum antibiotics and control SPF donor mice induced GVHD mortality at a similar rate, and reducing and altering the microbial flora in the donors had no effect on their T cell alloreactivity and induction of GVHD after allogeneic BMT (152). In human studies, the concept that gut decontamination prevents aGVHD is controversial given that some subsequent clinical trials have failed to demonstrate consistent benefits (68, 153–155). Prophylactic use of antibiotics is reported to be associated with more severe aGVHD that involves the intestinal tract and liver, impacting 1-y and 2-y overall survival (OS) in patients receiving myeloablative regimens (155). Earlier antibiotic treatment in patients prior to allo-HSCT was further associated with higher transplant-related mortality than no antibiotics (76). A potential role for anaerobic bacteria, in particular Clostridiales, was supported that Blautia abundance was inversely correlated with the risk of developing gastrointestinal GVHD (124), while clindamycin, an anti-anaerobic bacterial agent, increased the risk of GVHD by depleting anti-inflammatory clostridia (125). Reported previously, piperacillin-tazobactam and imipenem-cilastatin increased the incidence, severity, and mortality of GVHD, and piperacillin-tazobactam reduced Bacteroidetes and Lactobacillus abundance (102). Subsequent evidence further supports the adverse role of anti-anaerobic bacterial penicillin derivatives and carbapenems, as both are associated with a higher incidence of GVHD (156). Although seemingly negative, these findings may enlighten us that antibiotics preserving anaerobic bacteria may potentially reduce the risk of developing gut GVHD.

In conclusion, it is obvious that there remain many controversies for GVHD patients to undergo antibiotic therapy, which needs further exploration. Thus, it is of great necessity to identify new strategies to maintain the diversity and richness of the intestinal microbiota. Different attempts have been made in clinical practice involving narrow-spectrum antibiotics and modulating the timing and duration of treatment.

## FMT

Loss of microbiota diversity creates an opportunity to intervene in aGVHD by reestablishing diversity using microbes that modulate inflammation. FMT has been investigated as a potential therapeutic strategy for gut GVHD in both preventive and therapeutic strategies in recent years. The recent insight of FMT considers it the 'ultimate probiotic' for GVHD in allo-HSCT because it directly modifies the host's intestinal microbiome composition to restore eubiosis and gut homeostasis (157). Indeed, according to some previous reports, FMT significantly improves the outcome of GVHD. Kakihana et al. reported that 4 patients received FMT 92 days after HSCT, all patients responded to FMT within several days, with 3 complete responses and 1 partial response, and an increase in peripheral effector regulatory T cells during the response to FMT was also observed (158). Similarly, another study reported that two of three patients achieved a complete response with multiple FMTs, while the other obtained a partial response; the FMT response was correlated with increased microbial diversity and richness (159). More recent reports further illustrate the role of FMT in GVHD, with promising results (160–162). Currently, a number of ongoing clinical trials to investigate the role of FMT in preventing intestinal GVHD following allo-HSCT, summarized in **Table 4**, are being carried out to try to find the best treatment protocol.

Although many reports have shown that FMT is a safe and effective strategy used in different situations to modulate the microbiota and cure aGVHD, it should be noted that patients are usually immunocompromised with altered intestinal permeability, as infectious complications after FMT have been reported in other settings at the same time (163, 164). Moreover, the evidence mentioned above suggests a beneficial role of FMT in GVHD and needs to be confirmed in larger studies. The optimization of all practical aspects of FMT still needs to be addressed in the future.

## Predictive Marker

As reported in the current literature, a reduction in microbiota diversity and alteration of metabolites can predict transplantation outcomes and aGVHD, shedding light on the value of the microbiota. In 2015, Weber et al. detected 3-indoxyl sulfate (3-IS) in urine specimens within the first 28 days after allo-HSCT in 131 patients and found that a low level of 3-IS within the first 10 days was associated with significantly higher transplant-related mortality and worse overall survival 1 year after allo-HSCT. Furthermore, the class Bacilli was proven to be associated with low 3-IS levels (75). In 2017, Golob et al. showed that a gradient of 20 types of bacterial species could predict severe aGVHD by calculating a gradient of the sum of the relative abundance of positively correlated bacteria minus the sum of the relative abundance of negative correlates (126). Similarly, a study in 2019 by Lijie Han et al. showed that microbiota diversity combined with the gradients of 4 bacteria (Peptostreptococcaceae, Erysipelotrichaceae, Enterobacteriaceae, and Lachnospiraceae) can predict the development and severity of aGVHD (127). Geographic variations in the composition of human microbial communities and differences in clinical practices across institutions raise the question of whether relationships between microbiota composition and clinical outcomes after allo-HSCT are generalizable. In 2020, Peled et al. conducted a study with data from four clinical research institutions by comparing risk scores from regularized Cox regression with cross-validation; they

**TABLE 4** | Summary of ongoing microbiota-linked (not)recruiting clinical trials for GVHD.

| Number | Trail title | Interventions | Aims | Study design | Phase | Patients | Time |
|---|---|---|---|---|---|---|---|
| NCT03819803 | Fecal microbiota transplantation in aGVHD after ASCT | FMT | To explore the employment of FMT in GI-aGVHD. | Single group assignment Open label | III | 15 | 2017/3/1-2020/12/31 |
| NCT04285424 | FMT for steroid resistant gut acute GVHD | FMT | To evaluate safety and efficacy of FMT for the treatment of steroid resistant GVHD of the gut. | Single group assignment Open label | Early I | 30 | 2020/3/1-2022/3/1 |
| NCT03812705 | Fecal microbiota transplantation for steroid resistant/dependent acute GI GVHD | FMT | To evaluate the efficacy and safety of fecal microbiome transplantation in patients with steroid resistant/dependent acute gastrointestinal GVHD. | Single group assignment Open label | II | 30 | 2018/12/13-2022/12/31 |
| NCT04269850 | Fecal microbiota transplantation with Ruxolitinib and Steroids as an upfront treatment of severe acute intestinal GVHD | FMT; Ruxolitinib; Methylprednisone | To evaluate this combination treatment in the first line with FMT. | Single group assignment Open label | I/II | 20 | 2019/9/1-2023/9/1 |
| NCT03371667 | To compare the efficacy of the addition of Methotrexate (MTX) to current standard acute GVHD first-line treatment with corticosteroids | Methotrexate | To compare the efficacy of the addition of MTX to current standard acute GVHD first-line treatment with corticosteroids. | Randomized Multicenter Double blinded Parallel assignment | III | 102 | 2018/8/16-2021/9 |
| NCT03727113 | Optimization of antibiotic treatment in hematopoietic stem cell receptors | Antibiotics | To demonstrate that in ASCT receptors a predefined protocol of optimization of the antibacterial treatment will preserve the intestinal microbiota diversity which will correlate with decrease incidence of acute GVHD. | Observational Case-Control Prospective | Unknown | 180 | 2018/1/16-2020/5/31 |
| NCT03727113 | Choosing the Best Antibiotic to Protect Friendly Gut Bacteria During the Course of Stem Cell Transplant | Piperacillin-tazobactam | To compare the effects of different antibiotics on the community of friendly bacteria in the gut | Randomized Parallel Assignment Open Label | II | 144 | 2017/2/10-2021/2 |
| (not R) NCT04059757 | Fecal microbiota transplantation for the treatment of gastrointestinal acute GVHD | FMT | To see the efficacy and what side effects are seen with FMT as a treatment for GVHD. | Single group assignment Open label | II | 17 | 2020/7/1-2021/12 |
| NCT04280471 | Fecal microbiota transplantation for the treatment of severe acute gut Graft-Versus-Host Disease | FMT capsule | To explore side effects of using an investigational procedure (FMT) in treating patients with severe acute gut GVHD. | Single group assignment Open label | I | 10 | 2020/6/30-2022/7/1 |
| NCT04139577 | FMT In high-risk acute GVHD after allo-HCT | FMT | To evaluate the effectiveness of Fecal Microbiota Transplant (FMT) treatment in high-risk acute GVHD. | Single group assignment Open label | I | 11 | 2019/11-2023/5/1 |
| NCT03549676 | Fecal microbiota Transplantation for Treatment of Refractory Graft Versus Host Disease-a Pilot Study | FMT | To evaluate safety and efficacy of FMT for the treatment of refractory GVHD of the gut. | Single group assignment Open label | I | 15 | 2019/7/1-2020/12/31 |
| NCT03359980 | Treatment of steroid refractory gastro-intestinal acute GVHD after allogeneic HSCT With fecal microbiota transfer | FMT | To explore the employment of FMT in GI-aGVHD. | Single group assignment Open label | II | 32 | 2018/8/13-2020/12 |

*FMT, fecal microbiota transplantation; GI-aGVHD, gastrointestinal acute graft-versus-host disease.*

showed that not only a diversity metric but also a signature of specific bacterial abundances was informative about the risk of death after allo-HSCT across institutions (129). Similarly, another study analyzing data from stool and blood samples of 150 patients from two centers who underwent allo-HSCT also showed that gut microbiota score (GMS) from a LASSO (least absolute shrinkage and selection operator) model at neutrophil engraftment could predict aGVHD (165).

Thus, studies on the microbiota as a predictive marker for aGVHD are worth further exploration to provide assistance with the currently available tools for predicting the development of aGVHD.

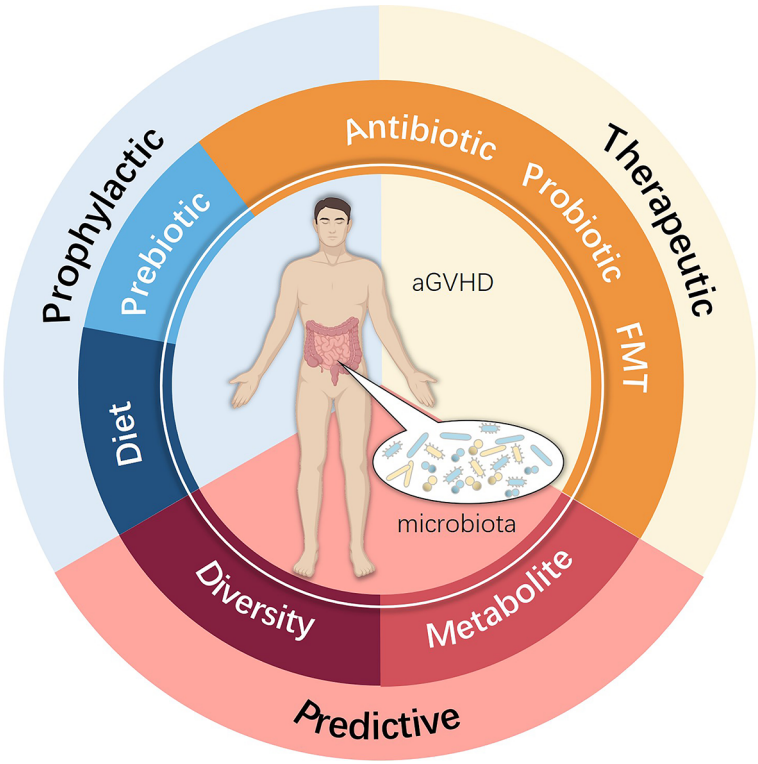


**FIGURE 3** | Potential clinical intervention associated with the intestinal microbiota used for preventing, treating and predicting aGVHD.

## CONCLUSION AND PROSPECTS

Alteration of the intestinal microbiota is a corollary given that the gut is an aGVHD target organ. Although we could further explore the intestinal microbiota by better dissecting compositional and functional microbiota structures, most of the research has concentrated on characterization, with data analyzed only *via* correlation. In this regard, determining which specific bacterial taxa are the main taxa affecting aGVHD is of great urgency and importance. In addition to descriptive investigations, mechanistic investigations are needed, which will help translate these associations into therapies for aGVHD if the microbiota is causal.

New approaches to prevent and cure aGVHD remain an unmet need that can be best addressed by understanding the complex pathophysiology, with increasing evidence indicating that the intestinal microbiota indeed participates in this process. Future studies are essential to explore further the role of the intestinal microbiota in aGVHD to elucidate the real impact of microbiota ecology. A promising approach may involve altering certain microbiota species by more precise and safer methods, which may consist of diet, prebiotics, probiotics, advanced antibiotic therapies, FMT, and microbiota metabolism analyses (**Figure 3**). Fully understanding the mechanism by which loss of microbiota diversity influences specific molecules and pathways and regulates the pathogenesis and development of aGVHD remains a top concern. Only by attempting to find better prophylactic and therapeutic schemes for allo-HSCT complications can we focus on what truly matters, which is also the appeal and value of the Human Microbiome Project and precision medicine.

## AUTHOR CONTRIBUTIONS

The manuscript was conceptualized by XZ and QG. TH wrote the majority of the manuscript. RW and XW co-wrote the manuscript. The figures were designed by TH and drawn by XW and SY. WW, RW, and TH summarized the tables. All authors contributed to the article and approved the submitted version.

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REVIEW
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# GVHD Prophylaxis 2020

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Graft-vs. host disease (GVHD), both acute and chronic are among the chief non-relapse complications of allogeneic transplantation which still cause substantial morbidity and mortality despite significant advances in supportive care over the last few decades. The prevention of GVHD therefore remains critical to the success of allogeneic transplantation. In this review we briefly discuss the pathophysiology and immunobiology of GVHD and the current standards in the field which remain centered around calcineurin inhibitors. We then discuss important translational advances in GVHD prophylaxis, approaching these various platforms from a mechanistic standpoint based on the pathophysiology of GVHD including *in-vivo* and *ex-vivo* T-cell depletion alongwith methods of selective T-cell depletion, modulation of T-cell co-stimulatory pathways (checkpoints), enhancing regulatory T-cells (Tregs), targeting T-cell trafficking as well as cytokine pathways. Finally we highlight exciting novel pre-clinical research that has the potential to translate to the clinic successfully. We approach these methods from a pathophysiology based perspective as well and touch upon strategies targeting the interaction between tissue damage induced antigens and T-cells, regimen related endothelial toxicity, T-cell co-stimulatory pathways and other T-cell modulatory approaches, T-cell trafficking, and cytokine pathways. We end this review with a critical discussion of existing data and novel therapies that may be transformative in the field in the near future as a comprehensive picture of GVHD prophylaxis in 2020. While calcineurin inhibitors remain the standard, post-transplant eparinsphamide originally developed to facilitate haploidentical transplantation is becoming an attractive alternative to traditional calcinuerin inhibitor based prophylaxis due to its ability to reduce severe forms of acute and chronic GVHD without compromising other outcomes, even in the HLA-matched setting. In addition T-cell modulation, particularly targeting some important T-cell co-stimulatory pathways have resulted in promising outcomes and may be a part of GVHD prophylaxis in the future. Novel approaches including targeting early events in GVHD pathogenesis such as interactions bvetween tissue damage associated antigens and T-cells, endothelial toxicity, and T-cell trafficking are also promising and discussed in this review. GVHD prophylaxis in 2020 continues to evolve with novel exicitng therapies on the horizon based on a more sophisticated understanding of the immunobiology of GVHD.

**Keywords: GvHD prophylaxis, calcineurin inhibitors, post-transplant cyclophosphamide, T-cell depletion, T-cell modulation, T-cell trafficking**

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## INTRODUCTION

GVHD prophylaxis has come a long way since the initial days of allogeneic transplantation. The improvement in GVHD outcomes has been one of the primary reasons for the reduction in non-relapse mortality over time (1) that has enhanced the success of allogeneic transplantation and allowed us to perform transplants in older patients as well as those with co-morbidities. GVHD comprises two distinct entities-acute GVHD (aGVHD) which typically presents in the first 3–6 months following transplant and manifests as a characteristic rash, secretory diarrhea, or cholestatic liver function abnormalities and chronic GVHD (cGVHD) which presents usually after the first 3 months and can affect virtually any organ system (ocular, oral, skin, musculo-skeletal, gastro-intestinal, pulmonary etc.,). Overlap syndromes are well-recognized, although relatively rare. These two entities have distinct pathophysiologies as well, however, prophylactic strategies generally try to prevent both acute and chronic varieties albeit with varying success depending on the strategy.

In HLA-matched transplantation, while the backbone of most widely used prophylactic platforms remains calcineurin-inhibitor (CNI)-based, a variety of new drugs have been added to CNI's in an attempt to improve efficacy and reduce toxicity. In this review, we discuss current standards and their evolution over time and highlight some of these translational advances. Further we touch upon novel pre-clinical advances developed on the foundation of a deeper understanding of transplant immunology and promising for translation to the clinic. We begin with a description of the immunobiology of GVHD, to better understand potential targets which have been exploited over the last few decades and currently to develop effective prophylactic therapies for GVHD.

## The Immunobiology of Graft-vs.-Host Disease

### Acute GVHD

One of the first models describing the biology of GVHD was proposed by Antin and Ferrara where they described a sequential cascade initiated by conditioning regimen mediated host tissue injury with the production of inflammatory cytokines (phase 1). This is followed by activation and proliferation of effector T-lymphocytes (phase II) which eventually lead to recruitment and activation of additional mononuclear effectors and amplification of a "cytokine storm" (Phase III) (2). Further extensive research has refined these concepts and identified targets for development of novel prophylactic strategies to prevent acute and chronic GVHD.

#### *Phase 1*

Both neutrophils and monocytes are involved in the initial inflammatory response in the pathogenesis of GVHD. Monocytes are activated by molecules called damage-associated molecular patterns (DAMPs) such as uric acid, ATP, Heparan sulfate, HMGB-1 or IL-33 which can initiate and perpetuate a non-infectious inflammatory response involving the innate immune system. In contrast pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides cause infection-associated inflammation. The DAMP and PAMP mediated inflammatory responses result in activation of the innate immune system (monocytes and neutrophils) which then cause local tissue damage mediated by reactive oxygen species. This eventually culminates in interaction of antigen-presenting cells (APCs) in the innate and adaptive immune and activation of cytokine cascades (IL-1, IL-6, TNF-α, etc.,) leading to the "cytokine storm (3)." Prophylactic strategies targeting these events have focused on arresting the cytokine storm through inhibition of particular cytokines or interrupting the interaction between APCs and PAMP.

#### *Phase II*

Primed by this cytokine storm, effector T-lymphocytes now migrate to lymphoid organs and host tissues mediated by L-selectin, CCR7. This culminates in APC mediated T-cell activation and engagement of the T-cell receptor complex and modulation by anti and co-stimulatory pathways. In particular, T-lymphocytes trafficking to the gut express high levels of integrin β7 (α4β7) which bind corresponding host tissue ligands presenting a potential target for intervention. The T-cell activation process and proliferation process is a crucial target for GVHD prevention.

#### *Phase III*

These events lead to a self-potentiating T-lymphocyte activation causing tissue damage via direct cellular cytotoxicity and indirectly via release of soluble mediators (TNF-α, IFN-γ, IL-1, and nitric oxide) (4).

A number of additional pathways have since been implicated in GVHD pathogenesis including the canonical NOTCH pathway (5). It has been shown via monoclonal antibodies that Notch-deprived T-cells proliferate normally but produce less inflammatory cytokines with a preferential increase in Tregs (6) and all the effects were dependent on NOTCH1/2 receptors on T-cells and Dll1/4 ligands in the recipient with dominant roles for NOTCH1 and Dll4 (6).

While the pro-inflammatory signals described above potentiate GVHD, there are also anti-inflammatory components of the immune system that try to dampen these inflammatory responses. Regulatory T-cells (Tregs) are important in immunologic tolerance, partly *via* release of anti-inflammatory cytokines such as IL-10 and TGF-β (7). Cytokine responses are often classified as effector T helper (Th) type 1 (IL-2, INF-γ) and type 2 (IL-4, IL-10) responses where type 2 cytokines can inhibit potent proinflammatory type 1cytokines, and a Th1 to Th2 shift could be beneficial in aGVHD (8). In addition a particular subset of CD4+ cells called Th17 cells have been identified which are characterized by the production characterized by production of *IL-17A* and F, IL-21, and IL-22 and which in murine models migrate to GVHD target organs causing severe pulmonary and GI lesions and GVHD deaths (9). These are postulated to be anatagonistic to Tregs (10) making them an interesting target. Invariant natural killer T (iNKT) cells are another cellular subset with putative immunoregulatory functions, in part via an increase Treg numbers and IL-4 secretion, that may be important in GVHD pathophysiology.

### Chronic GVHD

Chronic GVHD remains the most common late toxicity of allogeneic transplantation with significant morbidity and quality of life implications. cGVHD has its own distinctive immunobiology. Briefly we can conceptualize the pathophysiology of cGVHD in three phases: (1) Inflammation leading to tissue damage (2) chronic inflammation, thymic injury, dysregulated B- and T-cell immunity (3) tissue repair with fibrosis (11, 12). Although a more detailed discussion of these phases is beyond the scope of this review, we will focus on some of the known interventions that can prevent or reduce the incidence of cGVHD as well as some novel therapies being tested, particularly those targeting the B-cell axis.

Potential targets for developing novel prophylactic platforms have been identified based on our current and more comprehensive understanding of the biology of GVHD. In this review we discuss both current standards and important translational advances as well as exciting new potential therapies which may be translated to the clinic in the future.

## Current Standards in GVHD Prophylaxis

The effective prevention of GVHD is critical to the success of allogeneic transplantation. Based on the understanding that aGVHD is primarily mediated by effector T-lymphocytes, prophylactic strategies have focused on T-cell suppression in the recipient. Calcineurin inhibitors (tacrolimus/Tac and cyclosporine/CyA) inhibit the proliferation and activation of T-cells and have been used in combination with either methotrexate (MTX) or mycophenolate mofetil (MMF) as standard prophylaxis in HLA-matched HSCT. In two randomized controlled trials (RCT) in the 1990s, the combination of Tac/MTX was found to be significantly superior to CyA/MTX is the prevention of grade II-IV aGVHD and extensive chronic GVHD in HLA-matched sibling and unrelated donors, although a benefit in overall survival (OS) was not shown (13, 14). Furthermore, a single-center phase II RCT compared Tac/MTX with Tac/MMF and found that Tac/MTX was more effective in preventing severe aGVHD, particularly in matched unrelated donor (MUD) transplantation (15). CNI based prophylaxis remains the standard in HLA-matched transplantation. However, the recent advent of post-transplant cyclophosphamide (PTCy), has been revolutionary, not only allowing related donor haploidentical transplants to be performed but also making some inroads in the field of HLA-matched transplantation.

## Translational Advances in GVHD Prophylaxis

### *In-vivo* T-Cell Depletion/Modulation

T-cell depletion or modulation *in-vivo* has been the basis for the development of a number of novel GVHD prophylaxis strategies. These have typically been incorporated into regimens where the backbone comprises CNIs. We summarize some of these approaches below.

#### *Post-transplant Cyclophosphamide*

Transplantation across HLA barriers historically has been difficult due to high rates of graft rejection and severe GVHD secondary to strong bidirectional alloreactive responses between donor and recipient. The introduction of post-transplant cyclophosphamide (PTCy) in the context of haploidentical transplantation has been a gamechanger and allowed us to perform such mismatched transplants safely and effectively, typically from related donors.

First pioneered at Johns Hopkins, cyclophosphamide is given at doses of 50 mg/kg on days +3 and +4 following the infusion of haploidentical stem-cells. In initial studies reported by the Hopkins group, reduced intensity HSCT with PTCy along with Tac and MMF as GVHD prophylaxis resulted in engraftment in 87% of patients with acceptable rates of grade II-IV (34%) and III-IV aGVHD (6%). Further, rates of chronic severe GVHD were found to be particularly low. Relapse rates were in the 50% range (16). Numerous subsequent studies have shown similar numbers and more recently myeloablative haploidentical transplantation with PTCy based prophylaxis has also been widely adopted (17–19). It is interesting to note that the rates of grade II-IV aGVHD are not in fact significantly lower with PTCy based prophylaxis in haplotransplants compared with those seen in HLA-matched transplant with CNI based prophylaxis and the actual mechanistic implications of PTCy are being investigated.

The earlier more simplistic hypotheses postulated that PTCy results in the deletion of alloreactive T-cells with some debate regarding the effect it may have on the T-cell mediated graft-vs. leukemia (GVL) effect. However, more recently it has been shown in mice that Tregs are preserved and that T-effector cell exhaustion may play an important role (20). A comprehensive model has however not been defined yet.

The beneficial effect of PTCy on severe aGVHD and cGVHD has led to its adoption into the HLA-matched setting more recently. In a Phase II RCT (BMT CTN 1203) which compared three different GVHD prophylaxis regimens in RIC MUD HSCT (PTCy/Tac/MMF, Tac/MTX/bortezomib, and Tac/MTX/maraviroc) with standard Tac/MTX prophylaxis, the PTCy arm fared the best with comparable grade II-IV aGVHD (27%) but lower rates of grade III-IV aGVHD (2%) and cGVHD requiring immunosuppression (22%) outcomes. Relapse rates in the PTCy arm were 28%. Overall GVHD and relapse free survival (GRFS) was superior in the PTCy arm meeting the primary end-point of the trial (21). Again in an RCT from Europe, PTCy/Tac/MMF based prophylaxis fared better than CyA/MMF in the prevention of acute and chronic GVHD in HLA_matched RIC HSCT although the standard of care arm did not include Tac or MTX and numbers were limited (22). Some centers have been using low-dose ATG along with low-dose PTCy to try and improve grade II-IV aGVHD rates (23), however, this is not an universally accepted practice at this time due to limited data.

Bolstered by this data, PTCy/Tac/MMF is now being compared to standard Tac/MTX prophylaxis in RIC HLA-matched HSCT in a large phase III RCT (BMT CTN 1703). PTCy does have the potential to eliminate the effect of donor mismatch on GVHD outcomes, however, CNI based prophylaxis remains the standard until phase III data is available.

#### *Anti-thymocyte Globulin*

The polyclonal immunoglobulin product obtained from the sera of rabbits and horses immunized with human thymocytes or T-cell lines is called anti-thymocyte globulin or ATG. ATG has

been used as part of transplant conditioning to effect *in-vivo* TCD with the aim to reduce both acute and chronic GVHD with varying success.

Of the four RCTs that have evaluated ATG in combination with standard CNI/MTX prophylaxis, the first used horse ATG and showed a reduction in aGVHD; however, there were higher rates of infection with resultant no difference in NRM or OS. Importantly, there was a reduction in chronic severe GVHD (24, 25). The second RCT used rabbit ATG while the third mainly used PBSC grafts. In both of these trials, although there was no effect on aGVHD, a reduction in chronic GVHD was seen once again (26). Therefore, it seems that the use of ATG can reduce severe chronic GVHD with no deleterious effect on OS, however, aGVHD is not consistently reduced.

Rabbit ATG is generally considered to deplete T-cells more effectively as well as allow greater expansion of regulatory T-cells (Tregs) (27). The beneficial effect of ATG on extensive chronic GVHD was suggested in an retrospective analysis which compared ATG to no ATG containing GVHD prophylaxis regimens in matched unrelated donor transplantation (28). Subsequently, in a recent RCT which evaluated Tac/MTX ± anti T-lymphocyte globulin (ATLG), a form of rabbit ATG, in myeloablative unrelated donor transplantation, a significant reduction in grade II-IV aGVHD and moderate/severe chronic GVHD was seen. However, NRM and OS was impaired in the ATLG arm (29). It was suggested that a higher dose of ATLG in the trial may have contributed to increased infections and mortality.

In this context, there is evidence that increased doses or prolonged dosage schedules of ATG may have immunosuppressive toxicity with increased NRM and relapse (30). Individualized ATG dosing, based not just on weight but on absolute lymphocyte count has been proposed as a way to tailor doses of ATG for maximal benefit (31). Different doses of ATG have also been explored in the context of haploidentical transplantation (32).

In the realm of matched related donor transplantation, a recent multi-center randomized study from China demonstrated improved acute and chronic GVHD rates without compromising relapse or treatment-related mortality (33) and merits further study.

### *Sirolimus*

Sirolimus is a mTOR inhibitor which inhibits effector T-lymphocytes and in *in-vitro* studies appeared to spare regulatory T-lymphocytes. A favorable ratio of Tregs:Teff has been shown to be associated with better GVHD outcomes and hence sirolimus has an immunologic profile that was thought to be potentially beneficial for GVHD prevention. In addition, it has a distinct toxicity profile compared to tacrolimus and is not nephrotoxic. In a large RCT in myeloablative transplants with HLA-matched donors, sirolimus in combination with tacrolimus was compared with the standard Tac/MTX platform. There was no difference in grades II-IV aGVHD and cGVHD, but better grade III-IV aGVHD outcomes with sirolimus/Tac were seen. Non-relapse mortality (NRM) and OS were similar as well (34). Hence sirolimus appears to be an acceptable alternative to MTX when used with CNIs. In subsequent studies, sirolimus has been associated with higher rates of veno-occlusive disease (VOD) particularly when ablative busulfan is used (35) or when there are additional risk factors for VOD. In RIC transplantation, the addition of sirolimus to Tac/MTX resulted in better grade II-IV aGVHD outcomes without survival benefit in a phase II RCT (36). More recently, a phase II RCT found that the combination of sirolimus with CyA and MMF was superior to CyA/MMF; however, the comparator arm is generally considered inferior to Tac/MTX (37).

Sirolimus has been found to be particularly helpful in situations where nephrotoxicity is a concern such as in transplantation for sickle-cell disease. It is also being used with PTCy in patients with borderline renal function, with rates of engraftment and GVHD comparable to PTCy based regimens with CNI and may be a way to safely perform HSCT in patients with renal dysfunction (38).

Given its Treg sparing effects, novel combinations such as that with OX40L blockade are being investigated (39). This is discussed in greater detail in the section on OX40L blockade later in this review.

## *Ex-vivo* T-Cell Depletion/Modulation

*Ex-vivo* TCD has been used for decades in allogeneic transplantation as a prophylactic strategy to prevent GVHD. Methods of T-cell depletion have included (1) negative selection (removal of T-lymphocytes) through the use of monoclonal antibodies with or without complement (40, 41), counter flow elutriation (42), and immunotoxins (43) or (2) positive selection of CD34+ hematopoietic stem cells from the graft which (currently the preferred method) usually via immunomagnetic beads with the CliniMACS CD34 Reagent System (Miltenyi Biotech, Gladbach, Germany) (44). The two methods do differ in efficacy with greater TCD being achieved by positive selection.

### *Pan T-Cell Depletion*

An early concern with TCD was that it could affect the powerful GVL effect in HSCT which is also believed to be T-cell mediated. In a RCT in patients transplanted with marrow grafts, TCD was compared to conventional prophylaxis with CyA/MTX; the 3-year disease free survival (DFS) was similar in both groups with lower rates of grade III-IV aGVHD with TCD. Relapse rates were however, higher with TCD, particularly in patients with chronic myelogenous leukemia (CML) (45). A large registry analysis also showed higher relapse rates with TCD (46). Subsequently, in a phase II trial with peripheral blood stem-cell (PBSC) transplantation (BMT CTN 0303), immunomagnetic beads were used for CD34 selection of the graft and TCD and relapse rates appeared to be comparable to historic controls while rates of acute and extensive chronic GVHD were favorable (47). Another trial comparing CD34 selected HLA-matched sibling HSCT with conventional prophylaxis showed comparable rates of GVHD, relapse and overall survival (48). Whether these results will hold up in the setting of an RCT has been tested in the recently completed multi-center RCT (NCT02345850) comparing *ex-vivo* CD34 selection to PTCy + MMF and conventional Tac/MTX prophylaxis, the results of which are eagerly awaited.

The other notable issue with pan-TCD has been a higher incidence of graft failure and slower immune reconstitution (IR) leading to higher rates of infectious complications, particularly viral infections (CMV, EBV) (49). The use of additional *in-vivo* TCD in the form of anti-thymocyte globulin (ATG), particularly in the haploidentical setting can potentially be helpful in achieving better engraftment rates (50). Other strategies to improve IR have incorporated direct T-cell add back strategies post stem-cell infusion (51) or the use of megadoses of CD34 selected cells (Perugia group) which seem to have a tolerizing effect (52).

Given these issues with pan-TCD, more selective methods of T-cell depletion aimed at preventing GVHD while preserving GVL are being explored with the availability of sophisticated clinical grade cell separation techniques.

### *Selective T-Cell Depletion Strategies*

While a number of strategies have been attempted for selective TCD, they did not meet with lasting success. Depletion of CD5+ T-cells and CD8+ T-cells were tried in the 1990s; while rates of GVHD were encouraging, rates of relapse were high leading to the abandonment of these strategies. CD6 depletion is separately discussed in the section on T-cell modulation.

More recently Bleakley et al. have looked at CD45RA (naïve) TCD with the understanding that it is primarily the naïve T-cells in an allograft that are alloreactive. Unfortunately, this strategy has not produced encouraging results thus far. Bleakley et al. reported a first in human trial where they performed CD45RA (naïve) TCD via a two-step immunomagnetic bead-based procedure in 35 adult patients. Although 34/35 patients engrafted, rates of aGVHD were high in the 66% range. This was not improved when naïve TCD was combined with a/b TCD (described below) either and this approach is not widely used at this time.

### *α/β TCD*

While a majority of T-cells express α/β receptors T-cell receptors (TCR), 2–10% of T-cells express γ/δ TCR. These γ/δ T-cells are believed to have important innate immune effects characterized by rapid cytokine release and killing of viral infected and tumor cells (53). This makes them an attractive candidate to potentially mediate GVL without inducing GVHD by the selective depletion of α/β T-cells. In a prospective single-arm pediatric trial in patients with acute leukemia, an encouraging GRFS of 70% was seen (54). The median follow-up for surviving patients in this study was 46 months. This approach is being tested in a number of other trials in pediatric and adult patients; in one of these a CNI-free GVHD prophylaxis strategy for acute leukemia patients undergoing 1–2 locus MMUD MAC HSCT (NCT03717480) is being looked at.

## Modulating T-Cell Co-stimulatory/Co-inhibitory Pathways

During T-cell activation, following initial engagement of antigen by the TCR, a number of co-stimulatory and co-inhibitory signals come into play mediated by receptors on T-cells and APCs. This is true in acute GVHD as well. Hence the modulation of these co-stimulatory and co-inhibitory interactions is one of the new frontiers in the prophylaxis of GVHD.

### *CD28/CTLA-4 Blockade: Abatacept*

The most promising of these has been blockade of the CD28/CTLA-4 axis. CD28 and CTLA-4 are both receptors on the T-cell which bind to B7-1/CD80 and B7-2/CD86 ligands on the APC; however, while CD28 is co-stimulatory, CTLA-4 is co-inhibitory. Abatacept (CTLA4-Ig) is the soluble extra-cellular portion of CTLA-4 complexed with immunoglobulin heavy chain which blocks CD28 and CTLA-4, with more of an effect on CD28 leading ultimately to an inhibitory signal. Murine models from Blazar et al. showed that CD28/CTLA-4 blockade could reduce aGVHD lethality (55). Kean et al. performed a promising feasibility study in humans and reported results from a phase II RCT comparing standard of care (SOC) +abatacept to abatacept only in pediatric and adult patients. Grades III-IV aGVHD were significantly decreased and OS was improved in the abatacept arm (56). These impressive results have led to FDA breakthrough designation for this drug.

Since this approach blocks both stimulatory and inhibitory pathways, the concern for unwanted T-cell activation has been raised; hence more selective approaches to blocking CD28 are also being investigated. As an example, FR104 (CD28-specific pegylated-Fab') with and without sirolimus are being investigated in non-human primate models (57).

## Enhancing Regulatory T-Cells

Tregs (CD4+CD25+Foxp3+) comprise a unique subset of T-lymphocytes that can be derived from the thymus or converted from CD4+CD25- cells (inducible or iTregs). Tregs play an important role in immune homeostasis and a favorable balance between Tregs and effector T-cells may be important to prevent GVHD. While *ex-vivo* expansion of Tregs is possible (58), there are concerns about their stability *ex-vivo*. Some preliminary data in alternative donor transplantation has shown that infusion of such expanded Tregs can be beneficial (59).

Hence, rather than the direct infusion of Tregs, other approaches have been attempted which can upregulate Tregs or enhance their functionality in the post-transplant immune milieu. One such approach involves invariant natural killer T (iNKT) cells.

### *Invariant Natural Killer T Cells*

iNKT cells are unique in that they co-express both T and NK cell markers and therefore straddle both the innate and adaptive immune system with a semi-invariant TCR that recognizes glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule CD1d. They modulate the immune system via IL-4 and IL-10. In murine models, iNKT cells reduced GVHD both by a switch to a Th2 cytokine profile and/or IL-4-dependent Treg expansion. These mice were conditioned with a regimen incorporating total lymphoid irradiation plus ATG (TLI-ATG) (60, 61). This was then translated in a proof of concept study in humans with promising GVHD outcomes (62). An analysis of post-transplant immune reconstitution showed that low iNKT/T cell ratios were independently associated with

rates of acute GVHD (63) while another provocative study suggested that the larger numbers of iNKT cells in the donor graft correlated with improved GVHD free relapse free survival (64). Direct infusion of *ex-vivo* expanded iNKT cells is also an area of investigation.

In this contect, REGiMMUNE is a compound in which KRN7000, a synthetic alpha-galactosylceramide derivative and a CD1d ligand, is embedded in a lipid bilayer. REGiMMUNE has been shown to reduce aGVHD mortality by expanding Tregs via iNKT cells in murine models (65). In a Phase IIa trial REGiMMUNE in combination with sirolimus did reduce overall and acute GVHD although more mature data is awaited (66).

### Targeting T-Cell Trafficking

#### *Vedolizumab*

Alloreactive CD8+ T-cells bound for the intestines express high levels of integrin β7 (α4β7) that binds to its ligand mucosal addressin cell adhesion molecule 1 (MAdCAM 1) in Peyer's patches and gut-associated lymphoid tissue (GALT) in the intestinal mucosa. Vedolizumab is a humanized moAb which prevents T-cell trafficking to the gut by targeting α4β7integrins on the T-cells. Early proof-of-concept and restrospective analyses have shown promising efficacy with vedolizumab in steroid refractory aGVHD (67). Given its effect on T-cell trafficking, an early critical event in GVHD pathogenesis, it was then tested in the context of prophylaxis in a phase 1b study where it was moderately safe with low rates of acute and chronic GVHD (68). A phase III RCT comparing vedolizumab + SOC prophylaxis to SOC is currently underway (NCT03657160).

#### *Maraviroc*

Maraviroc is an antagonist of CCR5, a chemokine receptor that has been implicated in T-cell trafficking during GVHD pathogenesis. Maraviroc appears to block lymphocyte chemotaxis without actually affecting T-cell function which made it an attractive candidate as a prophylactic agent. However, in a prospective non-randomized study from the BMT CTN, maraviroc in combination with standard Tac/MTX was not superior to standard of care and in this trial the PTCy/Tac/MMF arm fared the best (21).

### Targeting Cytokine Pathways

#### *Tocilizumab*

Interleukin-6 (IL-6), an inflammatory cytokine has been shown to be one of the chief mediators of aGVHD in murine models (69). Therefore, IL-6 blocking agents could prevent aGVHD. In a phase II trial, tocilizumab, a humanized monoclonal antibody against the IL-6 receptor (IL-6R) was found to be promising (70); however, in a placebo-controlled phase III study from Australia, there was no significant difference in grades II-IV or III-IV aGVHD (71). This is a salient reminder that given the complex pathophysiology of aGVHD, with crosstalk between myriad cytokines and immune effector cells, targeting multiple cytokine pathways will be required for efficacy. These translational advances are summarized in **Table 1**.

## Novel GVHD Prophylactic Strategies on the Horizon

There are novel therapies which have not yet been successfully translated to clinical practice but hold great promise. These therapies are based on innovative targets based on a more intricate understanding of the pathophysiology of GVHD.

### Targeting Tissue Damage/Endothelial Injury

#### *Siglecs/CD24 Fc*

As mentioned above in the section on the immunobiology of GVHD, conditioning regimen associated tissue damage exposes antigens which comprise pathogens or Pathogen-Associated Molecular Patterns (PAMPs) and components of damaged cells (Danger-Associated Molecular Patterns or DAMPs) which trigger activation of the innate immune system. Conversely Sialic-acid-binding-immunoglobulin-like lectins (Siglecs) are a particular class of pattern recognition receptors that downregulate innate immune responses (72). A number of Siglec homologs have been identified in mice and humans and are all characterized by immunoreceptor tyrosine-based inhibitory motifs (ITIMs) or ITIM-like regions in their intracellular domains.

A role for Siglecs in modulating adaptive T-cell mediated immune responses has also been proposed. Reddy et al. have shown that Siglec-G interacts with CD24c in murine models and this interaction CD24 suppresses TNF-α, IL-1β, and IL-6 via NFkB and therefore is promising in the domain of GVHD prophylaxis (73).

#### *Defibrotide*

Defibrotide is a polydisperse mixture of predominantly single-stranded polydeoxyribonucleotides which in pre-clinical and human studies has demonstrated profibrinolytic, antithrombotic, anti-inflammatory and angio-protective effects ultimately resulting in stabilization of endothelial cells (74). Defibrotide is used in the treatment of veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS), another morbid complication of allogeneic transplantation. Endothelial activation is also associated with transplant conditioning regimens and prime the host for GVHD. In a randomized phase II pediatric trial of defibrotide in VOD/SOS, the incidence and severity of aGVHD at days +30 and +100 were significantly better in the defibrotide-treated arm in patients who underwent HSCT (75). This signal is being further explored in a phase II randomized, open-label study comparing defibrotide + SOC vs. SOC alone in pediatric and adult patients for the prevention of aGVHD (NCT03339297).

### T-Cell Modulation

#### *Notch Pathway*

The canonical NOTCH pathway has been shown to play a critical role in T-cell activation, differentiation, and function in aGVHD pathogenesis (5). Using humanized monoclonal antibodies, it has now been shown that Notch-deprived T-cells produce less inflammatory cytokines but proliferate normally, with a preferential increase in Tregs, without compromising GVL, mediated chiefly by NOTCH1, and Dll-4 (6). Selective

**TABLE 1 |** Translational advances and experimental strategies in GVHD prophylaxis.

| | Translational advances in GVHD Prophylaxis | | Experimental GVHD prophylaxis strategies | |
|---|---|---|---|---|
| 1. | *In vivo* T-cell depletion/modulation | | Targeting tissue damage/endothelial damage | |
| A. | Post-transplant cyclophosphamide | Phase II/III | Siglecs/CD24 Fc | Murine models |
| B. | Anti-thymocyte Globulin | Phase III | Defibrotide | Ongoing Phase II |
| C. | Sirolimus | Phase III | | |
| 2. | *Ex-vivo* T-cell depletion/modulation | | T-cell modulation | |
| A. | Pan T-cell depletion | Phase II/III | Notch pathway | Pre-clinical |
| B. | Selective T-cell depletion | | Mesenchymal stem-cells | Exploratory |
| | a/b T-cell depletion | Phase I/II | | |
| | CD45RA (naïve) T-cell depletion | Phase I/II | | |
| 3. | Modulating T-cell co-stimulatory/inhibitory pathways | | Modulating T-cell co-stimulatory/inhibitory pathways | |
| A. | CD28/CTLA-4 blockade/Abatacept | Phase III | OX40L blockade | Murine/non-human primate models |
| | | | ALPN101 | Murine models, Phase I/II ongoing |
| | | | CD6 blockade: Itolizumab | Xenograft models, Phase I/II ongoing |
| 4. | Enhancing Tregs (regulatory T-cells) | | Targeting T-cell trafficking | |
| | iNKT-cells | Murine models, proof of concept in humans | PSGL-1 | Murine models |
| | REGimmune | Phase IIa | | |
| 5. | Targeting T-cell trafficking | | Subset T-cell depletion | |
| | Vedolizumab | Phase 1b, Phase III ongoing | Xenikos/T-guard | Exploratory |
| | Maraviroc | Phase III (negative study) | | |
| 6. | Targeting cytokine pathways | | Targeting cytokine pathways | |
| | Tocilizumab | Phase III (negative study) | AAT (Alpha-1 antitrypsin) | Phase II/III ongoing |
| | | | JAK-STAT | Phase I ongoing |

NOTCH blockade is an exciting new frontier and offers potential for clinical translation.

### *Mesenchymal Stem-Cells*

Attempts have been made to utilize the immunomodulatory properties of MSCs for GVHD prophylaxis based on murine models of HLA-mismatched transplantation with co-transplantation of hematopoietic stem-cells and MSCs (76). Ning et al. showed in a small randomized trial, the incidence of grade II-IV aGVHD was 11.1% in the MSC group compared to 53.3% in the control group (77). However, the sample size in this trial was very small ($n = 25$) and larger studies are needed to further study the effect of MSCs on preventing GVHD. A randomized phase II trial has also shown some beneficial effect on cGVHD and need to be further studied (78).

## Targeting T-Cell Co-stimulatory Pathways (CD24 Fc)

### *OX40L Blockade*

OX40 (CD134) is a co-stimulatory receptor found on T-cells while its ligand OX40L is expressed on dendritic cells, B-cells, and endothelial cells. In 2003 Blazar et al. investigated the OX40 regulation of GVHD in murine models and found that antagonistic anti OX40L moAb or the use of $OX40^{-/-}$donor or recipient mice resulted in similar reduction in GVHD (79). Further, although OX40 was expressed on CD4 and CD8 cells, the effect of OX40 appeared to be mediated chiefly by CD4+ cells. OX40 is also a strong negative regulator of Foxp3(+) Tregs (80) and therefore blockage could enhance Treg reconstitution which could be beneficial in GVHD. Tkachev et al. (39) then have shown that in non-human primate models, the combination of KY1005 (OX40L blocking antibody) and sirolimus has synergistic activity in reduction of GVHD mortality associated with control of both Th/Tc1 and Th/Tc17 activity. In addition, there was a Treg sparing effect with the combination. This exciting approach is now being translated to the clinic.

### *ALPN101*

Inducible Costimulator (ICOS) is a member of the CD28/CTLA-4 family expressed on activated T-cells while the ICOS ligand (ICOSL), a B7 family member is constitutively expressed on B-cells, macrophages and dendritic cells and upregulated on APCs *via* TNF-alpha and lipopolysaccharides (LPS). In murine models of GVHD, blockade of the ICOS:ICOSL interactions via moAb or $ICOS^{-/-}$ mice resulted in significant decrease in GVHD and GVHD related mortality both mediated by CD4+ and CD8+ cells (81).

ALPN101 is a novel Fc fusion protein of a human ICOSL variant immunoglobulin domain (single domain vIgD™) binding both ICOS and CD28 at higher affinity than wild-type molecules, designed to inhibit both the CD28 and ICOS pathways to dampen co-stimulatory responses during alloreactive T-cell activation. In a murine dose-ranging study, ALPN101 inhibited

GVHD responses at all doses and more significantly than the comparator belatacept, an approved CTLA-4-Fc protein CD28 pathway inhibitor (82).

A phase 1/2 dose finding study (BALANCE) is ongoing in patients with steroid sensitive and steroid refractory aGVHD (NCT04227938) investigating this potentially transformative effect and this is another promising target for GVHD prophylaxis.

#### *CD6 Blockade: Itolizumab*

CD6 is a co-stimulatory receptor on T-cells that binds to activated leukocyte cell adhesion molecule (ALCAM), a ligand on APCs and is involved in T-cell activation and trafficking. Historically CD6 T-cell depletion using a monoclonal antibody (moAb) was evaluated in a single-arm trial ($n = 112$) with aGVHD rates in the 18% range using bone marrow grafts (41). More recently, itolizumab, a humanized anti-CD6 moAb was tested in xenograft models with some evidence that it could modulate T-cell activity (83). This molecule has also been fast-tracked by the FDA and is being tested in a phase I/II study for first-line treatment (with steroids) of severe aGVHD (NCT03763318) and may have a role in prophylaxis.

### Targeting T-Cell Trafficking

#### *PSGL-1*

P-selectin is one of a family of three glycosylated lectins (E, L, and P-selectin) which is constitutively expressed on the vascular endothelium of skin and bone marrow and inducibly expressed on other cells during inflammation. P-selectin is a receptor for PSGL-1, a glycoprotein strongly expressed on all leukocytes (84). PSGL-1 mRNA has been shown to be upregulated during GVHD in experimental models (85). P-selectin deficient mice were shown to have less GVHD morbidity and mortality; in addition T-cells were redirected from Peyer's patches and GALT to spleen and lymph nodes indicating that disruption of P-selectin interactions during GVHD pathogenesis can affect T-cell trafficking to target organs (84). Although it is likely that disruption of this pathway alone may not fully abrogate selectin interactions in GVHD, it is a promising new target.

### Targeting Cytokine Pathways

#### *Alpha-1 Antitrypsin*

AAT is a liver derived serine protease inhibitor which can inhibit proinflammatory plasma cytokines and induce anti-inflammatory IL10 among other somewhat protean immunologic functions. It has also been shown to be involved in the *in-vivo* induction of Treg. In preclinical aGVHD models, AAT reduced inflammatory cytokines, altered the ratio of effector and regulatory T-cells and reduced levels of DAMPs (86). AAT has shown promise in early phase trials for SR aGVHD (87) and is being tested in phase III trials. This drug is also being tested in the prophylactic setting in a phase II/III randomized, multi-center, placebo-controlled study for prevention of aGVHD (NCT03805789).

#### *JAK-STAT*

The Janus kinase family comprises intra-cellular signaling proteins (JAK-1, 2, 3, and tyrosine kinase 2) involved in downstream transduction of various cytokine pathways (88). They are fundamentally involved in all three phases of GVHD pathogenesis by regulating the activity of APCs, T- and B-lymphocytes (89). Pre-clinical studies showed that JAK-1/JAK-2 could reduce GVHD without affecting GVL (90, 91) including an effect on T-cell trafficking and enhancement of Tregs. JAK-1/JAK-3 inhibition also appears to reduce GVHD in murine models (92). Following on encouraging early phase studies with JAK-1/JAK-2 inhibitor ruxolitinib (Rux) in SR aGVHD, phase III data has now been reported (Rux vs. investigator's choice for SR aGVHD, REACH-2) which shows better overall response rates with Rux although a benefit in NRM could not be shown. JAK inhibition could be an exciting new frontier for GVHD prophylaxis as well. Choi et al. (93) showed that baricitinib (JAK1/JAK2 inhibitor) completely prevented GVHD in murine models without hampering GVL by multiple mechanisms including expansion of Tregs by preserving JAK3-STAT5 signaling; downregulation of CXCR3 and helper T cells 1 and 2.

So far clinical data is limited to a pilot study in myelofibrosis patients where a combination of ATG, ruxolitinib, and PTCy was used as GVHD prophylaxis with acceptable engraftment rates (94) and a single-arm study where Rux was used to replace CNIs in patients with CNI intolerance (95). Itacitinib, a selective JAK-1 inhibitor is being investigated in combination with CNI for primary prophylaxis of GVHD (GRAVITAS-119 trial, NCT03320642).

### Subset TCD

#### *Xenikos/T-Guard*

Monoclonal antibodies conjugated with immunotoxins is a method of selective TCD that has been attempted in the past with CD5 as a target among others as described above. T-guard is a immunotoxin combination comprised of a mixture of anti-CD3 and anti-CD7 antibodies separately conjugated to recombinant ricin A (CD3/CD7-IT), which induces *in vivo* depletion of T cells and natural killer (NK) cells and suppresses T cell receptor activation.

This was first evaluated in a phase I/II trial in humans in SR aGVHD with a 50% response rate and manageable toxicities albeit with evidence of capillary leak and thrombotic microangiopathy (96). This may be a potential target for prophylaxis in the future. These experimental methods are summarized in **Table 1**.

## Prevention of cGVHD

Although cGVHD is a distinct clinical and immunologic entity from aGVHD, there are limited interventions that specifically target the prevention of cGVHD. In general we know that patients who have less aGVHD will likely get less cGVHD and so the prevention of aGVHD is important in the prevention of cGVHD. In terms of donor and transplant related interventions, younger same-sex donors and the use of bone marrow product rather than PBSC has been shown to reduce rates of cGVHD (97).

T-cell directed approaches that have been quite successful include ATG and more recently PTCy as outlined above. B-cell directed approaches are an area of interest given our current understanding of the important role that B-cells play in the pathophysiology of cGVHD. Rituximab, a monoclonal antibody targeting CD20 was evaluated in a phase II trial with cGVHD rates in the 48% range cGVHD requiring immunosuppression in the 31% range (98). This was promising at the time and led to an ongoing randomized trial where obinutuzumab, another monoclonal B-cell directed antibody is being tested for cGVHD prevention (NCT02867384). It will be interesting to see how it will fare in comparison to PTCy.

Another area of interest is the augmentation of tolerance by the use of low-dose IL-2 (aldeskeukin) to enhance Treg reconstitution creating a favorable immunologic milieu for the prevention of cGVHD. This strategy has had success in the therapy of steroid-refractory cGVHD (99) and may have a role in prevention as well although remains investigational at this time.

## DISCUSSION

GVHD prophylaxis has evolved over the last few decades from direct *in-vivo* and *ex-vivo* pan T-cell depletion strategies to more directed immunomodulatory strategies based on a more comprehensive understanding of GVHD immunobiology. Nevertheless, the basic backbone of CNI based prophylaxis has survived the test of time. We have touched upon this current standard in this review and further discussed important translational advances and exciting pre-clinical strategies which may be a part of future prophylactic regimens.

Of these translational advances, PTCy is arguably the most exciting and a potential replacement for standard CNI/MTX based prophylaxis in multiple transplant settings. Sometimes considered to be an elegant method of *in vivo* T-cell depletion, mechanistic studies have indicated that PTCy has a far more complex impact on the post-transplant immune system including a Treg sparing role which is of great interest in the community (20). In haploidentical transplants, PTCy based GVHD prophylaxis is the standard, typically incorporating a CNI and MMF in the most widely used regimens. Although rates of grades II-IV aGVHD are comparable in haploidentical transplants with PTCy based prophylaxis compared to CNI/MTX prophylaxis in MUD transplants, the rates of severe acute and chronic GVHD are far lower without significantly compromising relapse rates which makes it a very attractive strategy (100). In fact, the results in the haploidentical setting with PTCy based prophylaxis have been so impressive that this platform is now being tested in the HLA_matched setting where it is being compared to standard CNI/MTX based prophylaxis. Data from a small RCT (22) as well as a larger prospective trial (BMT CTN 1203) (21) in the reduced intensity setting have already generated encouraging signals where the PTCy arm performed better than the standard CNI arm as well as other potential novel strategies. Based on this data, some centers have already migrated to using PTCy based prophylaxis for matched unrelated donor and in some cases even in matched related donor transplantation. However, from a purist's viewpoint, data from a well-powered RCT is still not sufficient to change practice standards and the results of BMT CTN 1703 comparing PTCy based prophylaxis to CNI/MTX are eagerly awaited. In the myeloablative setting, the standard remains CNI/MTX although PTCy based prophylaxis will likely be tested in this setting as well. Other *in-vivo* TCD strategies such as ATG are still widely used, although the most profound effect of ATG appears to be on severe chronic GVHD and comes at the cost of poorer T-cell immune reconstitution and therefore more infectious complications. Sirolimus is another drug that has had promising results in reducing severe acute GVHD without much impact on chronic GVHD and is a reasonable alternative to CNI/MTX (34).

In the domain of *ex-vivo* TCD, pan TCD is still performed routinely in certain centers; once again with gains in the realm of severe chronic GVHD at the cost of more infectious complications. There have been some concerns about higher rates of relapse with *ex-vivo* TCD as well. These three important methods of T-cell depletion for GVHD prophylaxis, namely CNI/MTX, PTCy and *ex-vivo* pan-TCD have been compared in a multi-center RCT (BMT CTN 1301), the results of which are eagerly awaited as well. In the last decade the spotlight has shifted to methods of selective *ex-vivo* TCD with limited success in the clinical setting. a/b TCD which attempts to reduce GVHD without affecting GVL and can be performed without the use of post-transplant immunosuppression is promising and may be an important modality in the future.

Separate from direct TCD (*in-vivo* or *ex-vivo*), a new frontier in GVHD prophylaxis is targeting immune checkpoints which regulate T-cell activation. Given the dramatic success of checkpoint inhibitors in the world of solid tumor oncology, there has been tremendous interest and a much better understanding of these checkpoints in recent years. In the case of GVHD of course, researchers have tried to downregulate rather than upregulate T-cell activation following initial antigen engagement by the T-cell receptor complex. Although there are a number of molecules being tested at the bench and detailed in this review, the most promising of these has been blockade of the CD28/CTLA-4 axis with Abatacept (CTLA-Ig) with an eventual inhibitory signal downstream to the T-cell. Results from a RCT with pediatric and adult patients has shown a dramatic reduction in severe acute and chronic GVHD including in mismatched unrelated donors (56). With FDA breakthrough status, this molecule has the potential to be an integral part of GVHD prophylaxis in the future although it is unclear if it is more effective than PTCy based prophylaxis. Certainly cyclophosphamide, a drug that has been used for decades, is far more affordable and therefore a platform easily generalizable in more resource poor settings.

Targeting T-cell trafficking, an early event in GVHD prophylaxis, is being tried with integrin blockers such as vedolizumab. It is logical that inhibiting the very movement of effector T-lymphocytes to target organs should better prevent GVHD rather than trying to arrest the widespread inflammation and cytokine cascades which characterize the final common pathway in GVHD pathogenesis. As a testimony to that, when tocilizumab an IL-6 blocker was evaluated, despite promising phase II single-arm data, was not more effective than standard

prophylaxis in a phase III RCT (71). This has in fact been the case with numerous promising prophylactic therapies which perform well in single-arm studies but have not been a home run in well-designed phase III studies.

Within the limitations of this review, we have highlighted some of the exciting pre-clinical science that has the potential to translate into effective prophylactic therapies which target GVHD pathogenesis beyond direct T-cell depletion. Targeting the interaction between DAMPs/ PAMPs on APCs and Siglecs with a downstream inhibitory effect on cytokine cascades as well as investigating a role for Siglecs in modulating adaptive T-cell mediated immunity are areas of interest (73). Endothelial damage, another inciting pro-inflammatory event in GVHD pathogenesis is being targeted by drugs like defibrotide which have enjoyed tremendous success in the therapy of VOD. Targeting selectin interactions such as PSGL-1 (84) is another developing area in the field of GVHD therapeutics and prophylaxis.

Pathways critical in T-cell modulation during activation and proliferation such as the Notch pathway has been an area of great interest although not ready for translation at this time (6). Bolstered by the success of Abatacept, other molecules targeting checkpoints such as OX40L blockade (including combination with sirolimus) (39), blockade of the ICOS: ICOSL interaction with ALPN101 (81) and CD6 blockade (Itolizumab) (83) are extremely exciting. The role of AAT in prophylaxis both as an immunomodulator as well as in opposing inflammatory cytokines is being looked at.

In conclusion, while GVHD prophylaxis in 2020 still incorporates the traditional paradigms of CNI based prophylaxis, PTCy is knocking on the door and a number of exciting new translational therapies and pre-clinical advances are on the horizon which promise to challenge the established paradigms.

## AUTHOR CONTRIBUTIONS

JHA supervised and edited manuscript along with MG. All authors contributed to the article and approved the submitted version.

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# Revisit of Optimal Donor Number Estimation in the Hong Kong Bone Marrow Donor Registry

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High resolution typing of the HLA-DPB1 locus for patient who requested for hematopoietic stem cell transplantation (HSCT) workup has recently become mandatory by the National Marrow Donor Program (NMDP) in order to facilitate matching between donors and recipients for better outcomes. The likelihood of identifying HLA matched donors in Hong Kong, on top of the existing HLA-A, -B, -C, and -DRB1 loci, is revisited in this study. HLA-A, -B, -C, -DRB1 and -DPB1 genotypes of 5,266 volunteer unrelated Chinese donors from the Hong Kong Bone Marrow Donor Registry (HKBMDR), were included in this study. Matching models were employed to determine the matching probabilities for 10/10(DPB1) and 9/10(DPB1) HLA match. The matching probabilities are 20% at 10/10(DPB1) HLA match and 55% at 9/10(DPB1) match, based on the existing 130,000 donors in the HKBMDR. The likelihoods of match become 27% and 65% respectively, by increasing the registry to 250,000. However, if DPB T-cell-epitope (TCE) model is considered in the matching, the probability will increase to 46% at 10/10 DPB1 permissive mismatching. Our findings provide vital information about the future planning on the targeted recruitment size, HLA typing and search strategies of the donor registry and arose the transplant physicians' acceptability to 9/10(DBP1) or 10/10(DBP1) HLA match. Nevertheless, the marrow donor registry has planned for increasing the registry size and bringing down the age of recruited donors which will ultimately enhance patient outcome.

**Keywords: alleles, frequency haplotypes, HLA antigens, Chinese, matching probability**

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## INTRODUCTION

The detrimental graft-versus-host disease (GVHD) remains a major challenge after curative hematopoietic stem cell transplantation (HSCT). Systemic outcome analysis has shown that HLA-DPB1 mismatch had resulted in increased risk of acute GVHD. Transplantation with non-permissive DPB1 mismatch was shown to be associated with higher transplant-related mortality (1). Starting from 27 February 2021, HLA typing of DBP1 loci has become mandatory for patients

requesting for HSCT workup from the National Marrow Donor Program (NMDP). In light of better outcome for HSCT, optimal matching between donors and recipients are recommended at high resolution in the HLA-A, -B, -C, -DRB1 and -DPB1 loci. Due to the population-specific allelic variation and the extremely high level of HLA gene polymorphism, the availability of optimal HLA-matched unrelated donors and cord-blood units has always been a concern (2, 3). As a result, donors with mismatched HLA antigens may also be considered in many situations. However, these HLA mismatches may lead to an 8% reduction per loci in the 5-year overall survival rate after HSCT (4). The additional information on DPB1 loci may help clinician on final donor selection by reviewing the matching at DPB1 to enhance the patient outcome when more than one potential donors are available for HSCT.

Volunteer unrelated donor database has been managed by the Hong Kong Bone Marrow Donor Registry (HKBMDR). At present, there are close to 130,000 stem cell donors in HKBMDR and 38 million donors in the Bone Marrow Donors Worldwide (BMDW) (5). Continual growth on the number of donors has been achieved globally. However, it accompanied with significant resource implication in donor recruitment and HLA typing. Therefore, strategic donor recruitment becomes very important account of the donor registry planning. Many crucial factors, including recruitment on more young male donors (6) or focus on the recruitment of donors with rare human leukocyte antigen (HLA) phenotypes (7), donors from ethnic minority (8–11), and recruitment activities based on HLA frequency differences at regional priority setting (12–15).

Estimation of matching probability, including mixed patient population, provides vital information for donor recruitment strategy planning and framework for international stem cell donor exchange (16). We have used the calculations based on HLA-A, -B, -C and -DRB1 loci high-resolution haplotype frequencies (HF) of our own population to estimate the donor pool size earlier (17).

The linkage disequilibrium between HLA-DPB1 and other loci are weak due to a hot-spot of recombination between HLA-DPB1 and HLA-DQB1 loci (18). A big proportion of unrelated donor HSCTs were performed across HLA-DPB1 mismatches (19, 20). HLA-DPB1 alloantigens are target of graft-versus-leukemia (GVL) or graft-versus-host (GVH) disease mediated by alloreactive T cells (21–24). However, only 3-57% of HLA-DPB1 were typed in the HLA DNA typed unrelated donors from varies registries (25). Since it was well known that racial and ethnic background play a profound role in adult-donor availability and match probabilities (26), the same phenomenon was proven in our previous study (17). We estimated the donor pool and matching probability on HLA 10/10(DBP1) matching with reference to our recent publication on the gene and HF of the Hong Kong population (27). To our knowledge, this is the first study to revisit the calculation of matching probabilities of our population and the estimation of donor size based on the additional DPB1 requirement.

# MATERIALS AND METHODS

## Sample Collection and Genotyping

The gene frequency and HF as reported previously were used in the analysis (27). In brief, Next generation sequencing supplemented with sequence-specific primer was used to define allele combinations and some specific alleles with 5,266 donors. HF was calculated from these results using Markov Chain Monte Carlo (MCMC) algorithm PHASE (28). Matching model was then utilized by using the calculated HF and effective adult-donor registry size for each group, with the assumption of genotypes in Hardy-Weinberg equilibrium (HWE) (29, 30).

HLA-DPB1 typing was assigned based on T-cell Epitope algorithms version 2.0 assignment (https://raw.githubusercontent.com/ANHIG/IMGTHLA/Latest/tce/dpb_tce.csv) and also the online tool at https://www.ebi.ac.uk/ipd/imgt/hla/dpb_v2.html (31). The TCE group assignment was reported for all HLA-DPB1 alleles according to the Release 3.38.0 of the IPD-IMGT/HLA Database, released 2019-10. The predicted immunogenicity of the HLA-DPB1 matching will be presented as Permissive, Non-Permissive GvH or Non-Permissive host-versus-graft (HvG).

## Statistics Analysis

The frequencies of HLA-A, -B, -C, -DRB1 and -DPB1 alleles were calculated from the number of observed genotypes. MCMC simulation from Guo and Thompson was utilized to assess the Hardy-Weinberg equilibrium for each loci *via* PHASE (32), and the deviance of genotype frequency within each loci was detected by PHASE invoking Arlequin (33). $P$ value of <0.01 was considered to be statistically significant.

Formulae described by Schmidt et al. has been utilized in this study with modification (16). In brief, the probability $p(n)$ for any patient from their own population to identify at least one matched donor in a registry including n individuals of a donor population is given $p(n) = \Sigma_i f_i[1-(1-f_i)^n]$ with $p(n)$ being the matching probability in "$n$" sample size, $f_i$ being the frequencies of the $i$-th genotype and $i$-th is any genotype from the rank of genotype frequencies in the order from the highest to the lowest in a donor population. The estimated HF was used to derive the genotype frequencies under the assumption of HWE.

# RESULTS AND DISCUSSION

Data from the recently published HLA genotype and haplotype frequencies of the HKBMDR (27) was applied in this study. Characteristics of these HLA haplotypes in Hong Kong were summarized in **Table 1**.

In concordance with our previous study (17), it was found that the number of haplotypes was significantly increased with number of donor samples. This increase is exclusive for our local population, as a plateau of number of haplotypes with increase in sample size was not observed in other ethnic groups, e.g. Caucasians and European populations (34). Mori et al.

**TABLE 1** | Characteristics of the haplotypes of Hong Kong.

| HLA loci available | | A-C-B-DRB1-DPB1 |
|---|---|---|
| Sample size (N) | | 5,266 |
| Sample size (2N) | | 10,532 |
| Number of haplotypes (>0.006%) | | 3,326 |
| Sum (%) of haplotype frequencies within the top | 10 | 10.7 |
| | 25 | 16.0 |
| | 50 | 21.1 |
| | 100 | 28.1 |
| | 250 | 40.2 |
| | 500 | 51.7 |
| | 1,000 | 65.6 |
| Number of haplotypes with frequency | ≥0.01 | 3 |
| | ≥0.005 | 9 |
| | ≥0.001 | 126 |
| | ≥0.0005 | 323 |
| | ≥0.0001 | 1,918 |

reported that a significant higher level of the occurrence of common haplotypes (0.01%) was observed in Asian Americans than in Caucasian Americans in the NMDP database. This suggested that the Caucasian Americans had a smaller degree of genetic diversity than Asian Americans (35). Similar findings from a large sample database that the occurrence of common haplotypes in Asian or Pacific Islanders (API) was also higher than Caucasians (34). However, whether the same phenomenon will be observed when HLA-DPB1 is considered requires further elucidation.

A similar methodology was applied in calculating the likelihood of finding a "matched" donor in US (26), likelihood of finding an 8/8 HLA match or ≥ 7/8 HLA match by different donor registry size in Hong Kong was reported in previous study for matching A, B, C, DRB1 loci only (17). With the increase in the number of donors in the HKBMDR to 130,000 as of December 2020, the likelihood of finding an available 8/8 HLA matched donor is 49% and 69% for finding 7/8 HLA matched donor (**Figure 1**). The results were comparable to those figures found among Asians, Pacific Islanders, and Native Americans (26). However, when taking into account of matching for HLA-DPB1 loci, the likelihood of finding an available 10/10(DBP1) HLA matched donor is 20% while 55% for finding 9/10(DBP1) HLA matched donor. Similar finding was observed in a Finnish retrospective study in which only 32.6% of local donors or 19.3% of both local and foreign donors were HLA-DPB1 matched with HSCT patients (36). In our data, the matching probability increases to 38% when taking into account of the DPB1 T-cell-epitope (TCE) permissive mismatching model.

TCE Groups has been utilized in classifying HLA-DPB1 mismatches that might be tolerated (permissive) or would increase risks (non-permissive) after unrelated HSCT. If HLA-DPB1 matching with TCE Groups is considered, beneficial effect during donor selection has been shown in various studies (37, 38). Donors with a permissive HLA-DPB1 group are preferred over those showing a non-permissive HLA-DPB1 group, among those 9/10(DPB1) and 10/10(DBP1) potential donors. "DPB1 TCE3 grading" has been implanted in OptiMatch with the

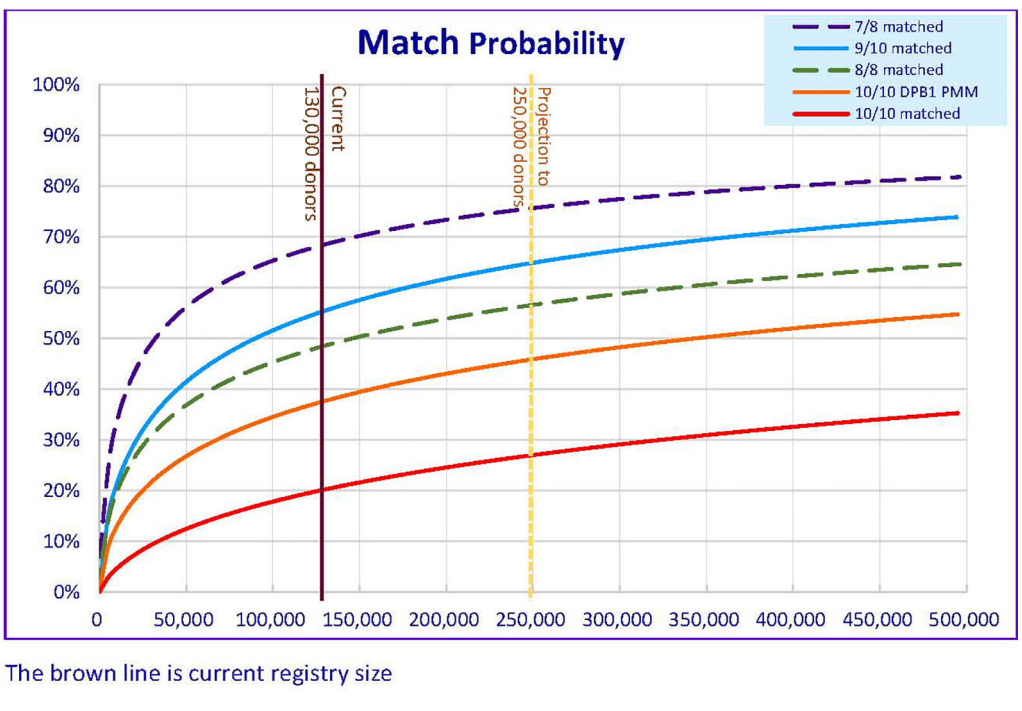


**FIGURE 1** | Matching Probability (MP) of varies level of HLA match against different donor registry size in HKBMDR. DPB1 PMM – with HLA-DPB1 permissive mismatch.

evident published by Zino et al. (39, 40). The new score was assigned based on the 3 TCE Groups algorithm according to the T cell cross-reactivity patterns (31).

IPD-IMGT/HLA website provided the original feature of DPB1 TCE3 algorithm and have been used in the BMDW Search & Match Service. The discrimination of permissive or non-permissive HLA-DPB1 mismatches is determined based on whether the donor and patient alleles belong to the same (permissive) or different (non-permissive) TCE Groups. There are total of 81 combinations of the HLA-DPB1 typing resulting for TCE version 2 assignment (**Supplementary Table 1**).

Greater heterogeneity in HLA typing of the Hong Kong Chinese was found where compared with other populations (34, 41, 42). Therefore, to enhance the chance of successful donor search, a larger donor pool is warranted. In concordance with the findings by Dehn and Buck, the likelihood of matching in HLA-A, -B, -C, -DRB1 and -DQB1 10 alleles for Asian Americans was also inferior than Caucasian Americans for 7/8 or 9/10(DQB1) matched unrelated donor search was also lower (98% vs 88%) (43–45).

In addition to the matching issue, attrition of donors due to age and contact unavailability may pose another negative impact on the likelihood of finding a donor. Based on the previous registry size of 100,000, the attrition rate was 2% or 2,000 per year. As shown in the projection (**Figure 1**), increasing the registry size to 250,000 in five-year time, 26,600 new recruitments per year is required to achieve matching likelihood at 46% for 10/10(DBP1) HLA-DPB1 permissible Match or 65% for ≥ 9/10(DBP1) HLA Match. An annual recruitment of 26,600 is a big rise compared to the current of 8,000 per year. Extra resources should be sought to cover the cost in donor recruitment and HLA typing. A survey was conducted to identify the crucial factors that affect the motivation of stem cell donation in Hong Kong (46). To enhance the recruitment ratio of the younger age group, recruitment program targeting a specific age group, especially for student at higher education may facilitate better recruitment rate and longer maintenance for donation to maximize the cost-effectiveness. Targeted educational activities such as Stem Cell Donation campaign, including educational talks to students and parents, promotion video on social media and social networking platforms and roadshows may help to enhance the recruitment of youngsters.

Racial and ethnic background in a donor registry has been reported to affect the adult-donor availability (26). The current analysis has not taken into the account of adult-donor availability which may have substantially lower match likelihoods. In addition, donors from the patient's own racial and ethnic group has shown to have the highest matching probability (47), this probability may also be enhanced if donors from other racial and ethnic groups could be available. Registry with donors that have a relatively low occurrence of inter-racial or inter-ethnic marriage might have less chance to have donors identified from other groups. The overall donor available rate is less than 30% (27) and it will expect to be lowered when additional loci is considered.

In the above estimation, the matching probability from around 3 million Chinese donors registered in China and Taiwan registries has not taken into account, which may provide extra donor matching. Furthermore, the matching probability of the cord blood units which are readily available and require less stringent HLA matching was not included in this calculation. Cord blood would be used as an alternative when adult donor is not readily available in many transplant centers. The issue of relatively low stem cell dose for adult size recipient has been resolved by the application of double cord blood units, and has been proven success clinically (48, 49). Whether cord blood can eventually substitute the need of a large registry is still debatable.

Although only 5,266 donors HLA haplotype frequencies have been included in the current study, some rare alleles may not be covered in the presence analysis and affect the accuracy of the estimation. Nonetheless, common haplotype for those with frequencies above 0.2% should be covered. The information provided in this study provided an overview of the matching probability for the local population and facilitate the formulation of donor recruitment target and planning for extra resources in order to support the cost in donor recruitment and HLA typing. Establishment of a cost-effective bone marrow donor registry with an expanded donor pool is utmost important to enhance the likelihood of matching, shorten donor search time in the same ethnicity as domestic donors are more likely to donate stem cells (47). Moreover, it circumvents the shipment restriction or border control especially during the COVID-19 pandemic. This will facilitate timely HSCT in order to catch the best timing during patient remission period, and thus enhance the success rate of HSCT and patient outcome. A more comprehensive model of analysis for inclusion of availability of donor, incomplete or discrepant donor typing and loss of contact would be desired. With the continuation of donor HLA typing by the NGS technology, a revisit of the analysis with a larger sample size would be warranted in the future in order to obtain a more accurate estimation to cover the rare HLA alleles.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The donors have provided their written informed consent to perform HLA typing for HKBMDR.

## AUTHOR CONTRIBUTIONS

The study was designed by WY and JK. Data was collected by JH, SC, IT, CKL and JK. The computation and statistical analyses were performed by IT, ZL, WY and JK. The samples were

provided by CKL and JK. The manuscript was written by JH, SC, IT, PI, CKL, DM and JK. 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.2021.638253/full#supplementary-material

**Supplementary Table 1** | 81 Combinations of DPB1 TCE v2 assignment and matching outcome.

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# Chimerism, the Microenvironment and Control of Leukemia

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Transplantation of allogeneic hematopoietic cells faces two barriers: failure of engraftment due to a host versus graft reaction, and the attack of donor cells against the patient, the graft versus host (GVH) reaction. This reaction may lead to GVH disease (GVHD), but in patients transplanted due to leukemia or other malignant disorders, this may also convey the benefit of a graft versus leukemia (GVL) effect. The interplay of transplant conditioning with donor and host cells and the environment in the patient is complex. The microbiome, particularly in the intestinal tract, profoundly affects these interactions, directly and via soluble mediators, which also reach other host organs. The microenvironment is further altered by the modifying effect of malignant cells on marrow niches, favoring the propagation of the malignant cells. The development of stable mixed donor/host chimerism has the potential of GVHD prevention without necessarily increasing the risk of relapse. There has been remarkable progress with novel conditioning regimens and selective T-cell manipulation aimed at securing engraftment while preventing GVHD without ablating the GVL effect. Interventions to alter the microenvironment and change the composition of the microbiome and its metabolic products may modify graft/host interactions, thereby further reducing GVHD, while enhancing the GVL effect. The result should be improved transplant outcome.

**Keywords: chimerism, microenviroment, microbiome, GVHD prophylaxis regimens, allogeneic transplant, Graft vs. Leukemia Effect**

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"… he first commanded Bellerophon to kill that savage monster, the Chimaera, who was not a human being, but a goddess, …" (Homer, *The Iliad*)

## INTRODUCTION

In modern medical terminology, particularly in transplantation, the term "chimera" is applied to the result of transplantation, specifically the transplantation of *cells* from one individual into another. This cell transfer will change the recipient composition (1) and may lead to adverse events by inducing a syndrome, termed graft versus host (GVH) disease (GVHD). While GVHD is undesirable, the transferred cells also aid in eliminating the disease for which the patient is being transplanted, via a graft versus leukemia (GVL) effect. In fact, conditioning with cytotoxic therapy alone generally will not eradicate the last malignant cells, as shown in early murine models (2). The donor cell-mediated GVL effect is an essential part of the curative potential of hematopoietic cell transplantation (HCT).

## GRAFT VERSUS HOST DISEASE AND GRAFT VERSUS LEUKEMIA EFFECT

A GVL effect was first reported by Barnes and colleagues in murine models in 1956 (3) and 1957 (4), just as Don Thomas et al. reported the successful transfer of normal blood-forming hematopoietic stem cells from healthy donors into human patients with leukemia (5). These reports were followed by publications considering immunotherapeutic approaches to treat leukemia (6, 7). Weiden et al. presented the first comprehensive analysis of clinical transplant results, which showed that patients with acute leukemia who were transplanted with marrow cells from human leukocyte antigen (HLA)-matched sibling donors and who developed GVHD, particularly in its chronic form, had a reduced incidence of relapse and superior survival (8, 9). The GVH reaction is triggered by the encounter of cells from two individuals, the transplant donor and the recipient, with prominent manifestations at the patient's boundaries, in particular the intestinal tract (1, 10). Since a patient's leukemic cells have the same basic genetic makeup as the patient's healthy organs and tissues, this GVL effect may not be surprising. However, the question that arises immediately is whether this effect could be achieved and exploited without the development of GVHD. It has been challenging to separate the GVL effect from GVHD, but animal models indicate that the post-transplant interaction of donor and host cells—conventional and regulatory T cells, donor and host dendritic cells of various lineages, and iNKT cells, along with components of the microenvironment—can be shifted such that GVHD is largely prevented while the GVL effect is maintained (11).

The probability of post-HCT relapse depends upon numerous factors, including disease characteristics, treatment received before transplantation, remission status, including measurable residual disease (MRD) at the time of transplantation, the transplant conditioning regimen, the source of donor cells, HLA mismatch between donor and patient, and the development of (chronic) GVHD. MRD, in particular, is currently an area of extensive research. The level of detection of MRD depends upon the methodology used (e.g., deep sequencing for DNA mutations vs. multi-color flow cytometric analysis) (12–14). While flow cytometry identifies immunophenotypic abnormalities that may serve as targets for the GVH reaction and the GVL effect, this is less likely to be the case for most mutations, unless they result in changes in protein expression. A head-to-head comparison of flow and mutation data in regard to their impact on post-transplant relapse is currently not available. Further, there has been a keen interest in the role of DNA polymorphism (and the respective differences between donor and patient) and the occurrence of GVHD and GVL reactivity. While some single-nucleotide polymorphisms associated with a limited number of genes and their possible role for GVHD have been described, no firm conclusions can be drawn (15). Considering an impact of cytogenetic risk and GVHD, we carried out an analysis (Radich and Deeg, unpublished) in patients transplanted for MDS, selecting cohorts, which by conventional criteria could be considered the two extremes for relapse risk: patients who had high risk cytogenetics (16) and did not develop GVHD (acute or chronic) and patients with good risk karyotype who did develop GVHD. Remission status at the time of transplantation, donor selection, conditioning regimen, and GVHD prophylaxis were comparable. Contrary to our hypothesis that there would be a high incidence of relapse in the first cohort and a low incidence in the second, we failed to observe a significant difference. While the analysis may have had limited statistical power, the lack of any difference was striking. Clearly, risk parameters such as DNA mutations (17) (not available for our analysis) and factors that have not been incorporated into currently used risk schemes are relevant for relapse or sustained remission. It is of interest in this context that a recent report suggests a higher incidence of chronic GVHD and possibly a reduction in relapse incidence in patients transplanted from donors with clonal hematopoiesis (18).

## T-CELL DEPLETION

Early data on global T-cell depletion of the donor cell inoculum before infusion into the patient showed substantial reduction of the incidence of GVHD but also resulted in a high rate of graft failure and disease relapse (19). More recent data using selective T-cell depletion appear to be more promising.

One strategy is the administration of post-transplant cyclophosphamide (CY), originally for HLA haplo-identical transplants but then extended to other donor/host combinations (20). The reduction of the incidence of GVHD, especially chronic GVHD, with this approach was interpreted as a result of the elimination of host-alloreactive donor T cells. However, more recent data from murine models show that treatment with CY favors the development of CD4+CD25+Foxp3+ regulatory T cells. In addition, some conventional alloreactive T cells persist, albeit with impaired function (21). It is this conjunction of an expansion of regulatory T cells, including those with alloantigen specificity, and altered immuno-competence of conventional T cells that is responsible for the observed prevention of GVHD (21). This mechanism was also functional in thymectomized mice, indicating that it does not require the generation or central selection of T cells. Whether the use of post-transplant CY is associated with an increase in relapse, particularly of myeloid malignancies, remains a matter of debate. Apparently, the modified donor-derived alloreactive T cells maintain GVL activity.

Another concept with similar aims, the prevention of GVHD without increasing the risk of relapse, is the depletion of CD45A+CD62L+ naïve T cells (22, 23). In murine models, the infusion of naïve T cells induced severe GVHD, while central memory T cells resulted in milder GVHD, and effector-memory T cells did not cause significant GVHD (24, 25). Memory T cells, however, conveyed anti-pathogen immunity and GVL reactivity (26). Naïve CD45+CD62L+ T cells appear to be "uncommitted" and, thus, are able to get activated by new (patient) antigens that they encounter, thereby triggering a cascade of signals that initiate GVHD. In the clinic, patients with myeloid or lymphoid malignancies conditioned with regimens

of various intensities and infused with hematopoietic cells from HLA-identical sibling donors that were *in vitro* depleted of CD45RA+ T cells achieved sustained engraftment, had a very low incidence of severe acute and chronic GVHD, and were not at a higher risk of relapse than patients transplanted with T cell-replete grafts (23). Further, in patients who did develop acute GVHD, generally grade II, corticosteroid treatment could be discontinued much earlier, at a median of 85 days, compared with 853 days in patients given T cell-replete grafts. No case of steroid-refractory GVHD has been observed so far after naïve T-cell depletion. This pattern of rapid response of acute GVHD to steroid therapy and the rare occurrence of chronic GVHD suggests a modified immune environment and a different biology of acute GVHD related to the removal of non-committed naïve T cells. The fact that regulatory T cells that express CD45RA are also eliminated suggests that those cells are not required for the establishment or maintenance of tolerance in this clinical model. In fact, one can speculate that elimination of those regulatory T cells might lead to a more potent GVL effect.

## MIXED DONOR/RECIPIENT CHIMERISM

What is the impact of *incomplete* donor cell engraftment? Available data indicate that the development of *mixed* chimerism, the concurrent presence of recipient and donor lympho-hematopoietic cells in the patient after transplantation, may attenuate or prevent the development of GVHD. Mixed chimerism was originally described in patients with *non-malignant disorders*, in particular immune deficiencies (27) but also in aplastic anemia (28). This mixed chimerism can persist for years. Studies in a canine model indicated that administration of sublethal doses of total body irradiation before and pharmacological immunosuppression after donor cell infusion resulted in stable mixed hematopoietic donor/recipient chimerism (29). These data underscore the importance of the intensity of the transplant conditioning regimens, which for non-malignant disorders tend to be less intensive, for the development of mixed chimerism.

Would mixed chimerism also be possible and consistent with transplant success in patients with *malignant disorders*? Stated differently, would the establishment of "tolerance" between patient and recipient cells include tolerance to the malignant cells and, thereby, eliminated the GVL effect? In fact, several reports have shown persistent antitumor responses *even after a loss of donor cell chimerism* (30, 31). What is the mechanism? The answer will at least in part depend upon which donor and patient cell sub-populations in the patient's marrow and immune system account for the mix and how the mix alters cell functions. We showed recently that in patients transplanted for myeloproliferative disorders, mixed CD33+ chimerism was associated with subsequent relapse, whereas mixed CD3+ chimerism was not and, in fact, did result in less GVHD without an increased incidence of relapse (32). We observed similar outcomes in two trials enrolling patients with acute myeloid leukemia (AML) or MDS who had been conditioned with busulfan/fludarabine and thymoglobulin (Yeh et al., unpublished observations, February 2021). The factors controlling this balance between patient and donor cells without leading to disease recurrence remain to be determined.

## GRAFT VERSUS LEUKEMIA EFFECTS WITHOUT CLASSICAL HEMATOPOIETIC CELL TRANSPLANTATION

If cells from healthy donors are able to induce a GVL effect after transplantation, can such an effect be achieved with the infusion of donor cells (DLI), *without carrying out an actual transplant*, as has been shown for patients who relapsed after transplantation (60)? Several investigators used leukocyte infusions from HLA-mismatched donors in an attempt to provide a direct GVL effect (33–35). In one study, DLI was given to patients with various malignancies to induce a GVL or GV tumor effect (33). These patients were pre-treated with interferon 2β and given DLI, and 4 weeks later, donor chimerism (determined by PCR for marker analysis) was detected in four of 11 evaluable patients. Of note, four patients who had previously received an autologous transplant developed acute GVHD, and the three patients who could be assessed did show anti-tumor responses. GVHD is a risk associated with DLI. However, the occurrence of GVHD in patients who had previously undergone a transplant is consistent with a modified microenvironment and a role of host cells in the GVHD pathophysiology (36). However, many patients given DLI for relapse after transplantation do experience tumor responses *without developing GVHD*, illustrating that *clinical* GVHD is not required for a GVL effect to occur. The GVL effect may be mediated by a subclinical reaction or, alternatively, might involve activity against antigens with limited expression, restricted to the tumor (37). Ongoing research is exploiting this possibility, for example, by generating effector cells against minor histocompatibility antigens (HA-1) primarily expressed on lympho-hematopoietic cells and for which patient and donor differ (61).

Guo et al. reported results with the infusion of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood progenitor cells (including CD34+, CD3+, and NK cells) at various doses, from HLA-mismatched donors in patients with AML in first remission who were not given any GVHD prophylaxis (38). Donor chimerism as determined by the identification of cells containing the Y chromosome (from the male donor) was present in 20 of 23 female recipients as late as 1,000 days after infusion. Leukemia-free survival was significantly prolonged in patients who received higher doses of CD3+ donor cells, while no GVHD was observed. The investigators also showed, further, that in addition to a *GVL* effect, these patients also experienced a *recipient vs. leukemia* effect, suggesting activation of the patient's immune system by the infused HLA non-identical donor cells (38). The same authors subsequently reported similar results in another 185 patients with *de novo* AML (35), but confirmation from other centers is currently not available.

## THE MICROENVIRONMENT

The marrow microenvironment is essential for the support of normal and malignant hematopoiesis. We have presented *in vitro* data from patients with MDS, which show a two-way signaling path between the clonal disease cells and non-clonal mesenchymal/stroma cells (39). Stroma cells exhibited altered gene expression and favored the survival of clonal MDS cells rather than healthy hematopoietic precursors. Exposure to the hypomethylating agent, 5-aza-citidine, normalized gene expression in stroma cells and restored their functional competence in support of normal hematopoiesis (39). It is intriguing to speculate that altered gene expression in the marrow microenvironment is a contributor to the frequently observed myelosuppression following DLI.

Data on the role of stroma in disease persistence or recurrence have also been presented for patients with AML (40, 41). Those studies show that malignant (clonal) myeloid cells trigger remodeling events in bone marrow niches, and this remodeled environment then favors the expansion of the malignant clone (39, 42). Other broad-acting contributors to the altered post-transplant milieu in the patient are the effects of endothelial cell activation (43).

Further, solid cancer models show that propagation of clonal tumor cells in the form of metastases was dependent upon the co-migration of stromal cells with those tumor cells (44). Consistent with that observation, we were not able to establish sustained engraftment of clonal MDS cells in a xenotransplant model of human MDS cells in immunodeficient mice, if MDS cells were injected by themselves. However, we did achieve long-term engraftment and expansion when MDS cells were injected along with the (transformed) human stroma cell line HS27a (45). The role of the microenvironment for effective hematopoiesis is undisputed, but what is of note in these models is the support of the *clonal disease* that is mediated at least in part by a quasi auto-feedback loop that leads to "preferential treatment" of the clone.

Possibly related to these data are observations on the development of donor-derived leukemia, i.e., the transformation of polyclonal, healthy donor cells into originator cells of a clonal myeloid disorder (assuming the absence of preexisting clonal abnormalities in donor cells). Several reports have postulated a "leukemogenic effect" of the marrow microenvironment (46, 47). Is the underlying mechanism related to signals provided by donor cells, viz., the chimeric status associated with a successful transplant?

## THE MICROBIOME

Exciting research has established that the microbiome plays a central role in the development of GVHD (48–50). We recently summarized data from several laboratories on the profound effects of donor/host interactions at the boundaries of the transplant recipient and the role of the patient's microbiome, particularly in the intestinal tract, in modifying those interactions (1). Shifts in the composition of the intestinal microbiome are associated with GVHD. While some bacteria, such as *Blautia*, appear to have a beneficial effect, others, for example, *Veillonella* or enterococcal species such as *Enterococcus faecium* or *Enterococcus faecalis*, favor the development or propagation of GVHD, leading to inferior transplant survival (51). These intestinal bacteria interact directly with patient cells, including GALT, L cells, and dendritic cells and thereby modify either tolerogenic or allo-reactive signals (52, 53). Various species, such as *E. faecalis*, can cross the intestinal barrier and migrate to intestinal lymph nodes, priming resident T and B lymphocytes. Bacterial metabolites, specifically the short-chain fatty acids butyrate or propionate, released into the bloodstream, have a protective effect against chronic GVHD (54, 55). One mechanism involves enhanced development of regulatory T cells. Conversely, a loss of species that produce high levels of butyrate would be associated with a higher incidence of GVHD. So far, there is no evidence that a shift in the composition of the intestinal microbiome impacted progression of the malignancy for which the patient underwent transplantation (51), although there is a profound impact of the mix of the gut microbiome on the response to immunotherapy in other models (56). Intriguing are some very recent observations (Chris Johnston PhD, personal communication, November 2020) indicating that bacteria can alter the methylation pattern of human DNA, thereby modifying gene expression. Conceivably, this may lead to alterations of potential targets for a GVL effect by donor cells.

Viral organisms such as picobirna viruses have also been shown to participate in these donor/host interactions (57), and the role of the cytomegalovirus (CMV) in GVHD development has been investigated extensively (58). Sellar et al. (59) studied patients with various lymphohematopoietic malignancies who were CMV+ and received transplants from CMV negative donors. The conditioning regimens were of reduced intensity and included *in vivo* T-cell depletion with alemtuzumab. The investigators showed that CMV-specific T cells were exclusively of *host* origin and protected the patients against recurrent CMV infections, indicating that the status of mixed donor/host chimerism in these patients was associated with increased immune protection. DLI to induced full donor chimerism did not trigger the development of symptomatic CMV infection, and in some patients, *donor-derived* CMV-specific CD8+ T lymphocytes further expanded. This conversion (from host to donor) occurred without clinical evidence of GVHD, suggesting the possibility that the presence of mixed chimerism, albeit temporary, facilitated the establishment of tolerance.

## SUMMARY AND CONCLUSIONS

The interactions between donor and recipient cells following allogeneic HCT are complex, and the cast of characters of this drama is not limited to donor and recipient immune cells. Additional actors include cellular and non-cellular components of the microenvironment and, importantly, the microbiome. Nature had not envisioned *Homo sapiens* trying to break down barriers that have evolved over millions of years. Doing so upsets the balance that we observe in healthy individuals. Of course, these therapeutic interventions are directed at the

eradication of a malignant disease, which has already changed the internal milieu. A better understanding of signals that trigger the development of malignant disorders such as leukemia would allow for earlier interventions and might permit their exploitation to restrict the reactions of donor cells to the GVL effect, while preventing GVHD. Can we direct the divine ability of the chimera against the malignancy and sever the ugly head of GVHD? Current research using state of the art tools, including systems biology and machine learning, may be able to pave the way.

## 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/s.

## AUTHOR CONTRIBUTIONS

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

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REVIEW
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# Allogeneic Stem Cell Transplantation for Acute Myeloid Leukemia: Who, When, and How?

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Although the majority of patients with acute myeloid leukemia (AML) treated with intensive chemotherapy achieve a complete remission (CR), many are destined to relapse if treated with intensive chemotherapy alone. Allogeneic stem cell transplant (allo-SCT) represents a pivotally important treatment strategy in fit adults with AML because of its augmented anti-leukemic activity consequent upon dose intensification and the genesis of a potent graft-versus-leukemia effect. Increased donor availability coupled with the advent of reduced intensity conditioning (RIC) regimens has dramatically increased transplant access and consequently allo-SCT is now a key component of the treatment algorithm in both patients with AML in first CR (CR1) and advanced disease. Although transplant related mortality has fallen steadily over recent decades there has been no real progress in reducing the risk of disease relapse which remains the major cause of transplant failure and represents a major area of unmet need. A number of therapeutic approaches with the potential to reduce disease relapse, including advances in induction chemotherapy, the development of novel conditioning regimens and the emergence of the concept of post-transplant maintenance, are currently under development. Furthermore, the use of genetics and measurable residual disease technology in disease assessment has improved the identification of patients who are likely to benefit from an allo-SCT which now represents an increasingly personalized therapy. Future progress in optimizing transplant outcome will be dependent on the successful delivery by the international transplant community of randomized prospective clinical trials which permit examination of current and future transplant therapies with the same degree of rigor as is routinely adopted for non-transplant therapies.

**Keywords: acute myeloid leukemia, allogeneic stem cell transplantation, graft-versus-leukemia, chemotherapy, minimal residual disease, measurable residual disease (MRD)**

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## INTRODUCTION

It is more than sixty years since allogeneic stem cell transplantation (allo-SCT) was pioneered as a novel and potentially curative therapeutic modality in patients with chemotherapy-resistant acute myeloid leukemia (AML) (1, 2). Subsequent analyses have confirmed the role of allo-SCT as the optimal treatment strategy in adults with AML in first complete remission (CR1) consequent upon

its ability to reduce the risk of disease relapse by more than 60% compared with intensive chemotherapy alone. Remarkably the magnitude of the augmented anti-leukemic activity of allo-SCT, result from both dose intensification and the genesis of a potent graft-versus-leukemia (GVL) effect, is similar in all biological subtypes of AML (3).

The survival benefit of the augmented anti-leukemic activity of allo-SCT is blunted by its attendant transplant related mortality (TRM). It is therefore essential to a) identify patients whose outcome with intensive chemotherapy (IC) is such that the enhanced anti-leukemic activity of allo-SCT is otiose b) identify patients whose outcome with IC is such that deployment of the enhanced anti-leukemic activity of an allograft should be considered and c) define as precisely as possible the patient population in which allo-SCT can be delivered with an acceptable morbidity and mortality. Thus the identification of patients likely to benefit from allo-SCT requires a dynamic assessment which incorporates both the predicted risk of disease relapse if the patient were to receive IC alone coupled with a prediction of the TRM were the patient to proceed to transplant (4). Accurate prediction of these parameters has been refined by progress in both risk stratification utilizing clinical, cytogenetic and molecular genetic data as well as advances in prediction of the risk of allo-SCT (5–9). Increasingly, randomized controlled trials are informing critical questions concerning relapse risk in patients treated with IC alone (10) and informing the personalization of transplant strategies (11–14). Cooperative transplant trials networks such as the US BMT CTN and the UK transplant cooperative IMPACT will play an increasingly important role in optimizing outcomes after allo-SCT in AML (15).

## Who and When Should Patients With AML Be Transplanted?

The focus of therapeutic endeavor in newly diagnosed AML in recent years has primarily been on improving induction chemotherapy (16, 17). However, the increasing availability of allo-SCT coupled with the recognition that a substantial proportion of patients treated with IC alone are destined to relapse has prioritized the development of algorithms designed to identify patients likely to benefit from allo-SCT in CR1. The advent of more accurate risk stratification, utilizing genetic and measurable residual disease (MRD) analysis, coupled with increased sophistication in predicting and reducing TRM has improved decision making concerning the delivery of optimal consolidation therapy in adult AML (18).

The importance of correctly identifying patients in first CR1 who are likely to relapse is predicated by the poor, incomplete rates of remission salvage, such that a significant proportion of patient who relapse do not reach a second CR (CR2) (19). Furthermore, the use of additional intensive chemotherapy and concomitant infections often result in patients with impaired fitness prior to an allo-SCT in CR2. Studies recurrently show that patients with active disease have poorer outcomes as compared to those patients transplanted in CR, thus this should be a critical goal prior to proceeding to transplant (20, 21). Whilst patients transplanted with CR with incomplete count recovery (CRi) have inferior outcomes to patients with AML in CR, this is as a result of increased non-relapse mortality (NRM) but not necessarily relapse risk (22). Other studies have shown the number of courses of consolidation chemotherapy delivered prior to transplant do not improve patient outcome (23).

### Who Should Be Transplanted With Refractory or Relapsed Disease?

The aim of therapy in fit adults with relapsed with AML is to proceed to allo-SCT once a 2$^{nd}$ CR has been achieved (24). This is based on studies demonstrating very poor outcomes in patients who are not allografted in CR2 (19, 25, 26). However, there may be a subset of patients with core-binding factor translocated AML who may achieve long term remission with autologous transplantation, or in a minority, salvage chemotherapy (19, 27). A number of prognostic systems exist for patients with relapsed/refractory AML (28, 29) which may help to identify subgroups of patients with AML who are likely to have long-term survival following an allo-SCT. Important factors identified in these prognostic systems include, duration of CR1, age at relapse and cytogenetic risk at diagnosis.

Retrospective analyses of allo-SCT for AML in CR2 have demonstrated overall survival (OS) of 30-60%, with acceptable rates of TRM despite intensive pre-treatment in this cohort of patients (30–32). Results have also been encouraging in the use of alternative donors in transplantation at CR2 (32). A formal comparison of myeloablative (MAC) versus RIC regimens in this setting is not possible, but registry studies show no significant differences in OS between patients treated with the differing conditioning intensities (32). Despite this, in fit younger patients who might tolerate a MAC regimen, this is probably the preferred treatment strategy to reduce further disease relapse which remains the major risk facing this patient cohort.

A particularly challenging group of patients with AML are those with primary refractory disease, defined as failure to achieve remission following two cycles of induction chemotherapy (33). Numerous studies have shown that patients transplanted with active disease have poorer outcomes than those in remission (20, 31, 34). However, studies have demonstrated approximately 20-30% of patients with primary refractory disease may have long term survival after an allo-SCT (35) and recent work has identified risk factors that may identify patients who are likely to have primary refractory disease at an earlier stage (36). In the evolving landscape of genetic stratification, these scoring systems are likely to be refined, and the long term impact of novel salvage options from targeted therapies remains to be seen (37, 38). One recent study underlined the particularly poor outcome of patients with *TP53* mutant AML, when they were transplanted with active disease (39). A challenge in assessments of such genetic risk factors will be the clonal evolution which occurs in patients with AML following treatment (40).

Finally, for patients who relapse following an allo-SCT, the outcome is very poor (41). However, for some patients, especially ones with a durable remission since transplant, and with disease control at the time of second allo-SCT, this procedure may provide an OS at 2 years of 25% (42). In patients who received an

unrelated donor transplant, no advantage for change in donor in this setting could be demonstrated.

## Who Should Be Transplanted in First Complete Remission?

Donor versus no donor studies were the first to demonstrate the ability of allo-SCT to increase disease free survival (DFS) and OS in patients transplanted using a myeloablative HLA matched sibling allo-SCT (43). A selection strategy to identify patients who should be transplanted in CR1 was articulated by Cornelissen and colleagues with the European LeukaemiaNet (ELN) AML working party (4) and is based on the competing risks of relapse with chemotherapy alone versus risk of relapse after an allo-SCT and the concomitant TRM (**Figure 1**). Underpinning this treatment algorithm is the observation that the risk of relapse following allo-SCT is more than halved as compared to that observed in patients treated with IC alone (3)- regardless of cytogenetic risk group. At the same time recent reductions in transplant toxicity permit delivery of an allo-SCT with an NRM of 15% or less in fit adults with a well matched sibling or volunteer unrelated donor. On this basis the ELN group recommend consideration of allo-SCT in fit adults with AML in CR1 who have a predicted relapse risk of 35-40% and a suitable donor (33). Thus adults with AML in CR1 who fulfill ELN criteria for good risk disease on the basis of cytogenetics or the presence of an NPM1 mutation without FLT3-ITD mutation, and demonstrate a good response to induction chemotherapy by MRD criteria are not routinely deemed eligible for an allo-SCT in CR1. Conversely, all other adults in CR1 in whom the predicted risk of relapse of >40% if they are treated with IC alone should, in principle, be considered transplant candidates providing a suitable stem cell donor is available (44).

Risk stratification in patients with AML in CR1 is based on clinical (5) factors, such as age and gender, as well as cytogenetic risk based on karyotyping results (6) (**Table 1**). This has been refined in recent years by the discovery of further mutations of prognostic significance in genes such as *FLT3* (45), *NPM1* (46), *ASXL1* (47), *RUNX1* (48) and *TP53* (49) as reflected in the 2017 ELN classification (33). Increasingly mutational information is available for patients as a result of next generation sequencing (NGS)

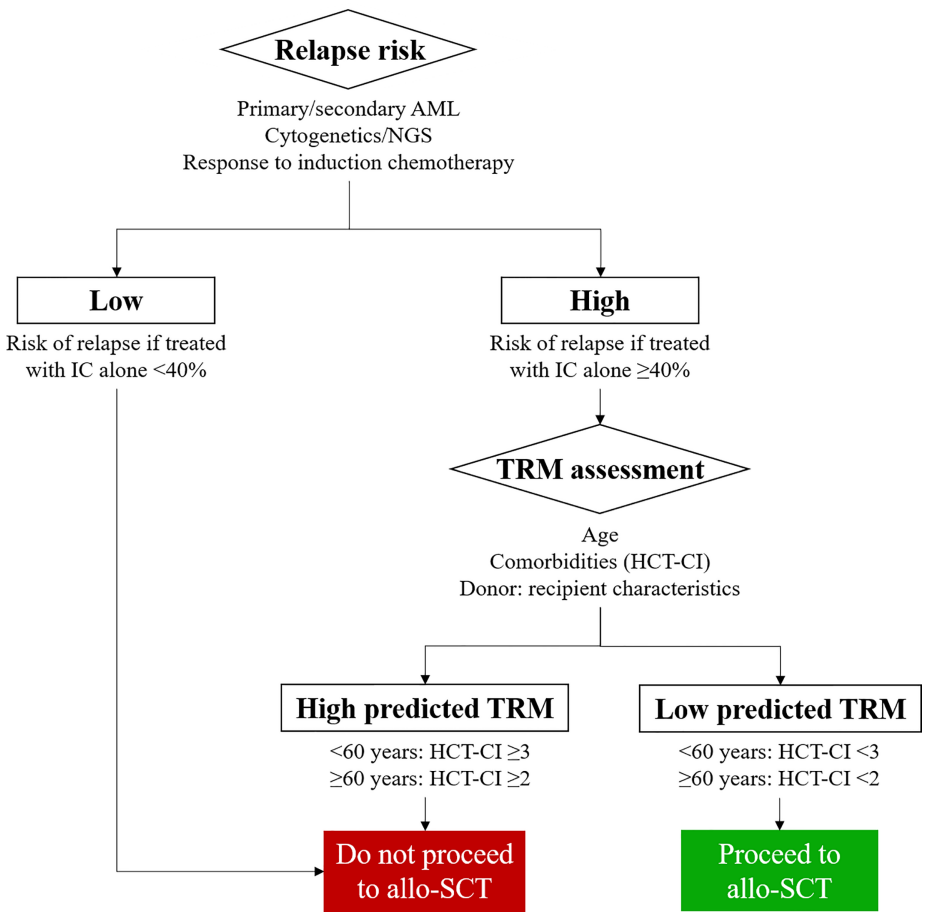


**FIGURE 1** | Identifying patients with Acute Myeloid Leukemia (AML) who are likely to benefit from allogeneic stem cell transplantation (allo-SCT). MRD, Measurable Residual Disease; TRM, Transplant related mortality; HCT-CI, Hematopoietic cell transplantation - specific comorbidity index; NGS, next generation sequencing.

**TABLE 1** | Factors determining disease risk in AML.

| Clinical Variables | Molecular variables | Dynamic variables |
|---|---|---|
| • Age | • Cytogenetic | • Response to course 1 by morphology |
| • Gender | • Next generation sequencing of genes e.g. *FLT3, NPM1, RUNX1, ASXL1, TP53* | • Response to treatment by MRD |
| • Presenting white cell count | | |
| • Primary versus secondary disease | | |
| • Performance status | | |

technology assaying panels of commonly affected myeloid genes (33). This is of further importance as these genetic markers are now commonly used as both therapeutic targets (50, 51) and as prognostic markers of response to therapies (52). The results of these large scale sequencing efforts of AML samples at diagnosis, in combination with data relating to treatment use and clinical outcome will likely refine these risk categories. This will provide a "personalized" risk score for individuals patients based on a number of these clinical factors and allow for incorporation of combinations of genetic mutations, such as that seen recently in the study of myeloproliferative neoplasms (53, 54). It is increasingly becoming apparent that both clinical and mutational characteristics determine the kinetics of disease relapse. Importantly patients with a *FLT3* mutation are amongst those likely to relapse early in whom the timing of transplant should not be delayed (55).

### *Incorporation of MRD Risk Stratification*

An important development in risk stratification has been the incorporation of MRD monitoring to routinely assess patients' response to chemotherapy (56) (**Table 2** and **Figure 2**). The kinetics and depth of response has been identified as being critical in re-assessing the risk of relapse in patients with otherwise favorable and intermediate risk disease. The impact of MRD monitoring appears to be the most important, independent prognostic factor in many scenarios (57, 58). The selection of the optimal MRD monitoring modality depends on the presence of leukemia specific molecular, cytogenetic or immuno-phenotypic dependent on the AML subtype. Each MRD monitoring technique has its own advantages and disadvantages, and all require expertise in the delivery of reliable results (**Table 2**).

### *Examples of Different Uses of MRD Risk Stratification*

Real-time quantitative polymerase chain reaction (RQ-PCR) monitoring of disease specific transcripts provides a sensitive and disease specific assay of MRD for patients with AML expressing a detectable fusion gene transcript (e.g. Core-binding factor (CBF) fusion gene, KMT2A fusion genes, mutant NPM1). In the case of AML with CBF translocation, although age can influence prognosis (59), the depth of response to course 1 and 2 of IC (57) are critical determinants of relapse risk. In cases with residual levels of CBF fusion transcripts at the end of treatment (60), relapse risk depends on level of transcripts, but low levels of CBF fusion gene transcripts may persist after end of treatment without affecting long-term survival. Failure to achieve a 3-log reduction in CBF fusion transcript after two cycles of chemotherapy is associated with an over 50% relapse risk in the monitoring studies of two large cooperative groups, suggesting possible benefit from an allo-SCT in these patients (57, 61).

In younger adults with NPM1 mutant AML, RQ-PCR positivity in the peripheral blood after two cycles of chemotherapy is an important predictor of relapse, identifying a population of patients who should be considered allo-SCT mandatory (58). This is supported by data which points to the beneficial effect of allo-SCT in patients with mutant NPM1 residual disease post induction chemotherapy (62). Recent studies have confirmed that, in younger adults at least, NPM1 is also a predictive biomarker. Patients with NPM1 mutant AML who have a less than 4-log reduction in peripheral blood NPM1 MRD levels demonstrated improved survival after allo-SCT compared with patients who received chemotherapy alone (62). The low relapse risk for patients who are negative for mutant NPM1 transcripts in the peripheral blood after two cycles of intensive chemotherapy outweighs other poor prognostic factors such as concomitant FLT3-ITD mutation or poor risk genotypes (7). The degree to which NPM1 mutations are a prognostic or predictive biomarker in older patients (over the age of 60 years) remains unclear (63). In part this may be due to the increased association of other poor risk cytogenetic features in more elderly patients with NPM1 mutant AML (64). Of note in patients with adverse risk cytogenetics, the presence of NPM1 mutation has no impact on survival outcomes.

**TABLE 2** | Relative merits of different MRD monitoring methodologies.

| Method | Multi-parameter Flow Cytometry (MFC MRD) | Quantitative PCR (RQ-PCR) | Next generation sequencing (NGS) |
|---|---|---|---|
| **Advantages** | Rapid results<br>Widely applicable to many patents | Sensitive<br>Easily compared with sequential results due to quantitative range<br>Widely accepted standardisation | Applicable to many patients<br>Error correction increases sensitivity |
| **Disadvantages** | Reliant on expertise of reporting lab<br>Phenotype of AML cells may change over time | Restricted molecular targets (e.g. Core binding factor translocations, NPM1c mutant) | Ongoing development of technology<br>Expense |
| **Examples of use** | Risk stratification in younger adults, post induction chemotherapy, with NPM1 negative AML. | Risk stratification post chemotherapy to determine relapse risk in NPM1 mutant AML. | Pre-transplant MRD monitoring. |

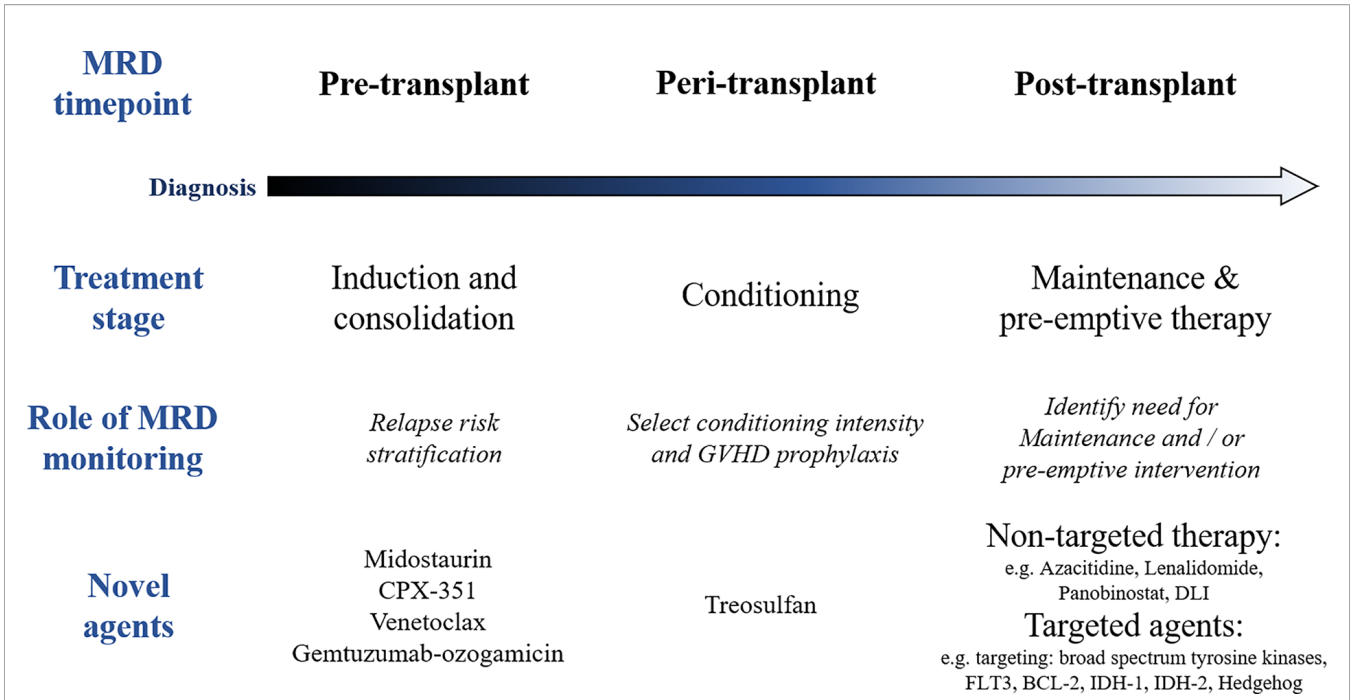


**FIGURE 2** | Role of measurable residual disease (MRD) and novel agents at different stages of the treatment pathway in acute myeloid leukemia (AML). GVHD, graft versus host disease; DLI, donor lymphocyte infusion.

A number of large prospective studies have confirmed the prognostic significance of multi-parametric flow cytometry (MFC) determined MRD in adults with newly diagnosed AML treated with IC. In younger adults, MFC MRD+ positive patients with standard risk, NPM1- mutated AML appeared to benefit from allo-SCT in CR1 (10) and data on this group of patients continues to be accrued, including the benefits of intensifying chemotherapy in patients with a suboptimal MRD response after first course of intensive chemotherapy. In older patients, a higher level of MRD after induction treatment is also prognostic of a worse outcome (65). However, in this age group, although MFC MRD negativity, offered improved overall survival, relapse rates remained high.

Early studies suggest a promise for NGS technology for MRD assessment (66), which has the advantage that it may be applicable for many forms of AML. Error correction methodology has become incorporated in this technology to enable higher levels of sensitivity (67), but is currently limited to research settings due to the costs. Furthermore, there has not yet been an upfront comparison of these different MRD technologies independently, or in combination, to compare technical specifications. A recent large study suggested there was an additive prognostic value of NGS MRD over MFC MRD, but interestingly the persistence of age related clonal hematopoiesis after treatment did not result in an increased relapsed rate (66)

### *Improving Assessment of Transplant Related Mortality in Patients With AML*

A critical factor to understand whether a patient with AML is suitable for an allo-SCT is estimation of the TRM associated with the procedure and whether it is outweighed by the improvement in relapse risk delivered by the transplant process (68–70) (**Figure 1**). Furthermore, these considerations are central to any discussion with patient and family as to whether the increased risk of an allograft is justifiable. The European Society for Blood and Marrow Transplantation (EBMT) risk score, originally developed in patients allografted for chronic myeloid leukemia (CML) (71), was subsequently shown to be applicable in other disease settings (72), and provided the first attempt to provide a quantifiable estimate of TRM and transplant outcome which could be routinely applied in clinic. However in patients allografted for AML more emphasis is now placed on the Hematopoietic cell transplantation-specific comorbidity index (HCT-CI) score which incorporates a weighted score based on the presence of pre-transplant comorbidities (8). This has been shown to be valid in patients undergoing an allo-SCT for myelodysplastic syndrome (MDS) or AML (9) and more recently combined with age (73), to demonstrate the varying effects of these comorbidities based on a patients' age. Of note, this analysis showed that younger patients with comorbidities were at a significant disadvantage to older fit individuals with no other significant comorbidities.

Unfortunately no scoring system for TRM can include the importance of a clinical assessment of patients based on the "end of bed" assessment and knowledge of how patients have tolerated recent intensive treatment. Thus despite improvements in mathematical modeling techniques to predict treatment related risk on a personalized basis to account for the dynamic interactions between different variables (74, 75), there remains a considerable limitation in the ability of these scoring systems to

predict TRM. Finally, the majority of these scoring systems were developed in the era of sibling or matched unrelated donor transplantation, thereby limiting their use for those with alternative donor sources, which are now of increasing use; such as for recipients of haploidentical donor or umbilical cord stem cells transplants.

### *What Is the Impact of Patients' Age in Considering Transplant Eligibility?*

It is commonly recognized that an important challenge in the management of patients with AML is the increased frequency of this disease with age. Furthermore, the older patient faces a combined challenge of increased frequency of comorbidities and higher risk genetic features (76). Nevertheless patients over the age of 70 years with AML are routinely transplanted with acceptable results (77) but careful assessment of transplant suitability is required. The widely used, updated HCT-CI score allows some adjustments due to age (73), and this analysis showed that younger patients with comorbidities were at a significant disadvantage to older fit individuals with no other significant comorbidities. Nonetheless the HCT-CI score is still of importance in this population, as it has been shown that in patients above 60 years of age a HCT-CI score of 2 or greater results in substantially higher TRM than otherwise expected (78). Future developments to improve assessment of transplant eligibility in this cohort should involve geriatric assessments that encompass an assessment of the functional status of the patient (79).

## HOW SHOULD PATIENTS WITH AML IN CR1 BE TRANSPLANTED?

The major causes of treatment failure in adults allografted for AML are transplant toxicity and disease relapse. Whilst significant progress has been made over recent decades in reducing TRM the risk of disease relapse remains stubbornly high. The key considerations in patients with allo-SCT-mandatory AML include identifying which patients should receive RIC as opposed to a MAC allo-SCT and, in patients lacking a well-matched sibling or unrelated donor, what is the preferential alternative donor stem cell source? The development of strategies with the ability to reduce the risk of disease relapse post-transplant also represents a major unmet need.

## Strategies to Improve Outcomes Pre-Transplant

The design of novel treatment strategies with the potential to reduce the risk of disease relapse post allo-SCT remains a priority if we are to increase the number of patients with AML who benefit from transplant. A number of questions remain regarding the optimal management of patients' pathway before, during and after an allo-SCT (**Figure 2**). This debate has been reinvigorated in recent years by two key innovations: the widespread use of MRD technologies in patients with AML (80) and the increasing availability of novel pharmacological agents that may be applied at different treatment stages (81) (**Figure 2**). The adverse impact of pre-transplant MRD on post-transplant outcomes has been increasingly widely recognized (14, 82) and this may inform pre-transplant treatment strategies. Furthermore, emerging data suggest that conditioning intensity and potentially graft-versus-host disease prophylaxis strategies may influence the poor prognostic impact of pre-transplant MRD (83). Finally, post-transplant monitoring of MRD may become important in identifying patients who should receive pre-emptive treatment (84) and is likely to be important in future maintenance strategies in patients post allo-SCT.

### How Important Is Pre-Transplant MRD?

A number of retrospective studies have demonstrated the adverse prognostic significance of patients with MFC MRD positivity prior to transplant (82), with some likening the outcomes of these patients post allo-SCT to those with active disease (85). This draws comparison to the outcomes of younger adults with a partial response to the first cycle of induction chemotherapy who have a similar overall outcome as compared to patients who have a CR or CRi but have MFC MRD positivity (10). Two prospective studies have demonstrated the importance of pre-transplant MRD (14, 83) in patients with AML or high risk MDS. The FIGARO study investigated the impact of pre-transplant MFC MRD in 244 patients entered into a randomized comparison between FLAMSA-Bu-RIC regimen and a control RIC arm. This identified a poor prognostic impact of a 0.2% threshold of residual disease. However, even in the MRD positive arm, only approximately 50% of patients relapsed: not only suggesting further strategies to identify patients at risk of relapse are required (14), but contrary to previously held opinions, this sizeable proportion of patients with high risk AML may be salvageable with an allo-SCT.

The importance of pre-transplant MRD persists regardless of the technique used to monitor MRD. RT-PCR monitoring of CBF fusion transcripts prior to allo-SCT for patients in CR2, show that those with MRD negativity have a reduced risk of relapse as compared to those with MRD positive disease pre-transplant (86).

### Can We Improve Transplant Outcomes in Patients With Evidence of Pre-Transplant MRD?

It remains unknown whether additional courses of chemotherapy or whether further alterations to transplant management in patients with pre-transplant MRD would be of benefit. However, in recent years a number of provocative results have provided impetus to design clinical trials to tackle the poor prognostic impact of pre-transplant MRD.

### Pre-Transplant Strategies to Alter Impact of Pre-Transplant MRD?

Studies of novel agents in recent years such as midostaurin and the liposomal cytarabine-daunorubicin preparation CPX-351, suggest that the benefits of these drugs may extend to patients who receive an allo-SCT (16, 50) (**Figure 2**). This provides interesting preliminary data that this may be through improving quality of remissions pre-transplant which may in

future studies be measured as pre-transplant MRD. In the case of the FLT3 inhibitor midostaurin which was added to intensive induction and consolidation, the overall survival benefit of the addition of midostaurin appeared to persist in the majority of patients who were allografted in first remission. Notably midostaurin was not administered as post-transplant maintenance in this study. Likewise, CPX-351 demonstrated improved remission rates and OS in patients receiving this drug over standard remission induction therapy in patients with secondary AML. In patients who subsequently received an allo-SCT, those who had received CPX-351 had improved survival as compared to those in the control arm, but the numbers in the study were small, and a smaller proportion were in a remission at time of transplant in the control arm (16). Definitive studies including the incorporation of pre-transplant MRD will be important in validating or refuting the role of pre-transplant therapy in influencing pre-transplant MRD status.

In patients with comorbidities and a high chance of induction related death following intensive chemotherapy, in whom a curative pathway is still intended (87, 88), a less intensive approach may be valid prior to transplant. With the increasing availability of venetoclax based regimens, data will likely emerge as to the transplant outcomes of patients who have a remission following these lower intensity approaches as compared to conventional intensive induction regimens. At present, data on this cohort remains limited, as these regimens have been developed in cohorts of less fit individuals in which the overall transplant rates have been low (89). Certainly, it is well established that patients with AML who have non-proliferative disease, or transformed MDS can have durable remissions with azacitidine alone (90), and patients who proceed to transplant in remission may have long term outcomes which is comparable to those who have remissions from IC (91–93). Although, remission rates for patients receiving non-intensive treatment such as Azacitidine are likely to be inferior as compared to conventional induction chemotherapy alone (94–96), it is unclear whether for patients who do remit, pre-transplant MRD levels are affected by treatment intensity, and whether this has subsequent impact on post-transplant outcomes.

### Can Changes in Conditioning and GVHD Prophylaxis Alter the Impact of Pre-Transplant MRD?

MRD as measured by error corrected NGS was performed in patients with AML who were enrolled onto the BMT CTN 0901 study which performed a randomized comparison of RIC versus MAC regimens (15). In a comparison of patients who were NGS MRD positive pre-transplant, patients who received a RIC regimen had an inferior outcome to those who were MRD negative at the same timepoint (83). In contrast, in patients transplanted with a MAC regimen, levels of MRD pre-transplant did not appear to affect outcomes post-transplant. This suggested that it was possible to alter transplant conditioning to improve outcomes of patients with MRD pre-transplant, but in practice would be limited to younger patients who would be eligible to receive a MAC regimen regardless (see below).

For those with NPM1 mutant transcripts pre-transplant, the risk of relapse post-transplant is increased. However, this is also dependent on the concomitant FLT3-ITD mutation status (97). The identification of T-cell depletion as an adverse risk factor in the whole cohort, and in those with positive NPM1 MRD pre-transplant, suggest a possible transplant strategy that may improve outcomes for this subset of patients.

## Improving Conditioning Regimens for Patients With AML

Transplant conditioning regimens have evolved since the establishment of allo-SCT as a pivotal tool in reducing relapse risk in patients with AML. MAC regimens established the benefits of an allo-SCT in patients with AML (43, 98) but patients over the age of 40 experienced excess toxicity historically. In the last two decades the increased use of RIC regimens has allowed the routine delivery of an allo-SCT to patients over the age of 70 (77). In recent years the efforts of a number transplant cooperative groups have delivered important randomized controlled trials to optimize transplant conditioning regimens to further inform choice of conditioning regimens (12, 15, 99, 100).

### What Is the Optimal Conditioning Intensity?

A MAC regimen by definition requires the infusion of donor stem cells to rescue recipients from permanent bone marrow aplasia. The original studies in allo-SCT used conditioning regimens based on radiotherapy (1). This established the basic principles required of any conditioning regimen in acute leukemia, which is to allow durable engraftment of donor hematopoiesis as well as the delivery of an anti-leukemic effect, which is in turn related to the intensity of conditioning (101).

Cyclophosphamide (Cy) based conditioning combined with total body irradiation (TBI) or busulphan are acceptable MAC regimens. The development of intravenous preparations of busulphan has improved the pharmacokinetics of this agent (102) and has practical advantages over TBI based regimens. Measuring busulphan pharmacokinetics may help predict optimal doses in conditioning (103). Cy/TBI regimens are still commonly used and may be better for patients with either central nervous system (CNS) disease or myeloid sarcoma. Nevertheless, a pivotal randomized controlled trial that demonstrated the superior tolerability of a Fludarabine/Busulphan (Flu/Bu4: 12.8 mg/kg over 4 days of IV busulfan) combination over a standard Cyclophosphamide/Busulphan combination, with acceptable tolerability in patients up to the age of 65 (12). This has resulted in the Flu/Bu4 regimen being accepted as a standard of care for fit patients where a MAC regimen is desired.

RIC regimens result in varying duration of cytopenias and are defined as containing less than ≤8 Gy Total Body Irradiation (TBI) or ≤8 mg/kg busulfan (104). The optimal RIC regimen has not been established. A number of RIC regimens have been developed over the last twenty years to enable a tolerable conditioning regimen to be delivered in patients due to either comorbidities or increased age, with varying levels of toxicity and anti-leukemic potency (e.g. Flu/Bu2: 6.4mg/kg, 2 days of IV

busulphan) (105), and Flu/melphalan (140 mg/m$^2$ of IV melphalan on 1 day) (106). The variability in the effectiveness of these regimens are exemplified by two randomized controlled trials (RCT) of RIC regimens. One study which compared the outcomes of a Flu/2Gy TBI regimen with a Flu/Bu2 regimen demonstrated increased TRM but notable decrease in relapse rates with the Flu/Bu2 regimen (107). In contrast, a recent Flu/Treosulfan study showed superior toxicity incidence to a Flu/Bu2 comparison, but is notable for a TRM in the Flu/Bu2 arm that is far in excess of historical expectations (108).

Given the improved tolerability of novel MAC regimens (12) alongside widespread experience with RIC regimens an important question arose as to whether a MAC or RIC regimen should be selected when either is available in high risk MDS and AML (109, 110). Despite this interest it was surprising that two RCTs comparing RIC and MAC regimens closed early to recruitment but did not demonstrate significant differences in relapse free or overall survival (99, 100, 111). In contrast, a Blood and Marrow Transplant Clinical Trials Network (BMT CTN) study (15) which studied a randomized comparison of RIC versus MAC regimens demonstrated a lower rate of TRM, but higher relapse risk resulting in an inferior relapse free survival (RFS) in patients receiving in the RIC arm as compared to those who received a MAC regimen. However, this study is notable for the higher than expected relapse risk in patients who received a RIC regimen.

The high relapse rates associated with RIC regimens, for patients with high risk AML resulted in the development of the FIGARO study, which compared the outcomes of a standard RIC arm with an augmented RIC schedule with sequential chemotherapy (FLAMSA-Bu) which had shown promising results in early studies in patients with primary refractory disease (112). However, this randomized controlled study demonstrated no improvement in relapse risk from the FLAMSA-Bu regimen as compared to a standard control arm (14).

### GVHD Prophylaxis Strategies

The introduction of Ciclosporin was critical in establishing the deliverability of allo-SCT in patients with acute leukemia (113, 114) reducing the risk of graft-versus-host disease (GVHD). However, studies that demonstrated an inverse relationship between GVHD and relapse risk form the basis of the evidence underlying the GVL (115, 116). Commensurate with this observation, further studies demonstrated a relationship between ciclosporin exposure and risk of relapse, in the context of T-cell depleted allo-SCT (21, 117). Tacrolimus (FK506) has also been compared with Ciclosporin in a number of randomized trials with varying results (118–120), suggesting a reduction in acute GVHD with the use of Tacrolimus but no significant effect on OS or RFS. Other agents such as Sirolimus (121, 122) and Mycophenolate mofetil (123, 124) have also been used either as an addition or substitute for historical Ciclosporin/Methotrexate combination without a definitive improvement in overall outcomes.

*In vivo* T-cell depletion can be achieved by either Anti-thymocyte globulin (ATG) or Alemtuzumab. Studies demonstrate an improvement in risk of acute GVHD without significant changes in OS (125, 126). However a US retrospective study suggested that ATG compromised relapse risk in patients undergoing a RIC allo-SCT (127) which has led to a discrepancy in the uptake of ATG on the two continents (128). More recent data suggest that variations *in vivo* levels of ATG may result in differences in relapse risk as well as NRM (129). It is also important to note that there appear to be different immunosuppressive properties dependent on the source of ATG, which is critical when different studies are compared (130). The humanized anti-CD52 antibody, Alemtuzumab has also been used extensively as a method of *in vivo* T-cell depletion (131, 132), with control of GVHD particularly notable in the HLA-mismatch setting (133). In more recent years, the use of post-transplant Cyclophosphamide which was pioneered for use in the haploidentical donor allo-SCT setting (134) has been used in the volunteer unrelated donor setting (135) but formal assessment in the clinical trial setting is awaited.

The variation in relapse rate from study to study for these different GVHD prophylaxis studies suggest the need to perform adequately powered studies with suitable endpoints, in order to determine the optimal GVHD prophylaxis strategies in AML.

## How to Improve Outcomes of Patients With AML Post-Transplant

### Improving Monitoring of Disease Post-Transplant

Whilst the cornerstone of post-transplant care remains careful clinical assessment and review, post-transplant disease monitoring to identify patients at risk of relapse, and timely intervention is becoming more important. This is particularly important with the increased use of RIC allo-SCT which is associated with a higher risk of relapse (15). Furthermore, the use of pre-emptive treatment before fulminant hematological relapse may increase the efficacy of interventions such as donor lymphocyte infusion (DLI) or Azacitidine (136–139).

#### *MRD Monitoring Post-Transplant*

Prior to hematological relapse, the prognosis of which is usually very poor, early disease re-emergence can be detected by several techniques. The ELN guidelines formally recommend monitoring for MRD post-transplant (33). Similar to pre-transplant, the optimal method for monitoring MRD will be dependent on disease characteristics, and availability of technology, and expertise in the treating center. Post-transplant MRD monitoring has prognostic value. For example, the (8, 21) fusion transcript RUNX1/RUNX1T1 is suitable for MRD monitoring and has been investigated post-transplant (60, 140, 141). Similar to pre-transplant, detectable RUNX1/RUNX1T1 transcripts at 3 months after transplant was a more potent predictor of relapse than presence of c-KIT mutations (141). The most prognostic threshold of MRD may be different after transplant, as compared to that of the pre-transplant setting. For example, one study determined the prognostic impact of NPM1 MRD pre- and post-transplant and found that 1% increase in transcripts pre-transplant and a 10% increase post-transplant were predictive of outcome (142). A combination of multiple methods to detect MRD may be

required to provide the most accurate prognostic information. For example combining NGS MRD for NPM1 with multicolor flow cytometry may improve relapse prediction over either modality alone (143).

Discrepancies between the most discriminatory MRD thresholds at different treatment stages illustrate how the pre- and post-transplant bone marrow environment is different; post-transplant, there is a complex immunological milieu of developing tolerance and GVL. As not all patients with MRD relapse, it is postulated that the GVL effect may eradicate residual disease without the need for further intervention. Although it is also logical that early intervention for patients with molecular MRD would be beneficial, there is limited evidence to support this strategy. In a sub-analysis of patients included in the UK AML17 trial, the provision of post-transplant MRD information to clinicians did not affect outcomes – although this was not a randomized comparison, and not a main aim of the study (97).

### *Chimerism*

Post-transplant monitoring of host-donor hematopoietic chimerism is a widely used post-transplant monitoring strategy, particularly after RIC allo-SCT. Chimerism can be measured in the whole blood, or specifically in T cells (CD3+ selected) or myeloid cells (CD33+). It is known that patients with mixed chimerism post-RIC allo-SCT do have an increased risk of relapse (144), although it should be noted that chimerism and residual disease are conceptually different. Mixed chimerism does not necessarily mean the presence of residual disease, nor does complete chimerism confirm its absence. In haploidentical allo-SCT disease relapse can occur due to acquired uniparental disomy of chromosome 6p leading to loss of the mismatched HLA-haplotype on leukemia cells and subsequent immune escape (145, 146). In this context, chimerism measurement by disparate methodologies can yield different results: recipient non-HLA marker based chimerism shows an increase during relapse, whilst HLA marker based chimerism remains low in disease relapse driven by a loss of HLA (147). Nevertheless chimerism monitoring, post RIC allo-SCT is an important way of identifying patients at high risk of relapse in whom intervention with pre-emptive DLI may be beneficial. Patients who achieve full donor chimerism (FDC) with DLI have a comparable outcome to those who reach FDC spontaneously (148, 149).

There may be ways to improve the performance of chimerism monitoring, including earlier use post-transplant (150), in CD34+ cells (151–154), and, in combination with monitoring for MRD. Waterhouse et al. compared the utility of chimerism and molecular monitoring including WT1 over-expression. Of 15/70 patients in whom increasing mixed chimerism was detected, all had a positive MRD marker and/or increased WT1 expression. They found that in half, detectable MRD and mixed chimerism occurred at the same time but in the other half, mixed chimerism preceded MRD positivity (155). The FIGARO study demonstrated that the risk of relapse following pre-transplant MRD positivity, is reduced by the achievement of full donor chimerism (14), and is a key finding that should direct future treatment strategies to identify methods of increasing the rate of achieving full donor chimerism.

## Post-Transplant Maintenance Strategies to Reduce Relapse

Post-transplant pharmacological interventions may have direct activity on malignant cells, and there is improving understanding that modulation of the complex immunological environment may provide additional benefit. There is improving interest in assessing the impact of routine, maintenance treatments, which do not significantly add to the burden of toxicity which includes infection, organ toxicity, and GVHD (**Table 3** and **Figure 3**).

### *Non-Targeted Agents*

Non-targeted agents which modulate the immune system and tumor microenvironment have the advantage that they are generalizable, are not dependent on specific mutations and may maintain efficacy across the patchwork of clonally heterogeneous disease which is rapidly changing in the post-transplant bone marrow (165, 166).

Azacitidine is an epigenetic modulator that has efficacy in AML both as sole therapy and in combination with other treatments. Post-transplant, in the RICAZA study, Azacitadine was shown to be well tolerated and may both reduce the risk of GVHD through regulatory T-cell expansion and augment the GVL through upregulation of cancer associated antigens on leukemia cells (139, 156, 167). Azacitadine has also been studied in the RELAZA (157) and RELAZA2 (84) studies whereby patients were with mixed CD34+ chimerism and MRD positivity respectively were offered single-agent Azacitadine. In RELAZA, 80% patients responded and Azacitadine delayed relapse. In RELAZA2, relapse free survival at 12 months was 46% in those who had MRD detected and received Azacitadine, suggesting a delaying of haematological relapse. Despite this, a phase 3 RCT of azacitadine versus observation did not show evidence of survival benefit when used as post-transplant maintenance for patients with high risk AML, although this study was limited by the short duration of time that patients remained on treatment (158). The oral formulation of Azacitidine (CC-486) and Panobinostat, another epigenetic modulator have also shown promise in early phase studies and are both the subject of on-going RCTs (NCT04173533 and NCT04326764 respectively) (168, 169). Lenalidomide, an immunomodulator, in combination with Azacitidine is also active in post-transplant relapse (170) but is associated with GVHD when used as monotherapy in the maintenance setting (159) thus indicating the importance of studying the effects of drugs in this specific treatment stage.

DLI can induce remission in patients with hematological relapse, eradicate MRD and promote reversion to full donor chimerism. Alternatively, prophylactic DLI can be delivered to patients at high risk of relapse regardless of detectable disease. A recent observational, matched-pair study found that prophylactic DLI in patients with high-risk AML increased OS at five years by 30% (164). The on-going prospective, 2-arm, phase II PRO-DLI randomized trial will add valuable further information in this area (171). There are also developing technology to manipulate DLI to improve efficacy and limit toxicity. These are reviewed elsewhere, and studies are on-going (172).

**TABLE 3 |** Examples of post-transplant maintenance strategies.

| | | Mechanism | Examples of use |
|---|---|---|---|
| **Non-targeted agents** | **Azacitidine** | Epigenetic modulator | RICAZA (2016)<br>Phase II trial, azacitidine single agent, n=37. Reduced GvHD (156).<br>RELAZA (2012)<br>Phase II trial, azacitadine single agent for mixed CD34+ chimerism, n=20. 80% responded (157).<br>RELAZA2 (2018)<br>Phase II trial, azacitadine single agent for MRD+ patients, n=55. Relapse free survival at 12 months 46% (84).<br>Oran et al. (2020)<br>Phase III trial, n=187. No difference in relapse free survival or overall survival (158). |
| | **Oral azacitidine** | Epigenetic modulator | On-going phase III trial<br>NCT04173533 (oral azacitidine *versus* placebo). |
| | **Panobinostat** | Epigenetic modulator | On-going phase II trial NCT04326764 |
| | **Lenalidomide** | Immunomodulator | LENAMAINT (2012)<br>Phase II trial, n=10. Stopped early due to high incidence of severe acute GVHD (159). |
| **Targeted agents** | **Sorafenib** | Broad-spectrum tyrosine kinase inhibitor | SORMAIN study (2020)<br>Randomised phase II, n=83, FLT3-ITD. Improved relapse free survival at 2 years (85% *versus* 53%) (160).<br>Xuan et al. (13)<br>Randomised phase III, n=202, FLT3-ITD. Reduced relapse at 1 year (7% *versus* 24%) (13). |
| | **Midostaurin** | Broad-spectrum tyrosine kinase inhibitor | RADIUS study (2020)<br>Phase II, n=60 (161). |
| | **Gilteritinib** | FLT-3 inhibitor | On-going phase III trial<br>NCT02997202 (gilteritinib *versus* placebo). |
| | **Venetoclax** | BCL-2 inhibitor | Kent et al. (2020) (abstract)<br>Phase II, n=23. 6 month leukemia free survival: 87% (162).<br>On-going trials<br>Venetoclax + azacitidine. NCT04161885 (phase III) and NCT04128501 (phase II). |
| | **Glasdegib** | Hedgehog inhibitor | Kent et al. (2020)<br>Phase II, n=31, high risk patients. No apparent benefit (163). |
| | **Ivosidenib** | IDH-1 inhibitor | On-going phase I trial NCT03728335 |
| | **Enasidenib** | IDH-2 inhibitor | On-going phase I trial NCT03564821 |
| **Cellular therapy** | **Prophylactic donor lymphocyte infusion (DLI)** | Graft-versus-leukemia effect | Schmid et al. (2019)<br>Retrospective matched-pair study of prophylactic DLI for high-risk disease. Overall survival benefit (69.8% vs. 40.2%) (164).<br>On-going phase II trial NCT02856464 |

## Targeted Agents and Future Areas of Development

Routine application of NGS for DNA mutations have allowed for the identification of dysregulated, druggable pathways in AML. Many are only applicable to a subset of patients, but may also offer the first rung on the ladder of personalized medicine. The major challenges include identification of suitable, druggable targets in the context of clonal heterogeneity (165), and proving clinical efficacy when patient subgroups are relatively small.

An ever-expanding list of targeted treatments directed against key pathways in AML have received Food and Drug Administration FDA approval in recent years. FLT3, as described above is a tyrosine kinase, mutations in which are known to be associated with poor outcomes. In patients with FLT3 mutations, the use of post-transplant sorafenib, a broad-spectrum tyrosine kinase inhibitor (including FLT3), was associated with improved survival compared with placebo (13), findings that were consistent with the phase II SORMAIN study (160). As discussed earlier, the use of another broad-spectrum FLT3 inhibitor, midostaurin along with induction chemotherapy improves outcomes in FLT3-mutated AML (50). In the post-transplant setting, evidence of benefit from midostaurin is limited to a randomized phase II study (RADIUS) which showed a reduction in relapse with midostaurin treatment post-transplant albeit compared with historical controls (173).

Despite some evidence of benefit, there remain concerns about the off-target toxicity and adverse events associated with the broad-spectrum tyrosine kinase inhibitors. The aforementioned SORMAIN study found that the patients most likely to benefit from sorafenib post-transplant were those in whom MRD was detectable (160). For treatments where there are concerns over toxicity, especially in patients with more comorbidities, it is clear that post-transplant disease monitoring can add vital information for assessment of the risk-benefit equation. Second generation drugs which are potent, more specific FLT3 inhibitors are now available and have efficacy as monotherapy in relapsed AML (37). Clinical evaluation of Gilteritinib for post-transplant maintenance is underway (174).

Other targets of small molecule inhibitors include the anti-apoptotic protein BCL2, the Hedgehog signaling pathway, and isocitrate dehydrogenase 1 and 2 (IDH1 & 2). Venetoclax is a selective BCL2 inhibitor which is currently licensed in combination with Azacitidine for the treatment of older patients who are not suitable for intensive treatment and was found to have a substantial survival benefit in this cohort when

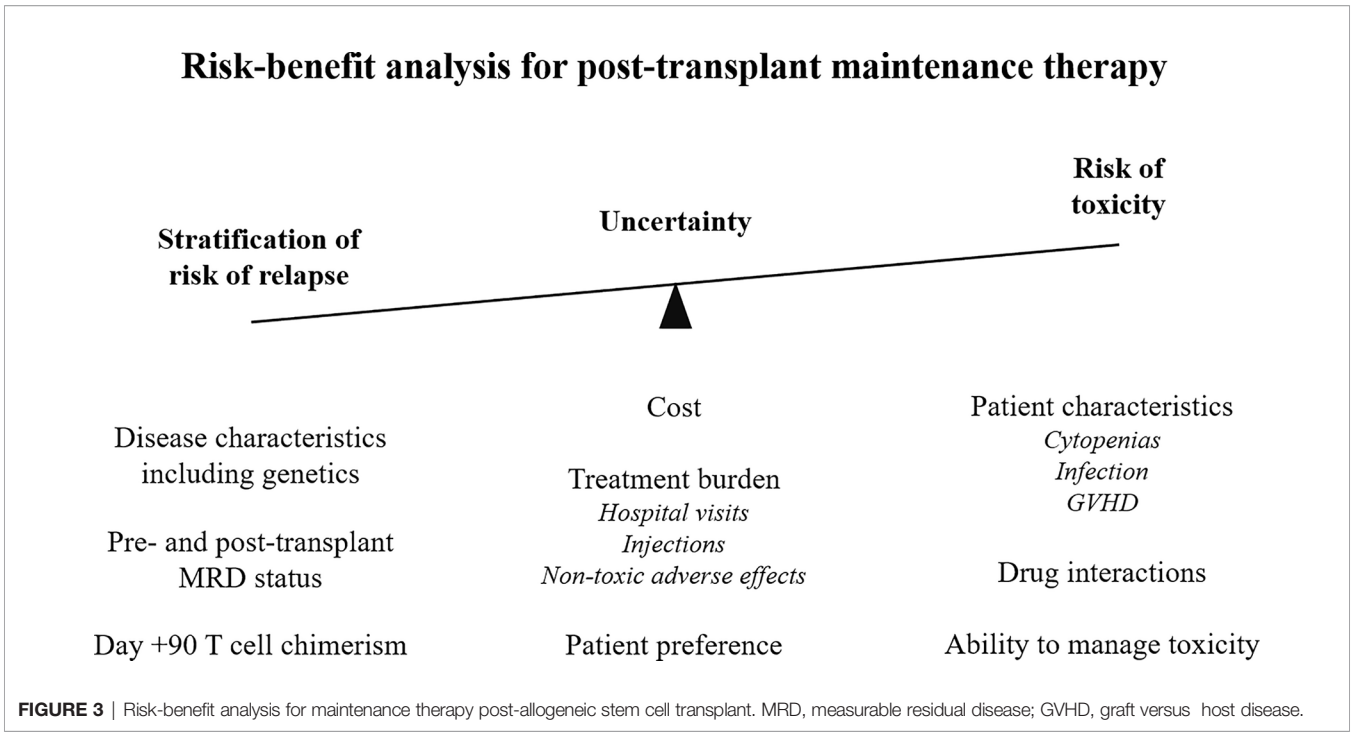


**FIGURE 3** | Risk-benefit analysis for maintenance therapy post-allogeneic stem cell transplant. MRD, measurable residual disease; GVHD, graft versus host disease.

compared with Azacitidine monotherapy (89). In a small study in the post-transplant maintenance setting, Venetoclax was reported to be safe and well tolerated but further studies are required to demonstrate benefit (162). Venetoclax is also being assessed in combination with Azacitadine as maintenance therapy post-transplant (175, 176) but its application may be limited by concerns over myelosuppression.

Glasdegib is an inhibitor of the Hedgehog signaling pathway which has evidence of modest benefit in combination with low dose Cytarabine for patients unfit for intensive treatment (38). It has been recently evaluated in a small single arm study in unselected high-risk patients in the post-transplant maintenance setting. However, there was no clear evidence of benefit either measured by MRD elimination, change in chimerism status, or clinical outcomes. Additionally, treatment was complicated by adverse events requiring pausing or cessation of treatment (163). Further studies in patients who are most likely to benefit as identified by genetic pre-stratification are required.

IDH1 and 2 are proteins which mediate the conversion of isocitrate to alpha-ketoglutarate. Gain in function mutations result in DNA and histone hypermethylation and altered downstream gene expression contributing to oncogenesis. Ivosidenib and Enasidenib, IDH1 and IDH2 inhibitors respectively both have evidence of efficacy in single-arm studies in AML (177–179) and are currently being evaluated for post-transplant maintenance (180, 181).

In summary, there is emerging, encouraging evidence that post-transplant maintenance therapies can reduce the risk of relapse, modulate the risk of GVHD, and improve survival. However, their use must be balanced in order to weigh up the additional toxicity and financial burden against the magnitude of the clinical effect. Detailed molecular analysis of a patient's disease and post-transplant disease monitoring will allow further stratification and potentially identify the patients who are most likely to benefit from treatment (summarized in **Figure 3**).

## CONCLUSION

The establishment of large transplant trial networks has improved the scientific rationale behind transplant practice at every stage of the treatment pathway. This has improved the identification of which patients who are most likely to benefit from an allo-SCT, and also provides a rigorous assessment of novel agents that may benefit patients. Finally, by embedding correlative translational science in these studies, this further improves our knowledge and understanding of the scientific basis of clinical practice. This is of direct benefit to patients, and subsequently provides a vital starting place for future studies.

## AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this review article. All authors contributed to the article and approved the submitted version.

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# Allogeneic Hemopoietic Stem Cell Transplantation for Myelofibrosis: 2021

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The aim of this review is to update the current status of allogeneic hemopoietic stem cell transplants (HSCT) for patients with myelofibrosis (MF). We have first summarized the issue of an indication for allogeneic HSCT, discussing several prognostic scoring systems, developed to predict the outcome of MF, and therefore to identify patients who will benefit of an allogeneic HSCT. Patients with low risk MF are usually not selected for a transplant, whereas patients with intermediate or high risk MF are eligible. A separate issue, is how to predict the outcome of HSCT: we will outline a clinical molecular myelofibrosis transplant scoring system (MTSS), which predicts overall survival, ranging from 90% for low risk patients, to 20% for very high risk patients. We will also discuss transfusion burden and spleen size, as predictors of transplant outcome. The choice of a transplant platform including the conditioning regimen, the stem cell source and GvHD prophylaxis, are crucial for a successful program in MF, and will be outlined. Complications such as poor graft function, graft failure, GvHD and relapse of the disease, will also be reviewed. Finally we discuss monitoring the disease after HSCT with donor chimerism, driver mutations and hematologic data. We have made an effort to make this review as comprehensive and up to date as possible, and we hope it will provide some useful data for the clinicians.

**Keywords: myelofibrosis, allogeneic transplantation, busulfan, thiotepa, fludarabine chimerism, splenectomy**

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## INDICATIONS FOR HSCT

In the era of JAK inhibitors, allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative treatment for patients with Myelofibrosis (MF) (1). The American Society for Transplantation and Cellular Therapy (ASTCT) considers an allogeneic HSCT "standard of care with clinical evidence" for patients with intermediate and high risk disease (2). In order to classify patients as intermediate or high risk several models have been developed. **Table 1** outlines some of the most commonly used scoring systems and the variables they are based on: IPSS (3), DIPSS (4), DIPSS-plus (5) and MIPSS70 (6). The first two are based exclusively on clinical data, the third incorporates cytogenetics and the fourth includes mutational analysis. Survival of patients with MF

**TABLE 1** | Prognostic scoring systems for patients with myelofibrosis.

| IPSS [3] | DIPSS [4] | DIPSS-plus [5] | MIPSS70 [6] | |
|---|---|---|---|---|
| | | | **Genetic variables:**<br>✓ One HMR mutation (1 point)<br>✓ ≥2 HMR mutations (2 points)<br>✓ Type1/like CALR absent (1 point) | ✓ |
| ✓ Age > 65 years (1 point)<br>✓ Constitutional symptoms (1 point)<br>✓ Hemoglobin < 10 g/dl (1point)<br>✓ WBC count > 25 × 109/l (1 point)<br>Circulating blasts ≥ 1% (1 point) | ✓ Age > 65 years (1 point)<br>✓ Constitutional symptoms (1 point)<br>✓ Hemoglobin < 10 g/dl (2points)<br>✓ WBC count > 25 × 109/l (1 point)<br>Circulating blasts ≥ 1% (1 point) | ✓ RBC transfusion (1 point)<br>✓ PLT count < 100 × 109/l (1 point)<br>Unfavorable karyotype [a] (1 point) | **Clinical variables:**<br>✓ Hemoglobin <10g/dl (1 point)<br>✓ Leukocytes >25×109/l (2 points)<br>✓ Platelets <100×109/l (2 points)<br>✓ Circulating blasts ≥2% (1 point)<br>✓ Constitutional symptoms (1 point)<br>✓ Bone marrow fibrosis grade ≥2 (1 point) | |
| • Low risk: 0 points (11.3 yrs)<br>• Intermediate-1 risk: 1 point (7.9 yrs)<br>• Intermediate-2 risk: 2 points (4 yrs)<br>• High risk: ≥ 3 points (2.3 yrs) | • Low risk: 0 point ( n.r.)<br>• Intermediate-1 risk: 1–2 point (14.2 yrs)<br>• Intermediate-2 risk: 3–4 points (4 yrs)<br>• High risk: 5-6 points (1.5 yrs) | • Low risk: 0 point ( 15.4 yrs)<br>• Intermediate-1 risk: 1 point (6.5 yrs)<br>• Intermediate-2 risk: 2–3 points (2.9 yrs)<br>• High risk: 4–6 points (1.3 yrs) | • Low risk: 0-1 points (n.r.)<br>• Intermediate risk: 2-4 points (6.3 yrs)<br>• High risk: ≥ 5 points (3.1 yrs) | • |

*IPSS, international prognostic scoring system; DIPSS, dynamic international prognostic scoing system; MIPSS70, mutation-enhanced international prognostic scoring system; HMR, high molecular risk (see text).*

can be predicted using one of those models, and thus eligibility for a transplant procedure. However eligibility must also include transplant related variables, such as patients age up to 70-75 years, a good performance status, low transfusion burden, absence of a massive splenomegaly and portal hypertension and donor type. Older patients also tend to have one or more comorbidities which may increase the risk of transplant related mortality (TRM) or even preclude a transplant approach. A Panel of experts recommends considering allogeneic HSCT for patients with IPSS/DIPSS/DIPSS plus high or intermediate-2 risk (7) **(Figure 1)**. The Panel also recommends considering an allogeneic HSCT for transplant-eligible patients with IPSS/DIPSS/DIPSS-Plus intermediate-1 risk score, who present with either refractory, transfusion-dependent anemia, a percentage of blasts in peripheral blood > 2% in at least two repeated manual measurements, adverse cytogenetics, or high-risk mutations, such as such as ASXL1, EZH2, IDH1/IDH2, SRSF2 (7)**(Figure 1).** In this situation, the transplant procedure should be performed in a controlled setting (registries, clinical trial) (7).

More recently a mutation-based prognostic model has been proposed to identify candidates for HSCT among low or intermediate-1 risk DIPSS, who are expected to have similar overall survival as patients with a high risk DIPSS (8). Patients who are triple negative (JAK2/CALR/MPL) or CALR wild type and ASXL1 mutated, irrespective of DIPSS risk scores, should be considered for HSCT (8). A combination of mutation-based prognosis together with clinical data has been compiled in a recent scoring system (9).

In conclusion, we are now able to identify MF patients with a different median survival: there is consensus on the eligibility to transplant for DIPSS intermediate2/high risk patients. The presence of high risk mutations in DIPSS intermediate1/low risk patients may also suggest eligibility for a transplant procedure. The clinical conditions of the patient, the degree of HLA matching of potential donors and the patient's choice must be considered in the final decision to transplant or not.

## HOW TO DEAL WITH SPLENOMEGALY

Splenomegaly is a common feature in patients with advanced myelofibrosis (MF) and it is a sign of extramedullary hematopoiesis (also known as myeloid metaplasia) (10). Patients may be severely symptomatic with abdominal pain, early satiety, weight loss, cytopenia, portal hypertension, and splenic infarction (10).

**Splenectomy** is effective in relieving symptoms, but is associated with a number of complications, as well as significant morbidity and mortality.

Peri-operative mortality is in the range of 5%-10%. The most common complications are infections, thrombosis and bleeding, occurring in up to 30% of patients (11). Patients with thrombocytopenia seemed to have an increased probability of post-splenectomy blast transformation, although this did not result in shortened survival. Leukemic transformation is more probably related to natural progression of the disease in advanced stage and to post-splenectomy redistribution of circulating blasts, not to true clonal evolution (12, 13). Hemopoietic stem cell transplantation (HSCT) offers the potential of cure for patients with intermediate or high risk myelofibrosis (14). Splenomegaly, characteristic of those patients, may lead to sequestration of transplanted stem cells and delayed hematologic recovery (3, 15) thus affecting the transplant outcome. Surgical removal of the spleen may be effective in reducing the time for neutrophil and platelet recovery (16) but its impact on relapse rate and survival is unclear (17, 18), calling for a prospective randomized trial. Pre-transplant splenectomy in MF patients was associated with

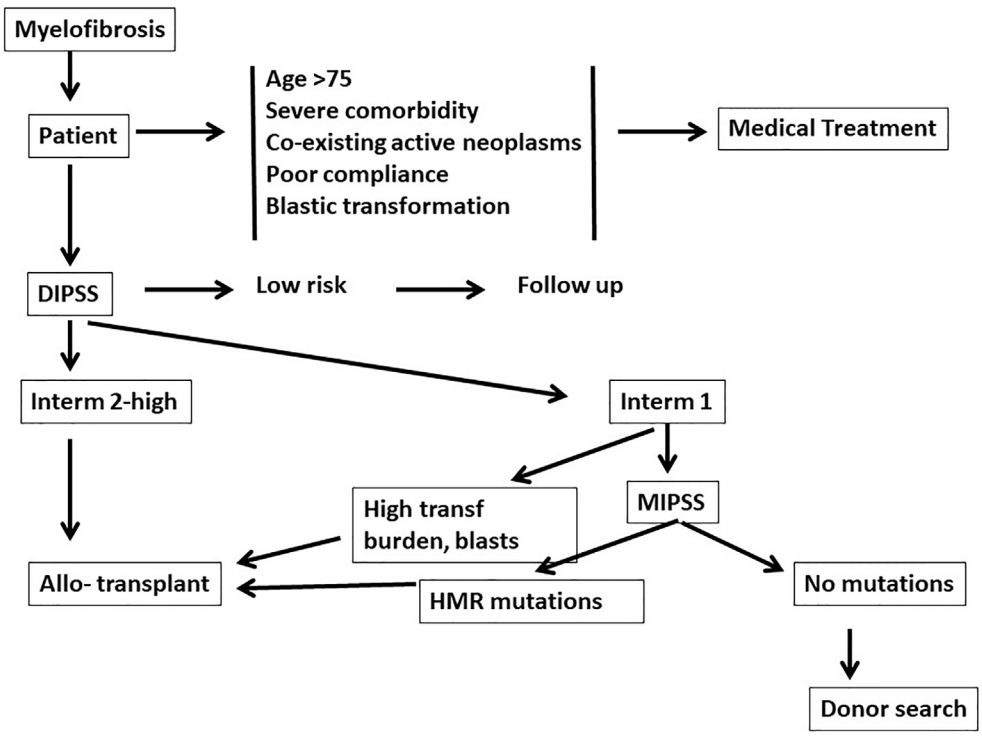


**FIGURE 1** | Eligibility for a transplant procedure in patients with myelofibrosis: medical treatment should be offered for older patients (>75 years) and/or patients with comorbidities. Dynamic international prognostic scoring system (DIPSS) will then identify patients low risk patients, who should be followed. DIPSS-intermediate 2 and high risk patients are who are strong candidates for an allogeneic transplant. DIPSS-Intermediate 1 patients with a high transfusion burden and blasts counts are also strong candidates for an allogeneic transplant. Patients may also be studied with a molecular international prognostic scoring system (MIPSS), and may be eligible for transplantation if high risk mutations (HMR) (see text) are identified.

a prolonged overall and event-free survival in a recently published study (19).

The advent of **Janus kinase (JAK) 1/2 inhibitors**, which decrease splenomegaly and alleviate MF-related symptoms, has had, as compared to old cytoreductive drugs, a major impact on the management of splenomegaly, removing some indications for splenectomy. However, in a proportion of patients, the splenic response is then lost. Many MF patients who proceed to allogeneic HSCT, are currently treated with JAK inhibitors, usually ruxolitinib: this should be tapered down over a 10- to 14-day period and should be discontinued just before the conditioning regimen (20). In one study, ruxolitinib was continued also during transplant in the attempt of preventing GvHD (21).

**Splenic irradiation (SI)** may also be used to reduce the spleen size and related symptoms; there are only few small studies on SI prior to transplant in MF patients (22, 23). It was demonstrated that SI alleviates splenic discomfort and reduces spleen size in a majority of MF patients, with a median duration of response of 6 months (24). Limitations of SI include prolonged pancytopenia with infectious complications. Comparable engraftment rate has been shown in patients receiving or not SI (25) as well as comparable acute and chronic GVHD incidence, post-transplant infectious complications and survival. The role of SI in leukemic transformation (LT) remains unclear and speculative. Radiotherapy may be indicated in patients who are not eligible for surgery or in patients who have lost their response to JAK2 inhibitors (26–28).

## PREDICTING THE OUTCOME OF HSCT

**Disease based risk score.** Survival of MF patients receiving medical treatment, with the exclusion of allogeneic HSCT, can be predicted by several scoring systems, reviewed in Indications for HSCT (3–7). Some studies have assessed whether these scoring systems can predict the outcome of patients after an allogeneic HSCT. DIPSS can predict post transplant survival (29), and the same has been shown for DIPSS-plus (30). In multivariate analysis, the DIPPS-plus score predicted survival, disease free survival (DFS) and TRM, together with conditioning regimen, comorbidity index (HCT-CI), patients' age and donor type (30). In 2019, a cohort of 159 patients with secondary myelofibrosis who underwent allogeneic HSCT was analyzed retrospectively to compare the predictive value of DIPSS and MYSEC (31). The four risk groups of DIPSS did not predict survival after allogeneic HSCT, whereas MYSEC maintained its predictive role also in the post-transplant setting.

**Transplant based risk score (TS).** Few scoring systems have been designed exclusively for allogeneic HSCT. In 2010, a study identified spleen size, transfusion history and donor type as

predictive of outcome: survival was 79% for low risk patients and 8% for high risk patients (18). In 2012 a predictive risk model including JAKV617F status, age and constitutional symptoms was proposed in the setting of 150 transplanted patients and resulted to be predictive for 5 years overall survival (OS) (32).

More recently, a scoring system has been devised, which incorporates HLA matching between donor and recipient, mutational analysis, and clinical data, at time of transplantation (MTSS), in patient with primary and secondary MF (9). This index is predictive of non-relapse mortality. In the last year we have revisited our transplant score (TS) including maximum spleen size and red blood cell transfusion burden before HSCT (18): the 5 year disease free survival (DFS) was 74% *vs* 36% (p=0.0001) for patients with low or high TS.

In conclusion, scoring systems designed to predict transplant outcome are available and can be used when counseling patients eligible for transplant procedures.

## DONOR TYPE, STEM CELL SOURCE AND GVHD PROPHYLAXIS

Donor type is an important predictor of outcome in myelofibrosis: a study from the Center for International Blood and Marrow Transplant Research (CIBMTR) on 233 transplants for myelofibrosis (33), showed that donor type was an independent risk factor for TRM, with a relative risk of death of 3.92 for matched unrelated donor (MUD) and 9.37 for mismatched unrelated donor (MMUD), when compared to matched related donor (MRD) (33). The 5 year overall survival was 56% for MRD, 48% for MUD and 34% for MMUD. The main causes of death were GvHD, infections and organ failure, in particular among MMUD grafts (33). Similar results are reported in other studies (17, 34–37). On the other hand, contrasting data exist regarding GvHD and donor type. Some studies show no significant difference among different donor types (17, 35, 38), whereas the CIBMTR shows a higher risk of GvHD for patients receiving MUD (RR 1.98) and MMUD (RR 1.52) as compared to MRD (33). Engraftment is reported to be comparable according to donor type (33, 35, 38), whereas significant differences have been described according to the stem cell source, with faster recovery with peripheral blood grafts (37, 38). Unrelated cord blood (UCB) transplants have been rarely used in myelofibrosis, and are associated with delayed engraftment and a high TRM, probably due to the significant risk for graft failure and infectious complications (39).

The addition of ATG to conventional GvHD prophylaxis, based on calcineurin inhibitor alone or combined to methotrexate or mycophenolic acid, reduces the incidence of GvHD, as one would expect (40). However, modified regimens of GvHD prophylaxis, including the use of post-transplant cyclophosphamide (PT-CY) have reduced post-transplant complications in alternative donor grafts, especially for HLA haplo-identical donor (36). A combination of calcineurin inhibitor with ATG and PTCy after reduced intensity conditioning may further reduce the risk of GvHD, improving TRM and survival, without an increased risk of relapse (41). Very recently, an interesting pilot study was conducted by Morozova and colleagues: GvHD prophylaxis with PTCY and ruxolitinib showed promising results in terms of GvHD control in a small cohort of patients with acceptable TRM (42).

In summary an HLA matched donor is the best option for myelofibrosis, in order to achieve optimal outcome: alternative donor grafts may be explored using modified regimens of GvHD prophylaxis.

## CONDITIONING REGIMENS AND RUXOLITINIB

Conditioning regimens in myelofibrosis were historically myeloablative (MAC), predominantly busulfan plus cyclophosphamide and total body irradiation with or without cyclophosphamide (15), but transplant related mortality (TRM) and GvHD rates were high, especially in older individuals (43).

Reduced intensity conditioning (RIC) has been increasingly used in MF, in consideration of the older age of MF patients. The first prospective EBMT multicenter phase II trial of RIC SCT consisted of busulfan (10 mg/kg) orally (or equivalent IV dose) plus fludarabine (180 mg/m$^2$) and *in vivo* T-cell depletion with anti-thymocyte globulin at a dose of 3 x 10 mg/kg (for related transplantation) or 3 x 20 mg/kg (for unrelated donor transplantation): this protocol resulted in low rates of primary graft failure and rapid hematologic recovery (17). Fludarabine 90 mg/m^2, combined with melphalan 140 mg/m^2 (FLU-MEL) is an alternative RIC regimen, and has been compared in a retrospective study with the BU-FLU regimen (44). Although the FLU-MEL was associated with increased early toxicity, the long-term outcome (OS and disease-free survival) was similar in the two groups. In both regimens the use of a HLA mismatched unrelated donor was associated with worse outcome, in terms of TRM, OS and progression-free survival. A randomized study comparing fludarabine in combination with busulfan 10 mg/kg i.v. or thiotepa 12 mg/kg, failed to identify significant differences in terms of clinical outcome (45): both regimens were associated with a significant degree of mixed chimerism.

In a retrospective comparisons of RIC versus MAC regimens for myelofibrosis, the latter do not appear to protect patients from relapse (46), neither there are differences within day +100 transplant-related mortality (47). A large retrospective analysis of the EBMT in 2224 patients with myelofibrosis, compared MAC regimens (781 patients) with RIC regimens (1443 patients) (48): there was no statistically significant difference in engraftment, GvHD, TRM and overall survival; there was a trend toward a higher relapse rate with RIC.

We have recently shown that a conditioning regimen including two alkylating agents (in our case busulfan and thiotepa) with fludarabine, significantly reduced the risk of relapse when compared to regimen with one alkylating agent (either busulfan or thiotepa or melphalan) in combination with fludarabine (36). Therefore, the choice of the conditioning regimen, may play a significant role in determining the control of the disease after an allogeneic HSCT.

The efficacy of the JAK1/JAK2 inhibitor ruxolitinib in reducing spleen size and systemic symptoms, in myelofibrosis, has been established (49, 50). Currently, most patients undergoing an allogeneic HSCT have been treated with this agent with the aim of reducing splenomegaly, improving the performance status and shorten time to engraftment. A phase II trial demonstrated the feasibility of ruxolitinib therapy followed by a RIC regimen for patients with myelofibrosis (51). Appropriate tapering should be scheduled (52), although recently peri-transplant ruxolitinib has been reported (42). There is no evidence, however, that the administration of ruxolitinib pre-transplant reduces the incidence of relapse after transplant.

## MONITORING DISEASE CONTROL (DONOR CHIMERISM AND MUTATIONS)

Patients with myelofibrosis may have one of three driver mutations (JAK2, CALR and MPL), or lack all three (triple negative patients). Ditschkowski et al. (53) showed that survival after transplantation was not significantly different for JAK2+ (75%) versus JAK2 negative (71%) patients. More recent retrospective studies have suggested a survival advantage for CALR mutation (54, 55). A large retrospective study has investigated the role of extensive mutational profiling with a targeted 16-gene panel, and has confirmed the favorable role of a CALR mutation (56). In the same study IDH2 and ASXL1 mutations confirmed their adverse prognostic role after allogenic HSCT, whereas a triple negative status (JAK2, MPL, CALR) did not appear to modify the outcome after transplant.

Minimal residual disease (MRD) should be used to identify patients achieving a complete remission after HSCT, as well as an early evidence of relapse. Alchalby et al. has shown that JAK2 negativity after allogeneic HSCT significantly reduces the risk of relapse (57). Similar results have been obtained with MPL and CALR mutations as MRD markers (58). A recent retrospective single-center study (59) has shown that that patients with detectable mutations on day +100 or at day +180 after allogeneic HSCT have a significant higher risk of clinical relapse at 5 years, as compared to molecular-negative patients (62% *vs* 10%, P<0.001 and 70% *vs* 10%, P<0.001, respectively): single different mutations have comparable predictive value on relapse.

However, 10% to 15% of patients are triple negative and cannot be followed after transplantation with a molecular marker: in these patients chimerism studies can be helpful to identify early signs of relapse. We have recently described 120 patients with chimerism data on day +30 (60), showing that early full donor chimerism is highly predictive of long-term disease control. The cumulative incidence of relapse at 5 years, was 14% *vs* 40% for patients with or without full donor chimerism (40). We found that a conditioning regimen including two alkylating agents (busulfan and thiotepa) induces a significantly higher rate of complete donor chimerism on day +30, as compared to patients prepared with one alkylating agent (either busulfan, melphalan or thiotepa) (87% *vs* 45%, p<0.0001).

MRD positive patients or patients with declining donor chimerism, who still are receiving immunosuppressive therapy, may discontinue immunosuppressive drugs and/or receive donor lymphocyte infusions (DLI), in order to achieve again full donor chimerism.

## PRIMARY GRAFT FAILURE (PRGF) AND POOR GRAFT FUNCTION (PGF)

Lack of engraftment of donor stem cells is referred to as primary graft failure (PrGF), and is characterized by neutropenia, combined with mixed or no donor chimerism on bone marrow and/or peripheral blood cells (61). PrGF should be distinguished from poor graft function, or cytopenia with full donor chimerism (62). The latter suggests inappropriate function of engrafted donor stem cells and can be treated with the infusion of selected CD34+ cells from the same donor, without a preparative regimen (62). Predictive factors have not been determined, but several conditions have been associated with unsuccessful engraftment, such as the intensity of the conditioning regimen, donor type, stem cells source, number of CD34+ cells infused, GvHD prophylaxis, degree of fibrosis, degree of splenomegaly, pre-transplant thrombocytopenia (63).

The incidence of PrGF ranges from 2 to 24%. A lower rate was reported in a large prospective study from EBMT (48), with only in 2 out 103 patients with PrGF. However, 11% of patients experienced poor graft function and required an additional stem cell boost. In a subsequent pilot study, PrGF was not influenced by the intensity of conditioning regimen (64) and no other predictors were found in other studies (17, 53). Donor type appears to influence the incidence of PrGF, which is lower in patients transplanted from HLA identical donors, as compared to transplants from family mismatched and unrelated donors (65–67). Contrasting data are reported on other factors: splenectomy before HSCT, peripheral stem cell use as source of stem cells and the absence of pre-transplant thrombocytopenia have been suggested to promote engraftment in some studied (18, 66, 68), but not in other studies (65).

Patients with full donor engraftment, may still have transfusion dependent low blood counts for variable periods of time, and this is referred to as Poor graft function (PGF). In a large retrospective analysis, the proportion of patients with less than 20x10^9/l platelets between day +50 and +100 after an allogeneic HSCT, is 10% and has not changed in the time period before 2000, 2001-2010 and beyond 2010 (unpublished). A diagnosis of myelofibrosis is a negative predictor for hematologic recovery: a low platelet count is seen in 18% *vs* 8% of patients with or without a diagnosis of MF (unpublished). For this reason, when looking at patients receiving a top up of CD34 selected cells for PGF, the proportion of patients with MF (26%) is higher than the proportion of MF in the transplant indications (7%) (62). These patients may remain transfusion dependent for long periods of time, and may be treated either with an infusion of CD34 selected cells from the same donor, or, more recently with high dose eltrombopag. Time to trilineage

recovery is however delayed with these approaches and long-lasting supportive care must be planned.

## TREATMENT OF MF RELAPSE AFTER ALLOGENEIC TRANSPLANT

Allogeneic hematopoietic stem cell transplant remains the only curative treatment for myelofibrosis (MF). A retrospective EBMT study on 1055 patients with MF transplanted between 1995 and 2014, alive and free of their disease at two years after HSCT showed that the most common cause of death (41-61%) was relapse of MF, for all time periods (2-5years, 5-10 years) (40). There is no standardized re-treatment of relapse after allogeneic transplant. Based on limited available literature, ruxolitinib, donor leukocytes infusion (DLI), and a second allogenic HSCT are three options for relapsing MF patients; obviously, the choice depends on patients age, fitness status, molecular or hematologic relapse, and the presence of GVHD.

The use of DLI and second transplant as salvage treatment for relapsed MF after allogeneic HSCT was reported in a retrospective study some years ago (69). Out of 26 relapsed patients, 39% achieved a stable response to dose-escalated DLIs. Seventeen patients, thirteen of which non-responders to DLI, underwent a second allogeneic HSCT, achieving an ORR of 80% (9 CR and 3 PR); incidence of relapse at 1-year was 24%. The 2-year overall survival and progression-free survival were 70% and 67%, respectively.

The most consistent data derive from a recent EBMT real-life retrospective study focusing on the treatment of 251/1371 (18%) MF patients, who relapsed after an allogeneic HSCT (70). DLIs were used in 23% of patients, whereas 20% underwent DLI combined with chemotherapy and 11% had chemotherapy alone. Fifty-one patients (25%) underwent second allogeneic HSCT alone and 26 (13%) underwent DLI and a second allogeneic HSCT. The median OS from the time of relapse for patients receiving DLI alone, DLI followed by a second allogeneic HSCT or second allogeneic HSCT were 76 months, 54 months, and 27 months respectively.

Recently Chabra et al. published a small number of MF patients, mostly treated with ruxolitinb pre-transplant (71): after a median follow up of >3 years, two patients out of 37 had relapsed after HSCT (5.4%), but the study lacked a strong control group of untreated ruxolitinib patients. Indeed other recently published data in the ruxolitinib era (72), have shown no improvement in survival nor in the incidence of relapse for MF. The use of ruxolitinib after allogenic HSCT is primarily attributable to the treatment of GVHD, and only in few cases for the treatment of the relapse, mostly in combination with DLIs. One study has reported peri-transplant use of ruxolitinib (21).

In conclusion, although based on a small number of studies, the best therapeutic strategy for MF patients relapsing after an allogeneic HSCT, seems to be dose -escalated DLI, or otherwise, for non-responders, a second allogeneic HSCT. The question remains whether DLI should be infused after a lympho-depleting treatment, as currently is being done for CAR-T cells.

## DESIGNING A TRANSPLANT STRATEGY FOR MYELOFIBROSIS

Patients with myelofibrosis need to be discussed to identify eligibility for transplant procedures **(Figure 1).** Patients over the age of 75 years, with severe comorbidities, coexisting active neoplasms, or poor compliance, should be addressed by medical treatment. Patients less than 75 years of age and fit, should be assessed for risk factors (DIPSS or other scoring systems): low risk patients should be followed regularly. DIPSS intermediate 2 or high risk patients are eligible for a transplant procedure **(Figure 1).** DIPSS int 1 patients should be studied with next generation sequencing (NGS): if no additional adverse mutations are found (ASXL1, EZH2, SRSF2, IDH1/2) then the search for a donor can be initiated, but the transplant may be postponed. If, on the contrary, additional adverse mutations are identified the donor search may be initiated and the transplant also programmed.

Once a transplant is programmed several facts need to be considered: in addition to patient factors such as age, comorbidities and disease phase (DIPSS), other facts need to be taken in to account, including transplant variables (donor type, stem cell source, conditioning regimen, GvHD prophylaxis), the psychological status of the patient, the presence of care givers, especially for the post-transplant discharge and logistics (transplant centers may be located at a distance from the patients' home). The combination of all these factors will then lead to a tailored strategy in terms of optimal timing and choice of a transplant platform.

## AUTHOR CONTRIBUTIONS

AB, SS and VS designed the study and overviewed the manuscript. Sections and authors: indications (II), splenectomy (ER), predicting outcome (FS, EG), monitoring disease (PC), Graft Failure (SG), Relapse (LL), Conditioning regimens (FA), donor type (EM), reviewed MS (GB). All authors contributed to the article and approved the submitted version.

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# Integrating CAR T-Cell Therapy and Transplantation: Comparisons of Safety and Long-Term Efficacy of Allogeneic Hematopoietic Stem Cell Transplantation After CAR T-Cell or Chemotherapy-Based Complete Remission in B-Cell Acute Lymphoblastic Leukemia

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Patients often undergo consolidation allogeneic hematopoietic stem cell transplantation (allo-HSCT) to maintain long-term remission following chimeric antigen receptor (CAR) T-cell therapy. Comparisons of safety and efficacy of allo-HSCT following complete remission (CR) achieved by CAR-T therapy *versus* by chemotherapy for B-cell acute lymphoblastic leukemia (B-ALL) has not been reported. We performed a parallel comparison of transplant outcomes in 105 consecutive B-ALL patients who received allo-HSCT after achieving CR with CAR-T therapy (n=27) or with chemotherapy (n=78). The CAR-T-allo-HSCT group had more patients in second CR compared to the chemotherapy-allo-HSCT group (78% *vs.* 37%; p<0.01) and more with complex cytogenetics (44% *vs.* 6%; p<0.001) but the proportion of patients with pre-transplant minimal residual disease (MRD) was similar. The median follow-up time was 49 months (range: 25-54 months). The CAR-T cohort had a higher incidence of Grade II-IV acute graft-*versus*-host disease (aGVHD 48.1% [95% CI: 46.1-50.1%] *vs.* 25.6% [95%CI: 25.2-26.0%]; p=0.016). The incidence of Grade III-IV aGVHD was similar in both groups (11.1% *vs.*11.5%, p=0.945). The overall incidence of chronic GVHD in the CAR-T group was higher compared to the chemotherapy group (73.3% [95%CI: 71.3-75.3%] *vs.* 55.0% [95%CI: 54.2-55.8%], p=0.107), but the rate of extensive chronic GVHD was similar (11.1% *vs.*11.9%, p=0.964). Efficacy measures 4 years following transplant were

all similar in the CAR-T *vs.* the chemotherapy groups: cumulative incidences of relapse (CIR; 11.1% vs.12.8%; p=0.84), cumulative incidences of non-relapse mortality (NRM; 18.7% *vs.* 23.1%; p=0.641) leukemia-free survival (LFS; 70.2% *vs.* 64.1%; p=0.63) and overall survival (OS; 70.2% *vs.* 65.4%; p=0.681). We found that pre-transplant MRD-negative CR predicted a lower CIR and a higher LFS compared with MRD-positive CR. In conclusion, our data indicate that, in B-ALL patients, similar clinical safety outcomes could be achieved with either CD19 CAR T-cell therapy followed by allo-HSCT or chemotherapy followed by allo-HSCT. Despite the inclusion of more patients with advanced diseases in the CAR-T group, the 4-year LFS and OS achieved with CAR T-cells followed by allo-HSCT were as remarkable as those achieved with chemotherapy followed by allo-HSCT. Further confirmation of these results requires larger, randomized clinical trials.

**Keywords: CD19 CAR T-cell therapy, relapse/refractory B cell acute lymphoblastic leukemia, allogeneic hematopoietic cell transplantation, survival, relapse**

## INTRODUCTION

Refractory/relapsed (R/R) B-cell acute lymphoblastic leukemia (B-ALL) is a leading cause of morbidity and mortality in children and young adults (1–3). Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is often undertaken for high-risk R/R B-ALL patients. However, many R/R patients are never able to achieve a complete remission (CR) following chemotherapy and are not referred for allo-HSCT. Therefore, relapse rates among these patients remain high despite the potential cure that is possible for B-ALL patients with an allo-HSCT. Achieving a CR prior to allo-HSCT has been shown to improve outcomes for these R/R B-ALL patients including improving leukemia free survival (LFS) following transplantation (4, 5).

In recent years, clinical trials with anti-CD19+ chimeric antigen receptor T-cell (CAR-T) therapy have demonstrated high CR rates of ~70% to 90% in patients with R/R B-ALL (6–12) and offer the hope of a potential cure for those patients who are otherwise refractory or relapsed following chemotherapy. However, remission following CAR-T therapy is often not durable with about half of CR patients relapsing within 1 year of therapy (6, 10, 13, 14). There is accumulating evidence from recent studies demonstrating that CAR-T therapy followed by allo-HSCT could potentially result in higher rates of durable, long-term remission for pediatric R/R B-ALL and reduce the relapse rates seen with CAR T-cell therapy alone (6, 8, 15–17). Yet there is still controversy around the safety and efficacy of allo-HSCT following CAR T-cell therapy and the ability to achieve long-term LFS with this sequential, dual immunotherapy. Some studies have reported high relapse rates and higher treatment related mortality following transplant after CAR-T therapy, resulting in no improvement of LFS and overall survival (OS) when compared to CAR T-cell therapy alone (11, 18).

In the present study, we conducted a parallel comparison of outcomes among R/R B-ALL patients who achieved remission from either CAR T-cell therapy or chemotherapy and who subsequently underwent allo-HSCT. We report safety and efficacy results in these two cohorts.

## MATERIALS AND METHODS

### Patients

We included 105 consecutive B-ALL patients who underwent allo-HSCT after achieving CR either from CAR-T therapy (n=27) or chemotherapy (n=78) at the Hebei Yanda Lu Daopei Hospital between November 2015 and August 2016. Details on the enrollment of the CAR-T group and chemotherapy group (including 13 B-ALL patients with *BCR/ABL* who received chemotherapy plus a tyrosine kinase inhibitor) are shown in **Figure 1**.

The study protocol was approved by the ethics committee of the Hebei Yanda Lu Daopei Hospital. Informed consent was obtained from the patients according to the Declaration of Helsinki.

### CD19+ CAR T-Cell Therapy and CAR-T-Related Side Effects

CD19+ CAR T-cell therapy was performed according to previously described methods (6). Briefly, we used a second generation CD19+ lentiviral vector that also expressed the co-stimulatory 4-1BB molecule (CAR-T clinical trials No: ChiCTR-IIh-16008711). Before CAR T-cell infusion, patients received lymphodepleting chemotherapy consisting of fludarabine (30 mg/m$^2$/day) and cyclophosphamide (250 mg/m$^2$/day) (FC) on days -5, -4, and -3. Cytokine release syndrome (CRS) and neurotoxicity grading were performed according to previously described methods (19–22).

### Clinical Transplant Protocol

Before transplantation, patients received intensive myeloablative conditioning regimens. Total body irradiation (TBI) plus cyclophosphamide/fludarabine-based chemotherapy or busulfan (Bu) plus cyclophosphamide/fludarabine-based were used according to each patient's status. TBI-based regimes were preferred if no contraindications such as severe pulmonary complications were observed. TBI was given using a horizontal beam in a linear accelerator. Patients in the TBI group received conditioning with fractioned TBI (200cGy Bid for 5-6 doses). Patients in the Bu group received Bu (0.8mg/kg i.v. Q6h for 16 doses) on days -8 to -6. TBI or Bu was followed by

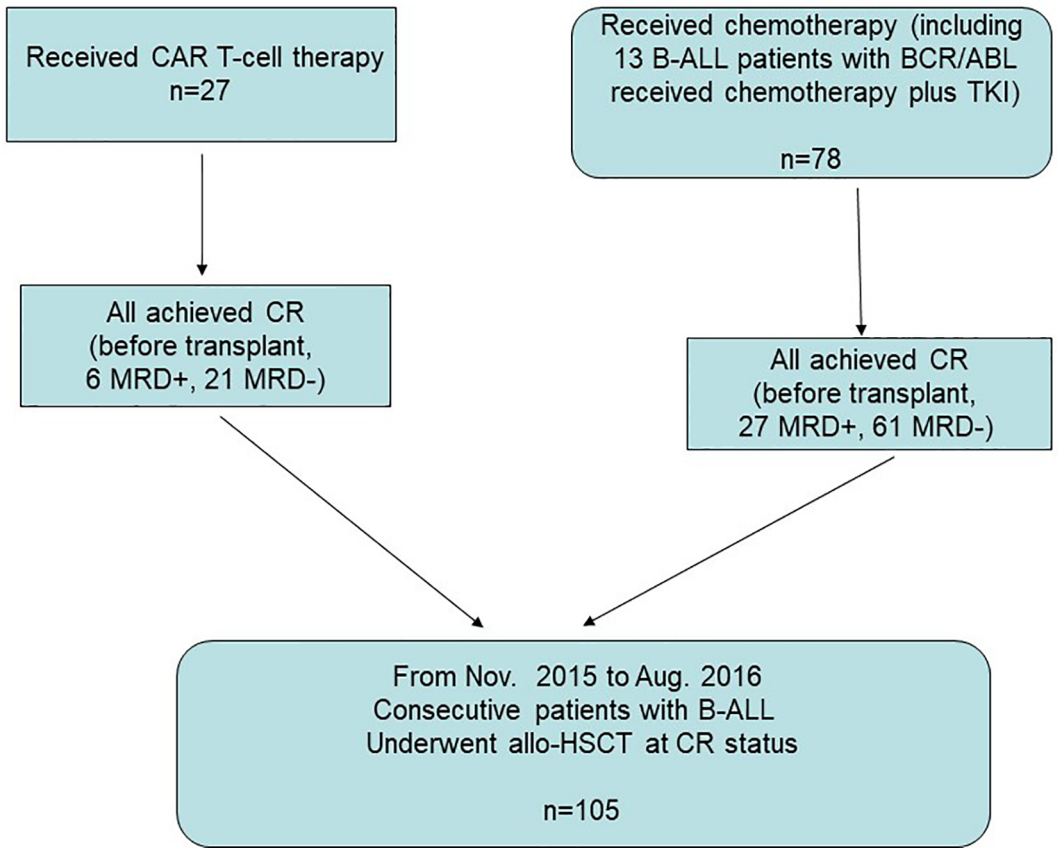


**FIGURE 1** | Enrollment, and parallel comparison. Between November 2015 and August 2016, 105 consecutive B-ALL patients who underwent allo-HSCT after achieving CR either from CAR-T therapy (n=27) or chemotherapy (n=78) were enrolled for parallel comparison.

cyclophosphamide 1.8 g/m$^2$/day for 2 days or fludarabine 30mg/m$^2$/day for 5 days. Rabbit anti-T Cell Globulin (ATG, Fresenius; totally 20mg/kg divided by 4 days) or thymoglobuline (ATG, Sanofi-Aventis, total dose of 7.5mg/kg divided by 4 days) were used on days -5 to -2 in mismatched unrelated transplants and haploidentical transplants (Haplo-HSCT). Cyclosporine, short-term methotrexate (15 mg/m$^2$ on day +1, then 10 mg/m$^2$ on days +3, +6, and +11 intravenously after transplantation), and mycophenolate mofetil were used for graft-versus-host disease (GVHD) prophylaxis. Grafts were granulocyte colony-stimulating factor (G-CSF) mobilized bone marrow (BM) and peripheral blood (PB) cells as described previously (23).

Acute GVHD (aGVHD) was diagnosed and graded according to modified Glucksberg criteria (24–26). Chronic GVHD (cGVHD) was evaluated using National Institutes of Health consensus criteria (27, 28) and aGVHD treatment was described previously (23, 24). Thrombotic microangiopathy (TMA) was diagnosed according to the Jodele criteria (29).

## Analysis of Chimerism

Hematopoietic chimerism was evaluated by PCR amplification of short tandem repeats (STR) using both bone marrow and CD3+ cells from PB samples collected at 1, 2, 3, 6, 9, and 12 months after transplant and at 6-month intervals thereafter. Complete donor chimerism was defined as the presence of ≥95% of the donor-type.

## Statistics

Comparisons of patient characteristics between the two groups were performed using the Mann-Whitney U-test for continuous variables and $\chi 2$ for categorical data. The probabilities of survival were calculated using the Kaplan-Meier method. Cumulative incidences were estimated for aGVHD, non-relapse mortality (NRM), and relapse to accommodate for competing risks. Death and relapse were competing events for aGVHD and death was a competing event for cGVHD. NRM was the competing event for relapse and vice versa. Hazard ratios (HRs) for clinical outcomes were estimated in a multivariate analysis using Cox proportional hazards regression with a backward stepwise model selection approach. The following variables were included: gender, patient age (<14 years *vs.* ≥14 years), pre-HSCT treatment (CAR-T *vs.* chemotherapy), disease status pre-transplant (≥CR2 *vs.* CR1), MRD status pre-transplant (positive *vs.* negative), poor risk chromosomes (yes *vs.* no), conditioning regimens (TBI-based

*vs.* Bu-based), donor type (alternative donor *vs.* identical unrelated or sibling donor), donor-recipient gender matching (female to male *vs.* others), course from diagnosis to transplant, and mononuclear, CD3+ and CD34+ cell counts (using the median value as the cut-off point). Independent variables with $P > 0.1$ were sequentially excluded from the model, and $P < 0.05$ was considered to be statistically significant. P values were 2-sided. The SPSS 16 (SPSS Inc./IBM, Armonk, NY, USA) and the R software package (version 4.0.0; http://www.r-project.org) were used for data analyses. Surviving patients were censored on April 30th, 2020.

## Definitions

CR and CR with incomplete count recovery (CRi) were defined in accordance with the National Comprehensive Cancer Network (NCCN) guideline, version 1.2018 (30). Minimal residual disease (MRD)-negative status was defined as the absence of leukemia cells in BM determined by multiparameter flow cytometry (FCM, sensitivity, 1:10,000), and the absence of leukemia-associated fusion gene in BM determined by real-time quantitative PCR (RT-PCR). Hypodiploidy, complex karyotype, t(v;11q23) or t(9;22) (q34;q11.2) determined by G band or FISH were defined as poor risk chromosomes according to the NCCN guideline, version 1.2018 (30). LFS and OS were calculated from the date of allo-HSCT to the date of relapse or death or the last follow-up time. The cumulative incidence of relapse (CIR) was calculated from date of allo-HSCT to the date of relapse.

# RESULTS

## Patient Characteristics

The detailed characteristics of the two groups are summarized in **Table 1**. Patients' median age was 13 years (range: 2–52 years) with a 58/42 male/female ratio. Fifty-three percent of the patients were pediatric (age <14 years) and 47% of patients were adults (age ≥14 years). High WBC counts were observed in 31 patients (30%) at initial diagnosis.

The median time from CAR T-cell therapy to HSCT was 84 days (range: 35-293 days). CRS was observed in the majority of the patients in the CAR T-cell therapy group. Grade 1 (56%) and Grade 2 (26%) CRS made up the majority of CRS cases. Severe CRS occurred in 15% of patients—11% of patients had Grade 3, and 4% of patients had Grade 4 CRS. A total of four patients had Grade 3 neurotoxicity with seizures.

In the CAR-T group, 22 (81%) patients had R/R B-ALL, and 5 (19%) had persistent or relapsed MRD after hematological remission. Among the 21 relapsed patients in the CAR T-cell group, the median time from diagnosis to first relapse was 17 months (range: 3-47 months). Following relapse, 17 patients failed to regain CR after a median 2 courses of chemotherapy (range: 1-5 courses) and afterwards underwent CAR T-cell therapy. Four patients who had relapsed during consolidation chemotherapy received CAR-T therapy directly. In the chemotherapy group, 48 (62%) had R/R B-ALL, and 12 (15%) had persistent or recurrent MRD. The median time from diagnosis to last relapse of the 33 relapsed patients was 31 months (range: 2-120 months). One patient relapsed 3 times within 10 years. Among 18 (17%) patients in the chemotherapy group, 9 presented with high risk ALL. As shown in **Table 1**, in the CAR-T group, prior to allo-HSCT, 22% (6/27) of the patients were in CR1 compared to 63% (49/78) of the patients in CR1 in the chemotherapy group. Compared to the chemotherapy group, the CAR-T group had more patients who were ≥CR 2 (78% *vs.* 37%, respectively; p<0.001).

As assayed by FCM and RT-PCR, 22% of patients in the CAR-T group and 35% of patients in the chemotherapy group had MRD detected pre-transplant (p=0.232). The proportion of patients with extramedullary diseases at diagnosis and at relapse before transplant were not significantly different between the CAR-T group and the chemotherapy group (p=0.927). There was no significant difference in the median time from diagnosis to transplant (13.5 months [range: 4-123 months]). Complex chromosomes were present in 44% of patients in the CAR-T group and 12% of the chemotherapy group (p<0.001). There was significant difference in the presence of fusion genes (p=0.023). Poor risk BCR-ABL1 (n=13) and MLL-AF4 (n=4) were exclusively observed in the chemotherapy group (**Table 1**).

## Donor Source, Graft, Conditioning Regimens and Engraftment

In the CAR-T group, 59% of patients received a transplant from haploidentical donors (Haplo-D), 30% from matched unrelated donors (MUD), and 11% from HLA-matched sibling donors (MSD). In the chemotherapy group, 64% of patients received a transplant from Haplo-Ds, 21% from MUDs and the remaining 15% of patients received a transplant from MSDs. There were no significant differences among different donor types, the donors' age and gender between the CAR-T and chemotherapy groups. In addition, there were no differences in the median mononuclear cells, CD34 and CD3 between the two groups (**Table 1**).

Myeloablative conditioning regimens were administered with TBI cyclophosphamide/TBI-fludarabine in 83% of patients and Bu cyclophosphamide/fludarabine in 17% of patients. There was no significant difference in conditioning regimens observed between the groups.

All patients achieved sustained neutrophil engraftment after a median of 14 days (range: 11-20 days) in the CAR-T group and 14 days (range: 10-29 days) in the chemotherapy group (p=0.97). Platelet engraftment failure occurred in 2 patients (7%) in the CAR-T group. One patient died of severe acute GVHD on day 27 after transplantation, and one died of infection at 68 days post-transplantation. All the 78 patients in the chemotherapy group achieved sustained platelet engraftment. There was a significant difference in platelet engraftment between the two groups (p=0.026). Post-transplant, the median day of platelet engraftment was significantly longer in the CAR-T group (14 days, range: 5-47 days) compared to the chemotherapy group (12 days, range: 4-32 days) (p=0.026).

No graft failure occurred (except that one patient had poor graft function) and rapid achievement of full donor chimerism

**TABLE 1** | Patient characteristics.

| Characteristics | Total | CAR-T group | Chemotherapy group | P value |
|---|---|---|---|---|
| No. | 105 | 27 | 78 | |
| Median age, years (range) | 13.0(2-52) | 11(3-44) | 14.5(2-52) | 0.441 |
| Age group, no. (%) | | | | 0.236 |
| ≥14 | 49(47) | 10(36) | 39(50) | |
| <14 | 56(53) | 17(63) | 39(50) | |
| Median donor age, years (range) | 33(8-63) | 31(16-54) | 33.5(8-63) | 0.550 |
| Gender, male, no. (%) | 61(58%) | 18(67) | 43(55) | 0.295 |
| With extramedullary disease (EMD), no (%) | 15(14) | 4(15) | 11(14) | 0.927 |
| Median duration from diagnosis to HSCT months (range) | 13.5(4-123) | 20(4-54) | 11(4-123) | 0.232 |
| Disease risk | | | | 0.042 |
| R/R B-ALL, no (%) | 70(67) | 22(82) | 48(62) | |
| From diagnosis to first relapse time, no. (%) | | | | |
| <18 months | 19(58) | 11(41) | 8(17) | |
| 18~36 months | 19(44) | 8(30) | 11(14) | |
| >36 months | 16(19) | 2(1) | 14(18) | |
| Primary refractory | 16(19) | 1(0) | 15(19) | |
| Persistent or relapsed MRD | 17(16) | 5(19) | 12(15) | |
| Others | 18(17) | 0 | 18(17) | |
| Disease status pre-transplant, no. (%) | | | | <0.001 |
| CR1 | 55(52) | 6(22) | 49(63) | |
| ≥CR2 | 50(48) | 21(78) | 29(37) | |
| Donor source, no. (%) | | | | 0.588 |
| Haplo-d | 66(63) | 16(59) | 50(64) | |
| MUD | 24(23) | 8(30) | 16(21) | |
| MSD | 15(14) | 3(11) | 12(15) | |
| FCM MRD-positive pre-conditioning, no. (%) | 33(31) | 6(22) | 27(35) | 0.232 |
| Fusion genes | | | | 0.023 |
| BCR-ABL1 | 13(12) | 0 | 13(17) | |
| TEL-AML1 | 9(9) | 2(7) | 7(9) | |
| E2A-PBX1 | 4(4) | 3(11) | 1(1) | |
| MLL-AF4 | 4(4) | 0 | 4(5) | |
| MLL-AF1P | 1(1) | 1(4) | 0 | |
| MLL-ENL | 1(1) | 0 | 1(1) | |
| Gene mutations | | | | |
| NRAS/KRAS | 12(11) | 5(11) | 7(9) | 0.16 |
| IKZF | 9(9) | 2(7) | 7(9) | 0.58 |
| TP53 | 5(5) | 2(7) | 3(4) | 0.383 |
| Flt3 ITD/KTD | 4(4) | 1(4) | 3(4) | 0.728 |
| High risk cytogenetics, no. (%) | 48(46) | 16(59) | 32(41) | 0.103 |
| Complex cytogenetic, no (%) | 17(16) | 12(44) | 5(6) | <0.001 |
| Donor-recipient gender match, n (%) | | | | 0.263 |
| Female to male | 19(18) | 7(26) | 12(15) | |
| Others | 86(82) | 20(74) | 66(85) | |
| Conditioning regimens, no. (%) | | | | 0.335 |
| TBI-based | 87(83) | 24(89) | 63(81) | |
| Bu-based | 18(17) | 3(11) | 15(19) | |
| Median CD34 cells,$\times10^6$/kg(range) | 4.45(1.76-12.23) | 4.6(1.76-10.18) | 4.41(2.02-12.23) | 0.428 |
| Median CD3 cells,$\times10^8$/kg(range) | 1.66(0.44-4.99) | 1.83(0.85-3.04) | 1.59(0.44-4.99) | 0.491 |
| Graft type, no. (%) | | | | 0.41 |
| BM+PB | 80(76) | 19(70) | 61(78) | |
| PB | 25(24) | 8(30) | 17(22) | |
| Neutrophil engraftment, days (range) | 14(10-29) | 14(11-20) | 4(10-29) | 0.973 |
| Platelet engraftment, days (range) | 12(4-47) | 14(5-47) | 12(4-32) | 0.026 |
| Median follow-up time in survivor, months (range) | 49 (25-54) | 49 (44-53) | 49 (25-54) | 0.831 |

was confirmed in all patients by day 30. No significant difference between the two groups was observed.

## Incidence of GVHD

The cumulative incidence of Grade II-IV aGVHD was higher in the CAR T-cell group compared to the chemotherapy group (48.1% [95% CI: 46.1, 50.1%] *vs.* 25.6% [95% CI: 25.2, 26.0%], respectively; p=0.016), while the incidence of Grade III-IV aGVHD were similar between the two groups (11.1% [95% CI: 10.3, 11.9%] *vs.* 11.5% [95% CI: 11.3, 11.7%], respectively; p=0.945) (**Figures 2A, B**). A low versus high grade CRS (Grade 0-1 *vs.* Grade 2-4) before transplant did not have significant effects on Grade II-IV aGVHD (47.4% [95% CI: 44.7, 50.1%] *vs.* 50.0% [95% CI: 42.6, 57.4%] among the CAR-

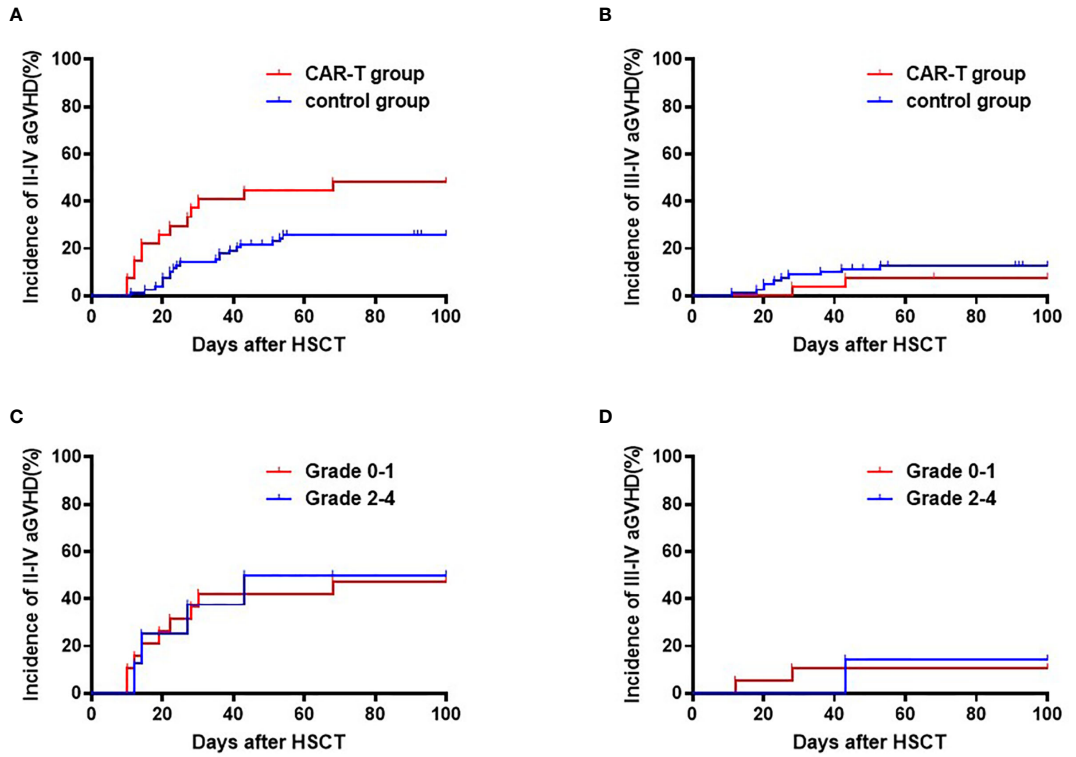


**FIGURE 2** | Cumulative incidences of Grade II-IV and Grade III-IV acute GVHD. **(A)** Cumulative incidences of Grade II-IV acute GVHD: CAR-T group: 48.1% (95% CI:46.1, 50.1%) *vs.* chemotherapy group: 25.6% (95% CI: 25.2, 26.0%); p=0.016. **(B)** Cumulative incidences of Grade III-IV acute GVHD: CAR T-cell group: 11.1% (95% CI: 10.3, 11.9%) *vs.* chemotherapy group: 11.5% (95% CI: 11.3, 11.7%); p=0.945. **(C)** Cumulative incidences of Grade II-IV acute GVHD: CRS Grade 0-I 47.4% (95% CI: 44.7, 50.1%) *vs.* CRS Grade II-IV: 50.0% (95% CI:42.6, 57.4%); p=0.95. **(D)** Cumulative incidences of Grade III-IV aGVHD: CRS Grade 0-I: 10.5% (95% CI: 9.5, 11.5%) *vs.* CRS Grade II-IV: 12.5% (95% CI: 9.4, 15.6%); p=0.92.

T and chemotherapy groups, respectively; p=0.95) or on Grade III-IV aGVHD (10.5% [95% CI: 9.5, 11.5%] *vs.* 12.5% [95% CI: 9.4, 15.6%]; p=0.92) after transplant (**Figures 2C, D**).

For patients surviving over 100 days after transplantation, cumulative incidence of cGVHD at 18 months were higher in the CAR-T group, but this difference did not reach statistical difference (71.3% [95% CI: 71.3, 75.3%] *vs.* 55.0% [95% CI: 54.2, 55.8%], p=0.107) (**Figure 3A**). Cumulative incidence of extensive cGVHD at 18 months was similar between the CAR-T and chemotherapy groups (11.1% [95% CI: 10.3, 11.9%] *vs.* 11.9% [95% CI: 11.7, 12.1%]; p=0.964) (**Figure 3B**).

## CIR After HSCT

The CIRs at 4 years following transplant were 11.1% [95%CI: 10.3,11.9%] for the CAR-T group versus 12.8% [95% CI: 12.0-13.6%] for the chemotherapy group (p=0.84) (**Figure 4A**). Univariate analysis showed that disease status (HR 3.87, [95% CI 1.09-13.7], p=0.027) and MRD before transplantation (HR 2.81 [95%CI 0.961-8.24], p=0.056) were predictive factors for relapse. The multivariate analysis confirmed these predictive effects of relapse (HR 4.10, [95% CI1.13-14.84], p=0.031 and HR 3.02, [95% CI1.02-8.96], p=0.046, for disease status and MRD before transplant, respectively) (**Table 2**).

A total of 13 patients relapsed after transplant, three in CAR-T group and 10 in the chemotherapy group. All except one patient died at a median time of day 283 (range: 48-1116) after transplant. The patient that survived relapsed following a second haploidentical transplant underwent donor CD19 CAR T-cell therapy and remains in remission.

In the CAR T-cell group, 6 patients were MRD positive (MRD+CR) before transplant, including 5 patients who were CD19 negative (CD19-) and MRD+CR. Two of the CD19-MRD+CR patients relapsed with CD19- leukemia at Day 60 and at Day 275, and consequently died at Day 270 and Day 336 after transplant, respectively. Another patient died of severe GVHD on Day 27. The remaining three patients survived in remission at Month 46, 47, and 49, respectively.

## Infection, TMA and NRM

No remarkable differences were observed in cytomegalovirus (CMV) reactivation (52% *vs.* 50%, p=0.93) between the CAR T-cell and chemotherapy groups, respectively. There were also no differences in rates of transplant-associated TMA (TA-TMA) between the CAR T-cell and chemotherapy groups, respectively (15% *vs.* 14%, p=0.51). In the chemotherapy group, three patients were diagnosed with viral pneumonia

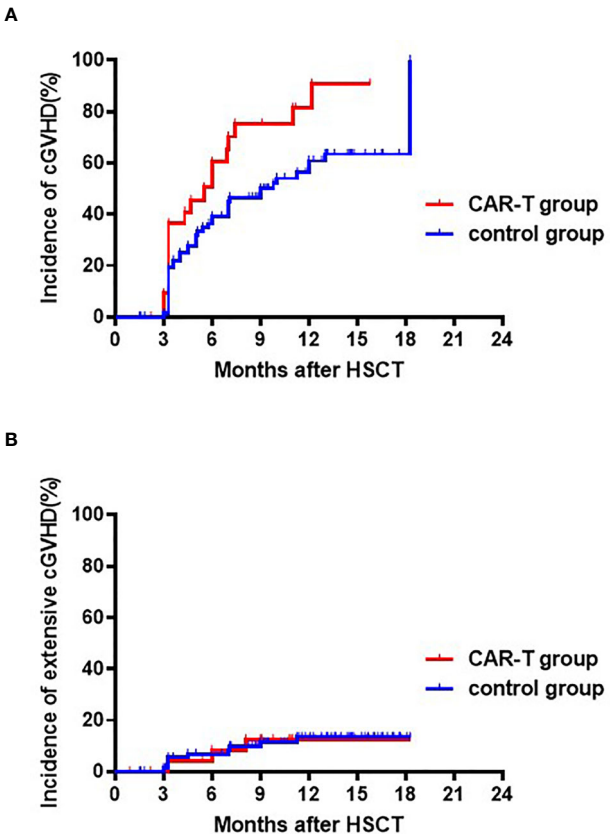


**FIGURE 3** | Cumulative incidences of chronic and extensive chronic GVHD. **(A)** Cumulative incidences of chronic GVHD: CAR T-cell group: 73.3% (95% CI: 71.3, 75.3%) *vs.* chemotherapy group: 55.0% (95% CI: 54.2, 55.8%); p=0.107. **(B)** Cumulative incidences of extensive chronic GVHD: CAR T-cell group: 11.1% (95% CI:10.3, 11.9%) *vs.* the chemotherapy group: 11.9% (95% CI: 11.7, 12.1%); p=0.964.

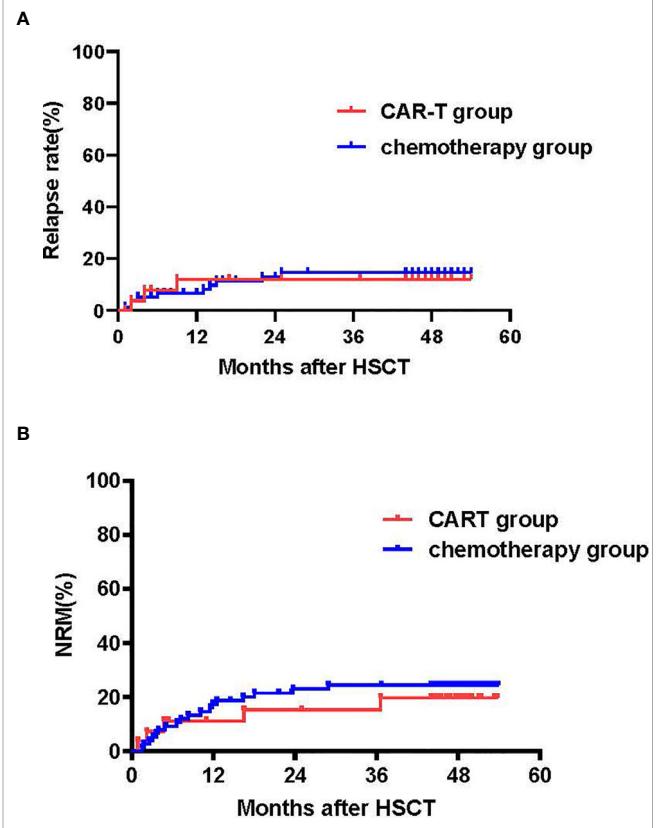


**FIGURE 4** | Cumulative incidences of relapse (CIR) and NRM. **(A)** Cumulative incidence of relapse: CAR T-cell group: 4-year CIR of 11.1% (95% CI: 10.3, 11.9%) *vs.* chemotherapy group: 12.8% (95% CI:12.6, 13.0%) (p=0.84). **(B)** Cumulative incidence of NRM: CAR T-cell group: 4-year NRM of 18.7% (95% CI:17.5, 19.9%) *vs.* chemotherapy group: 4-year NRM of 23.1% (95% CI:22.7, 23.5%); p=0.64.

and died. Incidences or non-relapse mortality (NRM) within 100 days were 7.4% (95% CI:6.8-8.0%) and 5.1% (95% CI: 4.9-5.3%) (p=0.64). The NRM at 1 and 4 years after transplantation was 11.1% (95% CI: 10.3-11.9%) and 18.7% (95% CI: 17.5, 19.9%), respectively, for the CAR-T group versus 16.7% (95% CI [16.3-17.1%] and 23.1% (95% CI: 22.7, 23.5%) for the chemotherapy group (p=0.64) (**Figure 4B**).

## LFS, OS and Cause of Mortality

With a median follow-up of 49 months (range: 44-54 months) for surviving patients, LFS and OS at 4 years were similar in the CAR-T and chemotherapy groups (LFS: 70.2% [95% CI: 53.0, 87.4%] *vs.* 64.1% [95% CI: 53.5, 74.7%], p=0.63; OS: 70.2% [95% CI: 53.0, 87.4%] *vs.* 65.4% [95%CI:54.8, 76.0%], p=0.681) (**Figure 5A**).

Univariate and multivariate analysis showed that MRD prior to transplant was a negative prognostic factor for LFS (p=0.024 for univariate-analysis and p=0.016 (HR 2.6 [95% CI: 1.2, 5.8] for multivariate-analysis). The 4-year LFS for the MRD-CR and MRD+CR groups was 72.2% [95% CI: 61.8, 82.6%] and 51.5% [95% CI: 34.4, 68.6%], respectively (p=0.024). The 4-year OS for MRD-CR patients was 73.6% (95%CI: 63.4, 83.8%) and MRD +CR of 51.5% (95%CI: 34.4, 68.6%) (p=0.02), respectively (**Figure 5B**).

For the 97 patients who survived over 100 days after transplant, 4-year LFS for no cGVHD, and limited and extensive cGVHD was 62.2% (95% CI: 46.5, 77.9%), 85.6% (95% CI: 75.8, 95.4%) and 36.4% (95% CI: 8.0, 64.8%), respectively (no *vs.* limited cGVHD, p=0.009; limited *vs.* extensive cGVHD, p<0.001; no *vs.* extensive cGVHD, p=0.20). The 4-year OS for the no cGVHD cohort was 64.9% (95% CI:49.6, 80.2%), 85.6% (95% CI: 75.8, 95.4%) for the limited cGVHD cohort and 36.4% (95% CI: 8.0, 64.8%) for the extensive cGVHD cohort (no *vs.* limited cGVHD, p=0.019; limited *vs.* extensive cGVHD, p<0.001; no *vs.* extensive cGVHD, p=0.123) (**Figure 5C**).

At the time of the latest follow up in April 2020, 29 patients had died. The primary causes of death were relapse (8 patients), GVHD (8 patients), infection (5 patients) and TMA (5 patients) (**Table 3**).

**TABLE 2** | Univariate and multivariate analysis of risk factors for clinical outcomes.

| | II-IV GVHD | | | | | III-IV GVHD | | | | | CIR | | | | | NRM | | | | | LFS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate | | | Multivariate | | Univariate | | | Multivariate | | Univariate | | | Multivariate | | Univariate | | | Multivariate | | Univariate | | | Multivariate | |
| | HR | 95% CI | p value | HR | 95% CI p value | HR | 95% CI | p value | HR | 95% CI | p value | HR | 95% CI | p value | HR | 95% CI | p value | HR | 65% CI p value | HR | 65% CI | p value | HR | 65% CI | p value |
| Age(≥14 vs. <14yrs) | 0.785 | 0.395-1.56 | 0.49 | | | 0.339 | 0.0921-1.25 | 0.088 | 0.506 | 0.1081-2.37 | 0.39 | 1.23 | 0.418-3.62 | 0.708 | | | 2.09 | 0.89-4.91 | 0.084 | | | 1.8 | 0.921-3.51 | 0.079 | 1.652 | 0.84-3.25 | 0.15 |
| Treatment pre-HSCT (CAR-T vs. control) | 2.36 | 1.18-4.75 | 0.016 | | | 0.956 | 0.263-3.47 | 0.945 | | | | 0.873 | 0.241-3.17 | 0.836 | | | 0.791 | 0.295-2.12 | 0.641 | | | 0.827 | 0.38-1.8 | 0.631 | | | |
| Disease status(≥CR2 vs. CR 1) | 1.09 | 0.557-2.15 | 0.769 | | | 0.494 | 0.152-1.6 | 0.239 | | | | 3.87 | 1.09-13.7 | 0.027 | 4.1 | 1.13-14.84 | 0.031 | 0.774 | 0.344-1.74 | 0.54 | | | 1.54 | 0.808-2.93 | 0.192 | | | |
| MRD(pos vs. neg) | 1.09 | 0.536-2.23 | 0.81 | | | 2.29 | 0.75-7.02 | 0.142 | | | | 2.81 | 0.961-8.24 | 0.056 | 3.02 | 1.02-8.96 | 0.046 | 1.45 | 0.638-3.32 | 0.38 | | | 2.21 | 1.16-4.24 | 0.017 | 2.105 | 1.09-4.06 | 0.027 |
| Poor risk chromosomes (yes vs. no) | 1.5 | 0.76-2.95 | 0.245 | | | 2.48 | 0.735-8.17 | 0.125 | | | | 0.709 | 0.236-2.13 | 0.543 | | | | 0.622 | 0.263-1.47 | 0.271 | | | 0.614 | 0.313-1.21 | 0.151 | | | |
| Conditioning(TBI-based vs. Bu-based) | 0.888 | 0.365-2.16 | 0.795 | | | 0.254 | 0.0815-8.17 | 0.015 | 0.387 | 0.0929-1.61 | 0.19 | 0.655 | 0.181-2.38 | 0.523 | | | | 0.937 | 0.314-2.79 | 0.903 | | | 0.883 | 0.384-2.03 | 0.765 | | | |
| Donor type (haplo vs. MUD/MSD) | 0.894 | 0.45-1.78 | 0.753 | | | 3.17 | 0.715-14 | 0.113 | | | | 1.33 | 0.413-4.31 | 0.629 | | | | 0.917 | 0.402-2.09 | 0.84 | | | 1.25 | 0.646-2.42 | 0.516 | | | |
| donor-recipients gender matched (female to male vs. others) | 1.16 | 0.464-2.89 | 0.761 | | | 3.37 | 1.07-10.6 | 0.0375 | 1.885 | 0.4163-8.54 | 0.41 | 1.15 | 0.271-4.89 | 0.853 | | | | 0.586 | 0.142-2.42 | 0.457 | | | 0.61 | 0.234-1.59 | 0.33 | | | |
| course from diagnosis to transplant (≥ median vs. <median) | 0.883 | 0.45-1.73 | 0.719 | | | 0.455 | 0.141-1.47 | 0.188 | | | | 2.34 | 0.732-7.48 | 0.144 | | | | 0.6 | 0.262-1.37 | 0.225 | | | 1.05 | 0.552-1.98 | 0.893 | | | |
| MNC (≥median vs. <median) | 0.59 | 0.303-1.15 | 0.3 | | | 0.996 | 0.351-2.82 | 0.934 | | | | 1.11 | 0.412-3 | 0.886 | | | | 0.926 | 0.433-1.98 | 0.871 | | | 1.12 | 0.62-2.01 | 0.616 | | | |
| CD3 (≥median vs. <median) | 1.17 | 0.596-2.29 | 0.655 | | | 0.713 | 0.228-2.23 | 0.561 | | | | 0.421 | 0.13-1.36 | 0.137 | | | | 1.13 | 0.506-2.53 | 0.766 | | | 0.824 | 0.432-1.57 | 0.559 | | | |
| CD34 (≥median vs. <median) | 1.01 | 0.511-1.98 | 0.986 | | | 0.682 | 0.219-2.12 | 0.51 | | | | 0.413 | 0.129-1.32 | 0.128 | | | | 0.916 | 0.408-2.06 | 0.834 | | | 0.576 | 0.303-1.1 | 0.096 | 0.644 | 0.34-1.22 | 0.18 |
| cGVHD (limited vs. extensive vs. | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

## DISCUSSION

Recently CAR-T therapy has shown dramatic initial responses with CR rates approaching 80-90% among R/R B-ALL patients (6–12). However, risk of relapse remains a major problem for these patients. Allo-HSCT after CAR-T therapy may have a consolidative role to further improve the durability of remission for these patients. However, whether prior CAR-T therapy can potentially increase the transplant-related mortality and toxicity remain a concern. In the present study, we compared the safety and efficacy of allo-HSCT in patients in patients after achieving CR either post CAR-T or after chemotherapy with a median follow-up of 4 years. To our knowledge, this is the first analysis comparing B-ALL patient outcomes after allo-HSCT following either prior CAR T-cell therapy or chemotherapy.

Although this is not a randomized trial, our parallel cohort study showed a similarly high LFS (70.2% *vs.* 64.1%) and OS (70.2% *vs.* 65.4%) after a median follow-up of 4 years in patients who received allo-HSCT after achieving CR from CAR-T therapy (n=27) or after achieving CR following chemotherapy (n=78), even despite having significantly more patients with advanced disease and refractory/relapsed status in the CAR-T group. There was no graft-failure in either group. The incidences of NRM, TMA and CMV reactivation within both groups were similar.

Hematopoietic reconstitution is one of the key issues in heavily pre-treated B-ALL patients after allo-HSCT. In our study, all patients achieved prompt and sustained neutrophil engraftment, at a median 14 days and achieved 100% donor chimerism in bone marrow and blood on day 28 in both the CAR-T and chemotherapy groups. Nevertheless, the engraftment of platelets was significantly slower in the CAR-T group compared to the chemotherapy group (Day 14 *vs.* Day 12, p=0.026). One possible reason may be due to the higher incidence of aGVHD and corresponding glucocorticoids treatment of patients in the CAR-T group. In addition, cytokine storm subsequent to CAR-T therapy might impair the endothelium system in transplant recipients (6–9), including the hematological microenvironment.

GVHD remains a major cause of morbidity and mortality following allo-HSCT. The reports of incidence and severity of GVHD after transplant post CAR T-cell therapy have been very limited. In a study by Shadman et al. from the University of Washington, it reported incidence of Grade II-IV and Grade III-IV acute GVHD of 69% and 25%, respectively (17). Jiang et al. reported no severe aGVHD except for mild skin rash and diarrhea (Grade ≤2) among 21 patients (31). In our study, Grade II-IV and Grade III-IV aGVHD were 48% and 11%, respectively, in the CAR-T group. Further analysis showed that the grade of CRS had no influence on the incidence and severity of aGVHD. Considering the limited size of the CAR-T group in our study, further clinical trials are necessary to verify the effect of CRS on aGVHD after transplantation. We found higher incidence of Grade II-IV aGVHD in the CAR T-cell group, but similar incidence of severe aGVHD compared to the chemotherapy group. Regarding the cGVHD, the overall incidence of cGVHD in the CAR-T group was higher, but the

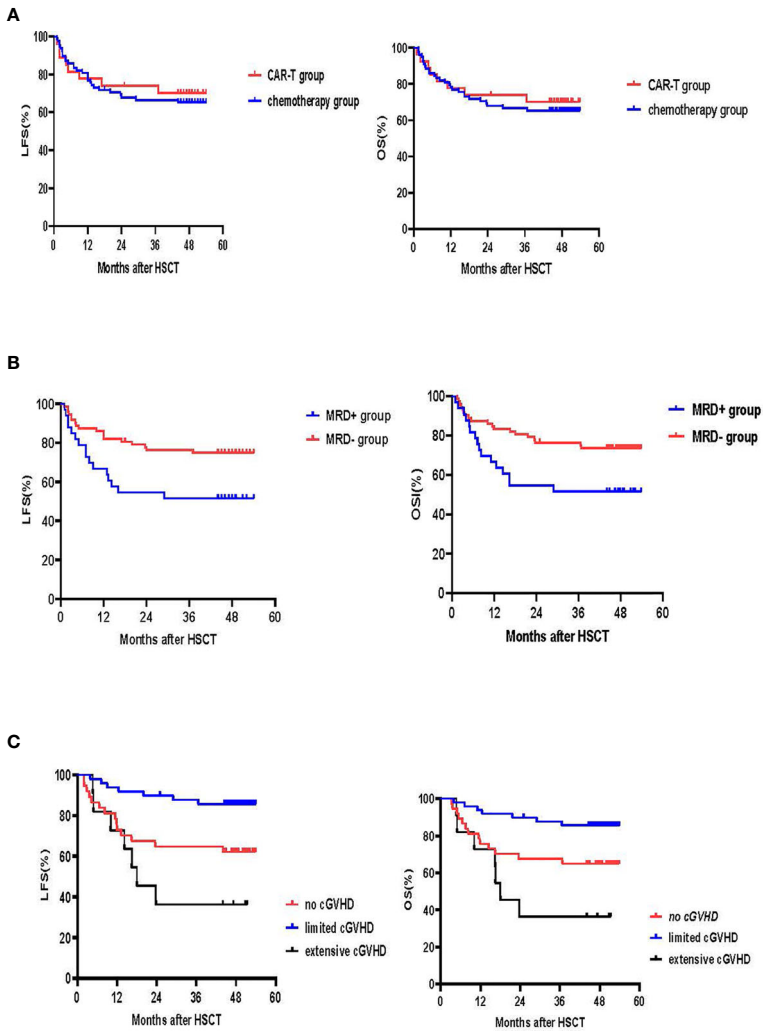


**FIGURE 5** | LFS and OS. **(A)** LFS and OS in the CAR-T and chemotherapy groups. The 4-year LFS for the CAR-T group was 70.2% (95% CI:53.0, 87.4%) *vs.* 64.1% (95% CI:53.5, 74.7%) for the chemotherapy group (p=0.63). The 4-year OS for the CAR-T group was 70.2% (95% CI:53.0, 87.4%) *vs.* 65.4% (95% CI:54.8, 76.0%) for the chemotherapy group (p=0.681) **(B)** LFS and OS according to MRD. The 4-year LFS for patients who achieved MRD- CR was 72.2% (95% CI:61.8, 82.6%) and 51.5% (95% CI:34.4, 68.6%) for those that had an MRD+ CR (p=0.024). The 4-year OS for the MRD- CR group was 73.6% (95% CI:63.4, 83.8%) and 51.5% (95% CI:34.4, 68.6%) for the MRD+CR group (p=0.02). **(C)** LFS and OS according to cGVHD. 4-year LFS for patients without cGVHD was 62.2% (95% CI:46.5, 77.9%), 85.6% (95% CI:75.8,95.4%) for those with limited cGVHD and 36.4% (95% CI:8.0, 64.8%) for those with extensive cGVHD (no *vs.* limited cGVHD, p=0.009; limited *vs.* extensive cGVHD, p<0.001; no *vs.* extensive cGVHD, p=0.20). 4-year OS for the no cGVHD group was 64.9% (95% CI:49.6, 80.2%), 85.6% for the limited cGVHD group (95% CI:75.8, 95.4%) and 36.4% for the extensive cGVHD group (95% CI:8.0, 64.8%) (no *vs.* limited cGVHD, p=0.019; limited *vs.* extensive cGVHD, p<0.001; no *vs.* extensive cGVHD, p=0.123).

**TABLE 3** | Causes of death in the CAR-T and chemotherapy groups.

| Causes | Total | CAR-T group | Chemotherapy group |
|---|---|---|---|
| All causes of death | 34 | 8 | 26 |
| Relapse | 11 | 3 | 8 |
| NRM | 23 | 4 | 18 |
| GVHD | 8 | 1 | 7 |
| Infection | 6 | 2 | 4 |
| TMA | 5 | 1 | 4 |
| Graft failure or rejection | 1 | | 1 |
| Malignant arrhythmia | 1 | | 1 |
| Acute pancreatitis | 1 | 1 | |
| Organic pneumonia | 1 | | 1 |

rate of extensive cGVHD (11.1% *vs.* 11.9%, in the CAR T-cell and chemotherapy groups, respectively p=0.96) was similar between the groups. There was a relative higher incidence of cGVHD in our study. It is likely because the major donor type was haplo donor (67%) in our study. In addition, considering the high risk of recurrence in this group of patients with advanced disease, immunosuppressants were withdrawn as soon as possible. And in some very high-risk patients, prophylactic DLI and interferon were applied to gain a limited chronic GVHD status in both groups. The incidence of the cGVHD was similar to our previous reports of haplo-HSCT with ATG (23, 32).

Neurotoxicity is a relatively common toxicity with CAR-T therapy (20–22). However, whether development of CAR-T-related neurotoxicity increases the neurotoxicity of fundamental immunomodulators such as cyclosporine and tacrolimus after allo-HSCT is still unclear. In our study, four patients experienced Grade III neurotoxicity with seizures after CAR-T infusion. However, none of the four patients had developed drug-induced encephalopathy or TMA after transplant. Nevertheless, patients who present with severe neurotoxicity after CAR-T should be followed up and treated with caution.

There are now more and more published studies confirming that consolidative allo-HSCT following CAR-T therapy could reduce relapse rates and improve LFS for R/R ALL patients (6, 10, 11, 14–16). However, studies comparing CIR, LFS and OS between patients post CAR T-cell therapy and post-chemotherapy have not been reported. In the present study, we found similar CIR, LFS and OS rates between our two cohorts. Among our 105 patients, there was only 11% CIR, which is lower than the CIR rate reported previously among ALL patients in CR (33–35). There are multiple potential reasons for the relatively lower CIR in our study. First, a haploidentical donor was the main donor source (67%) in our study. Mo et al. found that a haplo-donor was superior to a matched sibling donor in offsetting the detrimental effects of high-risk factors and pre-transplant MSD among ALL patients (13, 36). Second, TBI-based conditioning regimes were used in the majority of our patients (83%). TBI has demonstrated an advantage over Bu as a component of conditioning regimens for MSD, MUD, and haplo-HSCT in pediatric and adult patients with ALL (33–35, 37). Third, myeloablative conditioning regimens were used in all the patients in this study, which were more effective in eradicating residual leukemia disease.

Several studies have previously shown the prognostic relevance of disease status and MRD status among B-ALL patients (38, 39). In our multivariate analysis, we demonstrated that the CIR for MRD+ patients before transplant was 3 times as high as that of MRD- patients and a negative MRD status either after CAR-T therapy or after chemotherapy and prior to transplant predicted better results. We showed that MRD before transplant was an independent predicator for CIR after HSCT and that achieving an MRD-negative CR was crucial and equally important for optimal transplantation outcomes among both the chemotherapy and CAR-T therapy groups.

Among patients receiving CAR-T therapy, whether CR patients with an CD19-negative status before transplant will have an increased risk of relapse remains a concern and will require further investigation. The possible reason for a CD19-negative relapse could be due to a selective immune escape mechanism of the tumor cells (40). So far there is no evidence that CD19-negative leukemic clones may be more easily attained as a further escape from the graft-versus-leukemia (GVL) effect of donor cells. Allo-HSCT is an anti-HLA immunotherapy, which is independent from CD19. Excluding the one early treatment-related death within one month following transplantation, half (2/4) of the patients relapsed with CD19-negative clones in our CAR-T group. Due to the limited number of cases in our study, we cannot make any conclusions on whether patients with a CD19-negative MRD status before transplant are more likely to relapse. Nevertheless, caution should be taken for those patients with CD19-negative MRD status who are to undergo an allo-HSCT.

In addition to CAR-T therapy, other immunotherapeutic approaches have proven successful in R/R B-ALL such as blimatumomab and inotuzomab. Currently, it remains unclear whether it is better to treat R/R B-ALL with CAR-T cell therapy *vs.*. blimatumomab or inotuzomab. Thus, it is important to conduct a randomized clinical trial in the future to investigate this question. A head-to-head comparison trial of blimatumomab or inotuzomab *vs.* CAR-T cell is undergoing (NCT03628053). Despite high CR/CRi of 67% achieved with inotuzomab for pediatric R/R ALL patients (41), 44% with blimatumomab (42), relapse remains the major problem. Without consolidative allo-HSCT, long-term disease control was limited with both blimatumomab and inotuzomab, especially for patients with high leukemic burden (43). The incidence of sinusoids obstruction syndrome (SOS), previously known as veno-occlusive disease, has been reported after allo-HSCT following inotuzomab, at an especially high rate (52%) in pediatric patients (41). In addition to efficacy and safety considerations, cost, insurance coverage, and local availability of each immunotherapy are all factors that will influence clinical decision-making. One limitation of our study is that it is not a randomized clinical trial but it is not feasible at the present time to do such randomized clinical trial as the CAR-T therapy is currently only indicated to chemotherapy refractory or relapsed patients. Nevertheless, our long-term follow-up and parallel comparison results demonstrate that pre-transplant CR induced by CAR-T therapy in R/R B-cell ALL patients carry the same prognostic significance as CR induced by conventional chemotherapy for patients without refractory disease. Although the CAR-T group had a higher incidence of Grade overall aGVHD and cGVHD, the incidence of serve aGVHD and cGVHD was comparable in both groups. Importantly, no clear increased transplant related mortality was identified in our CAR-T group. We conclude that the strategy of CAR-T therapy followed by allo-HSCT in R/R B cell-ALL was safe and effective, exhibiting similar long- term NRM, CIR, LFS and OS as those achieved among patients in the chemotherapy group.

## 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.

## AUTHOR CONTRIBUTIONS

Y-LZ did the work of data collecting, data analysis and paper writing. D-PL and PL are the director of Lu Daopei Hospital and

respond for the whole paper. Y-LZ, D-YL, R-JS, J-PZ, J-RZ, Z-JW, MX, X-YC, and YL take care of the all the HSCT patients. And J-fY and XZ took care of the patients during CAR-T therapy. All authors contributed to the article and approved the submitted version.

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